University of Massachusetts Amherst

ScholarWorks@UMass Amherst

Masters Theses

Dissertations and Theses

November 2014

Increased Body Weight in Adulthood Following a Peripubertal Stressor and Proposed Mechanism for Effects of Increased Adiposity on Estrogen-dependent Behaviors

Christina F. Gagliardi
University of Massachusetts Amherst

INCREASED BODY WEIGHT IN ADULTHOOD FOLLOWING A PERIPUBERTAL
STRESSOR AND PROPOSED MECHANISM FOR EFFECTS OF INCREASED
ADIPOSITY ON ESTROGEN-DEPENDENT BEHAVIORS

A Thesis Presented

by

CHRISTINA FELDER GAGLIARDI

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE

September 2014

Neuroscience and Behavior Program

INCREASED BODY WEIGHT IN ADULTHOOD FOLLOWING A PERIPUBERTAL
STRESSOR AND PROPOSED MECHANISM FOR EFFECTS OF INCREASED
ADIPOSITY ON ESTROGEN-DEPENDENT BEHAVIORS

A Thesis Presented

by

CHRISTINA FELDER GAGLIARDI

Approved as to style and content by:

_____
Jeffrey D. Blaustein, Chair

_____
Mary E. Harrington, Member

_____
Jerrold S. Meyer, Member

_____
Jeffrey D. Blaustein, Director
Neuroscience and Behavior Program

ABSTRACT

INCREASED BODY WEIGHT IN ADULTHOOD FOLLOWING A PERIPUBERTAL

STRESSOR AND PROPOSED MECHANISM FOR EFFECTS OF INCREASED

ADIPOSITY ON ESTROGEN-DEPENDENT BEHAVIORS

SEPTEMBER 2014

CHRISTINA FELDER GAGLIARDI, B.A., MOUNT HOLYOKE COLLEGE

B.S., UNIVERSITY OF MAINE ORONO

M.S., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by Jeffrey D. Blaustein

Exposure to certain stressors during a sensitive period around puberty can lead to

enduring effects on an animal's response to estradiol. In estradiol-influenced behaviors,

such as sexual receptivity, hippocampal-dependent learning and memory, depression-like

behavior, and anxiety-like behaviors, exposure to a peripubertal stressor such as shipping

stress or an injection of lipopolysaccharide (LPS) can eliminate or even reverse the

normal response to estradiol. In addition to regulating these behaviors, estradiol play a

role in the regulation of body weight. While some of the previous studies touched on

short-term effects on body weight, no systemic long-term study of the effects of a

peripubertal stressor on body weight, particularly without interruption by ovariectomy,

have been undertaken. This paper introduces a hypothesis that proposes that increased

adiposity following exposure to a peripubertal stressor leads to the changes to estrogen-

dependent behaviors through altered levels of estrogens and changes to estrogen

receptors. The first chapter examines body weight data collected during studies with

other aims, and then proposes an experiment to test whether either of two peripubertal

stressors results in increased weight gain and body weight. The following chapter proposes further experiments designed to determine the proximate mechanisms leading to weight gain following peripubertal stressors and the role of diet on weight gain. The final chapter proposes experiments to test the effects of adiposity on peripheral levels of testosterone, aromatase, estradiol, and estrone; central levels of estradiol and estrone; and estrogen receptors in the brain.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

THE SENSITIVE PERIPUBERTAL PERIOD, STRESS, ESTRADIOL-

INFLUENCED BEHAVIORS, AND OBESITY: BACKGROUND AND

HYPOTHESIS

<u>**Previous findings from the Blaustein laboratory**</u>

Introduction

Exposure to certain stressors during a sensitive period around puberty can lead to
enduring effects on an animal's response to estradiol. Specifically, exposure to a
peripubertal stressor can eliminate or even reverse the normal response to estradiol for a
variety of behaviors, including sexual receptivity, hippocampal-dependent learning and
memory, depression-like behavior, and anxiety-like behaviors. This effect was first
examined and established in the Blaustein laboratory, with a series of papers published
beginning in 2009. Work on this phenomenon continues in the laboratory with
exploration on multiple levels into the mechanisms by which these changes occur. This
section will review the major papers from the lab, in the order in which they were
published, as well as give a brief mention to preliminary findings from studies in
progress. It will conclude with a short summary of the findings thus far.

Establishment of the sensitive peripubertal period

The initial work establishing an effect of peripubertal stressors on sexual
receptivity in response to exogenous hormones tested sex, age, and different stressors.
This initial work was published in two papers (Laroche, Gasbarro, Herman, & Blaustein,
2009a, 2009b) and all completed in C57Bl/6 mice. Female mice were all ovariectomized

and administered estradiol benzoate and progesterone weekly before sexual behavior testing. Sexual behavior testing was conducted weekly for five weeks, beginning the week after ovariectomy. Sexual receptivity was measured by assessing the animal's lordosis quotient, or the number of times that the female exhibited a lordosis posture in response to a mount from a male mouse.

Laroche *et al.* first established that female mice shipped at 6 weeks of age have significantly reduced sexual receptivity after ovariectomy followed by injections of estradiol and progesterone than mice shipped at 12 weeks or animals bred in the laboratory (Laroche et al., 2009b). These differences in sexual receptivity are not due to differences in age of behavior testing; female mice shipped at 6 weeks and tested beginning at 8 or 14 weeks each show significantly reductions in lordosis quotient contrasted with animals shipped at 12 weeks and tested beginning at 14. The sensitive period was identified by examining mice shipped at 3, 4, 5, 6, 7, 8, 9, 10, and 12 weeks of age and tested beginning at week 14. Mice shipped at 6 weeks showed lower sexual receptivity than those shipped at ages of 7 weeks or more. The mice shipped at 6 weeks also showed decreased sexual receptivity compared with those mice shipped at 3 or 4, but only in the fifth test session. No difference was detected between mice shipped at 5 or 6 weeks. Corticosterone response was assessed after each behavior test session by examining plasma corticosterone levels. Corticosterone decreased with each additional week of testing for both 6 week and 12 week shipped animals; the only difference found between animals shipped at 6 and 12 weeks was following the first week of testing when 12 week animals had a significantly higher corticosterone level. In conclusion this paper

established that the effects of shipping on sexual receptivity are specific to female mice shipped at 5 or 6 weeks.

The second publication (Laroche et al., 2009a), addressed other peripubertal stressors and their short-term effects on corticosterone levels and body weight and any enduring effects on sexual receptivity. Restraint stress, food deprivation, and a multiple stressor paradigm (three daily bouts of restraint under bright light) increased plasma corticosterone levels in response to the stressor after some or all applications of the stressor. These three stressors decreased body weight as measured for several hours to several days following the stressor. While animals from all three stress paradigms showed an increase in sexual receptivity by week of testing (increased receptivity with additional tests), there were no differences between stressed individuals and controls. Injections of several concentrations of lipopolysaccharide (LPS), a bacterial endotoxin from the cell membrane of *Escherichia coli*, increased plasma corticosterone levels, although only the highest dose (1.5mg/kg body weight) significantly decreased sexual receptivity over several test sessions. This highest dose of LPS specifically decreases sexual receptivity on the third, fourth, and fifth test sessions. Finally, animals were injected with the high dose of LPS at 3, 4, 5, 6, 7, 8, and 10 weeks of age and tested for sexual receptivity in order to determine the sensitive window. Animals injected at 3,7, 8, and 10 weeks showed no decrease in sexual receptivity when compared to controls. Those animals injected at 4 and 5 weeks decreased during only one or two test sessions, while animals injected at 6 weeks significantly decreased sexual receptivity for the third, fourth, and fifth test sessions. In summary this paper revealed that the effects of peripubertal stressor on sexual receptivity are dependent on the type of stressor and that

an injection of a high dose of LPS at 6 weeks results in decreased sexual receptivity in response to estradiol.

Further investigations of effects of peripubertal stressors

Following the initial publications, research extended to include effects on other estradiol-influenced behaviors including anxiety-like behavior, depression-like behavior, and memory in female mice. These studies were conducted either in C57Bl/6 mice with some experiments performed in parallel with CD-1 mice, or in CD-1 mice alone. The first study discussed also examined estrogen receptors in areas involved in sexual behavior.

Further work (Ismail, Garas, & Blaustein, 2011) replicated the effects of shipping and LPS injection on sexual receptivity in CD-1 mice, an unrelated outbred strain, and examined differences in estrogen receptor alpha (ERα). An injection of LPS at 6 weeks significantly reduced sexual receptivity as compared to saline injected controls; an injection of LPS at 8 weeks only reduced sexual receptivity in the fifth test session. Shipping mice at ages 6 or 8 weeks decreased sexual receptivity relative to those shipped at 3, 4, and 10 weeks in most test sessions. Finally, the effects of shipping on ERα were examined in mice shipped at 4 or 6 weeks and euthanized one week after their second sexual behavior test. Animals shipped had 6 weeks showed significantly fewer ERα – stained cells in the arcuate nucleus, medial preoptic area, and ventromedial nucleus of the hypothalamus, but not in the anteroventral periventricular nucleus. These experiments replicated previous findings from C57Bl/6 mice, of the effects peripubertal stressor on sexual receptivity, in CD-1 mice and established a vulnerable peripubertal period of 6 weeks of age for both shipping stress and LPS injection in CD-1 mice. Additionally, this

paper demonstrates for the first time a difference in estrogen receptors in the brains of peripubertally stressed animals.

Another paper expanding on the initial findings, (Olesen, Ismail, Merchasin, & Blaustein, 2011), examined the effects of an injection of LPS on anxiety-like behaviors under several conditions. In all three experiments both LPS and saline control animals were split into two groups: animals either received an injection of estradiol benzoate followed by progesterone or vehicle alone. The first experiment used only C57Bl/6 mice injected at 6 weeks, while the second and third studies used C57Bl/6 and CD-1 mice respectively injected at both 6 and 8 weeks. Although the results of the individual behavioral tests (light/dark box, elevated plus maze, and marble burying task) vary, together they show general trends for an effect of LPS injection, hormone, and age. In almost all cases, saline treated mice that received estradiol exhibited lower anxiety-like behavior than those administered oil, regardless of age of saline treatment. Similarly, mice treated with LPS at 8 weeks that received estradiol showed decreased anxiety-like behavior compared to those that received vehicle alone. Importantly, mice that received LPS at 6 weeks had increases, or no change, in anxiety-like behavior when administered estradiol rather than oil depending on the strain and task. In two tests, mice treated with LPS at six weeks and oil showed decreased anxiety-like behaviors, compared to those that received saline and oil. In conclusion, this paper demonstrated an effect of a peripubertal injection of LPS on anxiety-like behavior in two unrelated strains of mice. A peripubertal injection of LPS alters response to estradiol and progesterone with the mice showing no change or an increase in anxiety-like behaviors compared to oil controls.

A paper examining memory, including tests of object recognition, object placement recognition, social discrimination, and social recognition revealed further effects of peripubertal stress (Ismail & Blaustein, 2013). CD-1 female mice were injected at either 6 or 10 weeks of age with saline or LPS and implanted with Silastic© capsules containing either estradiol in sesame oil or sesame oil alone at the time of ovariectomy. In the tests for object recognition, object placement recognition, and social discrimination, estradiol improved performance in mice treated with saline, at either age, and the mice treated with LPS at 10 weeks. However, mice injected with LPS at 6 weeks had no improvement on memory with estradiol. There were no differences between any groups in a test of general locomotion, indicating that the differences in investigatory behaviors were not due to differences in locomotion. Results on the social recognition task were inconclusive. Therefore, this work provides evidence that a peripubertal injection of LPS eliminates the positive effects of estradiol on cognitive tasks.

The most recently published paper, (Ismail, Kumlin, & Blaustein, 2013), focused on depression-like behaviors and was conducted in both C57Bl/6 and CD-1 mice. For both strains, there were two age groups of animals, one injected in the sensitive peripubertal period (6 weeks of age) and one at a later period (8 weeks for C57Bl/6 and 10 weeks for CD-1). At ovariectomy half of the animals received a capsule containing estradiol in sesame oil, the other half received capsules containing only oil. The results of the tests were similar between strains. In both the forced swim test and tail suspension test, the saline treated animals improve and show less depression-like behavior when administered exogenous estradiol compared to those administered an oil vehicle alone, regardless of treatment age. The same result is seen in animals treated with LPS at an

older age. In the animals injected with LPS at 6 weeks, estradiol increased depression-like behavior compared to oil. Neither strain displayed any differences in sucrose preference. Unlike the forced swim and tail suspension test, this task is associated with anhedonia rather than behavioral despair. As the authors point out, the role of estradiol in the anhedonia in unknown. Thus, the lack of response may indicate either lack of a role for estradiol in anhedonia. Further, the results may have been due to depleted estradiol levels at the time of test administration, rather than indicating a lack of difference in depression-like behavior. The differences in performance in the forced swim test or tail suspension test are not due to changes in general locomotion as there were no differences within either strain in locomotor activity. A subsequent unpublished analysis of the CD-1 data shows that in animals treated with vehicle only, those exposed to LPS performed significantly better than saline controls in both tests (Holder, unpublished). In summary, this paper demonstrates that in both C57Bl/6 and CD-1 mice a peripubertal injection of LPS results in an increase in depression-like behavior rather than a decrease following administration of estradiol.

Microglial activation

Recent work in the laboratory has begun to address the role of neuroinflammation in effecting the changes observed in estrogen receptors and behaviors. This work, (Holder, 2014), is focused on the role of microglial activation following the peripubertal stressor. Preliminary results show differences in microglial activation in several brain areas between animals subjected to either an injection of saline or LPS, or shipping, at either 6 or 8 weeks of age. These findings indicate that in the arcuate nucleus and

ventromedial hypothalamus, which play a role in feeding and sexual behaviors, LPS exposure increases microglial activation more in 6 week mice than 8 week.

Summary

Together this body of work demonstrates the specificity of a period around puberty in which certain stressors can elicit long-term changes in estradiol-influenced behaviors. The existence of a sensitive peripubertal period has been demonstrated in two unrelated strains of mice. While the effects were first established using shipping as a stressor, the multi-faceted, variable nature of the stressor led to attempts to find a more reliable stressor. Injection of LPS during the peripubertal period had similar effects on the response to hormones in tests of sexual, anxiety-like, and depression-like behavior. The effects of estradiol on depression- and anxiety-like behaviors appear to be paradoxical in peripubertally stressed animals, insofar as these animals showed increased rather than decreased depression- and anxiety-like behaviors. Additionally, an LPS injection appears to alter some of these same behaviors in the absence of estradiol when compared with saline treated controls. Exposure to a peripubertal injection of LPS also eliminates the positive effects of estradiol on memory as demonstrated by hippocampal cognitive tasks. A decrease in estrogen receptor alpha expression in areas of the brain associated with sexual behavior points to a potential mechanism by which response to estradiol may be altered. Finally, current work has begun to show changes in neuroinflammation following the stressors at different ages, demonstrating a possible difference in early response to the stressors.

Obesity: connections with puberty, depression, estrogens, and inflammation

Introduction

In addition to the behaviors previously examined in the Blaustein laboratory, estradiol regulates body weight (Brown & Clegg, 2010; Wade, Gray, & Bartness, 1985). While some of the previous studies touched on short-term effects on body weight, no systemic long-term study of the effects of a peripubertal stressor on body weight, particularly without interruption by ovariectomy, have been undertaken. This area is a potential avenue both for exploration of the effects on body weight but also for the potential effects of increased adipose tissue on estrogen-influenced behaviors. Studies from both human and rodent literature have pointed to interrelations between inflammation, obesity, puberty, and depression with a complex relationship between obesity and estrogens.

Some of these links are explored in the human literature, and some in rodent models. While care must be taken not to try to directly relate results from one species to another, female mice are a relevant model group for puberty in girls as will be discussed. This section will first discuss connections between puberty, obesity, depression, and estrogens in the human literature, and then will review other connections between these factors, as well as inflammation, in mice. The section will conclude with a discussion of the similarities and differences in female puberty in humans and mice.

Selected findings from human literature

For women, the average age of onset for mental disorders, including depression and anxiety, falls during adolescence (Hayward & Sanborn, 2002). Puberty, timing of

onset of puberty, and stress during puberty in girls have all been implicated in the development of depression, vulnerability to later life stress, weight gain, and insulin sensitivity (Ellis & Garber, 2000; Ge, Conger, & Elder, 1996; McCabe, Ricciardelli, & Finemore, 2002; Patton et al., 1996). Women with severe mental illnesses are more likely to be obese (Daumit et al., 2003), and recent work indicates that obesity is predictive of depression (Luppino et al., 2010). A recent longitudinal study suggests that it is the metabolic dysregulations that often occur in obesity, rather than obesity itself, that increase the risk for developing depression (Hamer, Batty, & Kivimaki, 2012).

The relationship between obesity and depression is complex and reciprocal. While obesity increases the risk for depression over time, depression also predicts the development of obesity (M. S. Faith et al., 2011; Luppino et al., 2010). This is particularly relevant in adolescent girls where the onset of depression in early adolescence predicts the development of obesity in later adolescence, and development of obesity during adolescence predicts depression in adulthood (Marmorstein, Iacono, & Legrand, 2014). Specifically, increased visceral adipose tissue is associated with depression in both men and women (Rivenes, Harvey, & Mykletun, 2009).

Just as obesity and depression are interlinked, the associations between obesity, metabolic symptoms, reproductive difficulties, and estrogens are intertwined. Visceral fat is also associated with insulin resistance, inflammation, and anovulation (Androulakis et al., 2014; Melka et al., 2013). Not only is adipose tissue in adulthood associated with metabolic phenotypes, but the timing of pubertal onset and observed changes in body weight during puberty are predictive of obesity and metabolic syndrome in adulthood (Ferreira, Twisk, van Mechelen, Kemper, & Stehouwer, 2005; Lawlor, 2005; McGill et

al., 2000). Adipose tissue synthesizes testosterone in obese women without underlying metabolic syndrome (Quinkler, 2004) and has been implicated as a source of testosterone in women with polycystic ovary syndrome (Fassnacht et al., 2003). Complicating the matter further, adipose tissue, specifically the stromal cells, is known to have high aromatase activity and to produce estrogens, particularly estrone (Cleland, Mendelson, & Simpson, 1983; Simpson, Merrill, Hollub, Graham-Lorence, & Mendelson, 1989).

Selected findings from rodent literature

Rodent models allow investigation into processes that researchers are unable to address directly in humans due to time scale, practical, or ethical considerations. Most of the information below comes from work in mice, but for clarity the studies performed in rats or hamsters are noted as such. These studies show links between puberty, obesity, estradiol, and inflammation; these relationships are frequently complex and like the relationship between such elements in humans are far from fully understood. A few of these relationships are discussed here.

The relationship between obesity, estradiol, inflammation, and puberty is tangled. Removal of endogenous estradiol via ovariectomy increases adipose tissue, while estradiol replacement following ovariectomy decreases adipose tissue in both rats (Wade et al., 1985) and humans (Keith et al., 2006). Similarly, ERα knock out mice show increased adipose tissue (Heine, Taylor, Iwamoto, Lubahn, & Cooke, 2000). Of particular relevance to the work outlined below, lower than usual levels of estrogens during early development can also lead to obesity during puberty as evidenced that the offspring of dams fed low phytoestrogen feed during pregnancy develop obesity at puberty, indicating

that alterations in levels of estrogens during development can lead to enduring effects during puberty and adulthood (Grün & Blumberg, 2009; Ruhlen et al., 2007).

Obesity is commonly referenced as an inflammatory disease (Kennedy, Martinez, Chuang, LaPoint, & McIntosh, 2008). While numerous factors have been implicated, the inflammatory response and obesity are linked at least partially by the mediation of toll-like receptor 4 (*Tlr4*), a gene involved in innate immunity (Milanski et al., 2009; Saberi et al., 2009). A loss of function mutation in *Tlr4* has been shown to prevent obesity in mice fed a high fat diet that would normally induce obesity (Tsukumo et al., 2007). Male mice exposed to a prenatal stressor show increased body weight in adulthood as well as increased expression of Tlr4 as compared to unstressed males, or females regardless of prenatal stress, suggesting that early life stressors can affect both body weight and the expression of *Tlr4* in adulthood (Bolton et al., 2013; Bolton et al., 2012). Microglia also increase in number in the hypothalamus in mice exposed to dim light at night, although the specific mechanisms are still unclear (Fonken, Lieberman, Weil, & Nelson, 2013). This increase can be exacerbated by a high fat diet (Fonken, Lieberman, et al., 2013). Additionally, hamsters exposed to dim light at night exhibit a heightened neuroinflammatory response to an injection of LPS (Fonken, Weil, & Nelson, 2013). In male mice, the exposure to dim light at night has also been shown to lead to obesity (Fonken, Aubrecht, Melendez-Fernandez, Weil, & Nelson, 2013; Fonken et al., 2010) and depression-like behaviors (Fonken & Nelson, 2013); female hamsters also show depression-like behaviors following exposure to dim light at night (Bedrosian, Weil, & Nelson, 2012).

Puberty

Puberty is an important developmental stage in which many physiological and behavioral changes occur and have lasting effects on both physiology and behavior later in life (Forbes & Dahl, 2010; Marshall & Tanner, 1969). Key among these changes are altered levels of hormone production, altered sensitivity to hormones, and remodeling of the brain, due in part to steroid hormones (Schulz, Molenda-Figueira, & Sisk, 2009). In girls, puberty is an extended period beginning with the onset of breast development and ending with the first menses and takes approximately two to three years (Sizonenko, 1989; Tanner, 1962). In female mice, puberty begins with vaginal opening and ends with regular estrous cycle and reproductive competence; these events can be separated by several weeks (Vandenbergh, 1967, 1969). While several weeks may seem short in duration, in an animal that has a lifespan of less than two years (for C57Bl/6 (Russell, 1966)), this constitutes a prolonged period. Due to the similarly prolonged pubertal period, female mice are a relevant model for puberty in human girls.

Hypothesis for role of obesity in mediating estrogen-dependent processes following a peripubertal stressor

In light of the possibility of an increase in body weight, a new hypothesis emerges for the mechanism by which peripubertal stressors lead to the changes enumerated above. The overarching hypothesis is that peripubertal stress induces a metabolic syndrome and obesity, which in turn alters the hormonal environment, and leads to impaired response to estradiol. Because the human literature examines women and girls with ovaries, this hypothesis is proposed for intact females, although the enduring effects after ovariectomy

and with exogenous hormone administration are addressed as well. The following

hypothesis is also illustrated in Figure 1.
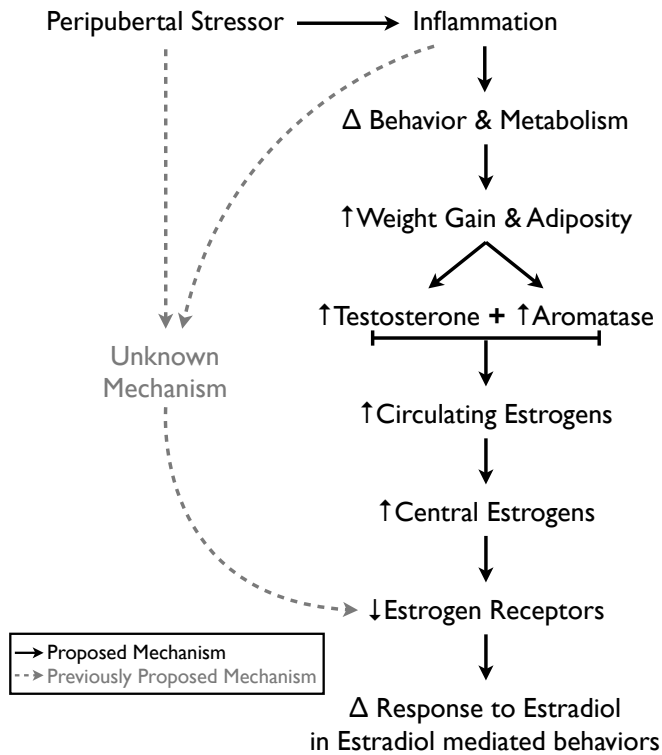
Figure 1. Hypothesis for obesity as a mediator of estrogen-dependent processes following peripubertal stress.

It is hypothesized that a peripubertal stressor leads to inflammation in the brain.

This inflammation leads to changes in behavior and metabolism. These changes in turn

lead to increased weight gain and adiposity. The increased adipose tissue produces

increased plasma levels of testosterone. Increased adiposity also leads to increased

aromatase activity. This increased aromatase activity results in increased peripheral

circulating estrogens. Increased levels of estrogens in the periphery lead to increased

levels of estrogens in the brain. These increased central levels of estrogens result in long-

term changes in the numbers, activity, and/or sensitivity of estrogen receptors in the

brain. The changes in receptors may be limited to certain receptor types or to specific regions. Finally, following ovariectomy, response to exogenous estradiol administration is altered due to the changes in receptors as well as enduring endogenous estrogen production by adipose tissue.

Overview of the following chapters

The hypothesis proposed above presents several unanswered questions and multiple avenues for investigation. Because other work already focuses on the links between peripubertal stressors and inflammation, the remaining sections here will address only the following areas.

1) Is there an increase in weight gain and body weight following peripubertal stressor?

2) What is the proximate cause of such weight gain?

3) Is obesity a mediator of estrogen dependent processes following pubertal stress and does it work through the proposed mechanism?

The first question will be addressed in Chapter 2, which will examine data collected during several studies each with other aims. The chapter will then propose a study to test the question directly while addressing limitations for analyzing weight data raised by the other studies from which data was collected. The second question will be addressed in Chapter 3 where a primary and alternative hypothesis will each be presented and followed by proposed studies to address the main points of the hypotheses. The third point will be addressed in Chapter 4 where the questions from the hypothesis above will be reviewed, and a study proposed to examine the effects of adiposity on several factors.

While each of these chapters builds on expected results from the previous chapters, each chapter will also briefly discuss possible alternative future directions.

CHAPTER 2

WEIGHT GAIN INCREASE FOLLOWING EXPOSURE TO PERIPUBERTAL STRESSOR

Introduction

Three experiments with different aims gathered weight data as part of the experimental design, mainly to monitor animal health. Several members of the Blaustein lab conducted these studies. The weight data from each of these three studies were analyzed to determine if there is an effect of peripubertal stress on body weight in adulthood. Because different experimental paradigms were used in each study, groups cannot be compared across studies nor combined for greater statistical power. However, the results of each study when examined side by side can provide insight into body weight change with age following different peripubertal stressors in two disparate strains of mice. Statistical analyses were conducted using HLM software to conduct hierarchical linear regressions. Hierarchical modeling was chosen to analyze the data for several reasons, most importantly because the data vary across both discrete (treatment) and continuous (age) predictors.

Effect of mouse strain

As each of these experiments had a discrete aim, they each used the strain of mouse most appropriate for the questions being asked. The first and third experiment used CD-1 mice, which are outbred and are expected to show variability between animals. The second experiment used C57Bl/6 inbred mice, which are expected to show less individual variability, as they are genetically identical. CD-1 mice are larger than

C57Bl6 mice at all age points studied and have other known differences as well, including differences in sensitivity to estradiol (Spearow, 1999). Because two different strains of mice were used across these three experiments, the results of the weight analyses may yield information about other differences in the response to peripubertal stress between strains or support previous demonstrations that strains are affected similarly.

Effect of type of peripubertal stressor

The three experiments also use two different types of peripubertal stressors, as well as two types of controls (Table 1). While the first and third experiment both use an LPS injection as a stressor, and each include a saline injection control, the first experiment also contains an non manipulated control group, which received no injection. The second experiment also contains an LPS injected group, with a saline control group, but also includes a group of mice, with the same birthdate, that were not injected but were instead shipped to the research facility on the same day as injections were administered to the other groups. The inclusion of two different stress types, as well as a non-injected control, may allow conclusions about the similarity in effect of the different stressors and support the use of a saline injection as an adequate control for LPS injection.

Table 1

Stressor and Control Groups for Previous Experiments

	Experiment 1	Experiment 2	Experiment 3
Peripubertal Stressors	LPS injection	LPS injection Shipping	LPS injection
Controls	Saline injection Non-injected	Saline injection	Saline injection

Experiment 1

Experimental aim

The aim of this study was to examine the effects on estrous cyclicity in animals administered a peripubertal stressor, in order to better understand the effects of such a stressor on the hormonal environment without an invasive measure of plasma estradiol. Animals were weighed before and after the peripubertal stress as well as before and after ovariectomy in adulthood.

Methods

Animal use was overseen by the Institutional Animal Care and Use Committee of the University of Massachusetts Amherst. 25 female outbred CD1 mice were purchased from Charles River Laboratories and arrived at 21 days of age (P21). Animals were housed in a temperature controlled, reverse light (14:10 LD, lights off at 10am) colony room. The animals were housed 4-5 to a cage lined with CareFRESH bedding, and were provided with a Nestlet at cage change. Cages were covered with a microisolator lid. All animals had *ad libitum* access to phytoestrogen-reduced mouse chow [Teklad 2014] and water.

All animals were monitored for vaginal opening by visual inspection beginning the day after arrival. Following vaginal opening, cells were collected by vaginal lavage from each animal daily (omitting P77-P83) until ovariectomy.

On P42 animals received an intraperitoneal injection of either sterile saline (n = 8) or 1.5 mg LPS/kg body weight (n = 9), LPS was dissolved in sterile saline at a concentration of 0.1mg/ml. Sickness behavior was monitored at 30 minutes, 4, 24, and 48

hours and was scored by assessing ptosis, piloerection, huddling, and lethargy (after (Ismail et al., 2011)). Body weight was also recorded. An additional group of eight animals received no injection.

Animals were weighed in a 2l glass beaker on an Ohaus animal scale. At P113 all animals were weighed prior to ovariectomy. Animal weights were tracked for several days post surgery to check that animals stabilized and regained the weight lost during and immediately following surgery.

Weights from six days between P28 and P115 were analyzed by hierarchical regression to determine if there is a difference in body weight over time between the LPS, saline, and not injected groups.

Results

Regression analysis shows a significant difference between the LPS and saline treated groups ($p < 0.05$) and between the control (non-injected) and saline treated groups ($p < 0.05$). No significant difference was detected between the LPS and control groups ($p = 0.90$). Analysis did reveal a significant difference in intercept between the saline and control groups ($p < 0.05$); there were no significant difference in intercept between LPS and saline ($p = 0.59$), although there was a trend towards difference between LPS and non-injected controls ($p = 0.06$). The analysis did detect a significant amount of variance in body weight that could not be explained by treatment effect at each age ($p < 0.05$). Mean body weights for each group at each age analyzed and linear trend are shown in Figure 2. As adults, LPS and control animals weighed significantly ($p_{LPS-Saline} < 0.05$, $p_{Control-Saline} < 0.05$) more than saline treated animals as exemplified at by the mean body weights

20

at P113 which were 32.1g for the LPS treated animals, 27.6g for the saline treated animals, and 33.1g for the animals that did not receive an injection.
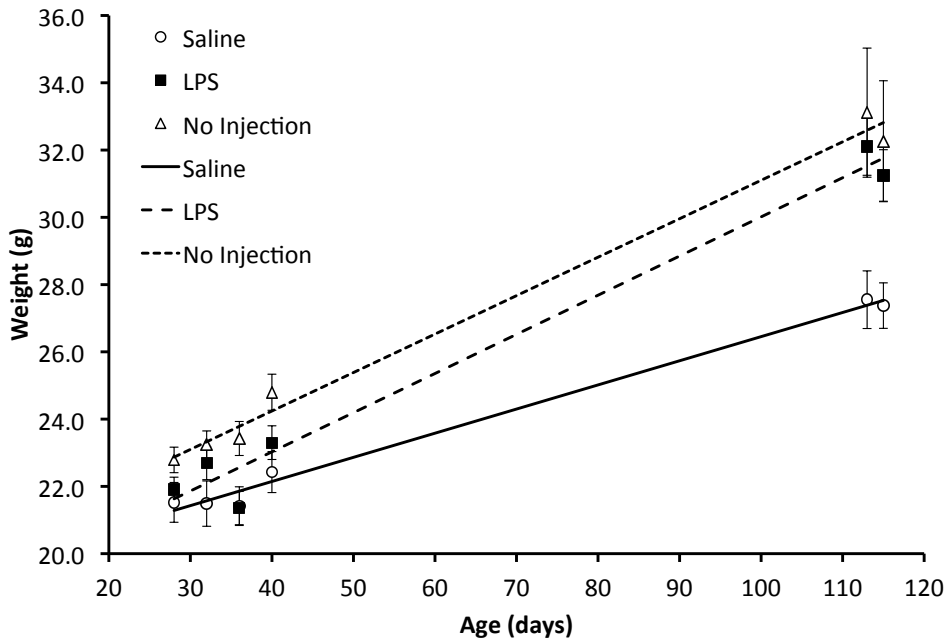
Figure 2. Mean body weights for Experiment 1 for each treatment group at each age analyzed with associated linear trend lines. Error bars represent standard error of the means.

Discussion

There is a difference in body weight gain between LPS and saline treated mice with LPS treated mice having a greater increase in weight than saline treated animals. While this increase was hypothesized, the difference in increase in body weight of the control group as compared to the saline treated was not. Indeed the similarity in body weight increase between the LPS and control groups is problematic. Rather than suggesting that peripubertal LPS treatment increased weight gain, these data indicate that the saline treated control mice have a slowed or decreased weight gain. Additionally, it is possible that the result of any injection during this sensitive period is more complicated than previously thought. Specifically, it is possible that any injection during this period

causes decreased weight gain compared to non-injected controls. However, the difference in intercept, that is to say the difference in the body weights on the first day weights were recorded, suggests that there may have been a difference in body weights between the injected groups and the non-injected control group prior to administration of saline or LPS.

The unexplained variance in the model is most likely due to the small sample groups. These small sample groups and the lack of data points between the peripubertal period and adulthood, make this analysis less valuable than it could be.

It should be noted here that this experiment was carried out under housing conditions that were new to the laboratory at the time, namely the inclusion of a static microisolator lid covering the cage. This practice was abandoned by the laboratory following adverse outcomes for animals housed under these conditions not previously seen in the lab. Moreover, the use of static microisolator lids has been demonstrated to increase heat, humidity, ammonia, and carbon dioxide levels inside such cages (Gonder & Laber, 2007). The use of these cages may have had detrimental effects on health and or feeding in all animals that received injections causing decreased weight gain over time, which may have been masked in LPS treated animals by the increased weight gain caused by the LPS treatment.

In summary the results here do not support the hypothesis and cannot be considered conclusive, as the two control groups (saline and non-injected) were significantly different from each other. The observation, that the control group more closely resembled the LPS group than saline group, cannot be explained.

Experiment 2

Experimental aim

The aim of this study was to replicate previous findings demonstrating decreased sensitivity to estradiol as demonstrated by decreased lordosis quotient in adulthood following a peripubertal stressor. The replication study was performed following several large-scale disruptions to the laboratory including an outbreak of murine parvovirus and construction surrounding the building, which resulted in sound and vibrations thought to disrupt mouse reproductive behavior (R. Faith & Miller, 2007; Rasmussen, Glickman, Norinsky, Quimby, & Tolwani, 2009; Turner, Bauer, & Rybak, 2007). Several previous replications were unsuccessful; although the reasons for this are unclear, the introduction of new housing conditions is thought to have played a role. This study was performed with the strain of mice used in earlier experiments. It also used both shipping during the peripubertal period and injection of LPS. Animals were weighed throughout the experiment in order to monitor animal health.

Methods

Twenty-four female inbred C57Bl/6 mice were purchased from Charles River Laboratories; of these, 16 animals arrived at P22 and the remaining 8 arrived at P36. Animals were housed with the same colony room, food, and water conditions as described in the first study. The animals were housed 4 to a cage lined with sanichips, and were provided with a Nestlet at cage change.

On P36 the animals that arrived at P22 received either a saline injection (n = 8) or an injection of LPS (n = 8), as previously described. The animals that arrived at P36

received no injection (n = 8). Sickness behavior and body weight were also recorded as described in the first study.

Animals were weighed as previously described, at 8-9 am (lights out at 10am) every 4-8 days. At P85, all animals were weighed prior to ovariectomy. Animal weights were tracked for seven days post surgery to check that animals stabilized and regained the weight lost during and immediately following surgery.

Beginning at P93 animals were injected and tested for sexual behavior weekly, for five weeks. Animals were injected weekly with 2µg estradiol benzoate in 0.1mL sesame oil subcutaneously followed 48 hours later with a subcutaneous injection of 100µg progesterone in 0.1mL sesame oil. Animals were tested for lordosis response six hours after progesterone injection.

Body weights from 13 separate days, beginning with P48 and ending with P123, were analyzed by hierarchical regression to determine if there is a difference in body weight over time between the LPS, saline, and peripubertally shipped groups.

Results

Regression analysis shows a significant difference between the shipped (peripubertally shipped) and saline treated groups ($p<0.05$) and between the shipped and LPS treated groups ($p<0.05$). No significant difference was detected between the LPS and saline ($p=0.997$). Analysis did not reveal a significant difference in intercept between any groups ($p_{LPS-Shipping}=0.48$, $p_{LPS-Saline}=0.67$, $p_{Saline-Shipping}=0.78$). Mean body weights for each group at the ages analyzed and linear trend lines can be seen in Figure 3. At P123, the final weight measured, shipped animals weighed significantly more than saline ($p<0.05$) and LPS ($p<0.05$) treated animals where the mean body weights were 23.6g for

24

the LPS treated animals, 23.9g for the saline treated animals, and 25.9g for the

peripubertally shipped animals.
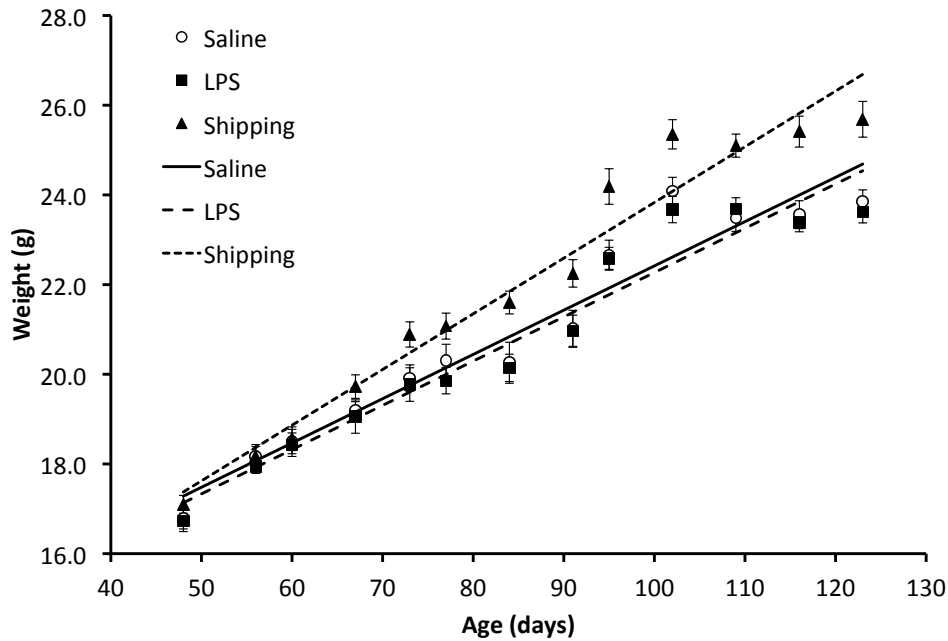
Figure 3. Mean body weights for Experiment 2 for each treatment group at each age
analyzed with associated linear trend lines. Error bars represent standard error of the
means.

Discussion

As expected shipped animals had an increased body weight gain contrasted with

the saline treated animals. No difference was observed in weight gain between saline and

LPS treated groups; in fact, the similarity between the slopes of the linear regressions of

the two groups is striking (see Figure 3). These results clearly demonstrate the effects of

shipping at P36 rather than P22 on body weight, but it is less clear that it is simply a

peripubertal stressor that produces these results. Indeed, as LPS failed to produce a

difference in body weight, it is clear that either the two peripubertal stressors are not

equal in their ability to produce weight gain, acting as they presumably do through

slightly different mechanisms or combinations of mechanisms, or that the LPS did not act as a peripubertal stressor.

Indeed, although the LPS animals lost more weight following injection (Appendix, Figure A1), they did not show levels of sickness behavior comparable to previous studies (Appendix, Figure A2). Additionally, the results of the sexual behavior testing showed no difference in lordosis quotient between any of the groups, although all groups displayed abnormally low lordosis quotients with high variability (Appendix, Figure A3). These results suggest that the LPS failed to make the animals as sick as expected, and hence the injection failed to elicit the previously observed decrease in sexual receptivity. In light of this, it is not surprising that the LPS treated animals failed to show an increase in weight gain as compared to the saline controls. It is believed that the method of preparing the LPS solution used for this study was unreliable and that the dosage may have been far lower than intended. In this study, the LPS was prepared in an extremely small volume by an inexperienced laboratory member. It is believed that the low mass of LPS used, static electricity, and inexperience led to the loss of a substantial portion of the LPS before saline was added.

It is interesting to note that although the animals that were shipped during the peripubertal period did not show the expected decrease in lordosis quotient, they did show increased weight gain. It is likely that the conditions in the laboratory were still not sufficiently similar to the conditions under which the previous results (decreased lordosis quotient in peripubertally stressed mice) were observed. The weight gain may either be very robust, persisting even where other effects do not, or may be due to some other element of the peripubertal stressor.

In conclusion, together these results demonstrate that shipping during the peripubertal period results in higher weight gain in C57Bl/6 mice compared to mice shipped prior to puberty even in the absence of other previously observed outcomes. The ability of an LPS injection to result in increased weight gain compared to saline controls could ultimately not be conclusively proved or disproved as the animals failed to become as sick as expected following the injection.

Experiment 3

Experimental aim

This study was conducted to replicate previous findings showing that exposure to an LPS injection during the peripubertal period resulted in altered expression of depression-like behavior in the presence and absence of estradiol. Additionally, this study aimed to examine microglial expression, as a marker of inflammation, in areas of the brain involved in depression-like behavior. In this study, animals were implanted with capsules containing either estradiol in oil or the oil vehicle alone at ovariectomy; therefore the examination of body weights was conducted beginning at ovariectomy at which time the animals become four distinct groups. In this experiment animals were weighed every four days.

Methods

Thirty-two female outbred CD-1 mice were purchased from Charles River Laboratories and arrived at P21. Animals were housed with the same colony room, food, and water conditions used in the previous two studies. The animals were housed 4 to a cage lined with sanichips and were provided with a Nestlet at cage change. Prior to

ovariectomy the animals were housed in cages topped with a microisolator lid in a ventilated cage rack; following ovariectomy, animals were housed in shoebox cages with a wire mesh lid on a wire rack.

On P42 animals received an injection of either saline (n =16) or LPS (n = 16), as previously described. Sickness behavior and body weight were recorded and assessed as in the previous two studies.

Animals were weighed as previously described at 8:45-9:45 am (lights out at 10am) every 4 days. At P78 all animals were weighed prior to ovariectomy. All animals were ovariectomized and implanted with a Silastic© capsule (length: 2.5cm, internal diameter: 1.57mm, external diameter: 3.18mm) containing either 50µg E2 in 25µl sesame oil or 25µl sesame oil vehicle alone. Animal weights were tracked for seven days post surgery to check that animals stabilized and regained the weight lost during and immediately following surgery.

One week following ovariectomy animals were tested in an open field test to assess differences in general locomotor behavior. Two and three weeks following ovariectomy the animals were then subjected to the tail suspension test and forced swim test to examine depression-like behavior.

Weights from eight days, beginning with P78 and ending with P106, were analyzed by hierarchical regression to determine if there is a difference in body weight over time between the groups, and specifically whether there is an interaction between treatment (LPS or saline) and hormone treatment (estradiol or vehicle).

Results

Regression analysis shows a significant main of effect of hormone ($p<0.05$) but not of treatment ($p=0.16$). There is no significant interaction between treatment and hormone ($p=0.25$). Analysis did not find a significant difference in intercept for any terms ($p_{Treatment}=0.95$, $p_{Hormone}=0.23$, $p_{Interaction}=0.39$). The analysis did detect a significant amount of variance in body weight that could not be explained by treatment, hormone, or interaction at each age ($p<0.05$). Mean body weights for each group at the ages analyzed and linear trend lines can examined in Figure 4. At the final weight measured on P106, there was no significant difference ($p=0.54$) in body weight between LPS and saline treated animals (when collapsed across hormone capsule type), but animals with an oil vehicle capsule weighed significantly ($p<0.05$) more than those with an estradiol-containing capsule (when collapsed across treatment).
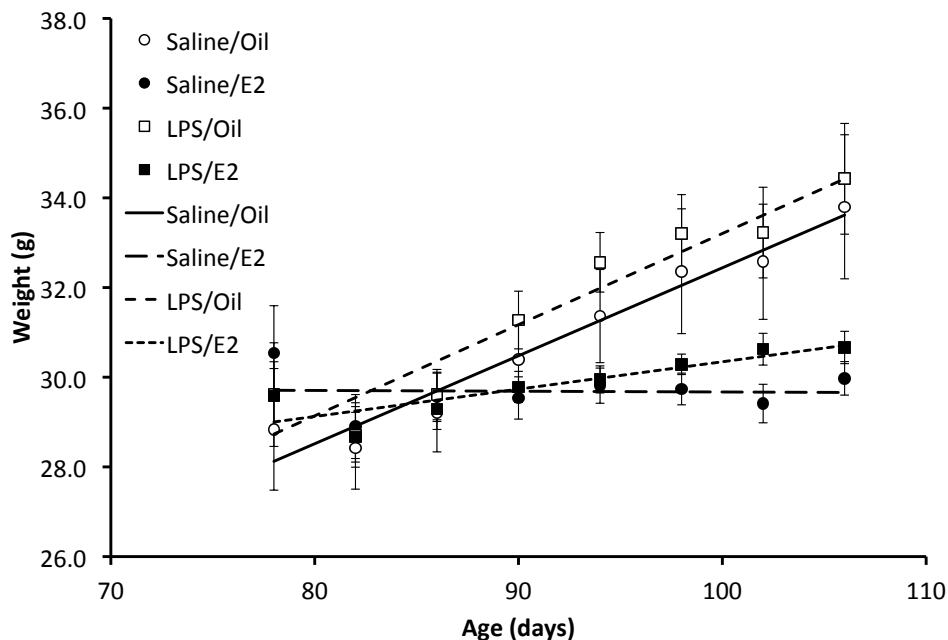
Figure 4. Mean body weights for Experiment 3 for each treatment group at each age analyzed with associated linear trend lines. Error bars represent standard error of the means.

Discussion

Animals implanted with an oil capsule had increased weight gain compared to those with estradiol capsules as predicted. The lack of increased weight gain in animals treated with LPS was unexpected, as was the lack of interaction between LPS treatment and capsule. A possibility for the lack of effect is that the age at which the animals were ovariectomized may have impacted weight gain. The animals in this study were ovariectomized five weeks earlier than the animals in the first study.

In conclusion these data do not support the hypothesis, nor do they replicate the results of the first study showing that an LPS injection during the peripubertal period results in increased weight gain and higher body weight in adulthood in CD-1 mice.

Conclusions

Taken singly these studies do not provide strong evidence for the universality of increased weight gain following a peripubertal stressor. However, together these results begin to paint a clearer picture of the impact of peripubertal stressors on body weight. While none of the studies individually can answer the question of whether different types of peripubertal stressors cause increased weight gain in genetically diverse strains of mice, the individual results tell us the following:

1) LPS injection may increase weight gain or may mask, or counteract, decreased weight gain following any injection during the peripubertal period in CD-1 mice

2) Shipping during the peripubertal period results in increased weight gain as compared to saline treated mice shipped prior to the period in C57Bl/6

3) LPS injection during the peripubertal period may result in increased weight gain as compared to saline injection in CD-1 mice but may be confounded by age of ovariectomy

Together these studies indicate that, although both LPS and shipping may result in increased weight gain as compared to saline controls, and that this weight gain might be seen in unrelated strains of mice, many details still need to be clarified.

Limitations and unanswered questions

Despite the conclusions that begin to emerge from the previous three studies, some of the initial questions remain unanswered due to limitations of each experiment. In order to better understand how ultimately to answer these questions, the limitations must be identified and addressed. They fit into three main areas: adequate controls for the purposes of comparing weight gain, reproducibility between and within strains and stressors, and the effects of timing of ovariectomy and administration of hormones.

First, because the second and third experiments lacked a non-injected control group, the groups in each of these experiments cannot adequately be compared. In the second study, the peripubertally shipped mice and mice shipped earlier in life should have been compared with a group shipped earlier in life but not injected; without such comparison it is unclear whether shipping truly increases weight gain. The results of the second study could indeed show a decrease in weight gain in the saline injected group as seen in the first study, with the peripubertally shipped mice showing normal weight gain. However, without a group shipped prior to puberty and not injected, this is impossible to determine. The third study also lacked a non-injected group and it is unclear how such a group would have compared to the saline or LPS groups.

Second, while there are LPS data from CD-1 mice and peripubertal shipping data from C57Bl/6 mice, there is no indication that either strain responds to both of the two different stressors in a similar manner in terms of weight gain, nor indeed that the two strains show similar increases in weight gain to one another following the same stressor. This point is key in the understanding of the effects of these stressors; regardless of other experimental limitations, the lack of these data alone prohibits drawing conclusions on the effect of peripubertal stress on weight gain.

Third, the increase in weight gain appears to begin in adulthood, rather than immediately following the stressor (as seen in Figure 3); therefore, ovariectomy at this age may alter results. The differences in study design make it difficult to draw conclusions about the effects that ovariectomy, or differing hormonal treatments, may have had on differences in weight gain between groups. In the first study the weight data were only recorded for two days following the surgery, making examination of longer-term effects impossible. In the second study, weight data were available for five weeks following ovariectomy, but animals were treated weekly with estradiol injections. It should be noted that, in these animals, the difference in weight was significant prior to ovariectomy. In the third study, weight data were available for four weeks following ovariectomy but because animals received either constant levels of estradiol or no estradiol, again it is difficult to piece apart the effects that the surgery itself might be having on body weight longer term (following the recovery period) versus the effects of treatment on body weight. These animals were ovariectomized five weeks earlier than the CD-1 mice from the first study (one week earlier than the C57Bl/6 mice in the second

study); it may be that the ovariectomy somehow interfered with an emerging difference in weight gain.

Together, these differences in the three studies, as well as the limitations of the individual studies, such as LPS failing to induce normal sickness in the second study, combine to produce a very tenuous basis for conclusions. In order to conclude that peripubertal stressors do indeed increase weight gain, and body weight in adulthood, additional studies are necessary.

Proposed experiment to test alterations to weight gain following a peripubertal stressor

Experimental basis

The final section of this chapter proposes a study, which should provide more conclusive evidence regarding the effects of LPS and shipping on weight gain. This section discusses how these experiments will overcome the limitations of the previous experiments.

First, this study addresses the lack of comparability between strains and stressors by including both stressors in a single experiment and then replicating the experiment in the other strain. Because both strains will be exposed to both stressors, the universality of the increased weight gain across strains should be evident despite the different body sizes of the two strains. Similarly, in the event that the stressors affect the strains differently, this should be evident both within and between the two experiments.

Second, these experiments address the lack of control group for the second and third experiments by including a non-injected control group shipped at P21 which will

serve both as a control for the animals injected with saline and LPS at P42, and as a control for the animals shipped at P42. In order to establish that it is not simply that an LPS injection at any age causes weight gain, the second part of the experiment tests an additional cohort that will be injected with either LPS or saline at P63, an age which does not cause enduring effects of LPS injections (Ismail et al., 2011; Laroche et al., 2009a). The non-injected control will serve as a control group for these animals as well.

Third, the possible effects of ovariectomy are examined by including both ovariectomized and unaltered subgroups in the third part of the study. All animals, regardless of treatment, will be kept until P119. Examination of intact animals and animals ovariectomized, but not receiving hormones, should help to determine whether ovarian secretions influence the difference in weight gain. The mice will be ovariectomized at the same age used in the third study and should help to determine whether ovariectomy alters the change in weight gain.

Experimental design

This series of experiments is large and involves the use of large numbers of animals; the breadth is necessitated by the myriad possible factors affecting weight gain with age as enumerated above and by the necessity of performing the experiments in both CD-1 and C57Bl/6 animals.

The following experiments will be performed first in CD-1 animals, and then repeated entirely in C57Bl/6. The study is divided into three parts; the experimental groups for each part of the study are shown in Table 2. The experimental procedure is outlined below and is followed by a figure depicting the timelines for each part (Figure 5).

Table 2

Experimental Groups for Parts 1, 2, and 3

Groups	Part 1	Part 2	Part 3
Non-injected control	Yes	Yes	Yes
LPS injection	P42	P42 & P63	P42
Saline injection	P42	P42 & P63	P42
Shipping stress	P42	–	–
Ovariectomized	–	–	Yes (½ each above)
Total groups	4	5	6

Part 1

Animals with the same birthdate will arrive at 3 weeks (P21) and 6 weeks (P42) of age. Twenty-four animals will arrive at 3 weeks; eight animals will arrive at 6 weeks. Animals will be housed with 3 conspecifics upon arrival. On P42 all animals will be weighed; thereafter all animals will be weighed every four days for experimental body weight data. Also on P42, at approximately 9 am, 16 of the animals that arrived at 3 weeks will be injected with either LPS (1.5 mg/kg) in sterile saline ($n = 8$) or saline vehicle alone ($n = 8$). The remaining animals will not be injected. Sickness behavior including lethargy, huddling, ptosis, and piloerection will be recorded at 30mins, 4, 24, and 48hrs following injection. Body weight changes will be monitored daily for seven days. These measures will be recorded for all animals regardless of treatment. Animals will continue to be weighed every four days until P119 when the experiment will conclude.

Part 2

   Forty animals will arrive at 3 weeks (P21) and will be housed and weighed as above. On P42 a subset of the animals will receive either an injection of LPS (n = 8) or saline (n = 8) as previously described. Sickness behavior and body weight changes will be recorded as described above. At 9 weeks of age (P63) a subset of the previously untreated animals will be injected with either LPS (n = 8) or saline (n = 8) following the same procedure as at P42. The remaining animals will remain as a non-injected control (n = 8). Sickness behavior and body weights will be recorded as previously described for all groups. Animals will continue to be weighed as previously described until the conclusion of the experiment at P119.

Part 3

   Forty-eight animals will arrive at 3 weeks (P21); the animals will be housed and weighed as above. On P42 the animals being injected will receive either an injection of LPS (n = 16) or saline (n = 16) as previously described. The remaining animals (n = 16) will remain as the non-injected controls. Sickness behavior and body weight changes will be recorded as described above. At 11 weeks of age half of each group will be ovariectomized (n = 8, for each peripubertal treatment group). Body weights will be recorded daily for one week to assess recovery. As in the previous parts, animals will continue to be weighed every four days until day 119 when the experiment will conclude.
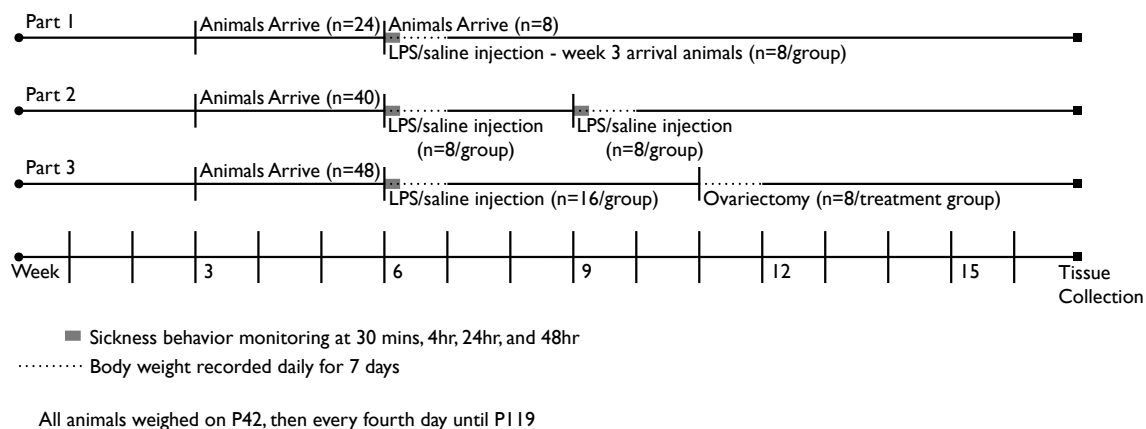
Figure 5. Experimental design for proposed study examining the effects of peripubertal shipping stress, LPS injection, and ovariectomy on weight gain and body weight.

Limitations and future directions

While these experiments endeavor to address all of the limitations encountered from the data analyzed from previous studies it cannot be fully comprehensive. Several of the identified limitations to this experiment are discussed. First, this experiment does not address the possibility that shipping at any age after P21 might cause increased weight gain; this question is, however, tested in an experiment in Chapter 3. Second, in the event that non-injected control animals more closely resemble LPS injected or shipped animals in weight, with saline animals showing a decrease in weight gain, this experiment will be inadequate to address whether it is specifically a saline injection that induces this gain or whether LPS masks an injection effect as no sham injected group (a group receiving an intraperitoneal needle stick but no injection) or group orally administered LPS is included. Third, in relation to ovariectomy and hormone administration, this experiment will not examine effects of exogenous estradiol on body weight gain, or address whether the results of surgery alone might affect weight gain as no sham ovariectomy group is included. However, both of these questions could provide the basis for future experiments

37

especially depending on any differences in weight gain detected between peripubertally

stressed animals that are ovariectomized and those that are not.

CHAPTER 3

PROXIMATE CAUSE OF INCREASED WEIGHT GAIN

Possible causes of increased weight gain

There are several possible mediators for increased weight gain: (i) behavioral changes such as an increase in feeding and/or a decrease in locomotor activity; (ii) a change in basal metabolic rate; and (iii) a change in the daily timing of feeding and activity which results in a change in metabolism but not total food consumption or activity. Mice exposed to dim light at night, a circadian disruption and stressor, show an increase in depression-like behavior (Fonken & Nelson, 2013) and increased body weight (Fonken, Aubrecht, et al., 2013; Fonken et al., 2010); in these animals, it has been demonstrated that the increase in body weight was not due to a change in total consumption or activity but instead to an alteration in the daily pattern of when the animals feed and were active (Fonken et al., 2010). Stressors such as shipping and an LPS injection are both thought to cause neuroinflammation; when administered in conjunction LPS has been shown to increase the neuroinflammation seen following circadian disruption (Fonken, Weil, et al., 2013). Because induction of neuroinflammation is the commonality between circadian disruption, LPS, and shipping, it is expected that both LPS and shipping will lead to increased weight gain through an alteration of timing of feeding and activity and resultant changes in metabolic rate. The experiments described in the following section attempt to disambiguate between the possible causes of increased weight gain by examining feeding and locomotor behavior as well as metabolic rate and body composition.

Specifically, the behavioral parameters of feeding, general locomotion, and wheel running will be examined using behavioral cages and analyzed for both total levels and patterns of activity over 24 hours. Mice supplied with a running wheel will run voluntarily. While general locomotor activity will reveal any differences in total locomotion, wheel-running activity will provide a more robust measure for determining differences in activity pattern over 24 hour periods (Verwey, Robinson, & Amir, 2013). These tests will be run separately from other behavioral behaviors because wheel-running behavior itself can induce other behavioral changes, and thus cannot be used as the sole measure of activity (Novak, Burghardt, & Levine, 2012). Metabolic rate will be assessed by daily energy expenditure as determined by gas exchange in a metabolic chamber, as well as by body temperature. Animals with lowered metabolic rate show lowered core body temperature (Pelleymounter et al., 1995), and these data will further support daily energy expenditure data in determining if metabolism is altered. Body composition will be examined by comparing fat, muscle, and bone mass calculated using live radiographic imaging, and by further analysis of amount of white adipose versus metabolically active brown adipose tissue. It is important to examine muscle, bone, and adipose mass to differentiate increases in overall growth from increases in adiposity. Similarly, by examining the proportion of white to brown adipose tissue it can be determined whether alterations observed in metabolism are due to alterations in amounts of metabolically active brown adipose tissue.

Expected outcomes

As discussed previously it is expected that the animals exposed to a peripubertal stressor will show altered feeding and activity patterns over the day as compared to

controls. The result of these altered behavior patterns is expected to be reflected in altered metabolic rate as measured by decreased energy expenditure and body temperature. The peripubertally stressed mice are also expected to have higher percentage of adipose tissue than the control animals. They are also expected to have less brown adipose tissue relative to white adipose tissue, although not a difference total brown adipose tissue.

Experimental design

The following experiments use C57Bl/6 mice and shipping as a peripubertal stressor. Following the full experimental methods are additional methods to be used to replicate the experiment using an injection of LPS during the peripubertal period as a stressor, as it may be instructive to determine whether LPS and shipping affect weight gain by the same proximate mechanisms. Figures illustrating the experimental timelines for both the shipping stressor (Figure 6) and LPS injection (Figure 7) experiments follow the descriptions. If the experiments described in Chapter 2 show differences in weight gain between strains and stressors these experiments should be repeated with CD-1 mice as necessary and the stressors chosen as appropriate to the strain being tested.

Metabolic cages

The following will examine behavior and metabolic rate by measuring feeding, locomotion, and energy use directly using a metabolic cage. 24 female C57Bl/6 mice will arrive at 3 and 6 weeks (n=12 per week). Animals will be housed with 3 conspecifics upon arrival, and will be isolated at 12 weeks for 96 hours of testing in a metabolic cage. This amount of time in the metabolic cages will be sufficient to collect accurate measures of activity and daily energy expenditure (Speakman, 2013), while minimizing stress on

41

the animals due to single housing. Feeding and drinking behavior will be monitored at regular intervals. Locomotor activity will be measured by beam break. Daily energy expenditure will be measured by calculating basal metabolic rate and respiratory exchange rate from oxygen and carbon dioxide gas exchange (Speakman, 2013). In a subset of 4 animals from each group, animals will be surgically implanted with temperature probe in the abdomen at 9 weeks of age to measure core body temperature. To eliminate inadvertently examining possible temperature changes due to exposure to the behavioral test cages themselves, temperatures will be recorded for 96 hours before and after the behavioral testing. Beginning at 6 weeks all animals will be weighed every fourth day.

Behavioral cages

Activity patterns over the day will be further examined using behavioral cages equipped with a running wheel. Animal numbers and protocol will mirror those given in the previous experiment with the exception that instead of metabolic cages, animals will be tested in a behavioral cage where only wheel running and feeding activity will be recorded and analyzed. In order to ensure that animals will recognize the running wheel, animals will be housed with an identical running wheel in their group housing conditions upon arrival. Body temperatures and weights will also be recorded as in the previous experiment.

Body composition

In order to establish that changes in body weight are due to increases in adipose tissue rather than increases in muscle tissue or general growth, the body composition of

all animals used the previous two experiments will be analyzed using a live radiographic imaging system 4 days after behavioral testing. Percentage body fat, bone, and muscle mass will be compared between all 4 groups. Additionally, distribution of white versus brown adipose tissue will be analyzed.

Figure 6. Experimental design for proposed experiments examining the proximate causes of increased weight gain following a peripubertal shipping stress.

Using LPS as a peripubertal stressor

24 female C57Bl/6 mice will arrive at 3 weeks of age. As in the shipping experiments, animals will be housed with 3 conspecifics upon arrival. The animals will receive an injection of LPS (1.5 mg/kg) or saline vehicle at 6 weeks of age. Sickness behavior including weight change, lethargy, huddling, and piloerection will be recorded at 4 time points. At 12 weeks the animals will be isolated for 96 hours of testing in a metabolic cage. Feeding behavior, locomotor activity, metabolism, and body weight will be tested by the same methods used in the shipping stress experiments with the exception that all animals will be weighed every fourth day beginning the day after arrival at 3 weeks.

As in the shipping stress experiments, activity levels will be further examined using behavioral cages equipped with a running wheel. Animal numbers and protocol

43

will mirror that of the previous experiment with the exception that instead of metabolic

cages, animals will be tested in behavioral cages as in the second shipping experiment.

Finally, body composition of animals will be examined in the same manner as the

shipping stress animals.

Animals Arrive (n=24) — LPS/Saline Injection — Body Composition Testing — Tissue Collection

* Temperature Probe Implantation (n=8)
Behavioral Testing (96hrs)
Sickness Behavior Monitoring (48hrs)
Body Temperature Recording (288hrs)

Body weights recorded every four days beginning one day following arrival, and for five days following Injection
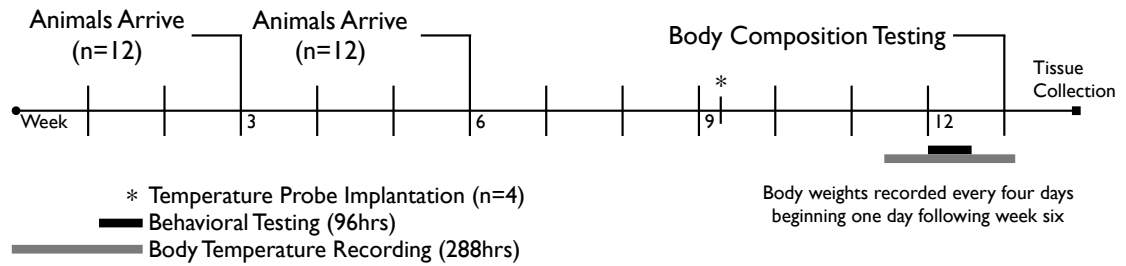
Week | 3 | 6 | 9 | 12
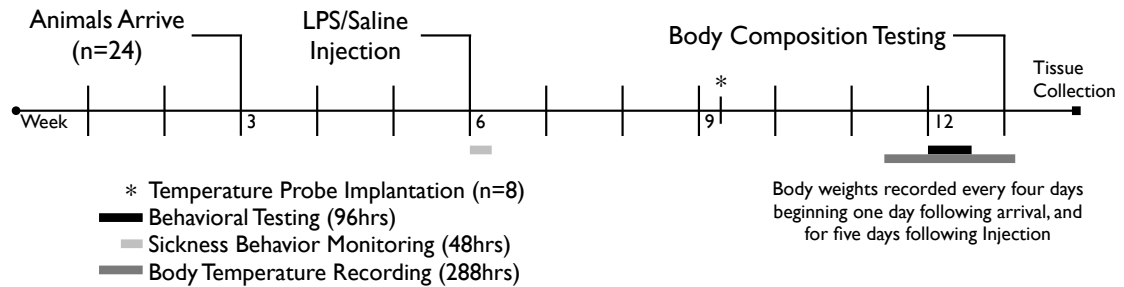
Figure 7. Experimental design for proposed experiments examining the proximate causes of increased weight gain following a peripubertal injection of LPS.

Potential pitfalls and future directions

Although no difference is expected in total feeding or locomotor activity,

alterations in either of these behaviors would indicate these behavioral changes as the

mediators of weight gain instead of, or in addition to, metabolic or daily pattern changes.

Future work would then examine the underlying mechanisms promoting these changes in

addition to mechanisms underlying metabolic changes; such studies are discussed in the

following sections of this chapter. In the event that radiographic imaging is unavailable,

brown and white adipose fat pad weights will be compared following gross dissection of

the pads of each group of animals and carcass analysis of the remaining animals. It is also

recommended that, if carcass analysis is unnecessary, blood and brains be collected and

preserved for future analyses, such as those discussed in Chapter 4.

Role of diet switch in increased weight gain

Findings indicating a change in total feeding or locomotor activity or in metabolic, but not activity patterns, would necessitate further exploration of the structural and hormonal underpinnings of the change in metabolism, or behavior, that directly cause the differences in weight gain in adulthood. Particularly when examining the mice shipped during the peripubertal period, one potential avenue for research concerns the change in chow that the mice experience when they are transferred from the breeder to the laboratory facility. While the animals experience a wide variety of changes (tap water, air quality, housing conditions, cagemates) upon arrival at the laboratory, the change in food is important to consider both in regards to long established and recent findings. It has long been understood that a change in food can have impacts on both body weight and reproduction, which can endure for several generations, and that different strains of mice are differently sensitive to changes in food (Hoag & Dickie, 1966). Much recent work has focused on the interactions between the microbiome and the body (McFall-Ngai et al., 2013), specifically on the microbiome and obesity (Cotillard et al., 2013; Everard et al., 2013; Ley et al., 2005). Not only do the animals experience a change in food when they arrive at the laboratory, but the chows are known to vary in two potentially important elements.

The two chows used by the animal supplier (Purina 5L79, 5% fat chow) and used by the Blaustein laboratory (Teklad 2014, phytoestrogen-reduced, 4% fat chow) differ in both phytoestrogen and fat content. These differences could elicit changes either alone or in conjunction with the stress of either shipping or an LPS injection during the sensitive period. The switch of chow before the peripubertal period might occur at a time when the

body can adapt to the changed nutrient or hormone content, while the animal may be less plastic in its ability to adjust to a new food during the peripubertal period. These changes in response may be due to classical physiological mechanisms or to alterations in the microbiome.

A switch from a high fat to lower fat diet may cause a fundamental shift in metabolism inducing a famine state in animals with subsequent weight gain and lower metabolic rate. Alternatively, the lower fat diet may cause an increase in food consumption and/or a decrease in either locomotor behavior or metabolic rate, which could also lead to increased body weight. The lowered concentration and variability of phytoestrogens present in the Blaustein laboratory food may also contribute, independently or in interaction with the change in fat content, to changes in weight by altering either behavior or metabolism. Estrogens have been previously demonstrated to cause changes in body weight in rodents – sometimes positively, sometimes negatively (Keith et al., 2006).

Alternatively, the change in feed may also point to a role for the microbiome in regulation of estradiol. Recent work has shown a link between an animal's hormone secretion, microbiome, and hormonal and metabolic response (Markle et al., 2013). It may not be the animal's inherent inability to adjust to switching the chow but the microbiome's inability to adapt or change of gut flora, which induces a change in metabolism and or behavior.

Examining effects of diet switch and shipping stress on body weight experimentally

In order to test the possible effect of change of diet on weight gain, the experiment will include groups of mice bred in the laboratory on either on the Purina

5L79, 5% fat chow or the Teklad 2014, phytoestrogen-reduced, 4% fat chow, and would examine the effect of switching chows, the timing of switching chows, and the presence or absence of an additional stressor at time of chow switch on body weight. A figure (Figure 8) outlining the experimental design follows the description. Although outlined only briefly here, future work should also explore possible differences and changes in the microbiome on different chows, following switches in chows, and in response to peripubertal stressors shown to elicit changes in body weight. Finally, this section closes with a discussion of the implications of results of the following experiment and what future experiments might be necessitated by certain results.

Experimental design

This study will use both female C57Bl/6 mice bred in the laboratory from dams purchased from the supplier and mice bred by the supplier and shipped to the laboratory at several ages. Animals bred in the laboratory will be raised on two different chows; eight animals will be raised on the laboratory's standard Teklad 2014, phytoestrogen-reduced, 4% fat chow, another 32 animals will be raised on the standard chow used by the supplier, Purina 5L79, 5% fat chow. At each of 3, 6, and 9 weeks of age, eight animals will be switched from the Purina chow to the Teklad. The animals will be weighed daily for a week following the change in chow, and then will be weighed every four days for the rest of the study. The animals raised in the laboratory that remain on their original chow will be weighed every four days beginning at week 3. 48 animals will arrive from the supplier, 16 each at 3, 6, and 9 weeks of age; upon arrival half of each group (eight animals) will be switched to Teklad chow, the other half will continue to receive the chow they received at the breeding facility. All animals will be weighed

47

every day for a week following arrival, then every four days for the duration of the study. All animals will be aged to P106 (15 weeks+) when the experiment will terminate.
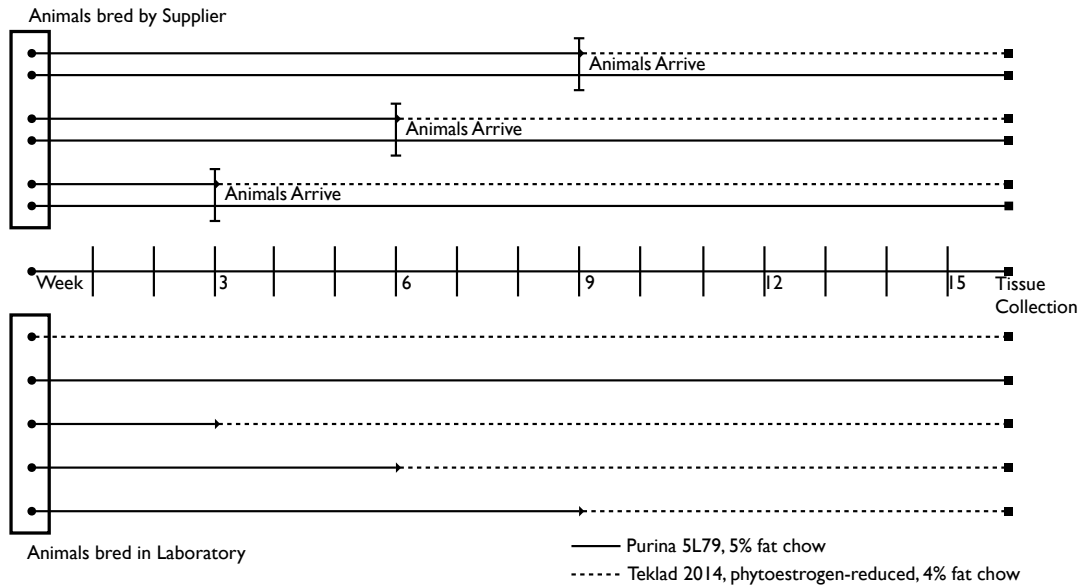

Figure 8. Experimental design for proposed experiment examining the effects of chow, a switch in chow, and shipping on weight gain, when chow switch and shipping occur before, during, or after the sensitive peripubertal period.

Microbiome and other future directions

Pending the results of the previous experiment, the next logical step may be to examine differences in the microbiomes of animals fed different chows, specifically those that show difference in weight gain. As this laboratory does not have expertise in the microbiome, a collaboration is suggested in which the flora of the different groups would be profiled – if planned, it may be possible to collect from the mice from the previous experiment at the conclusion of the study. Alternately, or perhaps additionally, a rescue of mice showing increased weight gain might be attempted by fecal transplant from animals showing normal weight gain in a future experiment, again, in collaboration with a laboratory with experience in these techniques.

Unexpected results and further experiments

Because the following chapter builds on expected results from the studies outlined here, namely that change in weight gain will be due either to the conjunction of switch in chow and peripubertal stressor or peripubertal stressor alone, it is important to discuss the other possible results. If the increase in weight gain is due the change in chow alone, then the increased adipose tissue may still result in alterations of estrogens and estrogen receptors. The experiments outlined in Chapter 4 could still be performed to establish whether these mechanisms explain the other changes seen in animals exposed to peripubertal stressors in the Blaustein laboratory. However, a more direct experiment to test the fundamental basis of this hypothesis would be to repeat earlier experiments on sexual receptivity, cognition, and depression-like behaviors using mice kept on one chow for their entire lifespan. This repetition might also be performed if both switch in chow and a peripubertal stressor result in increased weight gain. If on an unchanging chow, the same results are seen in the behavioral tests as previously, the role of chow switch can be abandoned as the alternation in response to estradiol. If, however, chow switch alone results in increased weight gain, and behavioral tests repeat previous results with an unchanging diet, the hypothesis established in Chapter 1 (that obesity mediates estrogen-dependent processes following a peripubertal stressor) should be rejected as behavior test results would be independent of diet induced adiposity.

CHAPTER 4

OBESITY AS A MEDIATOR OF ESTROGEN DEPENDENT PROCESSES

FOLLOWING PERIPUBERTAL STRESS

Introduction

The elements of the hypothesis to be discussed and tested in this chapter are those that occur as a result of increased adipose tissue. Because the difference in body weights occurred before ovariectomy in the two studies in which it was observed, all of the following experiments will be performed in non-ovariectomized animals. These experiments will address the portions of the hypothesis concerning the following.

1) Whether adiposity results in increased testosterone levels and aromatase activity

2) Whether adiposity results in increased levels of circulating estrogens

3) Whether adiposity results in increased central levels of estrogens and whether these levels correlate with altered peripheral levels of estrogens

4) Whether adiposity results in changes in expression of estrogen receptors in the brain

Experimental Overview

These experiments will address the questions above by examining differences in obese and average weight animals. First, plasma testosterone levels will be examined and aromatase activity will be examined by comparing aromatase activity in adipose tissue. Second, estrogen levels will be examined by assaying plasma levels of estrone and estradiol. These two estrogens have been chosen for analysis, as estrone is the primary estrogen produced by adipose tissue (Cleland et al., 1983), and estradiol is the most

active estrogen in reproductive aged females (Feder & Silver, 1974). Third, central estrogen levels will be compared in the hippocampus and the hypothalamus areas using homogenized tissue. The choice of these regions is due to the role of the hippocampus in cognition and depression-like behaviors, and the role of areas of the hypothalamus in feeding and sexual behavior. Finally, estrogen receptors in the brain will be examined by comparing regulation of ERα, ERβ, and GPR30 by examining mRNAs specific to each receptor type. Specifically, the hippocampus and medial basal hypothalamus will be examined for their roles in the behaviors listed above; the amygdala will also be examined.

The following experiments use one cohort of animals in order to reduce animal numbers and duration of experiment. If the results are positive the experiments should be repeated with a cohort of animals that experience a peripubertal stressor, in order to confirm that adiposity induced by peripubertal stressor is working by the same mechanisms.

Experimental design

32 female C57Bl/6 mice will arrive at 11 weeks of age. 16 of these animals will be standard C57Bl/6 animals, reared on Purina 5TJS, 5% fat chow ("Western Diet" control). The other 16 animals will be diet-induced obesity animals, animals that have been induced to obesity by being reared on the high fat diet Purina 5TJN, 20% fat chow ("Western Diet"). The animals will be allowed to recover from shipping for one week; body weights will be recorded daily. Because diet induced obese animals can rapidly begin to lose weight when placed on a standard chow (Parekh, Petro, Tiller, Feinglos, & Surwit, 1998), animals will be fed the same chows as at the breeding facility. At 12

weeks of age, animals will be deeply anesthetized with an injection of pentobarbital, blood will be collected by cardiac puncture, and the animals will be decapitated. Brains will be collected and preserved as described in sections below. Blood will be collected in EDTA coated tubes and plasma separated via centrifugation. Plasma will be aliquoted and stored at -80ºC. Abdominal fat pads will be removed, weighed, flash frozen, and stored at -80ºC. Collected tissues will then be analyzed as described in the following sections and in Figure 9.

Testosterone and aromatase

Plasma will be assayed for testosterone levels using a commercially available ELISA kit.

Aromatase activity will be measured in adipose tissue. Tissue will be thawed, homogenized and then aromatase assessed using either a commercially available ELISA kit to measure concentration of aromatase or following the methods established by Lephart & Simpson (Lephart & Simpson, 1991) to determine aromatase activity.

Peripheral estrogens

Plasma collected from the mice above will be assayed for both estrone and estradiol using commercially available ELISA kits.

Central estrogens

Brains collected from half of each group will be rapidly microdissected and the hippocampus and hypothalamus from each animal flash frozen and stored at -80ºC until use. Brain areas will be thawed then homogenized and estrogens extracted using an ether extraction followed by solid phase extraction as established by Chao (Chao, Schlinger, &

Remage-Healey, 2011). Central levels of estrone and estradiol will be determined using the same commercially available ELISA kits used in the above analysis.

Expression of estrogen receptors in areas key in estrogen-dependent behaviors

Brains collected from the remaining half of each group will be microdissected. The amygdala, hippocampus, and medial basal hypothalamus will be dissected and preserved in RNAlater®. Preserved sections will be incubated overnight at 2-8ºC, then transferred to -80ºC until used. Tissue will be thawed and homogenized, then the cells lysed and the lysate diluted. RNA will be purified using an Oligotex Direct mRNA kit. In separate assays, primers specific to mRNA for ERα, ERβ, and GPR30 will be used in RT-qPCR to amplify and quantify these mRNAs in the tissue homogenate.
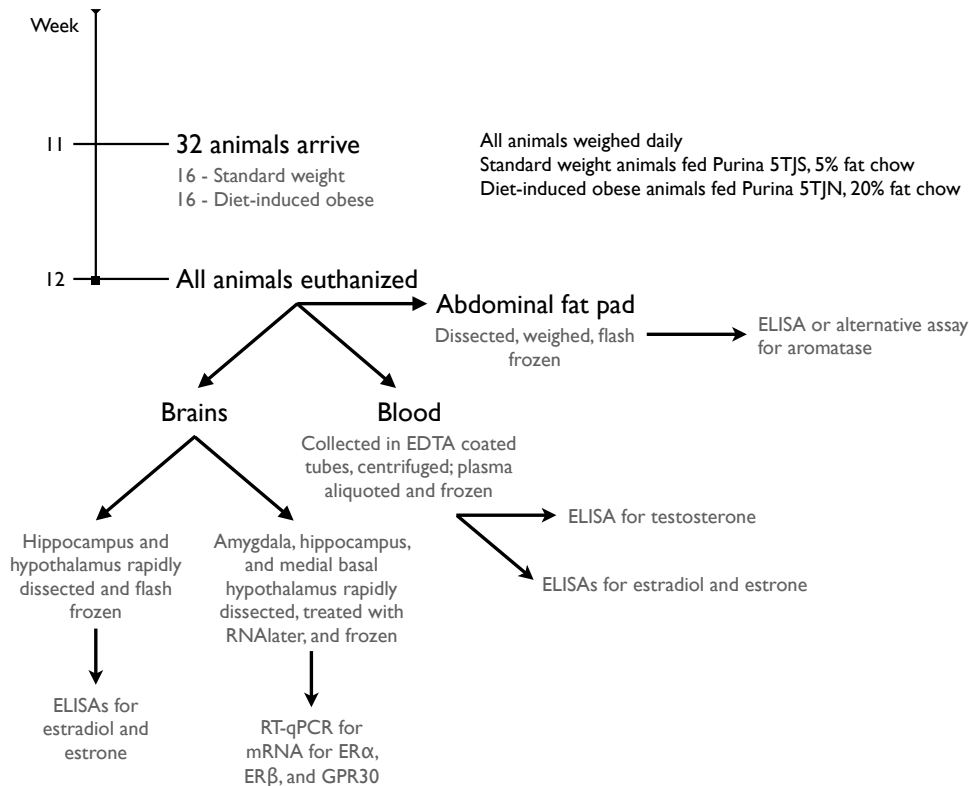
Figure 9. Experimental design for proposed experiments examining the effects of adiposity on: peripheral levels of testosterone, aromatase, estradiol, and estrogen; central levels of estradiol and estrogen; and ERα, Erβ, and GPR30.

Limitations and future directions

The experiments above contain limitations, but also avenues for expansion and extension in new directions. One of the most difficult limitations is that the accuracy of estrogen ELISAs in detecting physiological levels of estrogens is unreliable at best. Because of this limitation, exact results from individual samples will be examined not for statistical differences but for trends in differences between groups. While this limits analysis of estrogen levels, techniques using mass spectrometry to assay estradiol are becoming more accessible and might soon allow for more accurate measurements. When looking towards future directions the first step would be to repeat the above experiments using peripubertally stressed mice, rather than diet-induced obese mice. Some of these analyses could be performed using blood and brains collected from animals from the experiments described in Chapters 2 and 3. Not only could similar analyses be performed on those tissues are were performed in this section to further support these results, but the brains could also be examined for more region specific mRNA expression of estrogen receptors using either *in situ* hybridization or by further RT-qPCR of micropunches. Additionally, studies of altered response to estradiol in depression- and anxiety- like behaviors or in sexual receptivity or cognitive tasks should be carried out in animals with diet-induced obesity. This would build not only on findings in peripubertally stressed animals but also on studies of male diet-induced obesity mice which show increased depression- and anxiety- like behaviors (Sharma & Fulton, 2012).
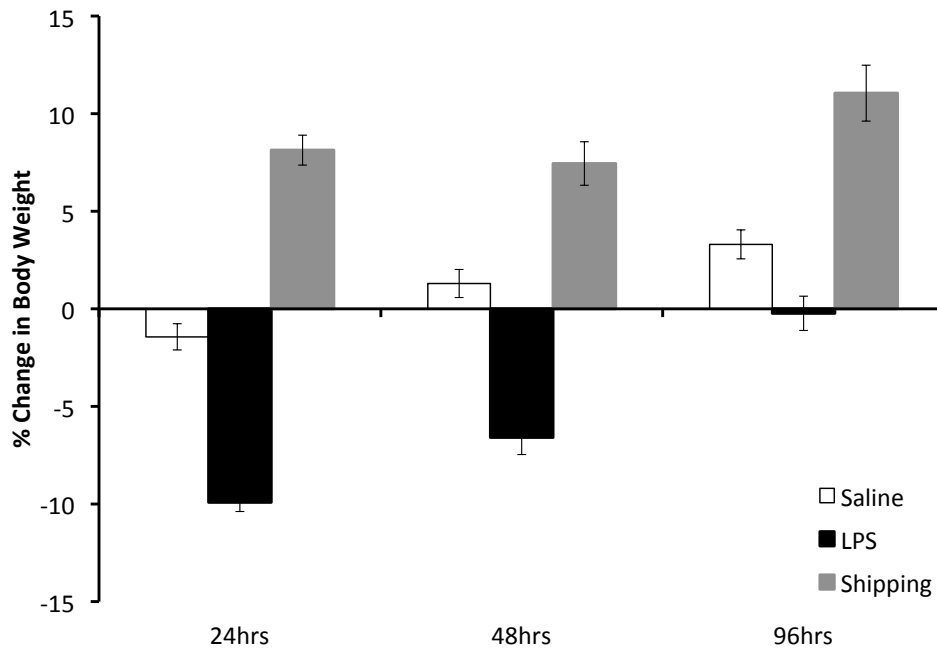
Figure A.1. Percent change in bodyweight following peripubertal stressors for Experiment 2. Error bars represent standard error of the means. Percent change in body weight is calculated by subtracting the weight before treatment from the current weight, then dividing by the weight before treatment. Note that for shipped animals, the initial weight used is the weight of animals upon arrival; the weight gain experienced by these animals results in a body weight which is not different from the saline or LPS animals until after P63.
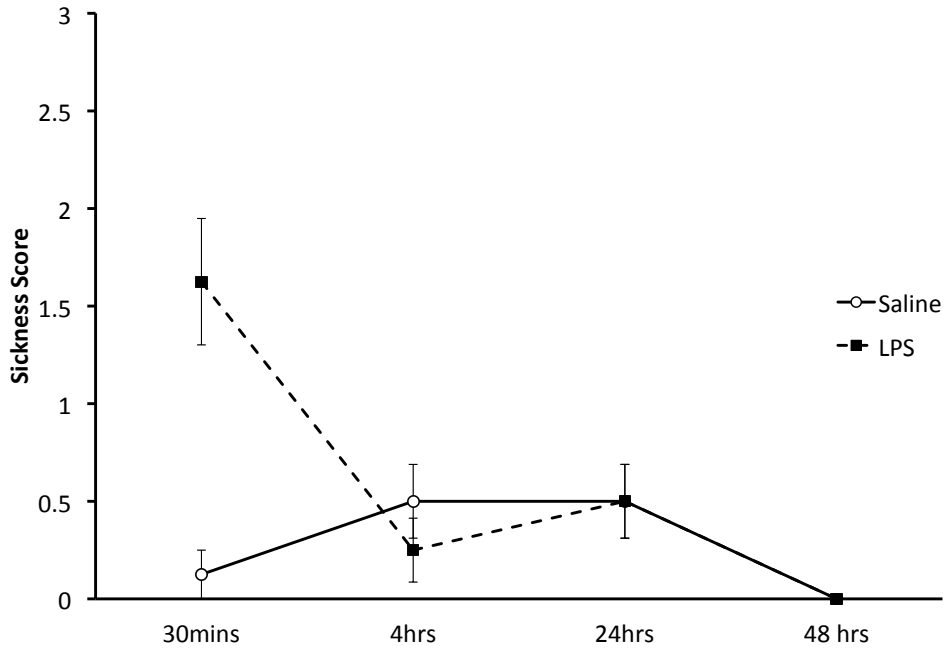
Figure A.2. Sickness behavior for Experiment 2 scored on a three-point scale for the saline and LPS injected groups at four time points following injection. Error bars represent standard error of the means. Sickness behavior scores for LPS treated animals were lower, and decreased more quickly than has been seen previously in C57Bl/6 (Ismail et al., 2013; Olesen et al., 2011).
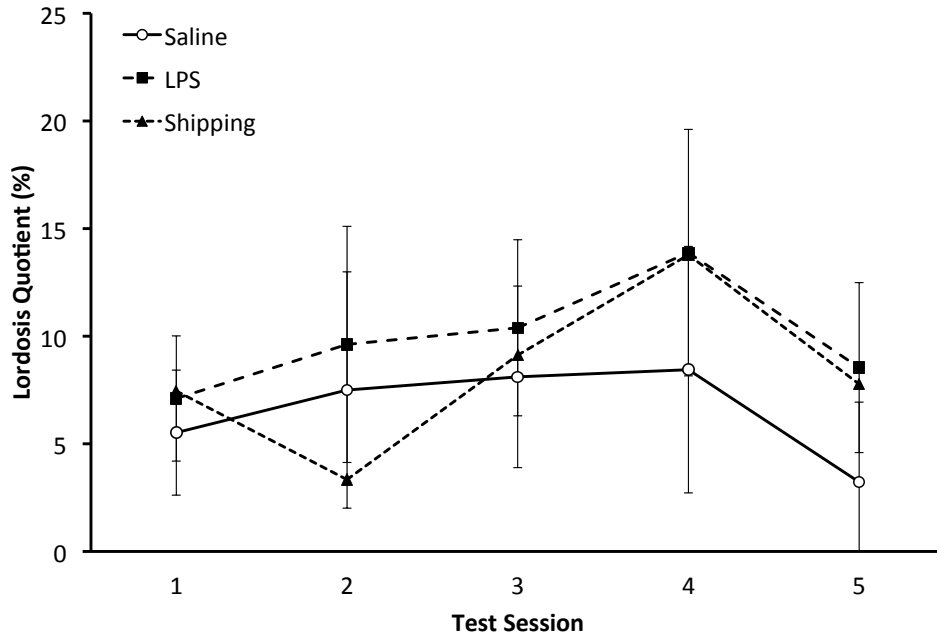
Figure A.3. Lordosis quotients for all treatment groups across five weeks of sexual receptivity testing for Experiment 2. Error bars represent standard error of the means. There were no effect of treatment, week of testing, or interaction on lordosis quotient. Lordosis quotients were far lower and showed much greater variability than seen previously in C57Bl/6 (Laroche et al., 2009a, 2009b).

BIBLIOGRAPHY

Androulakis, I. I., Kandaraki, E., Christakou, C., Karachalios, A., Marinakis, E., Paterakis, T., & Diamanti-kandarakis, E. (2014). Visceral adiposity index (VAI) is related to the severity of anovulation and other clinical features in women with polycystic ovary syndrome. *Clinical Endocrinology*, n/a-n/a. doi: 10.1111/cen.12447

Bedrosian, T. A., Weil, Z. M., & Nelson, R. J. (2012). Chronic dim light at night provokes reversible depression-like phenotype: possible role for TNF. *Molecular Psychiatry, 18*(8), 930-936. doi: 10.1038/mp.2012.96

Bolton, J. L., Huff, N. C., Smith, S. H., Mason, S. N., Foster, W. M., Auten, R. L., & Bilbo, S. D. (2013). Maternal Stress and Effects of Prenatal Air Pollution on Offspring Mental Health Outcomes in Mice. *Environmental Health Perspectives*. doi: 10.1289/ehp.1306560

Bolton, J. L., Smith, S. H., Huff, N. C., Gilmour, M. I., Foster, W. M., Auten, R. L., & Bilbo, S. D. (2012). Prenatal air pollution exposure induces neuroinflammation and predisposes offspring to weight gain in adulthood in a sex-specific manner. *The FASEB Journal, 26*(11), 4743-4754. doi: 10.1096/fj.12-210989

Brown, L. M., & Clegg, D. J. (2010). Central effects of estradiol in the regulation of food intake, body weight, and adiposity. *J Steroid Biochem Mol Biol, 122*(1-3), 65-73. doi: 10.1016/j.jsbmb.2009.12.005

Chao, A., Schlinger, B. A., & Remage-Healey, L. (2011). Combined liquid and solid-phase extraction improves quantification of brain estrogen content. *Front Neuroanat, 5*, 57. doi: 10.3389/fnana.2011.00057

Cleland, W. H., Mendelson, C. R., & Simpson, E. R. (1983). Aromatase activity of membrane fractions of human adipose tissue stromal cells and adipocytes. *Endocrinology, 113*(6), 2155-2160. doi: 10.1210/endo-113-6-2155

Cotillard, A., Kennedy, S. P., Kong, L. C., Prifti, E., Pons, N., Le Chatelier, E., . . . Ehrlich, S. D. (2013). Dietary intervention impact on gut microbial gene richness. *Nature, 500*(7464), 585-588. doi: 10.1038/nature12480

Daumit, G. L., Clark, J. M., Steinwachs, D. M., Graham, C. M., Lehman, A., & Ford, D. E. (2003). Prevalence and Correlates of Obesity in a Community Sample of Individuals with Severe and Persistent Mental Illness. *The Journal of Nervous and Mental Disease, 191*(12), 799-805. doi: 10.1097/01.nmd.0000100923.20188.2d

Ellis, B. J., & Garber, J. (2000). Psychosocial antecedents of variation in girls' pubertal timing: maternal depression, stepfather presence, and marital and family stress. *Child development, 71*(2), 485-501.

Everard, A., Belzer, C., Geurts, L., Ouwerkerk, J. P., Druart, C., Bindels, L. B., . . . Cani, P. D. (2013). Cross-talk between Akkermansia muciniphila and intestinal epithelium controls diet-induced obesity. *Proc Natl Acad Sci U S A, 110*(22), 9066-9071. doi: 10.1073/pnas.1219451110

Faith, M. S., Butryn, M., Wadden, T. A., Fabricatore, A., Nguyen, A. M., & Heymsfield, S. B. (2011). Evidence for prospective associations among depression and obesity in population-based studies. *Obesity Reviews, 12*(5), e438-e453. doi: 10.1111/j.1467-789X.2010.00843.x

Faith, R., & Miller, S. (2007). The Need for Sound and Vibration Standards in US Research Animal Rooms. *Animal Lab News*.

Fassnacht, M., Schlenz, N., Schneider, S. B., Wudy, S. A., Allolio, B., & Arlt, W. (2003). Beyond Adrenal and Ovarian Androgen Generation: Increased Peripheral 5α-Reductase Activity in Women with Polycystic Ovary Syndrome. *The Journal of clinical endocrinology and metabolism, 88*(6), 2760-2766. doi: 10.1210/jc.2002-021875

Feder, H. H., & Silver, R. (1974). Activation of lordosis in ovariectomized guinea pigs by free and esterified forms of estrone, estradiol-17 beta and estriol. *Physiol Behav, 13*(2), 251-255.

Ferreira, I., Twisk, J. W. R., van Mechelen, W., Kemper, H. C. G., & Stehouwer, C. D. A. (2005). Development of fatness, fitness, and lifestyle from adolescence to the age of 36 years: determinants of the metabolic syndrome in young adults: the amsterdam growth and health longitudinal study. *Archives of internal medicine, 165*(1), 42-48. doi: 10.1001/archinte.165.1.42

Fonken, L. K., Aubrecht, T. G., Melendez-Fernandez, O. H., Weil, Z. M., & Nelson, R. J. (2013). Dim Light at Night Disrupts Molecular Circadian Rhythms and Increases Body Weight. *Journal of Biological Rhythms, 28*(4), 262-271. doi: 10.1177/0748730413493862

Fonken, L. K., Lieberman, R. A., Weil, Z. M., & Nelson, R. J. (2013). Dim Light at Night Exaggerates Weight Gain and Inflammation Associated With a High-Fat Diet in Male Mice. *Endocrinology, 154*(10), 3817-3825. doi: 10.1210/en.2013-1121

Fonken, L. K., & Nelson, R. J. (2013). Dim light at night increases depressive-like responses in male C3H/HeNHsd mice. *Behavioural Brain Research, 243*, 74-78. doi: 10.1016/j.bbr.2012.12.046

Fonken, L. K., Weil, Z. M., & Nelson, R. J. (2013). Mice exposed to dim light at night exaggerate inflammatory responses to lipopolysaccharide. *Brain Behavior and Immunity, 34*(C), 159-163. doi: 10.1016/j.bbi.2013.08.011

Fonken, L. K., Workman, J. L., Walton, J. C., Weil, Z. M., Morris, J. S., Haim, A., & Nelson, R. J. (2010). Light at night increases body mass by shifting the time of food intake. *Proceedings of the National Academy of Sciences, 107*(43), 18664-18669. doi: 10.1073/pnas.1008734107

Forbes, E. E., & Dahl, R. E. (2010). Pubertal development and behavior: Hormonal activation of social and motivational tendencies. *Brain and Cognition, 72*(1), 66-72. doi: 10.1016/j.bandc.2009.10.007

Ge, X., Conger, R. D., & Elder, G. H. (1996). Coming of age too early: pubertal influences on girls' vulnerability to psychological distress. *Child development, 67*(6), 3386-3400.

Gonder, J. C., & Laber, K. (2007). A renewed look at laboratory rodent housing and management. *ILAR journal / National Research Council, Institute of Laboratory Animal Resources, 48*(1), 29-36.

Grün, F., & Blumberg, B. (2009). Minireview: The Case for Obesogens. *Molecular Endocrinology, 23*(8), 1127-1134. doi: 10.1210/me.2008-0485

Hamer, M., Batty, G. D., & Kivimaki, M. (2012). Risk of future depression in people who are obese but metabolically healthy: the English longitudinal study of ageing. *Molecular Psychiatry, 17*(9), 940-945. doi: 10.1038/mp.2012.30

Hayward, C., & Sanborn, K. (2002). Puberty and the emergence of gender differences in psychopathology. *The Journal of adolescent health : official publication of the Society for Adolescent Medicine, 30*(4 Suppl), 49-58.

Heine, P. A., Taylor, J. A., Iwamoto, G. A., Lubahn, D. B., & Cooke, P. S. (2000). Increased adipose tissue in male and female estrogen receptor-alpha knockout mice. *Proceedings of the National Academy of Sciences of the United States of America, 97*(23), 12729-12734. doi: 10.1073/pnas.97.23.12729

Hoag, W. G., & Dickie, M. M. (1966). Nutrition. In E. L. Green (Ed.), *Biology of the laboratory mouse* (2nd ed., pp. 39-44). New York.

Holder, M. K. (2014). *Microglial activation following the experience of an immune challenge during puberty and adulthood*. Manuscript in preparation.

Ismail, N., & Blaustein, J. D. (2013). Pubertal immune challenge blocks the ability of estradiol to enhance performance on cognitive tasks in adult female mice. *Psychoneuroendocrinology, 38*(7), 1170-1177. doi: 10.1016/j.psyneuen.2012.11.003

Ismail, N., Garas, P., & Blaustein, J. D. (2011). Long-term effects of pubertal stressors on female sexual receptivity and estrogen receptor-α expression in CD-1 female mice. *Hormones and Behavior, 59*(4), 565-571. doi: 10.1016/j.yhbeh.2011.02.010

Ismail, N., Kumlin, A. M., & Blaustein, J. D. (2013). A pubertal immune challenge alters the antidepressant-like effects of chronic estradiol treatment in inbred and outbred adult female mice. *Neuroscience, 249*, 43-52. doi: 10.1016/j.neuroscience.2012.09.047

Keith, S. W., Redden, D. T., Katzmarzyk, P. T., Boggiano, M. M., Hanlon, E. C., Benca, R. M., . . . Allison, D. B. (2006). Putative contributors to the secular increase in obesity: exploring the roads less traveled. *International Journal of Obesity, 30*(11), 1585-1594. doi: 10.1038/sj.ijo.0803326

Kennedy, A., Martinez, K., Chuang, C. C., LaPoint, K., & McIntosh, M. (2008). Saturated Fatty Acid-Mediated Inflammation and Insulin Resistance in Adipose Tissue: Mechanisms of Action and Implications. *Journal of Nutrition, 139*(1), 1-4. doi: 10.3945/jn.108.098269

Laroche, J., Gasbarro, L., Herman, J. P., & Blaustein, J. D. (2009a). Enduring Influences of Peripubertal/Adolescent Stressors on Behavioral Response to Estradiol and Progesterone in Adult Female Mice. *Endocrinology, 150*(8), 3717-3725. doi: 10.1210/en.2009-0099

Laroche, J., Gasbarro, L., Herman, J. P., & Blaustein, J. D. (2009b). Reduced Behavioral Response to Gonadal Hormones in Mice Shipped during the Peripubertal/Adolescent Period. *Endocrinology, 150*(5), 2351-2358. doi: 10.1210/en.2008-1595

Lawlor, D. A. (2005). Treatment and prevention of obesity--are there critical periods for intervention? *International Journal of Epidemiology, 35*(1), 3-9. doi: 10.1093/ije/dyi309

Lephart, E. D., & Simpson, E. R. (1991). Assay of aromatase activity. *Methods Enzymol, 206*, 477-483.

Ley, R. E., Backhed, F., Turnbaugh, P., Lozupone, C. A., Knight, R. D., & Gordon, J. I. (2005). Obesity alters gut microbial ecology. *Proc Natl Acad Sci U S A, 102*(31), 11070-11075. doi: 10.1073/pnas.0504978102

Luppino, F. S., de Wit, L. M., Bouvy, P. F., Stijnen, T., Cuijpers, P., Penninx, B. W. J. H., & Zitman, F. G. (2010). Overweight, obesity, and depression: a systematic review and meta-analysis of longitudinal studies. *Archives of general psychiatry, 67*(3), 220-229. doi: 10.1001/archgenpsychiatry.2010.2

Markle, J. G. M., Frank, D. N., Mortin-Toth, S., Robertson, C. E., Feazel, L. M., Rolle-Kampczyk, U., . . . Danska, J. S. (2013). Sex Differences in the Gut Microbiome Drive Hormone-Dependent Regulation of Autoimmunity. *Science, 339*(6123), 1084-1088. doi: 10.1126/science.1233521

Marmorstein, N. R., Iacono, W. G., & Legrand, L. (2014). Obesity and depression in adolescence and beyond: reciprocal risks. 1-6. doi: 10.1038/ijo.2014.19

Marshall, W. A., & Tanner, J. M. (1969). Variations in pattern of pubertal changes in girls. *Archives of disease in childhood, 44*(235), 291-303.

McCabe, M. P., Ricciardelli, L. A., & Finemore, J. (2002). The role of puberty, media and popularity with peers on strategies to increase weight, decrease weight and increase muscle tone among adolescent boys and girls. *Journal of Psychosomatic Research, 52*(3), 145-153.

McFall-Ngai, M., Hadfield, M. G., Bosch, T. C., Carey, H. V., Domazet-Loso, T., Douglas, A. E., . . . Wernegreen, J. J. (2013). Animals in a bacterial world, a new imperative for the life sciences. *Proc Natl Acad Sci U S A, 110*(9), 3229-3236. doi: 10.1073/pnas.1218525110

McGill, H. C., McMahan, C. A., Herderick, E. E., Malcom, G. T., Tracy, R. E., & Strong, J. P. (2000). Origin of atherosclerosis in childhood and adolescence. *The American journal of clinical nutrition, 72*(5 Suppl), 1307S-1315S.

Melka, M. G., Abrahamowicz, M., Leonard, G. T., Perron, M., Richer, L., Veillette, S., . . . Pausova, Z. (2013). Clustering of the Metabolic Syndrome Components in Adolescence: Role of Visceral Fat. *PLoS ONE, 8*(12), e82368. doi: 10.1371/journal.pone.0082368

Milanski, M., Degasperi, G., Coope, A., Morari, J., Denis, R., Cintra, D. E., . . . Velloso, L. A. (2009). Saturated Fatty Acids Produce an Inflammatory Response Predominantly through the Activation of TLR4 Signaling in Hypothalamus: Implications for the Pathogenesis of Obesity. *Journal of Neuroscience, 29*(2), 359-370. doi: 10.1523/JNEUROSCI.2760-08.2009

Novak, C. M., Burghardt, P. R., & Levine, J. A. (2012). The use of a running wheel to measure activity in rodents: Relationship to energy balance, general activity, and reward. *Neuroscience and Biobehavioral Reviews, 36*(3), 1001-1014. doi: 10.1016/j.neubiorev.2011.12.012

Olesen, K. M., Ismail, N., Merchasin, E. D., & Blaustein, J. D. (2011). Long-term alteration of anxiolytic effects of ovarian hormones in female mice by a peripubertal immune challenge. *Hormones and Behavior, 60*(4), 318-326. doi: 10.1016/j.yhbeh.2011.06.005

Parekh, P. I., Petro, A. E., Tiller, J. M., Feinglos, M. N., & Surwit, R. S. (1998). Reversal of diet-induced obesity and diabetes in C57BL/6J mice. *Metabolism: clinical and experimental, 47*(9), 1089-1096.

Patton, G. C., Hibbert, M. E., Carlin, J., Shao, Q., Rosier, M., Caust, J., & Bowes, G. (1996). Menarche and the onset of depression and anxiety in Victoria, Australia. *Journal of epidemiology and community health, 50*(6), 661-666.

Pelleymounter, M. A., Cullen, M. J., Baker, M. B., Hecht, R., Winters, D., Boone, T., & Collins, F. (1995). Effects of the obese gene product on body weight regulation in ob/ob mice. *Science, 269*(5223), 540-543.

Quinkler, M. (2004). Androgen generation in adipose tissue in women with simple obesity - a site-specific role for 17 -hydroxysteroid dehydrogenase type 5. *Journal of Endocrinology, 183*(2), 331-342. doi: 10.1677/joe.1.05762

Rasmussen, S., Glickman, G., Norinsky, R., Quimby, F. W., & Tolwani, R. J. (2009). Construction noise decreases reproductive efficiency in mice. *J Am Assoc Lab Anim Sci, 48*(4), 363-370.

Rivenes, A. C., Harvey, S. B., & Mykletun, A. (2009). The relationship between abdominal fat, obesity, and common mental disorders: Results from the HUNT Study. *Journal of Psychosomatic Research, 66*(4), 269-275. doi: 10.1016/j.jpsychores.2008.07.012

Ruhlen, R. L., Howdeshell, K. L., Mao, J., Taylor, J. A., Bronson, F. H., Newbold, R. R., . . . vom Saal, F. S. (2007). Low Phytoestrogen Levels in Feed Increase Fetal Serum Estradiol Resulting in the "Fetal Estrogenization Syndrome" and Obesity in CD-1 Mice. *Environmental Health Perspectives, 116*(3), 322-328. doi: 10.1289/ehp.10448

Russell, E. S. (1966). Lifespan and aging patterns. In E. L. Green (Ed.), *Biology of the laboratory mouse* (2nd ed., pp. 511-519). New York.

Saberi, M., Woods, N.-B., de Luca, C., Schenk, S., Lu, J. C., Bandyopadhyay, G., . . . Olefsky, J. M. (2009). Hematopoietic Cell-Specific Deletion of Toll-like Receptor 4 Ameliorates Hepatic and Adipose Tissue Insulin Resistance in High-Fat-Fed Mice. *Cell Metabolism, 10*(5), 419-429. doi: 10.1016/j.cmet.2009.09.006

Schulz, K. M., Molenda-Figueira, H. A., & Sisk, C. L. (2009). Back to the future: The organizational–activational hypothesis adapted to puberty and adolescence. *Hormones and Behavior, 55*(5), 597-604. doi: 10.1016/j.yhbeh.2009.03.010

Sharma, S., & Fulton, S. (2012). Diet-induced obesity promotes depressive-like behaviour that is associated with neural adaptations in brain reward circuitry. *International Journal of Obesity, 37*(3), 382-389. doi: 10.1038/ijo.2012.48

Simpson, E. R., Merrill, J. C., Hollub, A. J., Graham-Lorence, S., & Mendelson, C. R. (1989). Regulation of estrogen biosynthesis by human adipose cells. *Endocrine reviews, 10*(2), 136-148. doi: 10.1210/edrv-10-2-136

Sizonenko, P. C. (1989). Physiology of puberty. *Journal of endocrinological investigation, 12*(8 Suppl 3), 59-63.

Speakman, J. R. (2013). Measuring energy metabolism in the mouse – theoretical, practical, and analytical considerations. 1-23. doi: 10.3389/fphys.2013.00034/abstract

Spearow, J. L. (1999). Genetic Variation in Susceptibility to Endocrine Disruption by Estrogen in Mice. *Science, 285*(5431), 1259-1261. doi: 10.1126/science.285.5431.1259

Tanner, J. M. (1962). *Growth at adolescence, with a general consideration of the effects of hereditary and environmental factors upon growth and maturation from birth to maturity* (2d ed.). Oxford,: Blackwell Scientific Publications.

Tsukumo, D. M. L., Carvalho-Filho, M. A., Carvalheira, J. B. C., Prada, P. O., Hirabara, S. M., Schenka, A. A., . . . Saad, M. J. A. (2007). Loss-of-Function Mutation in Toll-Like Receptor 4 Prevents Diet-Induced Obesity and Insulin Resistance. *Diabetes, 56*(8), 1986-1998. doi: 10.2337/db06-1595

Turner, J. G., Bauer, C. A., & Rybak, L. P. (2007). Noise in animal facilities: why it matters. *J Am Assoc Lab Anim Sci, 46*(1), 10-13.

Vandenbergh, J. G. (1967). Effect of the presence of a male on the sexual maturation of female mice. *Endocrinology, 81*(2), 345-349. doi: 10.1210/endo-81-2-345

Vandenbergh, J. G. (1969). Male odor accelerates female sexual maturation in mice. *Endocrinology, 84*(3), 658-660. doi: 10.1210/endo-84-3-658

Verwey, M., Robinson, B., & Amir, S. (2013). Recording and analysis of circadian rhythms in running-wheel activity in rodents. *Journal of visualized experiments : JoVE*(71), e50186-e50186. doi: 10.3791/50186

Wade, G. N., Gray, J. M., & Bartness, T. J. (1985). Gonadal influences on adiposity. *International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity, 9 Suppl 1*, 83-92.