2020

The Feeding Pattern of C57BL/6J Mice

Yakshkumar Dilipbhai Rathod

Wright State University

Follow this and additional works at: https://corescholar.libraries.wright.edu/etd_all

Part of the Pharmacology, Toxicology and Environmental Health Commons

Repository Citation

Rathod, Yakshkumar Dilipbhai, "The Feeding Pattern of C57BL/6J Mice" (2020). Browse all Theses and Dissertations. 2429.
https://corescholar.libraries.wright.edu/etd_all/2429

This Thesis is brought to you for free and open access by the Theses and Dissertations at CORE Scholar. It has been accepted for inclusion in Browse all Theses and Dissertations by an authorized administrator of CORE Scholar. For more information, please contact library-corescholar@wright.edu.
THE FEEDING PATTERN OF C57BL/6J MICE

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

By
YAKSHKUMAR DILIPBHAI RATHOD
B. Pharm, University of Mumbai, India, 2017

2020
Wright State University
I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Yakshkumar Dilipbhai Rathod ENTITLED “The feeding pattern of C57BL/6J mice” BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

Mauricio Di Fulvio, Ph.D.
Thesis Director

Jeffrey B. Travers, M.D., Ph.D.
Chair, Department of Pharmacology & Toxicology.

Committee on Final Examination:

Mauricio Di Fulvio, Ph.D.

Khalid Elased, PharmD, Ph.D.

J. Ashot Kozak, Ph.D.

Barry Milligan, Ph.D.
Interim Dean of the Graduate School
Abstract
Rathod, Yakshkumar Dilipbhai, M.S, Department of Pharmacology & Toxicology, Wright State University, 2020. The feeding pattern of C57BL/6J mice.

The feeding pattern and control of energy intake in social mice are poorly understood. Here, we determined and quantify the normal nocturnal feeding pattern of group-housed male and female mice of the C57BL/6J genetic background fed a chow diet. Mice at 10 and 20 weeks of age showed the expected age-dependent increase in lean and fat mass being them more pronounced in males. Under *ad libitum* conditions, male and female mice ate comparable amounts of food per unit of body weight irrespective of gender. The analysis of the feeding microstructure revealed that energy intake in females is the result of significantly increased nocturnal meal size, but decreased meal frequency, relative to males suggesting that food has reduced satiation value to social females than to males. The intermeal interval was significantly increased in female mice relative to males, but only at 10 weeks of age, indicating increased satiety value of food in younger females. The increased meal size observed in females was accompanied by increased meal duration, which translated to significant reduction in the feeding rate of younger females but not in older mice fed *ad libitum*. In response to prolonged fasting, male and female mice lost comparable body mass, but females recovered it faster than males in spite of eating equivalent amount of food as males. Further, younger but not older female mice fasted 16hs robustly increased their meal size during the first hours of re-feeding whereas males did not, suggesting reduced inhibition of satiation in fasted young female mice relative to males. Both, fasted males and female mice significantly reduced their intermeal interval during the first hours of re-feeding to similar extents independently of their age suggesting that fasting inhibits satiety in mice. Therefore, when taken together these results demonstrate that satiation and satiety signals governing energy intake in mice are sex- and age-dependent.
## Table of Contents

1 Introduction ........................................................................................................................................... 1  
1.1 Energy homeostasis, definition and its components ................................................................. 1  
1.2 Humoral and behavioral control of energy homeostasis .......................................................... 2  
1.3 Enteroendocrine hormones and the control of meal size ....................................................... 3  
1.3.1 Cholecystokinin ......................................................................................................................... 3  
1.3.2 Ghrelin ......................................................................................................................................... 5  
1.3.3 Somatostatin ............................................................................................................................ 7  
1.3.4 Peptide double tyrosine (PYY) ............................................................................................... 9  
1.4 Incretin hormones and the control of meal size ...................................................................... 10  
1.4.1 Glucagon-like peptide-1 ........................................................................................................... 10  
1.4.2 Glucose-dependent insulinotropic polypeptide .................................................................. 13  
1.5 Pancreatic islet hormones and the control of meal size ......................................................... 14  
1.5.1 Glucagon ................................................................................................................................... 14  
1.5.2 Amylin or amyloid polypeptide .............................................................................................. 16  
1.5.3 Insulin ........................................................................................................................................ 18  
1.6 Adipose tissue hormones and the control of meal size ......................................................... 21  
1.6.1 Leptin ......................................................................................................................................... 21  
1.7 Conceptual summary .................................................................................................................. 24  
2 Hypothesis and Specific Aims ........................................................................................................... 26  
2.1 Hypothesis ....................................................................................................................................... 26  
2.2 Specific Aims ................................................................................................................................... 26  
3 Animals, materials, methods and procedures ............................................................................ 27  
3.1 Mice and diet ................................................................................................................................. 27  
3.2 Subcutaneous microchip implant for mice identification ....................................................... 27  
3.3 Automatic food and water intake monitoring system ............................................................... 28  
3.4 Microstructure of feeding ............................................................................................................. 28  
3.5 Fasting strategy to provoke a negative energy balance in mice ............................................... 29  
3.6 Quantitative magnetic resonance imaging (QMRI) ................................................................. 29  
3.7 Statistical Analysis ....................................................................................................................... 30  
4 Results .................................................................................................................................................. 31  
4.1 Body mass accrual and body composition of mice .................................................................. 31
4.2 The definition of a meal in social male and female mice ............................ 31
4.3 The feeding microstructure of male and female mice is different .......... 35
4.4 The feeding response to fasting ................................................................ 39
4.5 A negative energy balance differentially regulates satiation and satiety in social mice ................................................................. 43

5 Discussion ........................................................................................................... 47
6 References ........................................................................................................... 56
List of Figures

Figure 1: Body mass accrual and body composition of normal mice ..........................32
Figure 2: The feeding microstructure of mice and its definitions...............................34
Figure 3: Nocturnal energy intake of normal mice..................................................36
Figure 4: The feeding microstructure of normal mice feed ad libitum.........................37
Figure 5: Feed and metabolic efficiency in mice.....................................................38
Figure 6: Body mass and energy intake responses to acute negative energy balance..40
Figure 7: Feed and metabolic efficiency in responses to acute negative energy balance .................................................................................................................42
Figure 8: Meal size, meal duration and feeding rate changes in responses to acute negative energy balance..........................................................45
Figure 9: Number of meals and changes in the interval between meals in responses to acute negative energy balance...........................................46
List of Tables

Table 1: Net energy intake responses to acute negative energy balance.................41
Acknowledgements

“Hope is a good thing, maybe the best of things, and no good thing ever dies”

Creating knowledge is only possible when we are given the freedom of imagining things and working it out on bench. I am blessed and lucky to work and learn under such esteemed scientist Dr. Mauricio Di Fulvio who believed in me and gave me an opportunity to work with freedom in his laboratory. It is only after meeting him, I understood the concept of thinking in a critical manner and learning purely based on doubting the previous knowledge. Dr. Mauricio Di Fulvio have set an excellent example of being a role model, researcher and mentor.

I would like to express my deepest gratitude to my thesis committee members Dr. Khalid Elased and Dr. J. Ashot Kozak for always being supportive and helping me in building the project and sharing ideas which were valuable and helpful.

I have been lucky to be supported with our former lab manager Lisa Kelly who helped me from the very beginning and was always there for me whenever I needed help. This work would have not been completed without the help of my lab mate Rana Abdelgawad.

Finally, yet importantly, I offer my heartfelt thanks to my amazing family for the love, encouragement and support I have got over these years in pursuing my passion of studying and goddess Randal for showering her blessings on me in accomplishing my dreams.

This research was supported by funds from the American Diabetes Association and NIH Grants 1-17-IBS-258 and R21DK113446-01 to M. Di Fulvio.
1 Introduction
1.1 Energy homeostasis, definition and its components

Energy homeostasis is defined as a biological process involving the coordinated balance of regulating the inward flow of energy in the form of food (energy) intake (Ei) and the outward flow of energy in the form of its expenditure (Ee) (Morton, et al. 2014; Schwartz, et al. 2000). The latter, which is influenced by locomotor activity, ambient temperature and nutrition state, serves as a balancing factor for the amount of energy taken in the form of food (Munzberg and Morrison 2015). Therefore, food intake, which is invariably the product of the mean caloric content of meals i.e., meal size, and their number i.e., meal frequency in a given period of time (Geary 2005) represents a major component of energy homeostasis. In other words, the timing, caloric size and composition, duration and interval between meals all provide a complete description of Ei to address the questions of what, when, how and how much is eaten (Steinert, et al. 2017).

In the case of Ee, various components contribute to it, each of them to different extents. Almost 60-70% of total Ee is supplied by basal metabolism i.e., the energy used exclusively to maintain vital activities of the organism including respiration and circulation, which are in turn regulated by body weight (BW), age, sex, lean mass or fasting status (Pinheiro Volp, et al. 2011). Basal metabolism is usually estimated by measuring the basal metabolic rate (BMR) (Westerterp 2013). Approximately 5% to 15% of the total Ee is also supplied by diet-induced thermogenesis (DIT) i.e., the energy needed to process food (absorption, digestion and storage) (Pinheiro Volp et al. 2011). The adaptive or facultative thermogenesis (FT), which supplies 10-15% of the total Ee, is the energy necessary to regulate body temperature and to respond to changes in ambient temperature (van Marken Lichtenbelt and Schrauwen 2011). Lastly, in all forms
of physical activity, either exercise or non-exercise, thermogenesis contributes to Ee in a highly variable manner; 5-10% (Jequier 1983).

1.2 Humoral and behavioral control of energy homeostasis
From the previous definitions, it becomes apparent that energy homeostasis is maintained, and can be regulated, at multiple physiological and behavioral levels. In fact, the regulation of energy balance is accomplished by the interplay of at least three processes: Ei, substrate (energy) partitioning and Ee (Badman and Flier 2007). This inter-relationship can be seen at play by the sharp increase in Ei in response to negative energy balance e.g., that imposed by prolonged fasting. Indeed, the rate of BW loss is a function of the total Ee whereas the net amount of energy consumed after fasting contributes to the total recuperation of the BW lost (Schwartz et al. 2000). For example, less BW loss would be expected in fasted individuals with reduced total Ee, and less Ei would be required to recuperate the BW that has been lost. Energy homeostasis is therefore maintained and regulated by sophisticated and deeply interconnected physiological and behavioral systems (Qaid and Abdelrahman 2016) relying on sensing and signaling of food intake, circulating fuels, energy demands and energy stores, signals that can be generated from ingestion of a meal to a degree of reflecting adiposity or energy stores. These physiological signals require a combination of interactions which are mainly processed in the brain (Badman and Flier 2007) and behaviorally executed. Many responses are triggered right before initiation of a meal e.g., salivation in dogs expecting food or secretion of small amounts of insulin in anticipation for a meal. The latter is a physiological response for efficient insulin secretion and disposal of absorbed glucose, thus essential for normal postprandial glucose tolerance (Ahrén and Holst 2001; Eliasson, et al. 2017). Along with insulin, several gastric, enteric, adipose and sex hormones are involved in the acute and chronic regulation of satiation i.e., signals that promote the termination of a meal, satiety i.e., the time spent not eating or intermeal
interval, or both, and therefore, the physiology and regulation of the feeding microstructure become an integral component to properly assess and study energy homeostasis (de Graaf, et al. 2004; Eckel 2004; Steinert et al. 2017).

1.3 Enteroendocrine hormones and the control of meal size
1.3.1 Cholecystokinin

Of the many enteroendocrine factors considered involved in meal size regulation (Chaudhri, et al. 2006), cholecystokinin (CCK) has been the first to be extensively studied (Woods, et al. 1981). CCK is a small peptide hormone released to the bloodstream by enteroendocrine I-cells of the proximal small intestine, mainly in response to digested lipids and proteins (Donovan, et al. 2007; Liddle, et al. 1986). CCK harbors endocrine satiation signals by acting on receptors located in the vagus nerve on the wall of the gastrointestinal tract (Degen, et al. 2001). In doing so, CCK acutely and in the relatively short term reduces Ei by limiting meal size, *i.e.*, promoting meal termination, without affecting meal frequency (Ballinger, et al. 1995; Figlewicz, et al. 1992; Lieverse, et al. 1995a), effects that require a full stomach (Melton, et al. 1992). Notably, high doses of CCK (Sayegh, et al. 2015) or intragastric administration of CCK secretagogues (Lateef, et al. 2011) may prolong the intermeal interval by inducing aversion and nausea (Baldwin, et al. 1998). Therefore, CCK is not considered a satiety factor (de Graaf et al. 2004; Little, et al. 2005; Peikin 1989) as CCK does not appear to regulate the non-eating time spent between meals under physiological conditions (West, et al. 1987). However, several reports have recently suggested that CCK may play an acute role in Ei by promoting satiety *i.e.*, by prolonging the interval between meals and reducing their frequency (Dafalla, et al. 2019; Washington, et al. 2011). These apparent discrepancies are related, at least in part, to the fact that CCK precursors produced in intestinal I-cells give rise to many biologically active forms of CCK, all differing in their total number of amino-acids *e.g.*, CCK-58, CCK-39, CCK-33, CCK-22, CCK-8 and all
exhibiting potentially different effects on Ei, satiation and satiety (Lateef et al. 2011; Overduin, et al. 2014; Washington et al. 2011; Washington, et al. 2016). In fact, CCK-58 has emerged as the major plasma CCK form in mammals (Eberlein, et al. 1987; Eysselein, et al. 1987; Reeve, et al. 2003). Therefore, these data suggest the need for a re-assessment of CCK physiology and a re-evaluation of the conclusions from results obtained using different CCK forms (Green and Reeve 2008). An extra layer of complexity is also noted when considering the fact that CCK release from central and peripheral tissues is reduced in old male rats, but not in females (Miyasaka, et al. 1997). Moreover, further complications arise in the finding that mouse I-cells also secrete many other enteric hormones involved in the regulation Ei (Egerod, et al. 2012; Sykaras, et al. 2014).

The role of CCK in the long-term control of Ei and BW maintenance is not fully understood (Kopin, et al. 1999; Peikin 1989). In fact, individually caged male mice lacking all forms of CCK (CCK\(^{\text{KO}}\)) had increased Ei during the light period and reduced Ei during the dark cycle resulting in normal daily Ei, BW accrual, fat and lean mass (Lo, et al. 2008). Similarly, male mice lacking one of the two CCK receptors i.e., the CCK type 1 receptor (CCK1R\(^{\text{KO}}\)) had normal BW and Ei (Donovan et al. 2007; Kopin et al. 1999; Lo et al. 2008). In addition, analysis of the feeding pattern of CCK1R\(^{\text{KO}}\) mice fed chow diet revealed normal meal size, duration, frequency and intermeal interval, implying that CCK \textit{per se} may not play a major role in the regulation of meal patterns, or if it does, it is redundant (at least in mice fed a normal diet) (Donovan et al. 2007) and it is not essential for the long term maintenance of BW (Kopin et al. 1999). Contrary to CCK1R\(^{\text{KO}}\) mice, rats with spontaneous deficiency in the CCK1R have increased Ei associated to increased meal size and non-compensated decrease in their frequency (Moran and Bi 2006). The apparently disparate phenotypes of rats and mice lacking CCK1R appear
related to rat-specific hypothalamic responses to CCK (Bi, et al. 2004). They also appear related to subjective and different definitions used to measure meal size, which has become a recurrent problem. Although CCK has also been found in neurons (Calvigioni, et al. 2017; Dockray 1976), the role of central CCK in the control of appetite and Ei remains poorly understood (Little et al. 2005; Shiraishi 1990). Nevertheless, in humans, there is a clear association between polymorphisms in the gene encoding CCK1R and increased meal size, Ei and obesity (de Krom, et al. 2007; Marchal-Victorion, et al. 2002) implying that CCK may play a tonic role in Ei.

1.3.2 Ghrelin
The gastric A-cells (Tschop, et al. 2000), I-cells of the intestine (Egerod et al. 2012; Sykaras et al. 2014) and islet ε-cells (Andralojc, et al. 2009) secrete the hormone ghrelin. This hormone is considered a satiation factor by some authors (de Graaf et al. 2004), and as such, ghrelin regulates Ei, BW and glucose homeostasis. However, the role of ghrelin in the control of meal size remains unclear (Chaudhri et al. 2006). In fact, ghrelin appears to regulate energy homeostasis at many levels (Poher, et al. 2018), including gastric emptying or inhibiting insulin secretion (Broglio, et al. 2001; Delhanty and van der Lely 2011). The role of ghrelin in the initiation of a meal has been studied in relatively good detail (Yanagi, et al. 2018). Indeed, in humans, plasma ghrelin increases sharply immediately before a meal and drops quickly afterwards to gradually increase until the next meal (Cummings, et al. 2001; Hill, et al. 2012; Spiegel, et al. 2011; Tschop et al. 2000). In fact, plasma ghrelin levels oscillate as a function of time with scheduled meals (Ariyasu, et al. 2001; Drazen, et al. 2006). Therefore, this hormone has been proposed as a marker of hunger (Davis 2018; Osto, et al. 2007). However, ghrelin also appears to reduce satiety by increasing meal frequency and in the long term it appears to mediate BW balance by reducing Ee (Huda, et al. 2009) and promoting adiposity (De Vriese and Delporte 2007). In the short term, pharmacological inhibition of
the ghrelin receptor *i.e.*, the growth hormone secretagogue receptor-1a (GHSR1a) in individually housed and 16hs fasted male mice reduces Ei (Asakawa, et al. 2003) and systemic administration of ghrelin in satiated rodents and in humans results in increased Ei and obesity (Tschop et al. 2000 ; Wren, et al. 2001a ; Wren, et al. 2001b). Ghrelin also promote gastric emptying, but at supra-physiological concentrations (Levin, et al. 2006). Yet, whether physiological fluctuations on the plasma levels of this hormone impinge on gastric emptying, remains unknown. Also, relatively unknown, are the physiological mechanistic details involved in the stimulation of ghrelin secretion (Steinert et al. 2017). Better understood, is the role of insulin in the inhibitory control of ghrelin secretion. In fact, hyperinsulinemia inhibits ghrelin secretion in the absence of hypoglycemia or any other potential change in gut hormone secretion (Flanagan, et al. 2003 ; Murdolo, et al. 2003 ; Saad, et al. 2002).

At this point, it is worth mentioning the fact that many but not all physiological actions of ghrelin require acylation of the hormone and potentially different receptors (Poher et al. 2018). Indeed, the freely circulating des-acyl ghrelin, which accounts for ~90% of total plasma ghrelin (Patterson, et al. 2005) affects glucose metabolism and Ei when centrally administered through mechanisms that do not depend on GHSR1a, the only ghrelin receptor known to date (Thompson, et al. 2004 ; Toshinai, et al. 2006 ; Zhang, et al. 2008). In addition, when systemically administered, des-acyl ghrelin decreases (Chen, et al. 2005) or does not affect Ei (Toshinai et al. 2006) in rats. Further, the inhibitory effects of acyl-ghrelin, presumably the only ghrelin capable of activating GHSR1a (Gauna, et al. 2007 ; Kojima, et al. 1999) and insulin secretion (Broglio et al. 2001 ; Dezaki, et al. 2004) appear blocked by des-acyl ghrelin (Broglio, et al. 2004), a result not confirmed by recent studies (Tong, et al. 2014). These discrepancies could be related to the doses of ghrelin used and the facts that acyl-ghrelin does activate GHSR1a in the nanomolar
range (Kojima et al. 1999) whereas des-acyl ghrelin does it at the micromolar range (Gauna et al. 2007 ; Staes, et al. 2010).

Although it has been proposed that ghrelin is necessary to maintain plasma glucose during famine (Briggs and Andrews 2011 ; Mani, et al. 2019 ; Yanagi et al. 2018), elimination of ghrelin in mice (Ghrelin\(^{KO}\)) does not influence Ei (McFarlane, et al. 2014 ; Sun, et al. 2006). In addition, mice lacking GHSR1a (GHSR1\(^{KO}\)) have alterations in the circadian system (Lamont, et al. 2014) and blunted preference for sweet foods (Disse, et al. 2010). Therefore, ghrelin appears to have redundant, pleiotropic and often differential effects in energy balance (Edwards and Abizaid 2017 ; Muller, et al. 2015). However, the absence of changes in net Ei does not discard changes in meal size or frequency.

1.3.3 Somatostatin

Enteroendocrine D-cells of the gastric and intestinal mucosa produce somatostatin (STT), which is released in response to mechanical stimulation of the stomach, meal components and parasympathetic action. STT is not a hormone restricted to gastrointestinal D-cells; it is found in the brain (Johansson, et al. 1984), pituitary, δ-cells of the pancreatic islet, a subpopulation of C-cells in the thyroid, inflammatory and immune cells, some adrenal, prostate, kidney and placental cells (extensively reviewed in (Gunther, et al. 2018 ; Patel 1999)). STT has many central (Viollet, et al. 2008) and peripheral effects (Bray 1995). Central STT appears to play a role in cognitive functions and other processes related to memory and learning, locomotion and the control of food intake, and several others (Gunther et al. 2018). Peripheral STT regulates all gastrointestinal functions that have been studied so far (Kumar and Singh 2020). Interestingly, most of STT effects are anti-secretory of many hormones including insulin, growth hormone (GH), CCK, ghrelin, glucagon and glucagon-like peptide 1 (GLP-1) (Kumar and Singh 2020). Therefore, STT may also indirectly participate in the regulation
of feeding behavior or the control of meal size. Notable exceptions to that general rule are related to the fact that STT may directly increase Ei, body temperature and locomotion (Stengel, et al. 2013). Indeed, central STT potently and acutely stimulates Ei and that of water (Stengel, et al. 2010b; Stengel, et al. 2015; Stengel and Tache 2019), modulates normal body temperature in rats (Lin, et al. 1989) and promote ambulatory activity in mice (Vecsei and Widerlov 1990). These are all acute effects, that appear mediated by one of the five STT receptors known *i.e.*, STT receptor-2 (STTR2) expressed in the hypothalamus. Indeed, the use of stable and selective STT agonists and STT receptor antagonists (Beranek, et al. 1999; Danguir 1988; Stengel et al. 2010b) provided direct pharmacological support to the hypothesis that activation of hypothalamic SSTR2 in rodents reduces satiety by shortening the intermeal interval thus increasing meal frequency resulting in increased Ei without affecting meal size *i.e.*, satiation (*see* Stengel et al. 2010b, *reviewed* Stengel et al. 2015)). Interestingly, the acute intracerebroventricular (ICV) effect of STT agonists on Ei is accompanied by reduced BW in the face of increased Ee and basal body temperature (Stengel, et al. 2010a). However, central STT is also known to promote opposite behavioral adaptations to food consumption in rodents *i.e.*, increase or decrease in Ei, the latter related to high doses of STT (Stengel et al. 2013).

Interestingly, elimination of STT in mice (STTKO) does not alter growth curves or basal blood glucose (Hauge-Evans, et al. 2009; Low, et al. 2001) suggesting that STT is dispensable for somatic growth. Notably, the feeding microstructure and behavioral pattern of Ei in STTKO mice remains unknown. Also unknown is the relative contribution of non-central STT to the control of feeding behavior and of Ei and whether the rhythmic hypothalamic production of STT (Gardi, et al. 1999; Ishikawa, et al. 1997; Shinohara, et al. 1991) plays a role in nocturnal feeding behavior in a similar fashion as that suggested

1.3.4 Peptide double tyrosine (PYY)

The enteroendocrine cells responsible for most of the synthesis and secretion of PYY i.e., L-cells (Ekblad and Sundler 2002), frequently express and secrete GLP-1 and CCK and other hormones (Ali-Rachedi, et al. 1984 ; Egerod et al. 2012 ; Habib, et al. 2012 ; Svendsen, et al. 2015 ; Sykaras et al. 2014). The biologically active, often considered the anorectic form of PYY i.e., PYY3-36, results from local and peripheral dipeptidyl-peptidase-4 (DPP4)-mediated cleavage of PPY1-36 (McGowan and Bloom 2004 ; Medeiros and Turner 1994). This active PYY freely crosses the blood-brain-barrier (BBB) (Nonaka, et al. 2003) and is considered the physiological agonist of the Y2 receptor, one of the five known Y receptors targeted also by neuropeptide and pancreatic polypeptide (PP) (Batterham, et al. 2002 ; Larhammar 1996). Y2 is widely distributed in central neurons, particularly in the arcuate nucleus (Broberger, et al. 1997) and therefore potentially implicated in the control of Ei (McGowan and Bloom 2004). In humans, neural processes appear to promote PYY secretion after a single meal reaching a maximal concentration ~2hs after. This high concentration is maintained for several additional hours (Adrian, et al. 1985) and influenced by meal size and its composition, particularly meals rich in fats, at least in dogs (Lin and Chey 2003).

Supra-physiological or pharmacological infusions of PYY results in potent and acute inhibition of Ei in rodents and in humans (Batterham, et al. 2003 ; Batterham et al. 2002 ; Chelikani, et al. 2005 ; Pittner, et al. 2004). Apparently, PYY does so in rats by promoting satiety (Scott, et al. 2005) i.e., by prolonging the non-eating time between
meals. However, this effect has not been validated. In fact, PYY reduced Ei during the first hours of the nocturnal photoperiod of the day, and re-feeding following a severe food deprivation imposed by 24hs fasting in rats (Batterham et al. 2002; Halatchev, et al. 2004). However, according to a recent critical review, most studies performed using animal models and high doses of PYY do not provide strong evidence for a physiological role of PYY in Ei (Steinert et al. 2017), but do not discard them either. In fact, mice lacking PYY (PYY$^{\text{KO}}$) provided ambiguous results in the form of hyperphagia in males in one study (Batterham, et al. 2006) or in females, but not in males in another study (Boey, et al. 2006), or no eating phenotype at all (Schonhoff, et al. 2005; Wortley, et al. 2007). However, morbidly obese individuals exhibit suppressed PYY levels (Batterham et al. 2003) suggesting a potential contribution of PYY in the development of obesity. The opposite may also be true, as it is not known if obesity reduces PYY levels. Interestingly, over-expression of PYY in mice did not affect BW, but decreased Ei after prolonged fasting (Shi, et al. 2012). In the chronic setting, pharmacological administration of PYY reduces Ei and BW accrual in rodents in one report (Batterham et al. 2002), but not in another (Challis, et al. 2004). Therefore, the physiological role of any form of PYY remains an open question.

Although a small group of islet cells in the endocrine pancreas also produce PYY, its relative contribution to the control of Ei, beyond its paracrine positive effects on β-cell mass, is unknown (Persaud and Bewick 2014).

1.4 Incretin hormones and the control of meal size

1.4.1 Glucagon-like peptide-1

Enterendocrine L-cells secrete glucagon-like peptide-1 (GLP-1) in response to nutrients, hormones and neurotransmitters (Dumoulin, et al. 1995; Gameiro, et al. 2005). Like CCK cells, L-cells also secrete PPY (McGowan and Bloom 2004) and co-express CCK, GIP and other hormones to different extents (Egerod et al. 2012; Habib et
al. 2012 ; Svendsen et al. 2015). GLP-1, whose secretion is circadian (Gil-Lozano, et al. 2014) is negatively controlled by STT secreted from neighboring D-cells (Hansen, et al. 2000), is directly secreted into the portal system to have many systemic effects (Steinert et al. 2017). Indeed, the GLP-1 receptor (GLP-1R) is widely expressed in the brain, kidneys, heart, throughout the gastrointestinal tract or the pancreatic islet (Alvarez, et al. 2005 ; Bullock, et al. 1996 ; Campos, et al. 1994 ; Wei and Mojsov 1996). The latter being the site of action of GLP-1 to exert its "incretin effect" i.e., potentiation of insulin secretion from β-cells thereby contributing to the control of prandial plasma glucose concentration (Holst and Gromada 2004 ; Lamont, et al. 2012 ; Vilsboll and Holst 2004). GLP-1 also potently inhibits glucagon secretion from α-cells of the pancreatic islet (Orskov, et al. 1988) either indirectly through its effects on β-cells or directly and in a glucose-dependent manner via activation of GLP-1R recently discovered in α-cells (Zhang, et al. 2019). In addition, GLP-1 promotes STT secretion from δ-cells of the islet (Orskov et al. 1988) raising the possibility that insulin and glucagon secretion may be controlled by GLP-1 in a paracrine fashion.

GLP-1 is also produced and secreted by few pancreatic islet cells, a small neuronal population of the nucleus tractus solitarius (Holst 2007) and in the hypothalamus (Turton, et al. 1996). In fact, the discovery of GLP-1 in the brain (Jin, et al. 1988) suggested a potential physiological effect of this hormone in the control of appetite and Ei (Shah and Vella 2014 ; Trapp and Hisadome 2011), which was confirmed in experiments involving ICV administration of GLP-1 (Tang-Christensen, et al. 1996 ; Turton et al. 1996). In addition, GLP-1 appear to mediate its central metabolic effects through indirect mechanisms as well (Adams, et al. 2018 ; Shirazi, et al. 2013). Like CCK, GLP-1 is now considered a satiation factor that reduces Ei by decreasing meal size i.e., promoting meal termination (Flint, et al. 1998 ; Pannacciulli, et al. 2007). Unlike
CCK, however, GLP-1’s effects are independent of gastric emptying (Begg and Woods 2013; Bodnaruc, et al. 2016; Marathe, et al. 2011; van Dijk, et al. 1999). Notably, whether the satiating effect of GLP-1 is mediated by central and/or peripheral receptors is unknown. Nevertheless, in humans, GLP-1 acts as a classic hormone to regulate Ei at the central and peripheral levels by providing a synergistic effect on appetite suppression and regulation of satiation (Steinert et al. 2017; van Bloemendaal, et al. 2014). In rats and mice, however, GLP-1 appears to control appetite and Ei via local actions rather than endocrine or systemic ones. Indeed, GLP-1 does not increase during meal in rats (Shin, et al. 2010) but it does in mice (Gunnarsson, et al. 2006) whereas infusion of a brain-impermeable GLP-1 conjugate inhibits Ei in mice (Baggio, et al. 2004b). Nevertheless, it is important to note that GLP-1 does not need to increase its concentration in plasma of fed rats to have a physiological effect and that such an increase does not warrant a physiological effect. At this point, it is important to mention that the effects of endogenous GLP-1 on meal size and appetite are typically short-lived due to the very short half-life of the peptide (Kieffer, et al. 1995). In fact, DPP4-mediated inactivation of GLP-1 may start even before the hormone reaches the circulation, implying that GLP-1 actions could also be transmitted via intestinal and hepatic sensory neurons (Holst 2007; Punjabi, et al. 2011). Notably, and like CCK1RKO mice (Donovan et al. 2007), elimination of GLP-1R in the mouse (GLP-1RKO) does not alter satiety (Scrocchi, et al. 1996) suggesting that GLP-1 per se may be dispensable or redundant for appetite regulation. Regardless, however, chronic administration of the long-lasting and DPP4-resistant GLP-1 analogue exendin-4 (EX4) or liraglutide (LIR) to rats (Elfers, et al. 2012; Hayes, et al. 2011; Primeaux, et al. 2010; Yang, et al. 2014a) and humans (Astrup, et al. 2012), reduces Ei and BW gain. Given that EX4 and LIR promote weight loss by increasing satiety, and likely by
promoting gastric emptying as well (Nauck, et al. 1997), EX4 and LIR are approved drugs for the long-term treatment of obesity (Grill 2020 ; Pi-Sunyer, et al. 2015).

1.4.2 Glucose-dependent insulinoctropic polypeptide

Like GLP-1, glucose-dependent insulinoctropic polypeptide (GIP) participates in the incretin effect (Drucker 2006). Bioactive GIP\textsubscript{1-42} is produced by duodenal K-cells, some neurons and other cells types including L-cells, which coproduce GLP-1 (Fujita, et al. 2010). GIP is quickly secreted in response to a meal and quickly inactivated to GIP\textsubscript{3-42} by DPP4 (Kieffer et al. 1995). GIP activates at least two receptors (GIPRs), which are distributed in several organs and cell types including insulin-secreting \(\beta\)-cells of the pancreatic islet, adipocytes, cardiomyocytes and central neurons (Drucker 2006 ; Gasbjergr, et al. 2018).

Although the role of GIP or its receptors has not been thoroughly investigated, young male mice lacking GIPR (GIPR\textsuperscript{KO}) exhibit a mild phenotype characterized by a slight intolerance to oral glucose, but normal tolerance after intraperitoneal test, accompanied by normal basal blood glucose and BW up to 20 weeks of age (Miyawaki, et al. 1999). In line, chronic administration of GIPR blockers deteriorates glucose tolerance and insulin secretion in normal male mice (Irwin, et al. 2004). In addition, GIPR\textsuperscript{KO} male mice are resistant to diet-induced obesity whereas obese mice lacking GIPR exhibit reduced BW gain and improved glucose tolerance (Miyawaki, et al. 2002). Further, injection of GIP antagonists to obese mice reduces blood glucose and plasma insulin (Gault, et al. 2005). Together, these results suggest a role for GIP in insulin-mediated improvement of glucose homeostasis, at least in young male mice. Contrary to expectations, however, young mice of both genders lacking GLP-1 and GIPR (GLP-1/GIP\textsuperscript{KO}) did not show deteriorated glucose homeostasis beyond that found in GLP-1\textsuperscript{RKO} and GIPR\textsuperscript{KO} mice in one study (Hansotia, et al. 2004) but not in another, which demonstrated additive
glucose intolerance in older mice of both genders (Preitner, et al. 2004). Nevertheless, in humans with diabetes, the insulinotropic effect of GIP appears reduced (Nauck, et al. 1993; Vilsboll, et al. 2002) but preserved in obese individuals (Thondam, et al. 2017) strengthening the notion that functional β-cells are needed for the effects of GIP. However, at this point is important to keep in mind that GIP has the potential to stimulate secretion of glucagon from α-cells of the endocrine islet in normal and diabetic individuals (Christensen, et al. 2015; Christensen, et al. 2011; Lund, et al. 2011; Meier, et al. 2003).

GIP also regulates energy metabolism in coordination with GLP-1 at higher doses by decreasing food intake and BW. However, the mechanisms through which these effects are being mediated remain poorly known.

1.5 Pancreatic islet hormones and the control of meal size
1.5.1 Glucagon
The role of glucagon in feeding behavior and Ei appears related to a reduction in meal size in males and females (Langhans, et al. 1982; McLaughlin, et al. 1986), but these effects have not been validated. Glucagon is a hyperglycemic hormone secreted by α-cells of the pancreatic islets into the portal circulation to promote satiation i.e., by reducing meal size without affecting its initiation in rats and humans (Habegger, et al. 2010). Glucagon is thought to exert its functions through direct activation of a single receptor abundantly expressed in the liver and kidneys. Although glucagon can also activate its receptors in the heart, adipocytes, pancreatic islets as well as in the brain and other tissues (Svoboda, et al. 1994), the satiating effect of glucagon on short term Ei requires the hepatic branch of the abdominal vagus nerve (Geary, et al. 1993; Geary and Smith 1983; Martin, et al. 1978). In addition, glucagon reduces Ei by activating central neurons (Kurose, et al. 2009; Quinones, et al. 2015) and by stimulating resting Ee and thermogenesis in rats and in humans (Billington, et al. 1987; Davidson, et al.
In fact, acute glucagon administration increases core body temperature (Yahata, et al. 1983). Although the mechanisms involved in the thermogenic actions of glucagon remain not fully elucidated, this hormone appears to regulate non-shivering thermogenesis in several animal models by direct, and likely redundant, activation of adipocytes (Beaudry, et al. 2019; Townsend, et al. 2019) and/or indirectly via the sympathetic nervous system (Habegger et al. 2010). Regardless of the potential mechanisms involved, the long-term actions of glucagon in Ee has led to the hypothesis that this hormone could have beneficial effects in the treatment of obesity. Although that appears to be the case, at least in mice (Day, et al. 2009; Pocai, et al. 2009), some of the beneficial effects of glucagon, including BW regulation, appear mediated by hepatic secretion of fibroblast growth factor 21 (FGF21) (Habegger, et al. 2013; Kim, et al. 2018). Interestingly, chronic treatment of aged and obese male mice with the glucagon receptor (GCGR) agonist IUB288 resulted in an impressive FGF21-dependent reduction in BW, which was accounted for, at least in part, by reduced Ee and increased locomotor activity. These effects appear independent of Ei and were not observed in mice lacking GCG exclusively in the liver (Day et al. 2009; Habegger et al. 2013). Therefore, in the short term, glucagon appears to control energy metabolism and BW by directly reducing meal size and therefore Ei, whereas in the long-term, this hormone has a minor role in Ei. Instead, glucagon appears to regulate BW and energy metabolism by stimulating its expenditure, at least in part, through hepatic release of FGF21.

The role of glucagon or its signaling in long-term feeding behavior remains unknown. Yet, on one hand, mice lacking GCGR (GCGRKO) exhibit improved metabolic regulation in the context of reduced BW and Ei, in turn accompanied by massive increases in plasma GLP-1 (Conarello, et al. 2007) and FGF21 (Omar, et al. 2014). However, Ei and Ee were normal in a different GCGRKO model, as well as BW, which remained normal.
due to decreased fat mass but increased lean mass (Gelling, et al. 2003). Notably, blocking the action of both GLP-1 and FGF21 in GCGRKO mice prevented the beneficial metabolic effect of GCGR loss, thus involving, at least in part, GLP-1 and FGF21 as confounding mediators of that effect (Omar et al. 2014). On the other hand, mice with complete glucagon deficiency due to insertion of GFP in the mouse glucagon gene (GCGGFP) are phenotypically normal, but these mice are also deficient in GLP-1 and all hormones that derive from the glucagon gene (Hayashi 2011). This is relevant; in addition to GLP-1, GLP-2 and other peptides derived from the glucagon gene are considered anorectic (Baggio, et al. 2004a ; Tang-Christensen, et al. 2000). Unsurprisingly, Ei appears marginally affected in their absence (Hayashi 2011) and indeed remains normal even in the presence of elevated plasma GLP-1 and GLP-2 (Gagnon, et al. 2011).

1.5.2 Amylin or amyloid polypeptide

β-cells of the pancreatic islet produce, co-store (Lukinius, et al. 1989) and co-secrete (Kahn, et al. 1990) amylin (a.k.a., amyloid polypeptide) with insulin in a defined proportion after meals (Butler, et al. 1990) to potently increase the short-term satiation signals elicited by CCK at the central level (Lutz 2006) thus reducing meal size and Ei (Hay, et al. 2015). Although that is the general consensus, some of the effects of amylin in energy homeostasis are not mediated by CCK, particularly when amylin is chronically administered (Isaksson, et al. 2005 ; Roth, et al. 2006). These effects are likely related to increased Ee and reduced Ei (Mack, et al. 2007 ; Wielinga, et al. 2010). However, this awaits demonstration.

The role of amylin in the short-term control of Ei has been relatively well characterized (Woods et al. 2006). In rodents, for instance, ICV and intraperitoneal (IP) administration of amylin after a meal acutely reduces Ei in rats and mice during the first hours of
nocturnal feeding (Chance, et al. 1991 ; Lutz, et al. 1994) without changing blood glucose levels, at least in mice (Morley and Flood 1991). Interestingly, the acute effects of amylin in Ei were observed only in old male rats after 12hs fasting but not in young ones, who needed extended periods of food deprivation (Lutz et al. 1994). This effect could be related to differences in Ei during the circadian rhythm between old and young rats, as proposed (Del Prete and Scharrer 1993). In addition, IP amylin reduced the size and duration of the first meals without affecting the size of subsequent meals or their intermeal intervals in male rats fasted for 24hs (Lutz, et al. 1995 ; Reidelberger, et al. 2002) and in 4-22 month old obese fasted mice (Morley, et al. 1994). Further, the effects of amylin in the control of meal size and Ei were not blocked after vagotomy (Del Prete and Scharrer 1993 ; Morley et al. 1994) but were in rats with lesioned area postrema/nucleus of the solitary tract region (Lutz, et al. 2001). Furthermore, peripheral administration of amylin antagonists increases Ei in obese rats, but not in lean ones (Grabler and Lutz 2004 ; Reidelberger, et al. 2004). Therefore, these results suggest that amylin is a fast-acting peripheral (central) satiating hormone that reduces Ei.

In the long-term, amylin appears to act as an "adiposity signal" i.e., a hormone secreted in proportion to fat mass to convey central information about energy stores and maintain a relatively constant BW (Cancello, et al. 2004). In fact, chronic administration of amylin to rats results in anorexia when fed a normal diet (Arnelo, et al. 1996 ; Lutz et al. 2001) or when subjected to obesogenic diets (Eiden, et al. 2002), and 18-24 week old male mice with targeted disruption of the amylin gene (AMYKO) exhibit normal Ei, but increased BW mass (Gebre-Medhin, et al. 1998a). In addition, AMYKO mice have improved glucose tolerance due to increased insulin responses to intravenous (IV) or IP glucose in males, but not in females of mixed genetic backgrounds (Gebre-Medhin et al. 1998a). Therefore, it is possible that the increased BW observed in AMYKO mice could
be due to long-term insulin over-secretion in response to nutrients, and/or to small but chronic changes in meal size i.e., satiation, frequency or both. However, when male AMY\textsuperscript{KO} mice were backcrossed to the C57BL6 genetic background, no difference in BW accrual was observed relative to WT up to 27w of age. Similarly, Ei, meal size and frequency were apparently normal in AMY\textsuperscript{KO} male mice of both genders and under different diets (Olsson, et al. 2012 ; Turek, et al. 2010) thus suggesting that the role of amylin in the control of Ei is redundant or compensated.

Interestingly, it has been proposed that the absence of amylin observed in patients lacking insulin may contribute to their hyperphagia. In fact, administration of insulin together with the amylomimetic and the approved antidiabetic Pramlintide, appears to mitigate the high BW gain typical of diabetic patients subjected to treatment with insulin, insulin secretagogues or sensitizers (Hollander, et al. 2004 ; Weyer, et al. 2001). However, it remains unknown whether amylin secretion and/or its synthesis changes with age in normal or obese individuals (Hay et al. 2015). Also unknown is the physiological contribution of extra-insular amylin, as this hormone is also expressed in some neuroendocrine cells of the stomach and in neurons of the dorsal root (Gebre-Medhin, et al. 1998b ; Zaki, et al. 2002).

1.5.3 Insulin
Insulin, secreted by β-cells of the pancreatic islet and the only hypoglycemic hormone of the organism, is considered a long-term "adiposity signal" (Woods 2005). However, insulin is secreted into the portal vein during meals to quickly reduce meal size in some (Vanderweele 1993) but not all settings (Surina-Baumgartner, et al. 1995). The delivery of insulin antibodies into the portal vein increases meal size (Surina-Baumgartner et al. 1995) and when insulin is co-injected with glucose into the portal vein, meal size is reduced (Langhans, et al. 2001). These data indicate that insulin may have a short-term
effect in the control of meal termination and thus Ei. However, the previous results must be taken with caution; portal injection of insulin does not harbor the pulsatile nature of its physiological secretion, which is necessary for insulin’s normal hepatic function (Satin, et al. 2015).

In humans, there is no clear relationship between fasting plasma insulin and feeding behavior e.g., satiety in some studies (de Graaf et al. 2004 ; Heini, et al. 1998a) but not in others (Hallschmid, et al. 2012 ; Verdich, et al. 2001). In addition, the acute effects of exogenously administered insulin on meal size or Ei are confounded by its known fast hypoglycemic effect, which by itself provokes hunger and promote acute eating (Lotter and Woods 1977). Indeed, several early studies were not designed to test the separate effects of hypoglycemia versus hyperinsulinemia on feeding behavior (Briese and Quijada 1978 ; Mayer-Gross and Walker 1946), but others did. For instance, injection of 2-deoxy-D-glucose (2DG), an inactive analogue of glucose incapable of raising plasma insulin increased appetite and reduced satiety in young human males (Thompson and Campbell 1977 ; Welle, et al. 1980). Similarly, hyperinsulinemia, independently of hypoglycemia, increased Ei in humans (Rodin, et al. 1985). Further, acutely (Anika, et al. 1980) or chronically (VanderWeele, et al. 1982) administered insulin at doses low enough to leave glycemia intact results in reduced Ei in animal models. Similarly, when glucose is co-injected with insulin to avoid hypoglycemia, Ei is also quickly reduced in the baboon (Woods, et al. 1984). However, other authors did not find a relationship between post-meal plasma concentrations, satiation (meal size, duration) or satiety (meal frequency, intermeal interval) (Gielkens, et al. 1998 ; Lavin, et al. 1996 ; Lavin, et al. 1998 ; Woo, et al. 1984). Therefore, the role of insulin in the acute modulation of feeding behavior remains an open question (de Graaf et al. 2004). Nevertheless, it appears clear that insulin does have the potential to acutely modulate Ei in different
animal models and in humans under some circumstances. Yet, whether these effects are centrally and/or peripherally mediated remain unknown.

A small proportion of peripheral insulin crosses the BBB (Rhea, et al. 2018) and curiously, perhaps, some from the choroid plexus as well (Mazucanti, et al. 2019), to have central effects. In fact, basal, fasting plasma insulin is very low in normal individuals and as such, a decrease in its central availability is believed to convey a central signal indicating the degree of adiposity thereby regulating Ei and BW. Indeed, chronically increased central insulin reduces Ei (Schwartz, et al. 1992 ; Woods, et al. 1979 ; Woods and Seeley 2001) and chronically reduced insulin in the cerebrospinal fluid (Chavez, et al. 1995 ; Woods et al. 1979) promote Ei to regain lost BW and normalize central insulin signaling. Of note, the effects of chronic insulin administration in terms of Ei and BW regulation are clearly slow, taking in some cases several days to appear (Brief and Davis 1984 ; Plata-Salaman and Oomura 1986 ; Plata-Salaman, et al. 1986 ; Woods et al. 1979). Most notably, in humans, chronic intranasal administration of insulin sprays, which result in increased insulin concentration in the cerebrospinal fluid, but not in plasma (Born, et al. 2002), delays the reduction in Ei and BW, at least in males (Hallschmid, et al. 2004a ; Hallschmid, et al. 2004b). In the long term, therefore, insulin regulates Ei and BW through central mechanisms (Plum, et al. 2005) directly and in part by sensitizing the hypothalamus satiation and satiety signals elicited during and after meals, e.g., amylin and CCK (Woods et al. 2006).

The role of central insulin in long-term control of Ei and BW is further exemplified by the facts that male and female mice lacking insulin receptors (IR) selectively in neurons (NIR_KO) exhibit increased fat mass relative to wild-type (WT) mice under normal dieting (Bruning, et al. 2000). However, BW was only increased in female NIR_KO mice, a finding related to their increased Ei (Bruning et al. 2000). Similarly, rats with impaired IR
signaling in the hypothalamus are hyperphagic and obese (Obici, et al. 2002). At this point, it is worth noting that the feeding microstructure and feeding behavior of these and other rodent models with deficient IR signaling (Okamoto, et al. 2004) or mice models with primary defects in insulin secretion remain unknown.

1.6 Adipose tissue hormones and the control of meal size

1.6.1 Leptin

Leptin, the hormone product of the ob gene, is mainly synthesized in white adipocytes (Zhang, et al. 1994) in direct proportion to fat stores (Considine, et al. 1996; Sinha, et al. 1996) and as a major suppressor of Ei to reduce BW and to keep fat mass relatively constant over long periods of time (Myers, et al. 2008). The role of leptin in energy balance has been conclusively demonstrated in animal models and in humans with genetic defects in the leptin-signaling pathway (Dubern and Clement 2012; Farooqi, et al. 2007; O’Rahilly 2009; Zhang et al. 1994). For instance, rodents deficient in leptin e.g., ob/ob mice (LEPKO) or deficient in leptin receptor i.e., db/db mice (LEPRKO) and humans lacking functional LEPR are morbidly obese.

All known actions of leptin related to energy homeostasis occur centrally, via activation of the long form of the leptin receptor. Indeed, deletion of the receptor in the liver, adipose tissue and small intestines does not affect Ei, BW, temperature and composition (Guo, et al. 2007) consistent with the notion that those organs do not obviously participate in leptin-mediated metabolic effects. In addition, ob/ob or db/db mice are hyperphagic (Myers et al. 2008), but do not show changes in Ee due to thermogenesis (Fischer, et al. 2020). Indeed, leptin has a pyrexic effect i.e., it increases basal and defended body temperature at the central level independently of thermogenesis (Fischer, et al. 2016). Notably, in humans, leptin appears to be voided of acute or short-term effects in terms of feeding behavior before and after meals e.g., satiety (Joannic, et al. 1998; Karhunen, et al. 1997; Romon, et al. 1999). In fact, human plasma leptin
concentrations remain relatively normal for some time in response to nutrients (Romon et al. 1999) and follow a circadian rhythm, reaching higher levels at night (Sinha et al. 1996). However, a chronic negative energy balance imposed by prolonged food deprivation results in reduced leptinemia (Boden, et al. 1996 ; Keim, et al. 1998 ; Wisse, et al. 1999), which does not relate to reduced fat mass (Boden et al. 1996 ; Weigle, et al. 1997) but strongly correlates with increased Ei in humans (Heini, et al. 1998b ; Keim et al. 1998). Since low plasma leptin in response to prolonged fasting does not associate with changes in fat mass, the role of leptin in promoting robust Ei after fasting suggest that hypoleptinemia is a physiological signal aimed at restoring energy balance in response to fasting (Keim et al. 1998). These concepts must be considered within the context of additional results suggesting that leptin mRNA levels in adipocytes (Saladin, et al. 1995) and leptinemia (Dallongeville, et al. 1998) increase after meals in humans and decrease in fasted mice of both genders (Ahren 2000) and in sheep (Marie, et al. 2001).

Due to the effects of leptin in humans and animal models under chronic energy deficit, this hormone, like amylin and insulin, is now considered the prototypical "adiposity signal" that convey the message of energy replenishment to central nervous system (Bates and Myers 2003 ; Woods et al. 2006). Indeed, leptin directly activates hypothalamic and extra-hypothalamic circuits known to be involved in Ei and BW regulation (Elmquist, et al. 1999). In particular, leptin, at least in part and not exclusively (Dhillon, et al. 2006 ; Hayes, et al. 2010 ; Hommel, et al. 2006 ; Ring and Zeltser 2010 ; van de Wall, et al. 2008), differentially activates its receptors located in orexigenic neurons expressing neuropeptide-YY (NPY) and agouti-related peptide (AgRP), and anorexigenic neurons expressing pro-opiomelanocortin (POMC) and cocaine-and-amphetamine-regulated-transcript (CART), both in the arcuate nucleus (ARC) of the
hypothalamus (Aponte, et al. 2011 ; Krashes, et al. 2011 ; Schwartz et al. 2000). These neurons release their respective neuropeptides to activate or inhibit melanocortin-4-receptors (MC4R) expressed in neurons of the paraventricular nucleus (PVN) or lateral hypothalamic area (LHA), overall resulting in reduced Ei and BW. Notably, leptin like insulin, also controls glucose homeostasis at the central level but its effects appear independent of insulin (Hill, et al. 2010 ; Morton and Schwartz 2011). Indeed, ICV infusion or peripheral administration of leptin restores glycemia and glucose tolerance in rats or mice deficient in insulin signaling (German, et al. 2011 ; Kruger, et al. 2011 ; Yu, et al. 2008). These facts led to the concept that leptin can activate a "central glucoregulatory system" which in coordination with pancreatic islet hormones orchestrate and effectively regulate and maintain normal glucose homeostasis (reviewed in detail in (Schwartz, et al. 2013)).

Although the role of leptin as a lipostatic hormone maintaining energy homeostasis at the central level is well accepted, comparatively very little is known regarding the role of leptin in the regulation of feeding behavior and the feeding microstructure. Few studies have been performed to determine the role of a single ICV dose of leptin on the spontaneous meal pattern of rats, with different results. For instance, a single low ICV dose of leptin reduced mean meal size and feeding rate without influencing meal duration and frequency (Flynn, et al. 1998) whereas repeated ICV doses of leptin decreased meal frequency, size, duration and feeding rate (Hulsey, et al. 1998). These apparent discrepancies are related, at least in part, to different and subjective definitions of the threshold intermeal interval i.e., the minimum time necessary to distinguish two separate meals, as cleverly noted in (Zorrilla, et al. 2005b). This is a highly relevant distinction, different intermeal interval thresholds lead to disparate and inaccurate perceptions of baseline feeding behavior and changes in meal pattern in response to
treatments or interventions (Castonguay, et al. 1986 ; Castonguay, et al. 1982 ; Clifton 2000). In a more recent study adopting non-subjective, validated and mathematically defined intermeal interval thresholds to distinguish meals (Inoue, et al. 2003 ; Zorrilla, et al. 2005a), ICV increases in leptin concentration did reduce meal frequency and increased satiety \textit{i.e.}, it prolonged the intermeal interval, without changes in meal size (Zorrilla et al. 2005b), a conclusion supported by recent studies in humans (Chapelot, et al. 2000). These data suggest that subjectively long intermeal interval thresholds used as criterion to define meals have masked previous results, as it has been recently suggested (Demaria-Pesce and Nicolaidis 1998). Therefore, previous experiments aimed at understanding de feeding pattern and behavior of animal models may need re-evaluation.

1.7 Conceptual summary
Over the last decades, it has become clear that satiation and satiety are under the control of many signals generated immediately before, during and after meals, including hormones of the enteroendocrine system, pancreatic islet and adipokines, which by acting alone or in concert acutely control Ei and BW. In addition, some of these hormones are released in proportion to fat mass to modulate the central sensitivity of short-term signals to provide a long-term control of Ei and BW. For instance, amylin, insulin and leptin potentiate the satiation effects of CCK (Hay et al. 2015), but not all the effects of insulin, amylin and leptin are mediated by CCK (Lutz et al. 1995). Some hormones such as CCK or GLP-1 have been better characterized than others in the control of meal size, which is the most important indicator of Ei, or in the control of additional variables that define feeding behavior including meal duration, frequency and intermeal interval. Further, hormones can act either as classic endocrine signals in distant organs like the brain or as local ones on neighboring cells to control meal size and Ei. Yet, several questions remain, including the roles of many of these signals in the

That task requires consistent and systematic analysis of the feeding behavior and its microstructure. Indeed, with few exceptions (Inoue et al. 2003; Zorrilla et al. 2005a; Zorrilla et al. 2005b) most studies aimed at understanding the meal pattern of rodent models have used isolated mice/rats and arbitrary and discrepant meal definitions making difficult if not impossible the comparative and critical analysis of the literature (Castonguay et al. 1986; Castonguay et al. 1982; Clifton 2000; Demaria-Pesce and Nicolaidis 1998). This is not trivial; satiation signals define meal size and satiety signals define the intermeal interval. Further complicating the picture, age and sex are rarely considered as variables in the assessment of feeding microstructure, in particular meal size and intermeal interval. This has potential clinical and behavioral implications as subtle but chronic non-pathological or pathological changes in insulin secretion or any factor involved in the control or regulation of satiation and/or satiety, for instance, may change with aging in a sex-dependent manner. Moreover, whether satiation and satiety signals are overridden by negative energy balance or whether a correlation between fat mass and those signals exist, remain unknown. These concepts are, in essence, the foundation and rationale behind the hypotheses to be tested in this work.
2 Hypothesis and Specific Aims

2.1 Hypothesis
Satiation and satiety signals in social male and female mice are different and change with age.

To address that overarching hypothesis, we propose the following specific aims.

2.2 Specific Aims

1) To define a meal in group-housed social male and female mice. To accomplish this objective, we will mathematically determine the threshold intermeal interval necessary to separate two feeding events as part of two consecutive meals in male and female mice of 10 and 20 weeks of age fed *ad libitum* a normal diet. Hypothesis 1: The threshold intermeal interval of a meal in mice fed a normal diet is independent of age and gender.

2) To characterize the normal feeding microstructure of social male and female mice fed *ad libitum*. To accomplish these objectives, Ei will be continuously monitored for 3 weeks in mice of both genders and of 10 and 20 weeks of age to determine the following feeding parameters: meal size, meal frequency, meal duration and intermeal interval. Hypothesis 2: Male and female mice exhibit differential satiation and satiety signals in an age-dependent manner.

3) To determine if there is a functional relationship between meal size and intermeal interval to acute negative energy balance in social mice of both genders. To impose a negative energy balance, male and female mice of 10 and 20 weeks of age will be fasted for 16hs and allowed to re-feed to determine their feeding microstructure responses and BW changes to fasting. Hypothesis 3: Fasting overrides satiation and satiety signals in male and female mice independently of age.
3 Animals, materials, methods and procedures
3.1 Mice and diet
Male and female mice of the genetic background C57BL/6J of 10 and 20 weeks (w) of age were used in our studies. All the studies involving mice were approved and done in accordance to the regulations implemented by the Institutional Animal Care and Use Committee (IACUC) of Wright State University, School of Medicine. Mice were originally obtained from The Jackson Laboratory (Bar Harbor, ME) and bred in our animal facility to produce sufficient mice for experiments. Mice were weaned at post-natal days 19-21 and group-housed (4-5 mice/cage) in polycarbonate cages with sterilized bedding and nestlets for enrichment. Water and food (chow, Teklad 22/5 #8640, 3.0kCal/g) were provided ad libitum. Mice were monitored three times a week and cage bedding along with food and water were changed on weekly basis. Few days before experiments, mice were randomly swapped between cages to establish hierarchy and social context (Varholick, et al. 2019). Mice were housed in 12:12 hour light and dark cycle. That is, light phase: 6:30-18:30, zeitgeber: 1-12 and dark phase: 18:30-6:30, zeitgeber: 13-24). Ambient temperature was set to 21-22°C.

3.2 Subcutaneous microchip implant for mice identification
To identify mice, radio-frequency identification (RFID) chips (2.12x12mm, UNO-MICRO-12, Med Associates Inc.) were implanted under the dorsal skin, close to the neck of conscious mice. The procedure was carried out 1w before experiments. Mice are identified based on the RFID chip emitting a unique barcode that is interpreted by the radio-frequency (RF) sensor located near the feeders of the automatic feeding system (see below). Implanted (tagged) mice (9-11 per session) were then placed in the feeding system and allowed to acclimatize for at least 5 days. Food intake and its microstructure were continuously recorded for 21 days. During the length of the experiment, mice were minimally disturbed. Only BW was manually recorded before the initiation of the
experiments and every 2-3 days (14:00-15:00) until finished. Mice were weighed by using the RFID-equipped mouse scale connected to the feeding station. Food and water levels were monitored daily and refilled whenever necessary.

3.3 Automatic food and water intake monitoring system
All feeding events of mice were captured by using the HM2 Rodent Food and Water Intake Monitoring System (MBRose, Denmark) designed for group-housed rodents. A total of 9-11 age/sex-matched mice were placed in the HM2 system's cage (dimensions: 540cm x 400cm x 460cm = 0.1m³) equipped with two small tunnels connecting two independent feeders attached to scales. Each tunnel is surveyed by infrared beams that detect tunnel activity in the form of entry and exit of a single mouse while placing timestamps to each of them. The tunnels are also equipped with RF detector coils to detect and record the identity of a mouse implanted with a unique RFID chip. Atop the cage, the system is furnished with motion sensors that compute total mouse ambulatory activity.

3.4 Microstructure of feeding
The HM2 monitoring system continuously records the weights of feeders containing food (±0.001g) detecting "non-eating" events when the feeder's weight is stable or as "eating events" when unstable. Therefore, the interactions of the mouse with the feeders are recorded as differences in feeder's weight before and after a feeding event or bout (FB). Each FB recorded carries its size in grams and timestamps indicating initiation and termination of the feeding event. Thus, each FB is recorded as a vector with a start time, duration and amount consumed. The data obtained by the HM2 station is stored in a database (Firebird® relational database management system) which can be queried by using the HM2Lab software (MBRose, DK) of the HM2 station. The records of the food monitoring system are organized as raw data in the database that can be analyzed by
the HM2 software or exported to spreadsheets for manual analysis. We have performed the latter to corroborate and validate the automatic software-based analysis. Since mice are nocturnal animals which consume ≥75% of their daily Ei during the night, we have used only nocturnal raw data (1830-0630) to calculate the following parameters: meal frequency (MF, the number of meals in a defined period of time), meal size (MS, the caloric content of a meal), meal duration (MD, the time that a meal last), inter-meal interval (IMI, the time spent not eating), time spent in meals (the time spent eating) and the rate of ingestion or feeding rate (FR, the ratio between meal size and its duration, e.g., the number of calories consumed per second). Net nocturnal Ei was calculated as the product of the mean nocturnal MS and MF.

3.5 Fasting strategy to provoke a negative energy balance in mice
Prior to fasting, BW was recorded as described previously. Mice were not allowed to feed from 1600 to 800 (16hs fasting) by closing the gates of the feeders in the HM2 System while allowing mice to freely access water and the system to record spontaneous ambulatory activity. After fasting, mice were weighed. Mice were then allowed to re-feed at 800 to continuously monitor their feeding behavior and microstructure thereafter. BW was also determined at 2, 4, 6, 8 and 24hs.

3.6 Quantitative magnetic resonance imaging (QMRI)
Body composition of mice (fat and lean mass) was determined by QMRI (EchoMRI – 100H Analyzers, Houston, Texas, USA). This was performed in mice at 10 and 20 weeks of age as shown previously (Halm, et al. 2017). Briefly, prior starting the procedure, instrument was calibrated using a blank oil tube. Once instrument was calibrated and ready for use, body weight of mice was determined, and they were placed in a cylindrical tube-like barrel. To avoid movement of mice, which may interfere with the body composition reading, mice were secured with the help of plunger with head facing
upwards to prevent injury to mice. This tube-like barrel was then placed in the instrument and body composition was determined. This tube was sanitized with 70% ethanol after each use.

3.7 Statistical Analysis
Data are expressed as mean ± SEM. Differences in the means of two populations was determined by Students two-tailed t test and a preliminary F test to determine homogeneity of within group variances. Two-way ANOVA followed by Tukey’s post-hoc analysis was used to compare the mean differences between groups. A p<0.05 was considered statistically significant.
4 Results
4.1 Body mass accrual and body composition of mice
Body mass accrual in mice from weaning to 36 weeks of age is shown in Figure 1A. As expected, males accumulated more BW than females, which became significant after 6 weeks of age. The increase in BW is the combined result of increased lean and fat mass, as shown in Figures 1B-C. However, absolute fat and lean mass accrual in male mice was higher relative to females at 10 and 20 weeks of age. Notably, fat mass accumulation in 20w old males was disproportionately higher than that of females, whose fat mass accrual remained relatively constant at all ages tested (Fig 1C) including 30w old mice (not shown). These results, when normalized to total BW mass, translate to decreased and increased relative lean and fat mass, respectively, in 20w old males, as shown in Figures 1D-E and increased fat to lean ratio only in 20w old males (Fig 1F). Therefore, the increased BW mass observed in male mice relative to females is accounted for increased absolute lean and fat mass, being fat mass a relatively higher contributor to male BW.

4.2 The definition of a meal in social male and female mice
Based on published reports (Inoue et al. 2003; Zorrilla et al. 2005b) a meal unit is defined as a cluster of feeding bouts (FB) and drinking separated by short inter-bout intervals. Further, these clusters of FBs must be separated by an IMI that cannot be lower than a defined threshold (IMIt) to appropriately determine when a FB is the last one of a meal. In other words, each meal is a cluster of several FBs or mouthfuls separated by short inter-bout intervals and in turn, each cluster of FBs is separated from the next cluster by an IMI equal or higher than IMIt. These concepts are graphically represented in Figure 2A.

Therefore, to interpret the feeding microstructure of mice and process raw data obtained from continuously monitored feeding behavior it is necessary to define the minimum
Figure 1. Body mass accrual and body composition of normal mice. A. Weekly body mass in grams (g) of mice fed *ad libitum* a chow diet from weaning to 36 weeks of age. Mice were weaned at post-natal day 19-21. B-C. Absolute lean (B) and fat (C) mass of male and female mice at 10 and 20 weeks of age. D-E. Lean (D) and fat (E) mass of male and female mice at 10 and 20 weeks of age relative to their mean body mass. F. Absolute fat mass to absolute lean mass ratio of male and female mice at 10 and 20 weeks of age. Results are presented as mean values ± SEM (*n=15-20, *p<0.05).*
meal size (MS\textsubscript{m}) and the IMIt. To estimate MS\textsubscript{m}, we applied the following reasoning: when IMIt = 0, all single feeding events i.e., FB recorded by the automatic feeding system correspond to single meals. Therefore, FB = MS\textsubscript{m}. The mean MS\textsubscript{m} (in grams) of male and female mice of 10 and 20w of age fed \textit{ad libitum} was next determined by imposing an IMIt = 0. As shown in Figure 2B, the MS\textsubscript{m} of male mice of the indicated ages are of less caloric content than those of females, but equal or higher than 0.050g in all cases. Therefore, the MS\textsubscript{m} adopted for all our experiments was ≥0.050g.

To define a meal, we next determined the minimum time between the last FB ≥ 0.050g of a cluster must be separated from the next FB to be considered part of a next cluster, and therefore a new meal. Based on the definition of IMIt \textit{i.e.}, the IMI that effectively separates two meals, we determined the mathematical relationship between the number of single meals ≥0.050g \textit{i.e.}, meal frequency (MF) and progressively higher IMIt in male and female mice of 10 and 20w of age fed \textit{ad libitum}. As shown in Figure 2C-D, the relationship between MF and IMIt follows a typical double exponential decay in males (Fig 2C) and females (Fig 2D) mice. From this relationship, it can be seen that an IMIt ≥5min defines the minimum IMI that detect changes in the rate of change in MF and distinguishes changes in the rate of meal size change in males (Fig 2E) and females (Fig 2F). To further validate this IMIt, the rate of change in MS, predicted to be at its minimum when IMI = IMIt (Inoue et al. 2003 ; Zorrilla et al. 2005b) was verified as a function of increasing IMI. Therefore, to define a meal of social mice we adopted an IMIt ≥5min. In other words, a meal is recorded when the sum of single feeding events, each of a size ≥0.050g are clustered within ~5min of each other. Hence, our results demonstrate that IMIt ≥ 5min defines a meal in mice fed \textit{ad libitum} a normal diet irrespective of age and gender.
Figure 2. The feeding microstructure of mice and its definitions. A. Schematic representation of the feeding microstructure of mice. Each meal is represented by the sum of small feeding bouts (FBs), each of them of a minimum caloric size determined in B. Meals are in turn separated from each other by a threshold intermeal interval (IMIt) determined in C-D. B. Determination of the minimum meals size (MSm) in mice during the nocturnal period of the day. MSm of mice of both genders and of the indicated ages was determined by computing the meal size (MS) when IMIt = 0 and MS = 0. Under these conditions, each FB represents MSm (n=9-11, *p<0.01). C-F. Determination of IMIt in male (C, E) and female (D, F) mice during the nocturnal period of the day. The number of meals (MF, C-D) was determined as a function of increasing IMIt thresholds to establish that which distinguishes changes in the rate of MF or meal size (E, F). As shown, IMIt ≥ 5mins serve as a threshold to mathematically define a meal in 10-20w old male (C-D, n=9-11) and female mice (E-F, n=9-11).
4.3 The feeding microstructure of male and female mice is different

Since preliminary results demonstrated that more than 80% of the total feeding events occur during the nocturnal period of the day, we present here results obtained during the scotophase and refer to diurnal Ei when necessary. To determine whether age and gender affect Ei in mice, we first computed and analyzed the average nocturnal Ei recorded during 14 days in 10 and 20w old male and female mice. As shown in Figure 3A, Ei in males and females did not significantly differ from each other and no major changes were observed as a function of age. Because Ei is invariably the product between mean meal size and meal frequency, we next related Ei of males and females to those parameters. As shown in Figure 4A, meal size (MS, kCal) in males was significantly reduced relative to that of females at all ages tested. As expected for equivalent Ei, meal frequency (MF, counts) was significantly increased in male mice relative to females (Fig 4B). These results suggest increased satiation in male mice relative to females. Partially in line with that conclusion, as shown in Figure 4C, meal duration was significantly shortened in 10-20w old male mice when compared to females. In fact, MD decreased faster in older females than in male mice. This was reflected in feeding rate which is the calculated as the ratio between meal size and meal duration. Feeding rate in 10w male was higher as compared to female but increased to comparable levels in both genders at 20w as shown in Figure 4E. Therefore, satiation in social female mice decreases faster than that of males as their ages increase whereas satiety, which also decreases in social female mice as they age (Fig 4D), remains otherwise constantly low in males but increased in younger females (Fig 4D). Notably, male and female mice at 10 and 20 weeks of age maintained their BW stable when the feeding microstructure was being recorded and exhibited comparable Ei. Therefore, the feeding efficiency (FE) i.e., the change in BW per unit of energy (ΔgBW/ΔkCal) and the metabolic efficiency (ME) i.e., the change in Ei per unit of BW (ΔkCal/ΔgBW) in social
Figure 3. Nocturnal energy intake of normal mice. A. Mean nocturnal energy intake normalized to total BW in male and female mice at 10 and 20w of age. Results are presented as the mean ± SEM (n=9-11, p>0.999).
Figure 4. The feeding microstructure of normal mice feed *ad libitum*. A-E. Shown are meal size (A, kCal), number of meals (B, counts), meal duration (C, seconds), intermeal interval (D, minutes) and feeding rate (E, calories per second) of male and female mice aged 10 and 20 weeks. Data was recorded during 9 consecutive days and separated according to the two photoperiods of the day. Results are presented as the mean ± SEM (n=9-11, *p<0.05).
Figure 5. Feed and metabolic efficiency of mice. 
A. Feed efficiency (FE), calculated as the ratio between the changes in body mass (ΔBW) and the corresponding change in energy intake (ΔKcal) recorded in male and female mice of 10 and 20 weeks of age. 
B. Metabolic efficiency (ME) calculated as the inverse of FE recorded in male and female mice of 10 and 20 weeks of age. Results are presented as the mean ± SEM (n=9-11, *p<0.05).
mice remained stable and comparable in 10-20w old mice of both genders, as shown in Figures 5A-B.

4.4 The feeding response to fasting
Since EE can be inferred from the drop in BW after prolonged fasting and BW recovery depends on the net caloric intake upon re-feeding, we next determined BW loss when a negative energy balance is imposed by prolonged fasting and BW accrual after re-feeding. As shown in Figure 6A-B, BW drop relative to initial BW in response to 16hs fasting was comparable in males and females suggesting similar EE in 10-20w old social mice. In line, female mice recovered the lost BW within 2hs after re-feeding whereas males required more time (Figs 6A-B). In fact, female and male mice showed comparable net EI during the first 8hs of re-feeding, as shown in Table 1. Further, as shown in Figure 6C-D, EI adjusted to BW in social female mice was significantly higher during that period of re-feeding when compared to males. Therefore, EE in 10-20w old social male and female mice appears comparable. Of note, male and female mice had equivalent net EI during the first 8hs upon re-feeding whereas BW re-gain was clearly faster for females, thus predicting equivalent FE and ME. Indeed, as shown in Figures 7, these parameters did not show statistical significance between genders and age.
Figure 6. Body mass and energy intake responses of mice to acute negative energy balance. A-B. Shown are the body mass accrual recorded at the indicated times after allowing re-feeding in 16hs fasted male and female mice of 10 (A) and 20 (B) weeks of age. C-D. Energy intake normalized to body mass recorded at the indicated times after allowing re-feeding of 16hs fasted male and female mice of 10 (C) and 20 (D) weeks of age. Results are presented as the mean ± SEM (n=9-11, *p<0.05 vs. gender, •p<0.05 vs. baseline).
Table 1. Net energy intake of re-fed mice in response to acute negative energy balance. Energy intake (kCal) at the indicated re-feeding times in response to 16hs fasting in male and female mice of 10 and 20 weeks of age. Results are presented as the mean ± SEM (n=9-11, *p<0.05 vs. gender, †p<0.05 vs. ad libitum).

<table>
<thead>
<tr>
<th>Time after re-feeding (h)</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>24</th>
<th>Age (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>1.46 ± 0.27</td>
<td>3.10 ± 0.31</td>
<td>3.74 ± 0.60</td>
<td>4.49 ± 0.70</td>
<td>12.77 ± 0.49**</td>
<td>10w</td>
</tr>
<tr>
<td>Females</td>
<td>2.08 ± 0.32</td>
<td>2.72 ± 0.28</td>
<td>3.05 ± 0.30</td>
<td>4.00 ± 0.51</td>
<td>10.21 ± 0.68†</td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>2.04 ± 0.31</td>
<td>2.93 ± 0.24</td>
<td>3.95 ± 0.38</td>
<td>5.06 ± 0.42</td>
<td>13.63 ± 0.73*</td>
<td>20w</td>
</tr>
<tr>
<td>Females</td>
<td>2.33 ± 0.19</td>
<td>4.18 ± 0.26</td>
<td>5.02 ± 0.39</td>
<td>5.22 ± 0.42</td>
<td>13.36 ± 0.59*</td>
<td></td>
</tr>
</tbody>
</table>
Figure 7. Feed and metabolic efficiency of mice in response to acute negative energy balance. A-B. Feed efficiency (FE), calculated as the ratio between the changes in body mass (ΔBW) and energy intake (ΔKcal) recorded at the indicated times after allowing re-feeding of 16hs fasted male and female mice at 10 (A) and 20 (B) weeks of age. C-D. Metabolic efficiency (ME) calculated as the inverse of FE recorded at the indicated times after allowing re-feeding of 16hs fasted male and female mice of 10 (C) 20 (D) weeks of age. Results are presented as mean values ± SEM (n=9-11, *p<0.05).
4.5 A negative energy balance differentially regulates satiation and satiety in social mice

To correlate the net changes in Ei upon re-feeding to potential sex- and age-dependent changes in satiation and satiety, we next determined MS, MD, MF and IMI in response to fasting and re-feeding. As shown in Figure 8A, MS dramatically increased in 10w old females in response to fasting to gradually reach *ad libitum* levels after ~5hs of re-feeding whereas that of males did not change from baseline. Notably, on one hand, as shown in Figures 8A-B, the MS response of 10w old social males to fasting was lost in 20w old females when compared to males, which remained unresponsive (Fig 8B). Therefore, when taken together, these results suggest that a negative energy balance impose inhibition of satiation only to 10w old female.

Since fasting may increase the feeding rate (FR) in social mice, we next determined MD to compute FR. As shown in Figures 8C-D, MD did not change in response to fasting in males or females and remained close to *ad libitum* responses. When these results are related to those of MS to calculate FR, as shown in Figures 8E-F, it can be observed that 10w old males and 20w old female mice eat at a relatively constant rate and close to the *ad libitum* baseline upon refeeding in response to fasting whereas 10w old female mice showed increased FR responses relative to age-matched males. When taken together, these results suggest that meal duration is a proportional function of meal size in social males at 10 and 20 weeks of age and 20w old females whereas that of 10w old females is not. Therefore, the satiation value of food in response to a negative energy balance is reduced or inhibited in social 10w old females but not in males.

Since net Ei in social males and females in response to fasting was comparable (see Table 1) whereas MS (satiation) responses of 10w old female and male mice to the same intervention were not, we next determined MF and IMI to gain insights into their
satiety responses. As shown in Figures 9A-B, MF increased in males and females during re-feeding, as expected. However, during the first 8-24hs after re-feeding MF remained similar in both males and females. These data fully account for the net Ei recorded under similar conditions and further suggest that satiety may be inhibited to a similar extent in social male and female mice in response to a negative energy balance. To verify that, we next determined IMI in upon re-feeding in response to fasting. As shown in Figures 9C-D, male and female mice exhibited significant reductions in the IMI during the first hours of re-feeding, suggesting that food deprivation robustly inhibits satiety in social males and female mice to similar extents. Notably, the IMI responses of 10-20 old female mice to fasting followed similar sigmoidal kinetics reaching baseline *ad libitum* values within 6 and 2hrs, respectively, of re-feeding to reach 20-30% higher values than baseline in 20w old mice thus indicating that re-feeding gradually increased satiety in mice.
Figure 8. Meal size, meal duration and feeding rate changes in responses to acute negative energy balance. A-B. Meal size (kCal) recorded at the indicated times of re-feeding in 16hs fasted male and female mice at 10 (A) and 20 (B) weeks of age. C-D. Meal duration or time spent in each meal recorded at the indicated times after allowing re-feeding of 16hs fasted male and female mice of 10 (C) and 20 (D) weeks of age. E-F. Feeding rate was calculated as the ratio between meal size and meal duration at the indicated times after allowing re-feeding of 16hs fasted male and female mice of 10 (E) and 20 (F) weeks of age. Results are presented as mean values ± SEM (n=9-11, *p<0.05 vs. gender, •p<0.05 vs. ad libitum).
Figure 9. Number of meals and changes in the interval between meals in responses to acute negative energy balance. **A-B.** Meal frequency (counts per mouse) recorded at the indicated times after allowing re-feeding of 16hs fasted male and female mice of 10 (A) and 20 (B) weeks of age. **C-D.** The intermeal interval calculated as the time difference between the moment a meal is initiated and the time at which the previous meal has ended (min) recorded at the indicated times after allowing re-feeding of 16hs fasted male and female mice of 10 (A) and 20 (B) weeks of age. Results are presented as mean values ± SEM (n=9-11, *p<0.05 vs. gender, •p<0.05 vs. ad libitum).
5 Discussion

Attempts have been made to better understand the eating physiology of single- and group-housed mice. It has been previously reported that BW gain in mice of both genders is inversely proportional to the number of animals housed (Chvedoff, et al. 1980; DeFeudis 1974). For instance, isolated mice have significantly higher Ei and fat mass than mice in a social environment (reviewed extensively in (Schipper, et al. 2018)). Further, mice living in groups exhibit contact behavior i.e., huddling which contributes to the reduced Ee observed in social mice but not in isolated animals (Harshaw and Alberts 2012). This is reflected in their BW, lower oxygen consumption (Bryant and Hails 1975) and reduced Ei (Schipper et al. 2018). In addition, it has been demonstrated that single-housed male mice at 23 and 43 weeks have increased BW, fat and lean mass than single-house female mice and that fat mass increases with age in males whereas lean mass remains stable relative to females (Yang, et al. 2014b). We found that social males have apparently higher lean and fat mass than females at 20 weeks of age, but not at 10 weeks of age, where fat mass was comparable among genders (Fig 1B-C). These results suggest that fat mass contributes more to males' BW than to females. Notably, these differences are not related to changes in nocturnal Ei (Fig 3) but they could be potentially linked to diurnal differences in Ei and/or to age/sex-related differences in the circadian pattern of mice housed in groups. Further, the increased adiposity of 20week old social male relative to female mice may not be related to changes in ambulatory activity, which was shown increased in social males (Kosten, et al. 2014) or to overcrowding (Lin, et al. 2015). It remains to be demonstrated whether potential changes in Ee and/or age/sex-related differences in the susceptibility to develop obesity, as shown in isolated mice (Hong, et al. 2009) participate in a similar extent in grouped-housed mice.
One of the objectives of the present study was to understand and define the feeding microstructure of social male and female mice. This is rooted in the need to better interpret results published using different definitions of a meal, a subject that has only recently begun to be addressed in mice (Tabarin, et al. 2007). Indeed, in the absence of reliable definitions of a meal, the understanding of satiation and satiety becomes confusing and limited. For instance, it has been well recognized since a long time that many physiological systems participate in the maintenance of energy balance, mainly Ei through changes in satiation and/or satiety. In turn, changes in the energy intake lay the foundation of disorders like obesity or anorexia nervosa (Ali and Kravitz 2018). Further, until very recently, the methodologies used to determine and study the feeding pattern of rodents had several limitations and errors resulting from manual weighing of food, or the consideration of a single pellet as a single meal, or the mechanics of food dispensers, among others (Ali and Kravitz 2018; Ikeda, et al. 1986; Pan 1986). Moreover, almost all measurements of Ei published do not consider the fact that Ei intake is invariably the product of the mean MS and MF (Geary 2005) and that Ei computed during short intervals of time, or daily, weekly or on a monthly basis do not inform us about the quantitative aspects of Ei, satiation and satiety, the two most important modulators of Ei in the short and long terms. Furthermore, the determination of meal size as a marker of satiation or IMI as a marker of satiety in humans and rodent models of obesity produced conflicting results in part due to discrepant definitions used to determine meal size or IMI (Demaria-Pesce and Nicolaidis 1998; Leech, et al. 2015; Zorrilla et al. 2005a). For instance, the IMIt necessary to define a meal in rats was empirically determined (Castonguay et al. 1986; Langhans and Scharrer 1987; VanderWeele et al. 1982; Yeates, et al. 2001) even omitted in some studies (Furnes, et
al. 2009). Similarly, normal and mutant male or female mice models of different genetic background and age were used to determine meal size under several conditions using empirically (Bake, et al. 2014; Strohmayer and Smith 1987) or mathematically (Atalayer, et al. 2010; Richard, et al. 2011) defined IMIt. Moreover, in some instances, no IMIt criterion was used to define a meal in mice (Acosta-Rodriguez, et al. 2017; Goebel, et al. 2011). It is therefore important to note that different definitions used to categorize meals produced significant changes in meal parameters precluding their critical analysis and obscuring satiation and satiety as key parameters to understand feeding behavior (Castonguay et al. 1982; Kissileff 1970).

Recently, however, Zorrilla et al. provided for the first time a detailed analysis of the feeding pattern in the adult male rat by following an objective criterion validated mathematically to define a meal which included drinking as part of them (Geary 2005; Zorrilla et al. 2005a; Zorrilla et al. 2005b). The IMIt determined following those criteria reliably and consistently defined meals separated by an IMI with low probability to initiate a new meal initially, but increasing while approaching the next meal, thus objectively fulfilling the definition of satiety (Zorrilla et al. 2005a). This approach to determine the meal pattern in rats was also recently validated in a mouse model of mixed genetic background (Tabarin et al. 2007). Therefore, the work of Zorrilla et al. provided the conceptual framework to consistently assess and quantify feeding behavior and energy intake in animal models (Geary 2005). We have adopted the criteria validated by Zorrilla et al. in isolated rats (Zorrilla et al. 2005a) and in isolated mice (Tabarin et al. 2007) to determine the feeding pattern of social mice at different ages by using a sophisticated system to continuously capture single feeding events under minimal disturbance and for prolonged periods of time. Our results demonstrated that IMIt ≥5min defines a meal in social mice of both genders (Fig 2). Notably, this IMIt is very close to that determined by
Tabarin et al. who used that IMI ≥6min to describe the short-term feeding microstructure of single-caged male mice of mixed genetic background at 20-30 weeks of age (Tabarin et al. 2007). We also used IMI ≥6mins to determine the feeding microstructure of social mice, but the results were not different from those obtained by using IMI ≥5min (not shown). In fact, by imposing an IMI ≥5-6mins to define meals in social mice, we could visualize the behavioral satiety sequence between meals including grooming, wandering, playing, exploration, sniffing, resting and other non-feeding activities in an age- and sex-independent manner. These behaviors were observed during the nocturnal and diurnal photoperiods of the day, when the time separating meals is longer than 60min and 180min, respectively. Therefore, an IMI ≥5min appears to reliably define a meal in social mice while offering the conceptual and methodological framework to study satiation and satiety in socially housed mice of both genders.

Our results demonstrate that the average nocturnal Ei per unit of BW or the net daily Ei of male and female mice at 10 and 20 weeks of age is similar among genders and ages and corresponded to ≥80% of the total daily caloric intake (Fig 3). However, albeit Ei being comparable among genders and ages, the mean MS and MF were not. Indeed, social female mice ate larger meals than males, irrespective of age (Figs 4A). Of note, the nocturnal MS of 20w old social male mice were 0.642 ± 0.013 kCal, (Fig 4A) whereas those published by Tabarin et al. in isolated male mice of mixed ages around 20w was 0.486 ± 0.036 kCal (Tabarin et al. 2007). However, by using an empirically determined IMI ≥5mins, Strohmayer et al. recorded nocturnal liquid meal size from lean ~10w old male and female mice of 0.18 ± 0.04 and 0.25 ± 0.02 kCal, respectively, (assuming a caloric density of 1kCal/ml of a control rodent liquid diet, e.g., AIN-76) (Strohmayer and Smith 1987). These values are 3-4 times lower than those obtained by us (Fig 4A) and others (Tabarin et al. 2007) indicating potential methodological
differences. Although the caloric content of the liquid diet used by Strohmayer et al. was not reported, thus precluding proper comparison, it is worth noting that these discrepant results could also be attributed to methodological constraints introduced during the measurement of liquid diet, which was done to the nearest 0.5ml into a feeding tube (Strohmayer and Smith 1987). At any rate, Strohmayer et al. did demonstrate that the MS of a liquid diet is ~25% higher in 10w old female mice than in males, an estimation in close proximity to that we found in social mice at similar ages i.e., ~20%. Therefore, when all these results are taken together, MS in female mice is consistently higher than that of males at all ages tested and therefore, the satiation value of food is lower in females than that of males. These results may seem at odds with the well-documented effect that estrogens have in feeding behavior and the inhibition of Ei by reducing MS in animal models (Blaustein and Wade 1976; Eckel 2004; Geary and Asarian 1999; Zumpe and Michael 1970). However, recent data has not provided evidence that estrogens modulate the feeding pattern (Butera, et al. 2010) pointing out the need of better define MS to allow comparison among published data. At any rate, the role of estrogens in MS remains an open question.

An important point to be considered here is that mice housed in isolation usually have increased BW consequence of increased net Ei when compared to mice housed in groups (Chvedoff et al. 1980; Collier, et al. 1999). Interestingly, grouped mice tend to more efficiently conserve thermal energy than single-grouped mice, thus reducing heat loss and decreasing Ee (Chvedoff et al. 1980) thus potentially contributing to their net Ei. However, contrary to previous suggestions (Chvedoff et al. 1980; Collier et al. 1999), 20w old social males had 12.9 ± 0.9 nocturnal meals (Fig 4B) whereas isolated age matched males had 15.9 ± 1.1 (Tabarin et al. 2007). In addition, our results demonstrate that social male mice which had smaller MS exhibited increased MF whereas the
opposite was demonstrated for social females (Fig 4B) suggesting that MF compensates for changes in MS.

The regulation of meal frequency without affecting MS informs about the non-eating time spent between two meals i.e., satiety, which is reflected in the IMI (Strubbe and Woods 2004). Therefore, the fact that social males had more frequent meals than females (Fig 4B) does not imply that males are more satiated than females because their MS were accordingly different accommodating comparable net Ei. Notably, social males of 10 weeks of age ate a single meal every ~1h (i.e., IMI ~60mins) whereas age-matched females took ~2hs to eat a new meal (i.e., IMI ~120mins) (Fig 4D) indicating that the satiety value of food is twice as stronger for 10w old males than it is for aged-matched females. However, the nocturnal IMI of social females decreased at 20 weeks of age to male values (Fig 4D) indicating that the satiation value of food is comparable in older mice. Therefore, the mean nocturnal MF per se does not represent satiety in mice whereas IMI does. Of note, the IMI determined for 20w old social males (Fig 4D) are twice as higher as those obtained in isolated mice by Tabarin et al. (Tabarin et al. 2007). These apparent discrepancy is unlikely to be due to different meal criteria used to determine the meal pattern of mice but potentially to social factors such as isolation, which has been proposed to alter Ei leading to increased adiposity, at least in ~10w old C57BL/6J male mice (Sun, et al. 2014). Therefore, our results are compatible with the conclusion that nocturnal satiety under stable energy balance and BW is minimally affected by age in males, whereas that of social females decreases with age.

The determination of Ei under stable energy balance conditions such as when fed ad libitum does not reveal potential changes in Ee. However, BW changes after acutely imposing a negative energy balance by means of prolonged fasting indirectly does reflect Ee (Jensen, et al. 2013). In addition, driving energy balance towards the negative
side is expected to decrease satiation and/or satiety, leading to increases net Ei upon re-feeding, either by increasing MS, MF (or both) and/or by reducing IMI (Bellinger and Mendel 1975; Guss and Kissileff 2000; Schwartz et al. 2000). We tested these hypotheses in 16hs fated mice allowed to re-feed for 24hs. At this point it is important to mention that 16hs fasting is a harsh maneuver to single-housed mice resulting in the loss of fat and lean mass and up to 15% BW (Ayala, et al. 2006; Ayala, et al. 2010). As expected for house-grouped mice that better conserve energy, our results demonstrated less than 10% reduction in total BW after 16hs fasting (Fig 6A-B). Ee is expected to play a role in driving BW gain back to baseline and food deprivation is expected to increase Ei to compensate the deficit (Schwartz et al. 2000). In line, our results demonstrate that re-fed social female mice recovered their BW lost in response to prolonged fasting within the first 2hs of re-feeding whereas age-matched males required apparently longer time to attain baseline BW (Fig 6A-B). Notably, on one hand, the kinetics of BW recovery of 16hs fasted mice upon re-feeding was not reflected in their net Ei, which remained similar in males and females during the first 8hs of re-feeding (Table 1). On the other hand, however, BW recovery upon re-feeding was indeed reflected in Ei when adjusted to BW only in females of 10w during initial hours of re-feeding (Fig 6C-D). At any rate, when compared to the ad libitum daily Ei per unit of BW, Ei during the first 24hs of re-feeding was significantly higher demonstrating that a negative energy balance imposed by prolonged fasting increases net Ei and BW gained in males and females although at a different pace.

The increased Ei observed in social mice upon re-feeding was mostly the result of a transient increase in MS in 10w old female mice, which was significantly higher than that of males during first few hours following re-feeding (Fig 8A). In fact, our results demonstrate that the increased MS observed in response to fasting in 10w old females
decreases to reach *ad libitum* levels soon after allowing re-feeding whereas that of males remained close to baseline values throughout the re-feeding time. These results suggest that fasting minimally affects satiation in male and female mice, at least in the relative short term after re-feeding. Although this might appear counter intuitive for fasted mice, different mechanisms may contribute to the increased Ei observed in fasted mice, including satiety.

Indeed, the initial satiety responses of social mice to prolonged fasting were strong and unrelated to gender. In fact, the IMI in response to fasting was dramatically reduced during the first 2hs of re-feeding to a similar extent in males and females irrespective of age (Fig 9C-D) demonstrating that a negative energy balance triggers physiological mechanism aimed at potently inhibiting satiety. Further, social male and female mice recovered their satiety to baseline levels at a similar rate but reached different degrees of satiety 24hs after re-feeding (Fig 9D). Indeed, 20w old female mice reached baseline *ad libitum* values within 6-8hs of re-feeding to stabilize thereafter at 20-30% higher values than baseline thus indicating that re-feeding in response to fasting normalizes satiety within the time frame of maximal BW recovery (Fig 9 C-D). The IMI responses of social 20w old males to re-feeding also reached baseline *ad libitum* IMI values within 6-8 hours (Fig 9C). Notably, this recovery of satiety to baseline values occurred when male mice were gaining BW at a low rate (Fig 6A-B). Further, male mice showed higher satiety than females after 8hs of refeeding potentially contributing to the delay in BW recovery of males observed during re-feeding. Therefore, these results suggest that prolonged fasting inhibits satiety in social male and female mice to similar extents, that such inhibition is released upon re-feeding and that re-feeding increases satiety less efficiently in females than in males.
In conclusion, our results suggest that satiation and satiety in mice under stable or negative energy balance are under the control of sex- and age-dependent mechanisms. In addition, by using validated criteria to study the feeding pattern of social mice, our results offer for the first time a conceptual and methodological framework to test new hypothesis to elucidate the mechanisms involved in the control of satiation, satiety and Ei in mice.
6 References


6. Ahrén B & Holst JJ 2001 The cephalic insulin response to meal ingestion in humans is dependent on both cholinergic and noncholinergic mechanisms and is important for postprandial glycemia. *Diabetes* 50 1030-1038.


sustained weight loss over 2 years with the once-daily human GLP-1 analog, liraglutide. *Int J Obes (Lond)* **36** 843-854.


42. **Bray GA** 1995 Nutrient intake is modulated by peripheral peptide administration. *Obes Res 3 Suppl 4* 569S-572S.


44. **Bries E & Quijada M** 1978 Sugar solutions taste better (positive alliesthesia) after insulin [proceedings]. *J Physiol* 285 20P-21P.


46. **Broberger C, Landry M, Wong H, Walsh JN & Hokfelt T** 1997 Subtypes Y1 and Y2 of the neuropeptide Y receptor are respectively expressed in pro-opiomelanocortin- and neuropeptide-Y-containing neurons of the rat hypothalamic arcuate nucleus. *Neuroendocrinology* 66 393-408.


76. **Dangur J** 1988 Food intake in rats is increased by intracerebroventricular infusion of the somatostatin analogue SMS 201-995 and is decreased by somatostatin antiserum. *Peptides* **9** 211-213.


78. **Davis J** 2018 Hunger, ghrelin and the gut. *Brain Res* **1693** 154-158.


116. Furnes MW, Zhao CM & Chen D 2009 Development of obesity is associated with increased calories per meal rather than per day. A study of high-fat diet-induced obesity in young rats. *Obes Surg* 19 1430-1438.


of the type 1a growth hormone secretagogue receptor (GHS-R). Mol Cell Endocrinol 274 30-34.


133. **Goebel M, Stengel A, Wang L & Tache Y** 2011 Central nesfatin-1 reduces the nocturnal food intake in mice by reducing meal size and increasing inter-meal intervals. Peptides 32 36-43.


148. **Halm ST, Bottomley MA, Almutairi MM, Di Fulvio M & Halm DR** 2017 Survival and growth of C57BL/6J mice lacking the BK channel, Kcnma1: lower adult body weight occurs together with higher body fat. *Physiol Rep* 5


155. **Hayes MR, Kanoski SE, Alhadeff AL & Grill HJ** 2011 Comparative effects of the long-acting GLP-1 receptor ligands, liraglutide and exendin-4, on food intake and body weight suppression in rats. *Obesity (Silver Spring)* 19 1342-1349.


at multiple stages of virally induced type 1 diabetes in BB rats. *Autoimmunity* 44 137-148.


196. **Langhans W & Scharrer E** 1987 Role of fatty acid oxidation in control of meal pattern. *Behav Neural Biol* 47 7-16.


208. **Lin HC & Chey WY** 2003 Cholecystokinin and peptide YY are released by fat in either proximal or distal small intestine in dogs. *Regul Pept* 114 131-135.


McFarlane MR, Brown MS, Goldstein JL & Zhao TJ 2014 Induced ablation of ghrelin cells in adult mice does not decrease food intake, body weight, or response to high-fat diet. *Cell Metab* 20 54-60.


275. Primeaux SD, Barnes MJ, Braymer HD & Bray GA 2010 Sensitivity to the satiating effects of exendin 4 is decreased in obesity-prone Osborne-Mendel rats compared to obesity-resistant S5B/Pl rats. *Int J Obes (Lond)* 34 1427-1433.


