Fat as a Basic Taste: CD36 and its Role in Fat Taste

by

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ABSTRACT

Epidemiological studies have identified obesity as a risk factor for numerous chronic diseases such as adult onset diabetes, hypertension, and hypercholesterolemia. In both humans and laboratory animals, high-fat diets have been shown to cause obesity. Increases in dietary fat lead to increased energy consumption and, consequently, significant increases in body fat content. CD36 has been implicated in fat perception, preference, and increased consumption, but it is yet to be tested using a behavior paradigm. To study the effect of CD36 on fat taste transmission and fat consumption, four CD36 knockout (experimental) mice and four Black 6 wildtype (control) mice underwent 20 days of fat preference and perception testing. Both groups of mice were exposed to foods with progressively increasing fat content (10%, 12.5%, 15% 17.5%, 20%, 45%) in order to assess the effect of CD36 on fat preference. Afterward, the mice were subjected to an aversive conditioning protocol designed to test the effect of CD36 on fat taste perception; development of a conditioned taste aversion was indicative of ability to taste fat. Especially, knockout mice exhibited diminished preference for and reduced consumption of fat during preference testing and were unable to identify fat taste as the conditioned stimulus during aversive conditioning. A repeated measures ANOVA with Bonferroni correction revealed a significant main effect of group on fat consumption, energy intake, and weight. Linear regression revealed CD36 status to account for a majority of observed variance in fat consumption across both phases of the experiment. These results implicate CD36 in fat taste perception and preference and add to the growing body of evidence suggesting fat as a primary taste.

DEDICATION

This thesis was completed in memoriam of Dr. Elizabeth D. Phillips, PhD (1945-2017).

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CHAPTER 1

INTRODUCTION

Obesity is one of the most prominent health challenges facing the United States today; the Centers for Disease Control and Prevention (CDC) estimate that over one-third of American adults are classified as obese (Flegal et. al., 2010). Epidemiological studies have identified obesity as a risk factor for numerous chronic diseases such as adult onset diabetes, hypertension, and hypercholesterolemia (Mokdad et. al., 2003). Although the etiology of obesity has been shown to be multifactorial—often a result of genetic and psychological influences (Locke et. al., 2015; Stoeckel et. al., 2017)—there has been considerable interest and debate regarding the role of macronutrient and diet composition in initiating and propagating obesity in both humans and animals (Abete et. al., 2010; Berthoud et. al., 2012).

In both humans and laboratory animals, high-fat diets have been shown to cause obesity (Warwick & Shiffman, 1992; Buettner, 2007). In both rats and mice, increases in dietary fat have been shown to lead to increased energy consumption and, consequently, significant increases in body fat content (Ackroff & Sclafani, 2009; West & York, 1998). Fat has been shown to exert hedonic effects both during and after consumption (Ackroff & Sclafani, 2009; Sclafani & Ackroff; 2012). Therefore, an increased understanding of fat taste perception and preference can lead to both pharmacological interventions at the receptor level, as well as psychological interventions to modify the underlying dietary choices that lead to obesity. The fat preference model described in this thesis is a framework for understanding rodents' preferences for foods of differing fat content, using

a genetically modified mouse model. More specifically, this model presents mice with a simultaneous two-choice test of foods differing only in fat content, where quantification of fat preference is taken to be a function of fat consumption in grams—measured as a preference ratio, referred to now on as the 'fat preference score'. The fat preference score was calculated by dividing the intake of the higher fat food from the total food consumed over each 2-day period. In the context of the present two-part experiment, fat consumption is measured as a percent of total consumption, as fat content is known for all foods. In theory, animals able to detect fat should exhibit greater consumption in the first part of the experiment and decreased consumption in the second. Furthermore, such a model is highly conducive to the study of pharmacological and genetic effects of fat taste perception and preference (Kasper et. al., 2014).

The fat preference model described herein serves not only as a complement to other, already-established models of food intake (Velloso, 2009), but also confers significant advantages to these models. For instance, traditional models of fat consumption offer only one food to the animal subject, thereby eliminating the possibility of studying food choice, a vital component of human energy consumption. The model presented here, however, allows the researcher to specifically asses fat preference in a tightly controlled environment where only two food choices are made available to the animal subject (Kasper et. al., 2014). While previous studies have attempted to investigate food choice in rodent models using a "cafeteria" style diet (Rogers & Blundell, 1984), these studies suffer from poor reproducibility as ill-suited human food is often used in the assay, thereby confounding the results due to nutrient variability. In this

study, a modified version of the National Institutes of Health (NIH) 31 rodent diet was used (Clear H_2O DietGel 31M) to allow for easy manipulation of macronutrient content—in this case fat—and greater reproducibility. Since we know high fat intake is associated with obesity in humans (Warwick & Schiffman, 1992) and, as humans display a conditioned preference for high fat foods (Johnson, et. al., 1991), interventions that elucidate the physiological role of fat perception in rodent models and reduce fat preference in said models offer valuable insight into human fat-induced obesity. Although inferences to human populations can be made from such a study, a mouse model has been intentionally chosen as the experimental sample. In addition to the uncertainty and variance of innate human preferences for dietary fat, the high-cost and infeasibility of quantifying human CD36 taste receptor density using immunohistochemical methods necessitated the use of a mouse model in the current study.

Study Purpose

The purpose of this study is to evaluate the importance of receptor protein classification determinant 36—also known as CD36—in facilitating fat taste perception and mediating conditioned fat preferences. During the trial, two groups of mice—one CD36 knockout group, and one wildtype group—were assessed on their innate preference for high-fat foods as well as their ability to use fat taste as a learning cue during an aversive conditioning paradigm. An operative assumption here is that animal subjects capable of perceiving fat taste will act on their innate preference for fat and increase their consumption of foods with higher fat content.

In the first experimental phase, animals capable of perceiving fat taste should exhibit greater consumption of fat, increased energy intake, and—consequently—increased body weight in response to foods of progressively increasing fat content. In the second experimental phase, animals capable of perceiving fat taste should exhibit a progressively decreasing fat consumption trend, and consequently decreased energy intake and weight, in response to the aversive conditioning. Experimental phases were divided as such to show that, not only is CD36 is a unique transduction mechanism for fat taste, but that it is also capable of producing a cognitive signal sufficient of supporting associative learning.

Research Hypotheses

H_1: Wildtype mice in the control group will display a greater preference for high-fat foods as compared to the CD36 knockout mice in the experimental group.

H_2: Wildtype mice in the control group will exhibit faster acquisition of a conditioned fat-taste aversion as compared to the CD36 knockout mice in the experimental group.

H_0: Wildtype mice in the control group will exhibit no appreciable difference in innate fat preference or acquisition of fat-taste aversion as compared to the CD36 knockout mice in the experimental group.

Definition of Terms

- **Encephalization:** an evolutionary increase in the complexity or relative size of the brain, involving a shift of function from noncortical parts of the brain to the cortex

- **Metabolic syndrome:** a clustering of risk factors for cardiovascular disease and type 2 diabetes, including elevated blood pressure and obesity
- **Triacylglycerol or triglyceride (TAG):** an ester formed from glycerol and three fatty acid groups
- **Classical conditioning:** a learning process that occurs when two stimuli are repeatedly paired, such that the response initially elicited by the second stimulus is eventually elicited by the first stimulus alone
- **Operant conditioning:** a learning process through which the frequency of a behavior is either positively or negatively modified through reinforcement or punishment, respectively
- **Conditioned taste aversion (CTA):** occurs when an animal associates the taste of a certain food with symptoms caused by a toxic, spoiled, or poisonous substance
- **Avoidance**: a type of negative reinforcement in which performing a response prevents an aversive stimulus from occurring in the first place
- **Oleogustus:** fat detection by means of receptor-ligand interaction and neuronal transduction to the primary gustatory cortex
- **Conditioned response (CR):** the response that is elicited by a conditioned stimulus after classical conditioning has taken place
- **Conditioned stimulus (CS):** an initially neutral stimulus that develops the capacity to elicit a conditioned response after it is paired with an unconditioned stimulus

- **Unconditioned response (UR):** In classical conditioning, an innate response that is elicited by an unconditioned stimulus
- **Unconditioned stimulus (US):** In classical conditioning, a stimulus that naturally elicits a specific response
- **Taste:** the sensation produced when a substance in the mouth reacts chemically with taste receptors embedded in taste buds, mostly on the tongue
- **Odorant:** a substance giving off a smell
- **Generalization:** the transfer of a learned response from one stimulus to another, similar stimulus
- **Aversive conditioning:** a treatment in which a preferential substance is paired with an aversive stimulus, such as an illness-producing drug, designed to condition an aversive response to the paired substance
- **Acquisition phase:** the period in the learning process when an individual is learning a new behavior
- **Extinction:** In classical conditioning, presenting the conditioned stimulus without the unconditioned stimulus; in operant conditioning, no longer presenting the reinforcer when the operant response is made. In both cases, responding decreases and eventually disappears
- **Obesity:** the condition of being grossly fat or overweight
- **Punishment:** a behavior reduction procedure in which the occurrence of a behavior is followed by an aversive stimulus

Delimitations and Limitations

Delimitations:

- Healthy, naïve, adolescent mice between the ages of 7-12 weeks were purchased for this study to ensure efficacy of the conditioning protocol.
- Other receptors implicated in fat taste signaling were not considered, due to heightened complication of research design.
- Only male mice were purchased for the study, due to the hormonal/temperamental fluctuation of female mice.

Limitations:

- Although preferable, a knockout rat model was not used in this study due to the significant cost associated.
- Also, due to cost considerations, this study is significantly under-powered.
- Textural and olfactory cues of fat could not be controlled for.
- Due to time constraints, extinction time following aversive conditioning was not measured.
- Results of this study may not be applicable to some mammalian models.

CHAPTER 2

LITERATURE REVIEW

Evolutionary Perspective on Fat Consumption and Metabolism

The evolution of the large human brain has had many important implications for the energy needs of the species. It has been shown that, compared to other mammals and primates, humans expend a much larger proportion of their total energy expenditure on brain metabolism and function (Navarrete et. al., 2001). Comparative diet analyses reveal this increased energy demand is supported in large part by energy derived from fat (Leonard & Robertson, 1997). Relative to their larger ape cousins, humans still display a dietary preference for fat. Among primates, a clear correlation has been shown between increased diet quality and increased allocation of energy to the central nervous system (Leonard et. al., 2003). Humans observe both a high-quality diet and have evolved to possess large, intelligent brains capable of higher-order reasoning (Kety, 1957).

Our large brain to body ratio—a high degree of encephalization—is reflected in our body composition, particularly during early development. Human infants have more adipose tissue than infants of any primate relative (Holliday, 1986). These stores of energy are used to fuel the growth of a large brain during infancy and early adolescence (Leonard & Robertson, 1992). Under conditions of nutritional inadequacy, infants and toddlers have been shown to preserve body fat for brain metabolism, usually at the expense of linear bodily growth (Leonard & Robertson, 1994). This type of growth stunting has also been shown to lead to decreased fat oxidation and increased fat storage.

These adaptations are indicative of the critical role of fat in meeting the energy demands of the brain during early development (Popovich, et. al., 1997).

Fossil records indicate the emergence of large brains and a distinctly human diet around 2 million years ago (Crawford et. al., 1999). With the emergence of archaic human species, such as *Homo erectus* roughly 1.8 million years ago, we see the concurrent emergence of the first hunting and gathering societies characterized by increased protein and fat consumption, transport of scarce food resources across vast distances, and the sharing of food within social groups (Cordain et. al., 2001). *H. erectus*—our closest extinct relative—had a much larger brain than any primate of that time, one approaching the size of our brains today, suggesting a much higher energy requirement. The increased energy need of *H. erectus* is further evidenced by reduced facial size, teeth better adapted to grinding fat and sinew, and sophisticated technology designed to fall large, fatty prey (Sclafani, 2002). In summary, the consumption of fat was a necessary but insufficient condition to increasing brain size and cognitive performance (le Coutre & Schmitt, 2008).

To better detect and preferentially consume fat, humans developed distinct molecular pathways suited to fat taste (Gaillard et. al., 2008b). Humans show near-ubiquitous preference for foods with higher fat content and greater energy, and this preference is reflected in our genetic code. Important genetic mutations made digestion of increasingly higher amounts of animal flesh possible (Finch & Stanford, 2004). Recent evidence has even shown the remarkable ability of the human brain to perceive and accurately assess the fat and energy content of foods (Toepel, 2009). All things

considered, the ability to perceive, consume, and store fat was a key adaptation that conferred significant reproductive advantages to our early hominid ancestors (Milton, 1987). More research is needed to reveal the exact diet composition of our ancestors and the relative contributions of carbohydrate, protein and fat on human brain development.

Pathophysiology of Dietary Fats Across the Life Cycle

Early *Homo sapiens* observed a predominantly plant-based diet high in alpha-linolenic acid (ALA), arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid; protein sources, usually fish, were low in linoleic acid and saturated fatty acids (Gluckman et. al., 2005). Although the composition of this diet has been in constant flux for the last 10,000 years, the globalization of diets has significantly accelerated these changes since the mid-19th century. It is hypothesized that diseases typically attributed to Western diets (i.e. metabolic syndrome) are caused by this departure from our evolutionary diet. In their critical analysis of adult disease models, Gluckman and colleagues (2005) discuss—among other things—the causal relationship between low levels of ALA, fish oil, and coronary artery disease as well as the correlation between long-chain polyunsaturated fatty acids and depression. It is no surprise, therefore, that interest in returning to diets more akin to the ones observed by our ancestors, and more congruent with our genetic code, has gained much attention in recent years. Such a shift, however, would require a complete reassessment of the typical Western diet and nutritional recommendations (Anderson & Millen, 2017). In fact, Western dietary philosophy has become so pervasive worldwide that any study of natural human homeostasis necessitates the study of remote populations whose food supply has

remained endemic. The use of randomized controlled trials (RCTs) to study the effects of single macronutrients has considerable drawbacks including, but not limited to, large numbers of participants, long observation times, constant dose-response testing, and high rates of interactions with micronutrients that are often difficult to interpret. The careful study of an appropriate dietary recommendation to maximize health outcomes will require perspectives from anthropology, archaeology, biochemistry, nutrigenetics, and metabolomics (Gidding et. al., 2006). Considering the association between early diet composition and later disease risk (Butte et. al., 2004), the establishment of a healthful diet fitting our evolutionary background is of the utmost importance.

In human nutritional research, there has been considerable debate regarding the relative contribution of macronutrients—specifically carbohydrates and fats—to visceral and subcutaneous adiposity. Although prolonged overfeeding of either carbohydrates or fat will most likely result in overweight or obesity, the physiology literature better supports the notion that excess fat consumption is the more likely culprit. Jequier (1993) demonstrated that fat overfeeding increased the rate of fat storage in people consuming a mixed diet. Specifically, this study showed that overconsumption of fat resulted in decreased fat utilization and increased storage, while excess carbohydrate intake resulted in increased carbohydrate utilization. Horton et. al. (1995) overfed isocaloric amounts of fat and carbohydrate to nine lean and seven obese men and found that carbohydrate overfeeding produced proportional increases in carbohydrate oxidation and total energy expenditure (TEE) while fat overfeeding exerted a negligible effect on fat oxidation and TEE. As a result, the researchers estimated that 75-85% of excess energy derived from

carbohydrate is stored as fat, compared to 90-95% of excess energy derived from fat being stored as fat. While overfeeding of either will result in fat storage, excess dietary fat leads to greater fat accumulation than does excess dietary carbohydrate.

Gustatory Mechanisms for Fat Taste

There is a growing body of literature implicating the peripheral gustatory system in fat taste detection. While fat is yet to be recognized as a primary taste, there is mounting evidence that free fatty acids are responsible for primary fat taste sensation. Although early research into fat taste focused primarily on essential fatty acids (Gilbertson, 1998; Dransfield, 2008), recent investigations have looked at taste response to a wider variety of free fatty acids (Degrace-Passilly & Besnard, 2012). To date, however, no studies have used behavioral assays to study fat taste beyond the detection of *cis*-polyunsaturated fatty acids (PUFAs). Clearly, any assertion of fat as a sixth taste modality would require a thorough molecular and behavioral investigation of all types of fatty acid ligands for which a gustatory receptor has been identified for (Besnard et. al., 2015).

Our knowledge of fat taste transduction is, at best, misinformed and, at worst, woefully incomplete. Although some studies seem to suggest delayed rectifying K+ (DRK) channels as being involved in the transmission of chemosensory fat cues, there is a widespread consensus among researchers that detection of triacylglycerol requires detection of its constituent parts (i.e. glycerol and free fatty acids) and that studies focusing on fat taste would do better to consider fatty acid (FA)-activated G-protein coupled receptors (GPCRs) and other FA-binding proteins such as CD36. Studies of

certain FA receptors remain unfeasible due to lack of a viable knockout model. As such, a global understanding of the fat taste signaling cascade is impeded by lack of available technology.

Conversely, results linking receptors for which a knockout model has been established—such as CD36—are indisputable. Studies involving knockout mice have repeatedly demonstrated the involvement of CD36 in fatty acid binding and fat taste signaling (Gaillard et. al., 2008a; Laugerette et. al., 2005; Sclafani et. al., 2007a; Sclafani et. al., 2007b; El-Yassimi, 2008). How this receptor-ligand interaction affects global feeding behavior, however, is currently unknown. Studying CD36 at the molecular level has proven especially difficult as it can be expressed as a transmembrane FA transporter, or as a cytosolic chaperon protein. Experiments involving CD36 knockout models are not conducive to the study of CD36 receptor subtypes as current gene knockout technology does not allow for selective suppression of CD36.

The recent discovery of FA-activated GPCRs and identification of their respective ligands holds significant potential for elucidating the full signaling cascade of fat taste. Although little information on these GPCRs is readily available (Pittman et. al., 2008), it is believed they are involved in fat taste acuity and FA chain length differentiation.

In the last 20 years, food science has transitioned from the widespread belief that fat is tasteless to active study of the cellular and behavioral responses to fat. During this transition, a number of purported fatty acid receptor proteins have emerged in the literature. Of these, CD36 is the most upstream and has therefore garnered the most attention in recent years. The evidence, however, suggests CD36 may not be wholly

responsible for fat taste detection (Spector & Glendinning, 2009), accounting for only one-third of the observed variance in fat taste response. Instead, fat taste perception is thought to be a product of many receptors and associated ion channels (Running et. al., 2015), casting doubt on whether a unifying model of fat taste exists. Biomechanically, CD36 is thought to work in two ways. First, it may be that CD36—when embedded in the cellular membrane—acts as a receptor for fatty acids, either free or by the action of lingual lipase (Gilbertson et. al., 1997). It is equally plausible, however, that cytosolic CD36 acts merely to orient free fatty acids for detection by embedded GPCRS such as GPR120 (Gilbertson et. al., 1997). There is some doubt regarding the latter signaling mechanism as GPR120 and other GPCRs have been shown to account for less variance than CD36 models (Ichimura et. al., 2012; Cartoni et. al., 2010). Immunostaining methods of GPCRs in general and GPR120 in particular is made even more difficult by its high degree of co-localization with other fat-taste implicated proteins such as phospholipase-Cβ2 and α-gustducin (Matsumara et. al., 2009). As with other taste stimuli (i.e. salty, sweet, sour, bitter, and umami), the release of calcium (Ca^{2+}) is essential for neuronal depolarization and signal transduction (Roper, 2007). As such, interest has grown in studying cation channels as a means of understanding fat taste (Abdoul-Azize et. al., 2014). Were it not for the actions of associated cation channels such as transient receptor potential cation channel subfamily M member 5 (TRPM5) (Liu et. al., 2011), cell depolarization and consequently fat taste transmission would be impossible even in the presence of active CD36 and free fatty acids. Without the actions of these cation channels, there would be no cellular output or signal to the afferent nerve. It is for this

reason that fatty acid receptors and cation channels cannot be studied in the same knockout model.

The current model of fat taste perception (i.e. primary gustation) is highly tentative. What little we know about the interaction between GPR120, CD36, and DRK channel signaling remains speculative. The evidence does suggest, rather unequivocally, that these receptors do bind dietary fat and do elicit behavioral responses. A more complete understanding of fat taste detection and preference holds potential for the development of new fat substitutes and drug targets for the treatment of obesity.

Primary Gustation of Fat Taste

Ability to detect fat allows animals, including humans, to preferentially consume high-fat foods (Drewnowski, 1997). As mammals are not able to synthesize double bonds beyond carbons 9 and 10, linoleic acid (18:2 cis-9,12) and *alpha*-linolenic acid (18:3n-3) are essential in the diet (Laugerette et. al., 2007). Therefore, the ability to discriminate between foods of varying fat content not only makes the consumption of high-energy foods possible, but also helps identify good sources of essential fatty acids. CD36 is expressed on the circumvallate papillae of mammalian tongues which, in humans, is innervated by cranial nerve IX, the glossopharyngeal nerve; fat taste signals are then interpreted by the primary gustatory cortex (Herness & Gilbertson, 1999). Research with rat models has also shown ability of peripheral fat taste detection through textural and olfactory cues as well (Smith et. al., 2000). Studies examining fat taste through conditioned taste aversions (CTA) have had little success as no one receptor is thought to

be responsible for global fat detection (Warwick et. al., 1990). To date, no studies have examined the role of CD36 using a CTA study.

Taste receptors of the tongue, mechanical receptors of the mouth, and olfactory receptors of the nose and throat are responsible for the detection of fat taste, texture, and smell (Drewnowski & Greenwood, 1983). The development of multiple sensory modalities for fat is indicative of the evolutionary and nutritional importance of consuming high-energy foods and essential fatty acids. Some researchers have found evidence for post-ingestive detection of fat via flavor nutrient learning and activation of the dopamine reward system (Warwick & Synowski, 1999). In fact, investigations of fat taste detection via fatty acid receptors is a relatively recent trend in fat taste research (Plesnik et. al., 2018). In Black 6 mice, Smith and colleagues (2000) demonstrated a distinct cephalic response to fatty acid detection: increased pancreatic secretions in anticipation of lipid digestion. In the last decade, primary gustatory detection of free fatty acids in humans has been the center of much research (Chale-Rush et. al., 2007a; Chale-Rush et. al., 2007b). In humans, fat taste detection has been shown to mediate post-prandial triglyceride levels (Mattes, 2001b). Fat substitutes, by contrast, do not produce the same elevation in TAG levels (Mattes, 2001a). The significance of such discoveries to human obesity research has urged others to study the characterization of the taste of dietary fat in animal models (Mattes, 2005). A more complete understanding of the neural encoding of fat taste would allow for the development of fat substitutes designed to simultaneously retain the palatability of high fat foods while reducing the energy density of dietary fat that contributes to the human obesity epidemic (Mattes, 2007).

Secondary Detection of Fat Taste

The study of peripheral fat taste processing is still very much in its infancy and, as of now, very little is known. In addition to taste, fat also possesses a distinct smell and texture; elegant data by Verhagen and colleagues (2003) showed that neurons in the primate orbitofrontal cortex respond to fat texture independently of viscosity. In rats, mechanoreceptors in the mouth detect fat texture after the degradation of fat into free fatty acids by lipase enzymes (Kawai & Fushiki, 2003). Kinney and Antill (1996) showed the degradation of triglyceride to release odorants critical to the olfaction of fat in mice. Sense of smell is so important to oleogustus that anosmic rats are incapable of differentiating between foods of vastly disparate fat content (Fukuwatari et. al., 2003). Unlike bitter compounds (Bartoshuk et.al., 1995), there is no easy method for typifying ability to perceive fat, complicating the construct of a universal dose-response curve in humans (Perna et. al., 2018). One interesting difference that has been noted is the heightened sensitivity of female mammals to fat content (Mela, 1988). Perhaps for purposes of reproduction and fetal security, mammalian females seem to be almost 20% more sensitive to free fatty acids than males.

As fat preference is influential in shaping food choice, fat perception has far reaching implications for the pathogenesis of obesity (Treesukosol & Moran, 2018). Given the high cost of obesity-related chronic health outcomes many Western nations face, fat taste research has enjoyed surprisingly little attention—particularly when considering the important role high-fat diets play in the initiation and propagation of obesity (Dixon, 2010). Given the evidence at hand, three things are clear: 1) the etiology

of obesity is multidimensional and thus requires a dynamic, adaptive approach, 2) one important facet of obesity is fat preference and consumption, and 3) a thorough investigation of fat taste demands receptor-specific study design, rather than global detection of free fatty acids (McCormack et. al., 2006).

Orosensory Mechanisms for Fat Detection

Animal studies involving fat taste predominantly use emulsified corn oil mixtures of varying fat content or high-fat, cafeteria-style diets using human foods (Smith et. al., 2000). Unfortunately, results from studies using fat emulsions do not generalize well to humans and results from studies utilizing human food are poorly suited to rodent consumption and often suffer from a lack of reproducibility (Smith, 2004). To date, no study has used accurately measured rodent food with known macronutrient content in a gene-specific knockout model to identify tasting phenotype using a CTA paradigm.

That being said, studies involving oil mixtures have elucidated the role of fat and sweet taste interactions and have uncovered the effects of post-ingestive feedback. Orosensory detection of fat is defined as perception of all known constituents of the construct of "flavor"—olfactory cues, texture identification, and primary gustation. Early appetite studies identified the orosensory properties of fat in animal trials; both mice and rats showed preferential consumption of high-fat emulsions even before tasting available samples (Smith, 2001), indicating the important role of olfaction in fat preference. These effects were paralleled almost a decade earlier by Rhinehart-Doty and colleagues (1994) when studying sucrose; rats in this study used non-taste cues to identify energetically dense solutions with greater amounts of sucrose. Rodents are also able to detect fat using

textural cues. Mindell, Smith and Greenberg (1990) showed that rats initially confused corn oil mixtures with essential oil mixtures, likely due to the similarity in texture, and only through repeated trials were the rats able to discern between the two—presumably through differences in taste, smell, and post-ingestive effects. In an attempt to replicate the concentrations of free fatty acids experienced during feeding and to contextualize future results parsimoniously with available data, Kawai and Fushiki (2003) showed that lingual lipase released from the rodent Von Ebner's gland was necessary for lipolysis and fatty acid taste. In fact, these researchers noted a 34% reduction in fat detection ability in rats following resection of Von Ebner's gland. Interestingly, this same reduction was not seen in detection of essential fatty acids, suggestive of specialized compensatory mechanisms designed to ensure adequate intake of essential nutrients—independent of energy content. Indeed, fat taste is a product of multiple sensory modalities, again supporting the evolutionary importance of fat consumption.

Takeda and colleagues (2000) showed the inability of mice to regulate caloric intake from corn oil whereas, with high-energy solutions such as 20% sucrose, mice limited energy intake to levels comparable to baseline feeding. With prolonged exposure, mice given high-fat corn oil routinely overfeed and gain substantial weight (~25%), whereas mice given sucrose asymptotically limit energy intake and experience modest weight gain (~10%). These results suggest that not only does fat consumption lead to weight gain, but that fat is more palatable than sweet taste and is therefore preferentially overconsumed even in the presence of sugars.

When using a CTA design to study fat detection, it is highly recommended that extinction time be made a dependent variable (Spector et. al., 1981). The duration an aversion lasts can be taken as an index of the importance and sensitivity of the sensory pathway under investigation. Generalization of taste aversion to other stimuli signify similarity between those stimuli. In the 1981 study by Spector and colleagues, conditioned aversions to sucrose solutions were quite fragile and aversive responses were extinguished in a matter of days, whereas aversions to corn oil persisted for almost three weeks. In fact, aversions to corn oil were so pervasive that responses were generalized to texturally-similar mineral oils. In this study, the extinction times between corn and mineral oil was cleverly used as a proxy variable for taste acuity.

It is worth mentioning the serious limitations of studies using fat solutions as a testing vehicle. In these studies, lipids—which are inherently hydrophobic—are usually dissolved in ethanol prior to being administered to animal subjects. Unfortunately, mounting evidence has shown ethanol to be a serious confounder of results (Smith, 2010). When comparing a 0.43 mM concentration of linoleic acid in ethanol to an equivalent concentration of ethanol in water after completion of a CTA protocol, near-identical aversive responses were recorded. These results denote a significant effect of generalization to the vehicle (ethanol). Simply put, there is too much transference from fat to the solvent to reliably indicate fat taste ability. Therefore, behavioral studies hinging on ingestive testing results would do well to consider the effect of their medium. Furthermore, this necessitates the application of macronutrient-specific lab diets.

Future research into orosensory mechanisms of fat detection must offer both proximate and distal explanations of fat feeding behavior. To this end, studies must resolve to understand the microstructure of feeding behavior (i.e. rates of licking or feeding behavior/day-night patterns). In rats, the microstructure of sucrose intake has been well-typified and discussed (Smith, 2000). On the other hand, virtually nothing is known about the microstructure of fat feeding, other than some limited knowledge about ordering effects (Smith et. al., 1992). Understanding how an animal preferentially feeds is just as, if not more, important than understanding how much it consumes. A better understanding of the microstructure of fat feeding behavior would not only elucidate mechanisms of fat intake regulation (Davis et. al., 1995), but also offer translationally relevant knowledge of human fat-induced obesity (Beheshti et. al., 2018).

Conditioned Taste Aversions

The current experiment utilizes a combination of both classical and operant conditioning to produce conditioned taste aversions in the cohort of animal subjects. Together they form the process of associative learning and it is only through both mechanisms whereby sensitivity and specificity to fat taste is observed and quantified. Therefore, a sufficient discussion regarding both theories is demanded by experimental design and protocol. To better understand the conceptual underpinnings of the methods described in this thesis, the basics of classical conditioning—including both acquisition and extinction—in addition to behaviorism must be discussed.

Initially described in detail by Ivan Pavlov in the early 20th century, classical conditioning involves the repeated pairing of a biologically relevant stimulus such as

food (known as the unconditioned stimulus, or US) with a neutral stimulus such as the sound of a bell (known as the conditioned stimulus, or CS). Originally studying the digestive secretions of dogs, Pavlov noticed that dogs who normally only salivated when given food (known as the unconditioned response, or UR), began to salivate in the presence of the technician who usually administered the food (known as the conditioned response, or CR). Designing simple studies with food and the sound of a metronome, Pavlov demonstrated that dogs who heard the sound of a metronome before being given food would—after successive pairings—salivate at the sound of the metronome, rather than the presentation of the food (Mazur, 2016). He also noticed that some animals developed this association quicker than others and named the process of developing this CS-US association as 'acquisition'. Furthermore, he observed inhibition of the conditioned response when the CS was delivered without the US and termed this phenomenon 'extinction'.

During acquisition, a biologically preferred stimulus is paired with an unconditioned stimulus, usually the most chronologically continuous and perceptually salient neutral aspect of the environment. In research design, the period in the learning process when an individual is learning a new behavior is known as the acquisition phase.

Conversely, extinction primarily involves the disappearance of a learned response. Continuing the previous example, if the sound of the metronome is not followed by delivery of the food, the dog will no longer associate the former with the latter and will no longer salivate in response. Extinction time (i.e. number of trials

required to observe extinction in a sample of previously conditioned subjects) is taken as a measure of the strength of a CS-US association.

Expanding on the principles of classical conditioning, operant conditioning is focused primarily on operant responses. The fundamental principles of operant conditioning were outlined by Thorndike (1898) six years before Ivan Pavlov was awarded by the Nobel Prize for Physiology or Medicine in 1904: "Of several responses made to the same situation, those which are accompanied or closely followed by satisfaction to the animal will, other things being equal, be more firmly connected with the situation, so that, when it recurs, they will be more likely to recur; those which are accompanied or closely followed by discomfort to the animal will, other things being equal, have their connections with that situation weakened, so that, when it recurs, they will be less likely to occur. The greater the satisfaction or discomfort, the greater the strengthening or weakening of the bond." Here we see the earliest reference to the interrelationship between behavior, consequence, and the psychological associations made by the organism experiencing the consequence.

In contrast to Thorndike, subsequent work conducted by B.F. Skinner (1974) strived to make no speculation as to the internal psychological goings-on of the organism and, instead, strived to only measure the frequency of observed behaviors as they related to the stimulus applied. Very briefly, Skinner observed some stimuli to increase the frequency of certain behaviors while others decreased the probability of occurrence. He termed these stimuli reinforcement and punishment, respectively. Armed with these fundamental tools of operant theory, Skinner demonstrated the versatility of shaping

behaviors by successive approximations—whether that behavior be lever pressing in a rat or promoting desirable behaviors in a classroom of children.

The aforementioned principles of associative learning are the driving force behind the development of conditioned taste aversions (CTA). In species capable of taste, a CTA is developed when an animal experiences nausea or vomiting after ingesting a particular substance (Mazur, 2016). The ability to associate taste and sickness in order to modify future consumption is thought to be an evolutionary adaptation and protective of overall health. Although the simple (discreet) occurrence of this phenomena can be correctly categorized as classical conditioning, it is considered operant conditioning when the delivery of the aversive stimuli is contingent on an operant response (as is the case with the present thesis experiment).

Fat as a Primary Taste

As of now, only five primary tastes are widely accepted by the scientific community. Criteria for determining primary taste modalities is well-defined. For a taste to be considered a primary, it must 1) possess a unique transduction mechanism, 2) bind directly to a specialized set of receptors, 3) activate taste-specific regions of the cortex, 4) be stimulated independently of other tastes, and 5) produce a signal capable of supporting associative learning (Chandrashekar et. al., 2006; Liu et. al., 2016). For fat to be a basic taste, it must meet all five of the aforementioned criteria.

A number of fat taste transduction mechanisms have been identified (Mattes, 2010) such as DRK, CD36, GPR40, GPR41, GPR43, GPR120, and FATP. Each is specialized to detect fatty acids of varying chain length (C6-C22) and saturation.

Unfortunately, receptor-level studies have been thus far confined to electrophysiological recordings, controlled sensory exposures, and gene knockout-models. No studies have investigated associative learning in CD36 KO subjects. However, to answer this question definitively in humans, future studies must show targeted stimulation via receptor-specific agonists.

To be considered a primary taste, fat must be shown to not be itself a composite of other primary tastes. Some results from rat studies suggest fat might be only a dimension of umami (Gilbertson et. al., 2005), since fat has traditionally accompanied protein (Pittman et. al., 2006). These findings, however, were not corroborated by neither subsequent work done by this group (McCormack et. al., 2006), nor results from human studies (Mattes, 2007) which both report fat taste as being perceived distinctly by subjects. Although fat taste is a primary driver of fat preference and consumption, no dose-response relationship to commonly consumed fatty acids has been established in humans. Challenges faced by fat taste researchers are chiefly related to the multimodal sensory information conveyed by fat. Unlike the other established primary tastes, fat detection is achieved through integration of taste, texture, and olfactory cues. Therefore, the study of fat taste is impeded by our inability to isolate the tastant component of fat. Evidence from human and animal studies suggest gustation to be the primary means of fat detection, whereas somatosensory and olfactory detection of fat is thought to modulate intensity.

Fat taste information is transduced alongside the same neurological pathway as the established five primary tastes (Hamilton & Norgen, 1984). Ablation of the

glossopharyngeal nerve significantly diminishes animal response to oral exposure of free fatty acids. Although implicated in the detection of salty and sour tastes, there is no evidence of vagus nerve involvement in fat taste. However, evidence linking exposure to free fatty acids and activation of the gustatory cortex is limited to rodent and primate models—not including humans. Nonetheless, the results are to date very assertive of neuronal involvement in fat detection.

Some evidence has shown fat taste detection to be advantageous for the detection of spoiled food. It has been reported that short-chain fatty acids, volatile compounds with two to six carbons, are indicative of spoiled meat and are accordingly perceived by humans as sour (Forss, 1973). Unfortunately, no systematic study of this evolutionary claim has been conducted since Forss' early discourse. As this is a burgeoning field of research, one difficulty faced by researchers is the development of a universally accepted jargon for the description of fat taste. What is clear from the data, however, is that fat taste does produce a neurological signal capable of supporting conditioning and stimulus-specific learning (Smith et. al., 2000). McCormick and colleagues (2006) reported stimulus generalization across fatty acid chain lengths with chemical structures within five carbons of each other, suggestive of modulatory pathways designed to differentiate between fatty acids. Such a careful examination of fatty acid differentiation has not been conducted in a human sample. There is still considerable work to be done in order to characterize fat taste as having a distinct quality in humans.

Chemical signals can also modulate post-ingestive responses and affect homeostasis (Mattes, 1997). These post-ingestive responses are usually specific to the

nature of the substance being consumed and are often reflective of digestion and substrate utilization (Zafra et. al., 2006). For instance, stimulation of sweet taste sensations increases carbohydrate absorption from the lumen (Giduck et. al., 1987) and improved post-prandial glycemic control (Teff, 2000). In humans, ingestion of a hypertonic saline solution causes almost instantaneous antidiuresis (Akaishi et. al., 1991) and the perception of umami modulates insulin secretion and global endocrine functioning (Nijima et. al., 1990). There is also substantial evidence regarding the post-ingestive nutritional consequences of fat taste. In rats, oral stimulation by fat enhances intragastric digestive secretions (Ramirez, 1985). In humans, oral fat exposure has been shown to alter postprandial lipid metabolism by increasing fat oxidation (Mattes, 1996), elevating postprandial triacylglycerol concentrations (Mattes, 2001a), and triggering the release of stored lipids (Mattes, 2002). Oral fat sensory exposure has also been shown to cause a hormonal shift in subjects (Crystal and Teff, 2006), leading to elevated chylomicron-TAG content (Mattes, 2001b) and delayed glucose metabolism (Parks, 2008).

Taste research is still an underdeveloped niche, and it is general consensus that more primary taste modalities are yet to be discovered (Chaudhari & Roper, 2010). Initially, four taste modalities were recognized as fitting the criteria of a primary taste: bitter, salty, sour, and sweet. Each of these primary tastes are thought to fulfill a unique evolutionary need; bitter is thought to convey the presence of poisonous compounds, salt is perceived in order to promote sodium consumption for the maintenance of a hypertonic extracellular environment, sour is thought to indicate unripe or spoiled foods, and the ubiquitous palatability of sweet taste denotes energetically dense food sources

(Drewnowski & Specter, 2004). Umami was later recognized as a primary taste modality and is thought to signal the presence of protein in foods. It is thought that fat taste perception developed in order to better detect this essential macronutrient in potential food sources. Considering the designated criteria for inclusion as a primary taste outlined in the beginning of this review section, there is strong evidence suggesting fat should be acknowledged as the sixth taste modality.

CHAPTER 3

METHODS

All experimental procedures were conducted with approval of the Institutional Animal Care and Use Committee (IACUC) at Arizona State University (Appendix B). A modified version of Kasper and colleagues 2014 protocol for evaluating fat preference was adapted for genetic and pharmacological intervention and used in the first experimental phase, hereby referred to as the 'fat preference model'.

In the second experimental phase, a CTA paradigm is used to test fat taste perception. During this time, animals were placed on a continuous punishment schedule, whereby an aversive stimulus was delivered after every occurrence of a specific response. In the present context, the aversive stimulus was an injection of lithium chloride that was delivered to any animal which consumed more than 50% from the high-fat food offering in 24 hours—the specific response. The rationale is that animals capable of tasting fat would preferentially consume more of it and, after experiencing the aversive stimuli, would learn to associate the taste of fat with the sickness-inducing effects of lithium chloride. Therefore, it can be inferred that animals that did not inhibit their consumption of high fat food during the aversive conditioning procedure were incapable of tasting fat.

Materials

Experimental foods of varying fat content were purchased from ClearH2O® (Westbrook, ME, USA). A gel diet was chosen since available dry diets reported fat content only as a minimum percentage guaranteed—thereby unable to offer a definite fat content for research purposes—in addition to the similar appearance of gel diets when

modified to widely varying fat contents (i.e. 10% vs. 45%). Foods of varying fat content were prepared by mixing gel diet amounts proportionally with vegetable shortening to derive various percentages. Vegetable shortening was used due to its low cost, precedence in feeding and appetite research, and high content of palmitic acid—a saturated, 16-carbon fatty acid uniquely suited to detection by CD36. These modified gel foods of varying fat content were the sole source of nutrition for all animal subjects during all experimental phases. All food hoppers were replaced with crock dishes to prevent spillage of gel foods. Non-standard bedding and animal enrichment were requested and implemented to minimize the addition of the environment to food weight and vice versa. A 13.3 M aqueous lithium chloride solution and 5 M sodium chloride solution, in addition to 0.5 cc insulin syringes, were all purchased from ThermoFisher Scientific (Waltham, MA, USA). The lithium chloride solution was diluted to 7 M concentration. Animals had unrestricted access to water for the full duration of all experiments.

Subjects

All animal models (*Mus Musculus*) were purchased from the Jackson Laboratory, 8 male subjects in total (Appendix D). Of the 8 animal subjects, 4 were CD36 KO mice assigned to the experimental group while 4 were wildtype B6 control mice. Both CD36 KO (JAX stock #019006) and B6 WT (JAX stock #000664) were bred and engineered at the Jackson Laboratory (Febbraio et. al., 1999). Both groups were age-matched and arrived at 7 weeks old. Each subject was single housed at 21 ^{o}C and 30-50% humidity with a 12-hour light-dark cycle (lights on at 6:00 a.m. and off at 6:00 p.m.).

Upon approval of an amended protocol (Appendix C), the animals were habituated to the singly housed holding room for 10 days prior to the start of experimentation. All animals were maintained on standard NIH 31 dry lab diet throughout these first 10 days. The subjects were handled and weighed at exactly 6:00 p.m. for these initial 10 days. The first day after the habituation period was Training Day 1.

Members of both the experimental and control groups were born on 05/07/2017 and arrived together on 06/13/2017. Habituation began on 07/01/2017 for 10 days. Training began on 07/11/2017 and continued for 10 days. Testing began on 07/21/2017 and data collection ended at 6:00 a.m. on 07/31/2017. The mice were euthanized on 08/14/2017.

For the duration of data collection, mice had physical access to both food dishes simultaneously (Figure 1) in order to demonstrate true preference for foods with differing fat content. Leftover food was weighed daily during training and testing phases and any spillage was easily found and accounted for (Figure 2). Spillage of food and/or removal by animal was deducted from the total and found to be minimal and did not significantly influence the data.

Figure 1. A mouse in its homecage with two food choices. The mouse had physical access to the two crock dishes and to water. The food dishes were filled with 5 g of food each to ensure *ad libitum* access to both foods.

Figure 2. Food spillage was highly visible and easily accounted for. While weighing leftover food daily, any food that was not contained in the dish was found and weighed.

Adaptation

The purpose of this phase is to determine if any positional feeding bias exists among members of the cohort. This determination allows for statistical correction of any directional predisposition. This phase also serves to habituate the animals to the dosing vehicle in order to prevent misattribution (or generalization) of future feelings of sickness to the injection procedure itself.

At 6:00 a.m. on Training Day 1, two clean, ceramic crock dishes were filled with 5 g of 10% fat food for each cage. The food was weighed carefully, ensuring the measurement was within + or – 0.05 g. The food-filled crock dishes were placed in the cages, ensuring each animal had access to both foods. Afterward, animals were habituated to the dosing vehicle, sodium chloride (NaCl; 5 M aq. soln.). At 6:00 p.m., the animals were weighed, and their weights recorded.

On Training Day 2, all leftover food was removed from the crock dishes—taking care to examine the home cage for food removed from the dishes by the animal—and the leftovers weighed. The leftover weight was recorded, leftover food was thrown out, and the dishes were refilled with 5 g of 10% fat food, and the positions of dishes A and B were switched to mitigate position feeding bias. Food intake (y) was measured as $y = 5 - x$, where (x) equals the amount of food left over in grams. The animals were habituated once more to the dosing vehicle (sodium chloride) at 6:00 a.m. At exactly 6:00 p.m., all animals were weighed for the day. Animal weight and food intake were measured and recorded daily. It should be noted here that animals were not water or food deprived during any phase of the study.

At 6:00 a.m. on Training Day 3, the leftover food weights were recorded as before. The amount of food consumed in each crock dish for each animal between days was examined. Animals should consume roughly 40-60% from each dish; if an animal fed more than 60% from a specific dish, this was indicative of a position bias or specific dish preference. No animal exhibited such a position bias. Any remaining food was thrown out and the animals were habituated again to the dosing vehicle (sodium chloride). At 6:00 p.m., the animals were weighed, and their weights recorded.

Training

In this phase, animal groups were sequentially tested with foods of varying fat content (12.5%, 15%, 17.5%, 20%, and 45%) to determine the role of CD36 in fat taste perception. Percentages were chosen to be in keeping with previous literature and structured in a way to provide empirical data on both fat taste sensitivity and specificity. Habituation of animals to the dosing vehicle continued during this phase as well.

At 6:00 a.m. on Training Day 3, two clean food dishes—again, one marked A and another B—were filled with 5 g of 12.5% and 15% fat food, respectively (Table 1). Care was taken to use clean crock dishes in order to prevent confounding scents from previous foods. At this time, the animals were habituated to the dosing vehicle using sodium chloride. At 6:00 p.m., animal weights were measured and recorded.

At 6:00 a.m. on Training Day 4, the leftover weights of each dish were recorded and refilled with 5 g of food as necessary (see Table 1). Their positions were switched and placed back in the cage. Also, at 6:00 a.m., the animals were habituated to the dosing vehicle, sodium chloride. At 6:00 p.m., animal weights were recorded.

At 6:00 a.m. on Training Day 5, the leftover weights of each dish were recorded. After determining the grams of each % fat food consumed each day, a preference score was calculated (see Data Analysis below). The dishes were refilled with 5 g of both types of food as necessary, their positions switched, and placed back in the cage. Also, the animals were once more habituated to the dosing vehicle—sodium chloride—at this time. At 6:00 p.m., animal weights were taken and recorded.

The aforementioned steps were repeated for Training Days 6-10 multiple times with the same cohort of animal subjects, comparing 12.5% fat food to foods with increasing fat content: first 15%, next 17.5%, then 20%, and lastly 45% fat. On each day, consumption and weight data were collected for each animal.

Table 1. Preference Acquisition Schedule (Training Phase)

Training Day	Injection[1]	% Fat Food		kcal/gram	
		Left	Right	Left	Right
1	NaCl	10%	10%	3.85	3.85
2	NaCl	10%	10%	3.85	3.85
3	NaCl	12.5%	15%	3.9	3.95
4	NaCl	15%	12.5%	3.95	3.9
5	NaCl	12.5%	17.5%	3.9	4.01
6	NaCl	17.5%	12.5%	4.01	3.9
7	NaCl	12.5%	20%	3.9	4.06
8	NaCl	20%	12.5%	4.06	3.9
9	NaCl	12.5%	45%	3.9	4.73
10	NaCl	45%	12.5%	4.73	3.9

[1]Volume of NaCl was equivalent to 2% of the animal's body mass.

Testing

During this phase of the experiment, animals that preferentially fed (>50%) from the higher fat food option in a given 24-hour period received an injection of lithium chloride to induce sickness. For 10 days, animals were given a choice of 10% and 45%

fat food and group differences in fat consumption, energy intake, body weight, and subsequent injection amounts were recorded. Here, evidence of decreased fat consumption, energy intake, weight, and injection type signify a learned avoidance response and can be taken as evidence of CD36's role in fat taste transmission and acuity.

All experimental procedures were conducted at 6:00 a.m. On Testing Day 1, leftover food from the day before was weighed out and consumption calculated. Any mouse that consumed more of the 45% fat lab chow in the past 24 hours was injected with lithium chloride (LiCl; 7 M aq. soln.) to induce sickness (aversive conditioning to the high fat diet), in an amount equaling 2% of that animal's body weight (i.e., body mass to mass of LiCl) (Yamamoto et. al., 1995). Animals that consumed more of the 10% fat food received an intraperitoneal (IP) injection of sodium chloride (NaCl). Punishment (i.e. IP injection of LiCl) was contingent on fat-feeding behavior. Two clean food dishes marked A and B were filled with 5 g of 10% and 45% fat food, respectively (see Table 2 for full testing schedule). Clean crock dishes were used in order to prevent confounding scents from previous foods. At 6:00 p.m., animals were weighed, and their weights recorded.

On Testing Day 2, the leftover weights of each dish were recorded and refilled with 5 g of food as necessary. Their positions were switched, and they were placed back in the cage. Animals that consumed more of the higher fat food (45%) were injected with LiCl and those that consumed more of the lower fat food (10%) were injected with NaCl. At 6:00 p.m., animals were weighed, and those weights recorded.

On Testing Day 3, the leftover weights of each dish were recorded. After determining the grams of each % fat food consumed each day, a preference score was calculated (see Data Analysis below). The dishes were refilled with 5 g of food as necessary, their positions switched, and placed back in the cage. The higher fat consuming animals were dosed with LiCl and the lower fat consuming animals with NaCl. At 6:00 p.m., all animal weights were recorded.

The aforementioned steps were repeated for Testing Days 4-10 multiple times with the same cohort of animal subjects, comparing 10% fat food to 45% fat food. On each day, consumption, injection, and weight data was collected for each animal.

Table 2. Perception Testing Schedule

Testing Day	Injection[2]	% Fat Food		kcal/gram	
		Left	Right	Left	Right
1	NaCl or LiCl	10%	45%	3.85	4.73
2	NaCl or LiCl	45%	10%	4.73	3.85
3	NaCl or LiCl	10%	45%	3.85	4.73
4	NaCl or LiCl	45%	10%	4.73	3.85
5	NaCl or LiCl	10%	45%	3.85	4.73
6	NaCl or LiCl	45%	10%	4.73	3.85
7	NaCl or LiCl	10%	45%	3.85	4.73
8	NaCl or LiCl	45%	10%	4.73	3.85
9	NaCl or LiCl	10%	45%	3.85	4.73
10	NaCl or LiCl	45%	10%	4.73	3.85

[2] Mass of LiCl was equivalent to 2% of the animal's body mass, not exceeding a volume of 0.5 mL. Injection type was contingent on animal consumption of high/low fat food.

Data Analysis

For each animal, daily food intake was calculated by subtracting the final mass of the food from the starting mass. Each food was measured over 2 days, and the 2-day total was added together for each % fat content.

Preferences scores for each diet (12.5%, 15%, 17.5%, 20%, and 45%) were averaged and graphed (see Figures 3 and 4). Total fat and energy intake was also plotted as grams and kcals (see Figures 5-8).

Average animal weight was also a reliable indicator of fat consumption and, as such, was averaged and graphed for both groups of animals (see Figures 9, 10, and 12). Frequency of lithium chloride injections was recorded—along with total volume in mL—averaged, and graphed (see Figure 11).

As more than one group of animals were used, a repeated measures ANOVA followed by a Bonferroni post-test was performed to correct for multiple comparisons. Linear regression was also used to differentiate intervention effects from testing effects (i.e. the proportion of variance accounted for by group versus practice). The Statistical Package for Social Sciences (SPSS) software (SPSS Incorporated, Chicago, IL, USA) version 24 was used to complete ANOVA testing of the data. RStudio software (Boston, MA) version 3.4.0 was used to conduct linear regression analyses. Power analysis indicated that n=8 animals per group were sufficient for detecting a 70% difference between treatment groups (see Appendix A).

CHAPTER 4

RESULTS

Descriptive Statistics

For every percentage of fat food offered during training, the Black 6 wildtype (B6 WT) mice preferentially consumed more of the higher fat food, while the CD36 Knockout (CD36 KO) mice only consumed more of the 20% fat food (Figure 3). During testing, this trend was reversed; consumption of the higher fat option was tempered in the B6 WT mice, while the CD36 KO mice—rather paradoxically—displayed an even greater preference for the 45% fat option than observed during training (Figure 4).

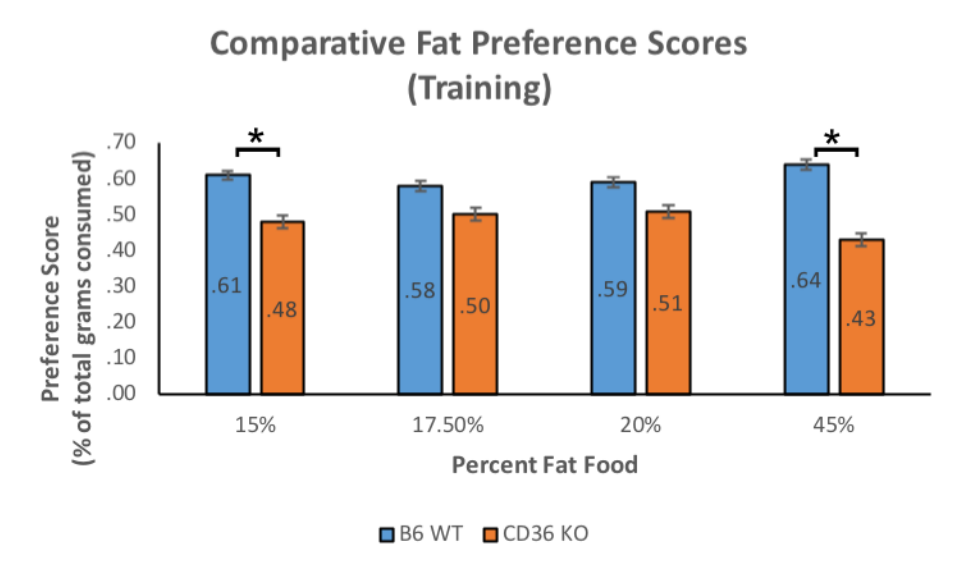

Figure 3. B6 WT mice demonstrated an increasing preference for foods with higher fat content, while CD36 KO mice displayed near-random feeding behavior. Error bars represent the standard error of the mean. Asterisks denote significant between-group differences.

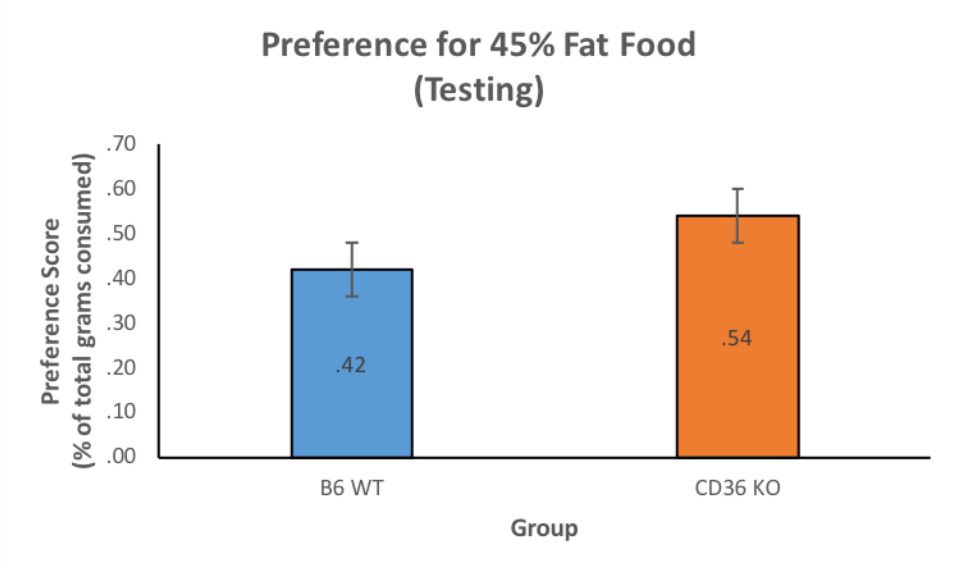

Figure 4. B6 WT mice demonstrated a clear reduction in fat preference in response to the aversive conditioning protocol. CD36 KO mice, on the other hand, displayed an increased preference for the higher fat option than observed during training. Error bars represent the standard error of the mean.

Similar results can be obtained by calculating the average fat consumption in grams for both groups during training and testing. As shown in Figure 5, very little difference between groups is observed during training. Both groups display little to no preference for foods ranging from 10%-20% fat. A between-group difference was only observed when mice were offered 12.5% and 45% fat food. For 45% fat food, the B6 WT mice consumed almost twice as much as their knockout counterparts. The opposite trend was observed during testing (Figure 6). B6 WT mice demonstrated an increasing aversion to the higher fat option, while the CD36 KO mice consumed twice as much of the 45% fat food than observed during training.

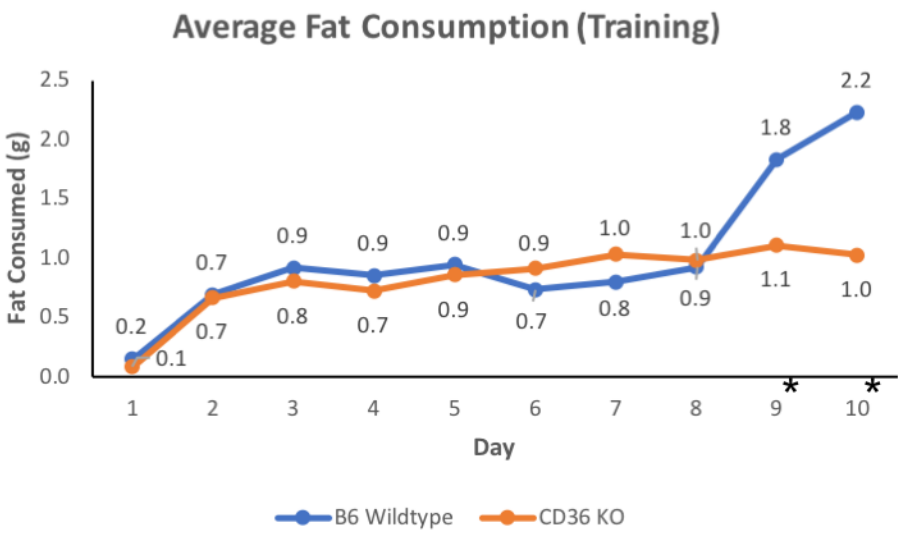

Figure 5. Average fat consumption in grams per group during training. A clear difference in means is observed during Days 9 & 10, when the animals were exposed to the 45% fat food. Asterisks denote days on which a significant between-group difference was observed.

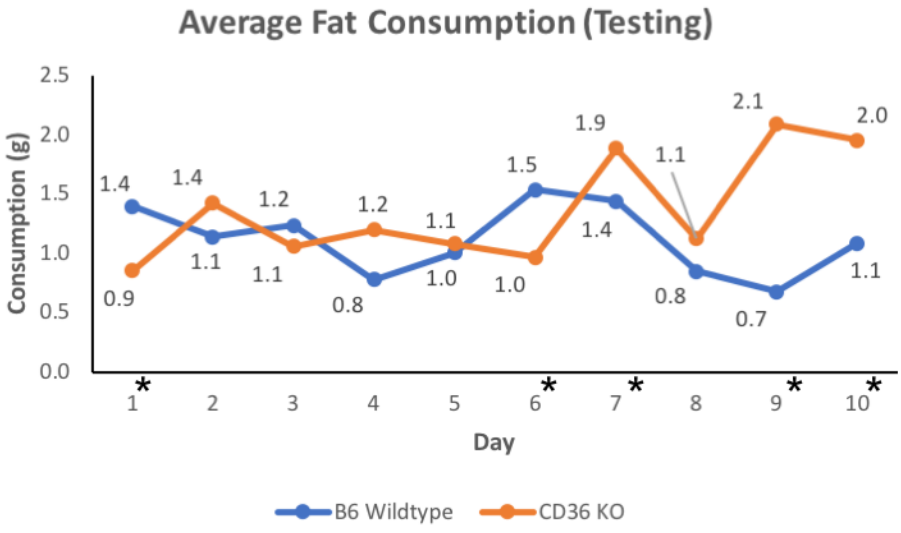

Figure 6. Average fat consumption in grams per group during testing. A weak trend line can be observed for the B6 Wildtype mice and is accentuated during the last 4 days of testing. No discernable trend is observed in the feeding habits of the CD36 KO group. Asterisks denote days on which a significant between-group difference was observed.

Total energy intake was calculated for both groups and plotted (in kcal) separately for training (Figure 7) and testing (Figure 8). Echoing previous results discussed herein, the B6 WT mice consumed far more calories than their experimental counterparts, especially as the food offerings increased in fat content. There was no pattern in the feeding behavior of the CD36 KO mice. During training, energy intake was reduced in the control group in response to the aversive conditioning protocol while, again, no clear trend emerged in the experimental group.

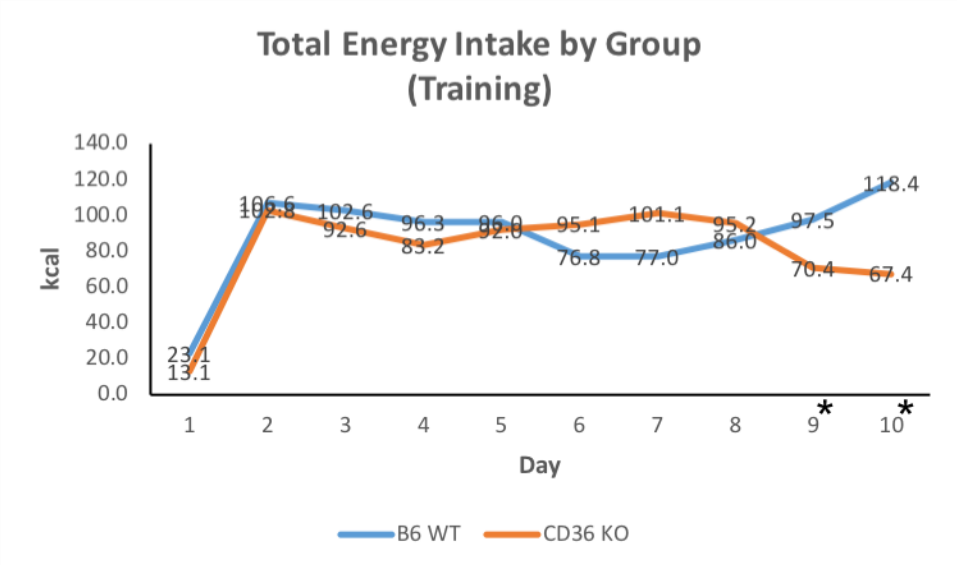

Figure 7. Total energy intake by group. During the 10 days of training, the B6 WT group consumed 67.5 kcal more than the CD36 KO group. Asterisks denote days on which a significant between-group difference was observed.

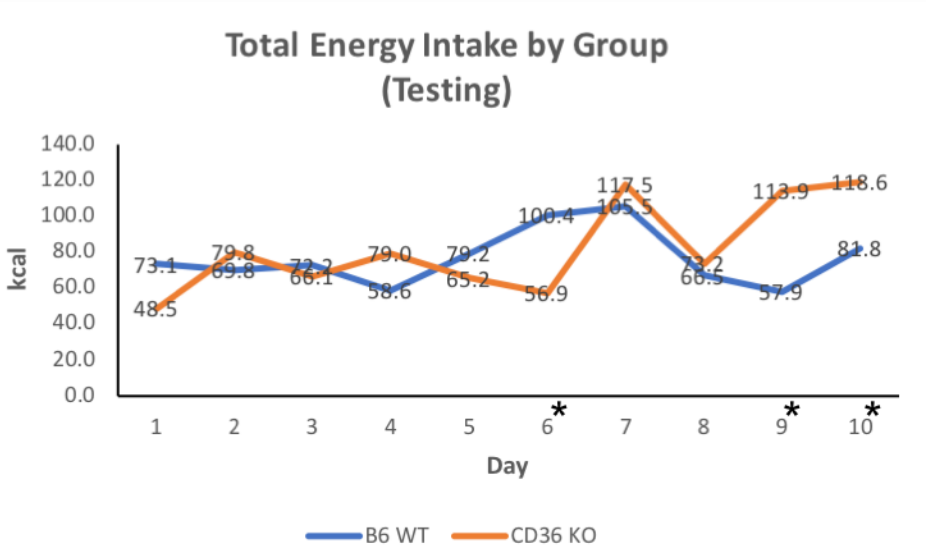

Figure 8. Total energy intake by group. During the 10 days of testing, the CD36 KO group consumed 53.8 kcal more than the B6 WT group. Asterisks denote days on which a significant between-group difference was observed.

These differences in fat preference, consumption, and energy intake were also reflected in animal weight. Both groups of animals gained weight during the acquisition phase, but the control group weighed 11.7% more at the end of training (Figure 9). However, the control group went on to lose an average of 3.9 g in response to the aversive conditioning protocol whereas the experimental group actually gained 2.5 g; at the end of testing, the experimental group weighed 13.5% more than the control mice (Figure 10).

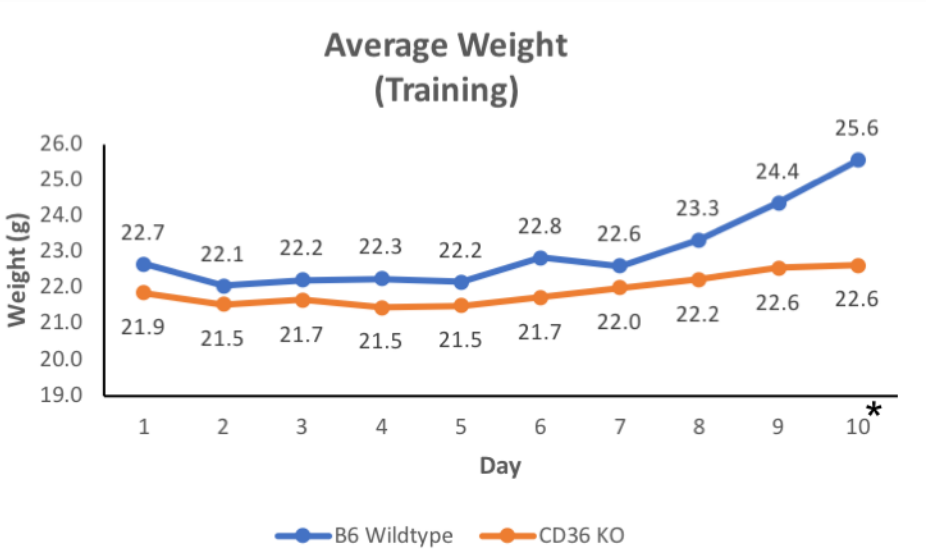

Figure 9. Average weight by group during training. B6 WT mice gained weight as a function of increased fat food offerings. Asterisk denotes day on which a significant between-group difference was observed.

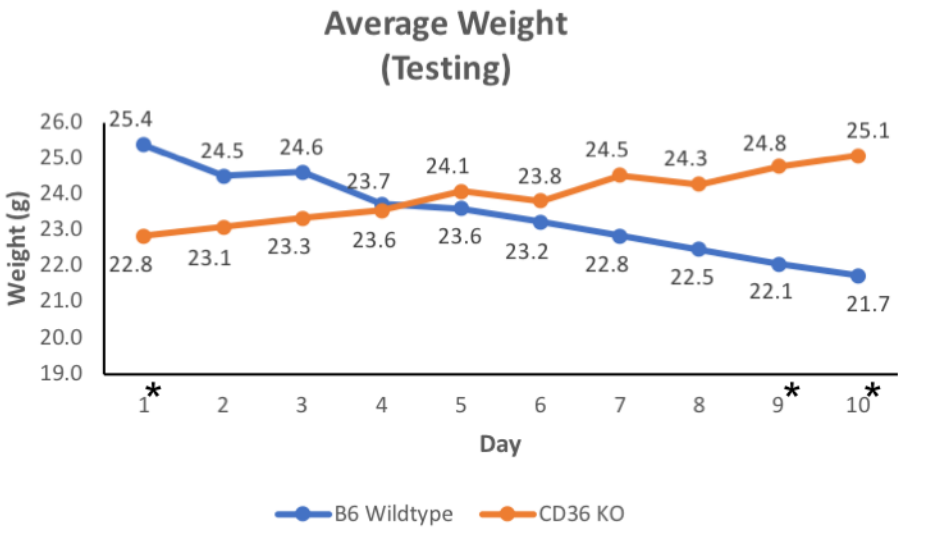

Figure 10. Average weight by group during testing. B6 WT mice lost weight in response to contingent LiCl injections. Asterisks denote days on which a significant between-group difference was observed.

The difference between groups in ability to perceive fat taste is shown in Figure 11. As can be seen, the wildtype mice gradually received less injections of LiCl, eventually receiving no injections for the last 4 days of testing. On the other hand, the knockout mice received variable amounts of LiCl with no indication of a trend or pattern.

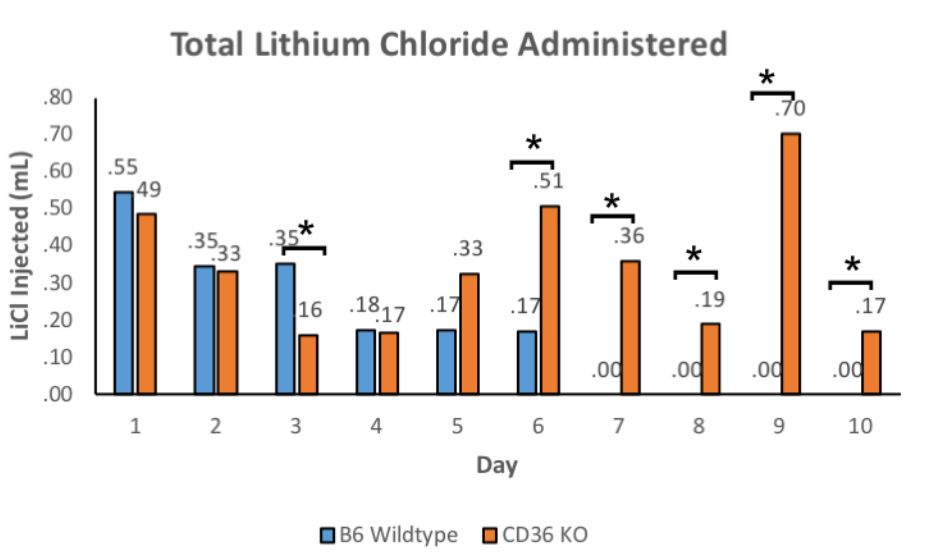

Figure 11. A bar graph of the total amount of LiCl administered per group, per day. Injection of LiCl was contingent on preferential consumption (more than 50%) of the higher fat food (45% fat); amount injected (mL) was contingent on animal weight (g). Asterisks denote significant between-group differences.

Treatment effects are best reflected in average weight differences between groups, across phases (Figure 12). For the first 10 days (Habituation), there is a negligible difference in weight between groups as they both seem to gain weight concomitantly. On days 11-20 (Training), the difference in weight between groups is accentuated—the B6 WT group clearly weighs more on day 20. Between days 21-30 (Testing), a sharp decrease in weight can be observed in the control group, whereas the average subject weight of the experimental group increases, still. Viewing the average animal weight by group across these 30 days allows for better global assessment of the treatment effect.

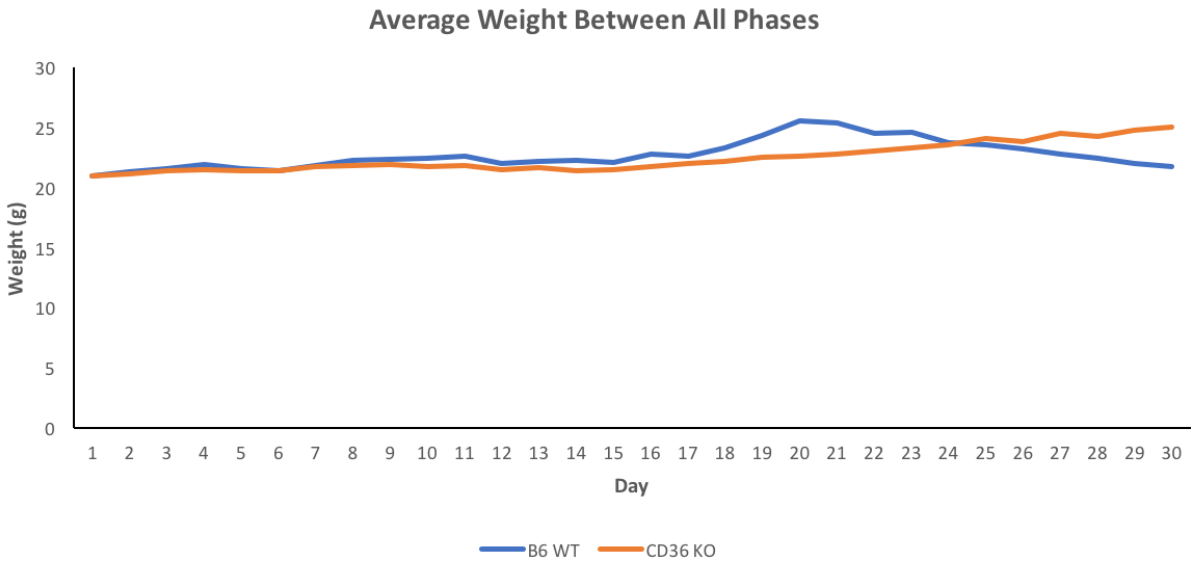

Figure 12. Average weight per group, by day. Days 1-10 represent the Habituation phase where no treatment was given. On days 11-20, subject preference for fat was tested using a schedule of foods with increasing fat content. On days 21-30, subject perception of fat was tested using a schedule of contingent LiCl/NaCl injections.

Inferential Statistics

A repeated measures ANOVA with Bonferroni correction was used to determine significant main effects, interaction of main effects, and subject differences in fat perception and preference. The overall ANOVA revealed a significant main effect of group on fat consumption [$F(1, 7)= 29.51$, $p < .001$, $\eta_p^2= .29$], energy intake [$F(1, 7)= 10.85$, $p < .01$, $\eta_p^2= .16$], and weight [$F(1, 7)= 6.987$, $p =0.039$, $\eta_p^2= .05$]. Mean group differences in volume of LiCl injections during testing was also found to be significant ($p= 0.042$). Two, two-way interactions were also found to be significant: group x phase ($p < .001$, $\eta_p^2= .19$), and group x day ($p < .01$, $\eta_p^2= .07$). As both experimental phase and day are variables of time (i.e. every phase lasted ten days), it was unclear whether significant between-group differences were due to testing effects or the primary

intervention (i.e. knockout of the CD36 gene). Linear regression was conducted to determine the proportion of variance accounted for by time and CD36 status. CD36 significantly outperformed time—measured as day number—in regression analyses. During training, CD36 status was over 6x more predictive of average fat consumption in grams (adjusted R^2= 0.5988). A strikingly similar relationship between CD36 and time was seen during testing (adjusted R^2= 0.5697). Taken together, these findings clearly support the acceptance of alternative hypotheses H_1 and H_2 and rejection of the null (H_0).

CHAPTER 5

DISCUSSION

The comparative preference scores shown in Figure 1 show a marked difference between groups. While the B6 WT mice showed a sustained preference for the higher fat offering at all levels, the CD36 KO mice only showed a slight preference for 20% fat food. This, however, should not be taken as a sign of true preference as the CD36 KO group preference score for 45% fat food was only 0.43. Indeed, this final preference score was lower than that for 15% fat (0.48). These data support the hypothesis that CD36 is responsible for primary fat taste transduction, as mice without the receptor were unable discern fat content and consequently did not display innate preference for higher fat foods.

The inability of the CD36 KO group to detect fat is also highlighted in the results. The mice in the control group lowered their consumption of 45% fat food by 22% during testing, while the experimental group actually increased their consumption of 45% fat food by 11%. As observed, the total amount of LiCl administered to the control group decreased daily—and no injections were administered during the last 4 days of testing—while no pattern emerged in the experimental group. It is clear from these results that the CD36 KO group could not learn the contingencies of the conditioning paradigm as a result of their inability detect primary fat taste.

Failure to detect fat taste is also evidenced by statistically significant between groups differences in grams of fat consumed, total energy intake, and weight. In the first phase of the experiment, the CD36-positive mice showed preference for higher fat food

options whereas the CD36-negative mice showed no such preference. This relationship was reversed in response to the CTA protocol. These results clearly demonstrate the cellular involvement of CD36 in fat taste detection. Fat consumption in the control group exhibited a clear trend line during training, but testing data was less discernible. This was most likely due to the time associated with learning the contingency-shaped behavior.

Fat consumption—itself a proxy variable for fat preference—led to significant increases in energy intake and, subsequently, significant differences in weight between groups. The inability of the knockout group to detect fat, either when presented in increasing gradations or presented as a conditioned stimulus (CS), are evidenced by their continuous weight gain throughout both experimental phases. Neither fat consumption nor preference can be said to have been influenced by any latent factors inherent in prolonged exposure to a repeated protocol as CD36 status explained 50% more variance than time.

The fat preference model described herein serves as a simple and informative assay of fat detection and subsequent feeding behavior. By design, this protocol yields itself especially useful in identifying the cognitive and receptor-mediated mechanisms underlying food preference and overfeeding which, although important to obesity research, remains understudied. Changes in fat preference resulting from genetic differences (B6 WT or CD36 KO) and pharmacological manipulation (LiCl or NaCl) were easily visualized and interpreted. To better depict shifting preference as a function of the genetic and pharmacological interventions applied, more than two levels (%) of fat food were tested. In this paradigm, preference is interpreted similarly to classic two bottle

choice tests (Takeda et. al., 2000; Takeda et. al., 2001), where increased consumption is taken to signify increased preference. As mice innately prefer higher fat food, a significantly altered fat intake is indicative of a genetically or pharmacologically related change in food choice. A recent study using rats adopted a similar model of feeding behavior to implicate neuromedin U receptor 2 in fat preference (Benzon et. al., 2014). In this present study, the color of all food offerings was as similar as possible to one another, to prevent any confounding as a result of perceptual differences inherent in the mice. All spillage was collected before weighing leftovers, to ensure as much measurement accuracy as possible. The method described here was quick to perform and was easily repeated with varying concentrations of fat food.

Since genetic and pharmacological manipulations were necessary in this investigation, special considerations were made to properly assess dietary fat preference. Subjects were habituated to injections using the dosing vehicle for 10 days prior to the start of testing to prevent generalization of induced sickness to the injection itself. Although the half-life of lithium chloride is relatively long, subjects were injected daily, as necessary. As the experimental group was created by viral knockout, mice did not begin experimentation until adolescence to ensure stable gene expression.

Foods used in this assay varied in fat and carbohydrate. Protein was kept constant due to its high satiety to kcal ratio (Berthoud et. al., 2012). That being said, the composition of foods used in this assay can easily be modified to study carbohydrates or protein. It should be noted, however, that studying more than one macronutrient simultaneously would require significantly more subjects. With the current cohort of

animals, preference for fat food was seen at 15%. Preference testing in this study was limited to a maximum of two choices, rather than a cafeteria style option. If all levels of the fat food (10%, 12.5%, 15%, 17.5%, 20% and 45%) were offered simultaneously, the overwhelming preference for 45% fat food would naturally reduce the sensitivity of the assay.

This preference model does suffer at least one major drawback: the high amount of labor associated with the protocol. Animals must be weighed, injected—and their food dishes weighed and cleaned—daily.

The method also suffers from some conceptual limitations. Foremost among them, there is risk of an ordering effect as foods are presented in increasing concentrations. This order, however, is still preferable to the alternative, as high fat food consumption may affect future food choice. As Lucas et. al. (1989) demonstrated, high levels of dietary fat can induce hyperphagia in rats. If the order of the fat foods were reversed—that is, if the mice were first exposed to 45% fat food—preference data of all % fat foods given after would be, at best, difficult to interpret. A second limitation concerns the use of 12.5% fat food as the baseline comparator. Some would argue that repeated exposure to 12.5% fat food would induce novelty-seeking behavior and naturally lead to increased consumption of progressively higher fat food. This, nonetheless, is preferable to the alternative of influencing fat intake and/or failing to produce comparative results. Also, these results could be construed as indicating a lower preference for carbohydrate, rather than an increased preference for or ability to perceive fat. Such interpretations, however, would not be in accordance with available literature

showing an innate preference for carbohydrate in mice (Sclafani et. al., 2007c). Furthermore, it should be noted that extinction time was not recorded. Future studies should take care to afford sufficient protocol time to measure this variable of interest. Finally, rates of licking should be observed in future studies in order to resolve the microstructure of fat feeding behavior.

In conclusion, this fat preference model is a reliable indicator of mice's perception of and preference for two foods of differing fat content. The design of the study allows for clever manipulation of genetic and pharmacological parameters and careful observation of their effects on fat taste perception and preference. The results of this experiment not only implicate CD36 in fat taste detection and provide further evidence for the inclusion of fat as a basic taste, but also offer a translationally relevant model of fat feeding behavior in humans.

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APPENDIX A

POWER ANALYSIS INPUT PARAMETERS

Effect size f	Citation	Intervention	α err prob	Power (1-β err prob)	Number of groups	Number of measurements	Corr among rep measures
0.7	Gaillard et. al., 2008a	Fat preference	0.05	0.8	2	6	0.2
	Laugerette et. al., 2005	Fat preference+ conditioning					
	Sclafani et. al., 2007a	Fat preference+ conditioning					
	Sclafani et. al., 2007b	Fat preference					

Sample size was calculated using G*Power 3.1 software.

APPENDIX B

IACUC APPROVAL

Institutional Animal Care and Use Committee (IACUC)
Office of Research Integrity and Assurance
Arizona State University
660 South Mill Avenue, Suite 312
Tempe, Arizona 85287-6111
Phone: (480) 965-6788 *FAX:* (480) 965-7772

Animal Protocol Review

ASU Protocol Number:	**17-1587R**
Protocol Title:	**Fat as a Basic Taste: CD36 and its Role in Fat Taste**
Principal Investigator:	**Elizabeth Capaldi Phillips**
Date of Action:	**5/25/2017**

The animal protocol review was considered by the Committee and the following decisions were made:

The protocol was approved.

If you have not already done so, documentation of Level III Training (i.e., procedure-specific training) will need to be provided to the IACUC office before participants can perform procedures independently. For more information on Level III requirements see https://researchintegrity.asu.edu/training/animals/levelthree.

Total # of Animals:	**8**	
Species:	**Mice**	**Pain Category: C**
Protocol Approval Period:	**5/25/2017 – 5/24/2020**	
Sponsor:	**N/A**	
ASU Proposal/Award #:	**N/A**	
Title:	**N/A**	

Signature: Gregory Bramble for C. Miller Date: 6/1/2017
IACUC Chair or ***Designee***

Cc: IACUC Office
IACUC Chair

APPENDIX C

REQUEST FOR CHANGES (RFC) APPROVAL

Institutional Animal Care and Use Committee (IACUC)
Office of Research Integrity and Assurance
Arizona State University
660 South Mill Avenue, Suite 312
Tempe, Arizona 85287-6111
Phone: (480) 965-6788 *FAX:* (480) 965-7772

Animal Protocol Review

ASU Protocol Number:	**17-1587R RFC 1**
Protocol Title:	**Fat as a Basic Taste: CD36 and its Role in Fat Taste**
Principal Investigator:	**Elizabeth Capaldi Phillips**
Date of Action:	**6/15/2017**

The animal protocol review was considered by the Committee and the following decisions were made:

The request for changes was approved to change the type of food given to animals and modify the husbandry parameters of the protocol.

If you have not already done so, documentation of Level III Training (i.e., procedure-specific training) will need to be provided to the IACUC office before participants can perform procedures independently. For more information on Level III requirements see https://researchintegrity.asu.edu/training/animals/levelthree.

Total # of Animals:	**8**	
Species:	**Mice**	**Pain Category: C**
Protocol Approval Period:	**5/25/2017 – 5/24/2020**	
Sponsor:	**N/A**	
ASU Proposal/Award #:	**N/A**	
Title:	**N/A**	

Signature: Angela Bruckert for C. Miller Date: 6/16/2017
IACUC Chair or ***Designee***

Cc: IACUC Office
IACUC Chair

APPENDIX D

DESCRIPTION OF USE (DOU) FORM

DACT DESCRIPTION OF USE FORM (DOU)

For DACT Use Only:
Order #:
Locations:

To save this form, please use the "save as" function on the File tab.

I. Contact & Ordering Information

Protocol #: 17-1587R Billing Account #: MGB-1034

Principal Investigator Name: Elizabeth Phillips Phone: (480) 965-1684

Individual(s) performing procedures: Paniz Jasbi

Please list whom we should contact in case of emergencies.

	Name	Phone	Email
Primary Contact	Paniz Jasbi	602.502.7346	pjasbi@asu.edu
Secondary Contact			
After Hours Emergency Contact	Paniz Jasbi	602.502.7346	pjasbi@asu.edu

Deliver by: Project Start Date: Duration:

Species	Strain	Vendor	Sex	Age/Weight	Quantity	Special Considerations (e.g. vasectomized, splenectomy)
Mus Musculus	CD36 KO	ckson Laboratc	M	see comments	4	N/A
Mus Musculus	Black 6	ckson Laboratc	M	see comments	4	N/A
			Select			
			Select			
			Select			

Additional comments:
We need the Black 6 and CD36 KO mice to arrive together and be age-matched.

Please provide a brief explanation of the experiment(s) being performed (e.g., performing multiple vaccine challenges, addiction extinction studies):
We are implementing a novel model of rodent fat feeding behavior to determine whether CD36 i s reponsible to primary fat taste detection.

II. Husbandry and Housing

Primary animal holding site: ISTB1 Light Cycle: 12:12 Standard Number of Animals per Cage: 1

If animals will be transported outside the vivarium, please list locations:

Will they be returning once removed from the vivarium: Select

Please note: Animals may not be taken out of the vivarium and returned without PRIOR approval of the Attending Veterinarian.

Husbandry and housing will follow DACT SOPs. If deviations are requested, please indicate below:

☐ None ☑ Special Diet ☐ Special Bedding ☐ Nonstandard Caging ☐ Special Watering System
☐ Immunocompromised ☐ Other – Please list below

Brief details of changes requested:
Upon arrival, they may be given stadard chow. After 10 days, they will be fed a special gel-diet.

Animals found dead are to be: Discarded

If you need DACT veterinary or veterinary technician assistance, please submit a request to vet.team@asu.edu.

Possible symptoms or effects for each experiment:

☐ None ☐ R – Rectal Prolapse ☐ A – Anorexia, Thin ☐ T – Tumor, Mass ☑ D- Diarrhea ☐ L – Lesion
☐ S – Scruffy Animal Syndrome (scruffy, hunched, slow-moving) ☐ H – Head Tilt
☐ DAE – Category E Death (on protocol) ☐ O – Other, for all other symptoms or concerns, please list below:

III. Safety

If you are using any hazardous materials (e.g., chemicals, infectious agents, radioactive materials, carcinogens, cell line) that will be used in or with the animals please list them below or select "no hazards":

Type (select)	Item Name	Animal Biosafety Level (ABSL)	Special instructions or comments for the use of this item
Hazardous Ch	Lithium Chloride	ABSL1	
Select		Select	
Select		Select	

Person completing this form: Paniz Jasbi Date: 06/01/2017

BIOGRAPHICAL SKETCH

Paniz Mohajer Jasbi was born in Tehran, Iran on September 5, 1992. His secondary education was completed in 2010 at Westwood High School in Mesa, Arizona. In 2015, Paniz graduated Summa Cum Laude from Arizona State University's College of Liberal Arts and Sciences with a Bachelor of Science in Psychological Science and a minor in Philosophy. Upon graduation, he joined Dr. Elizabeth D. Phillips' conditioned food preferences laboratory as a research and lab manager. In August 2016, he entered the Graduate College at Arizona State University to pursue a Master of Science in Obesity Prevention and Management. Throughout the course of his graduate studies, he was mentored by Dr. Elizabeth D. Phillips and Dr. Carol Johnston. Paniz will begin a PhD in Exercise and Nutritional Sciences at Arizona State University in Fall 2018.