UNIVERSITY OF CALIFORNIA SAN DIEGO

The Relationship between the Gut Microbiome and Metabolic Dysregulation Mediated by E2 Insufficiency

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

in

Biology

by

Lillian Sau

Committee in charge:

Professor Varykina Thackray, Chair
Professor Kimberly Cooper, Co-chair
Professor Stephanie Mel

2020

The Thesis of Lillian Sau is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-chair

Chair

University of California San Diego

2020

DEDICATION

I’d like to dedicate my thesis to:

My parents, Vivian, and Tim

The Thackray lab

And my supportive friends

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LIST OF ABBREVIATIONS

HPG	hypothalamic-pituitary-gonadal axis
ARC	arcuate nucleus
KNDy	neurons that coexpress Kiss1, NKB, and Dyn
Kiss1	kisspeptin
NKB	neurokinin B
Dyn	dynorphin
GnRH	gonadotropin-releasing hormone
LH	luteinizing hormone
FSH	follicle-stimulating hormone
E2	17-β estradiol
T	testosterone
AVPV	anteroventral periventricular nucleus
ERα	estrogen receptor α
ERβ	estrogen receptor β
αERKO	ERα knockout
FBG	fasting blood glucose
FBI	fasting blood insulin
OVX	ovariectomy
HFD	high-fat diet
AR	androgen receptor
DHT	dihydrotestosterone
PCOS	polycystic ovary syndrome
LET	letrozole
SHAM	sham-operated
ITT	insulin tolerance test
SVs	sequence variants

PD	phylogenetic diversity
PCoA	principal coordinate analysis
OVX^{ch}	cohoused OVX mice
$SHAM^{ch}$	cohoused SHAM mice

ACKNOWLEDGEMENTS

I would like to acknowledge the chair of my committee, Dr. Varykina Thackray, for her unconditional support and guidance over the last 4 years. Her help has shaped me into the researcher that I am today. I would also like to acknowledge Bryan Ho and Laura Cui for being exceptional lab managers, mentors, and friends. I am very grateful for all their advice both inside and outside of lab.

I would also like to acknowledge my committee members, Dr. Kimberly Cooper and Dr. Stephanie Mel, for their interest in my research as well as for being outstanding professors at UCSD.

Lastly, I would like to thank the previous and current members of the Thackray lab who have supported me and given me many laughs and lasting memories.

Chapter 1, in part, is a reprint of the material as it appears in Alterations in Gut Microbiota Do Not Play a Causal Role in Diet-independent Weight Gain Caused by Ovariectomy. *Journal of the Endocrine Society*. 2020. Lillian Sau, Christine M. Olmstead, Laura J. Cui, Annie Chen, Reeya S. Shah, Scott T. Kelley, and Varykina G. Thackray. The thesis author was the primary investigator and author of this chapter.

ABSTRACT OF THE THESIS

The Relationship between the Gut Microbiome and Metabolic Dysregulation

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Lillian Sau

Master of Science in Biology

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Professor Varykina Thackray, Chair
Professor Kimberly Cooper, Co-chair

Estrogen is involved in many important reproductive and metabolic processes in mammals. Since evidence suggests that gut microbes may facilitate the protective effect of estrogen on metabolic dysregulation, we investigated whether the gut microbiome plays a role in the diet-independent weight gain that occurs after ovariectomy (OVX) in adult female mice. We

found that OVX was not associated with changes in alpha diversity but was correlated with a shift in beta diversity of gut bacteria. Additionally, we observed differences in the relative abundance of a few bacterial taxa such as *Turicibacter* 3-5 weeks post-OVX.

A complementary cohousing study was performed to determine whether exposure to a healthy gut microbiome was protective against development of the OVX-induced metabolic phenotype. We found that cohousing OVX mice with sham-operated mice did not improve OVX-induced metabolic phenotype. Altogether, these results indicate that post-OVX changes in the gut microbiome are unlikely to play a causal role in the weight gain observed after OVX.

Due to the strong metabolic phenotype of a letrozole-induced (LET) polycystic ovary syndrome mouse (PCOS) model that we previously investigated, we compared the metabolic phenotypes of pubertal LET mice with pubertal OVX mice to determine which phenotypes may be mediated by low estrogen. We found that pubertal OVX mice and pubertal LET mice both had metabolic dysregulation including elevated weight gain, fasting blood glucose, fasting blood insulin, and parametrial fat. However, only LET mice developed insulin resistance, indicating that estradiol deficiency alone is not sufficient to induce insulin resistance in pubertal mice.

INTRODUCTION

The Hypothalamic-Pituitary-Gonadal Axis

The hypothalamic-pituitary-gonadal (HPG) axis regulates neuroendocrine function and plays a major role in development and reproduction[1–3]. The HPG axis is composed of three main regions: the hypothalamus, the pituitary gland, and the gonads. The arcuate nucleus (ARC) of the hypothalamus contains KNDy neurons that express three main neuropeptides: kisspeptin (Kiss1), neurokinin B (NKB), and dynorphin (Dyn)[4,5]. KNDy neurons secrete Kiss1 which binds to Kiss1 receptors on gonadotropin-releasing hormone (GnRH) neurons and stimulates the production of the neuropeptide, GnRH in a pulsatile manner[6,7] (**Figure 1**). GnRH is secreted into the portal vasculature where it binds to GnRH receptors on gonadotrope cells in the anterior pituitary[8,9]. This results in the synthesis and secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) into the bloodstream[8] (**Figure 1**). LH and FSH target the gonads (testes in males and ovaries in females) and stimulate the production of sex steroid hormones such as 17-β estradiol (E2) and testosterone (T)[8,10,11] (**Figure 1**). Sex hormones produced by the gonads negatively feedback on the hypothalamus and pituitary to regulate production of GnRH, LH, and steroid hormones[12–15] (**Figure 1**). In females, E2 is also involved in a positive feedback loop with Kiss1 neurons in the anteroventral periventricular nucleus (AVPV) of the hypothalamus in which it promotes the LH surge that causes ovulation[16,17] (**Figure 1**).

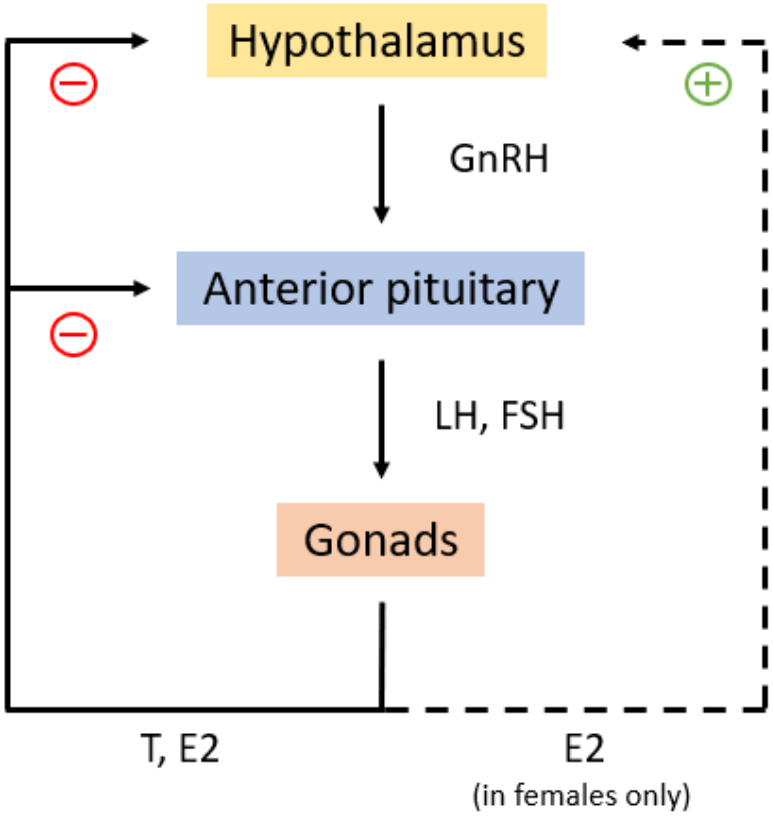


Figure 1: The hypothalamic-pituitary-gonadal (HPG) axis regulates reproductive function. Neurons in the arcuate nucleus (ARC) secrete kisspeptin (Kiss1) which binds to the Kiss1 receptor and stimulates gonadotropin-releasing hormone (GnRH) neurons to produce GnRH. GnRH targets gonadotrope cells in the anterior pituitary which leads to the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH and FSH then act on the gonads to produce sex steroids such as testosterone (T) and estradiol (E2), which feedback negatively on the hypothalamus and anterior pituitary. In females, high concentrations of E2 also lead to positive feedback on Kiss1 neurons in the anteroventral periventricular nucleus (AVPV) of the hypothalamus.

Estrogens

The sex steroid hormone, estrogen, plays a key role in regulating physiological processes involved in reproduction, metabolism, the cardiovascular system, and bone in mammals[18–21]. Of the three estrogens (estrone, estradiol, estriol) produced in females, E2 is the primary estrogen circulating in pre-menopausal women. Estrogens bind to and activate intracellular signaling from two related estrogen receptors: estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ)[22]. In animal models, knockout of ERα (αERKO) in female mice caused increased weight and adiposity[23,24], insulin resistance[24–26] as well as elevated fasting blood glucose (FBG)[25] and fasting blood insulin (FBI) levels[25–27]. In contrast, knockout of ERβ in female mice did not cause obesity[25,27], suggesting that estrogen signaling via ERα primarily regulates metabolism in females.

In addition to the gradual reduction in estrogen levels that occurs during menopause, acute hypoestrogenism or estrogen deficiency in women can occur as a consequence of hyperprolactinemia, chemotherapy, GnRH agonist treatment, and removal of hormone replacement therapy[28–31]. Various rodent models of estrogen deficiency have been used to study the role of estrogen in female metabolism. In ovariectomy (OVX) mouse models, OVX was reported to result in increased weight and adiposity but not insulin resistance[32]. However, OVX mice that were fed a high-fat diet (HFD) developed insulin resistance along with elevated weight and fat mass[32–34]. Interestingly, hormone replacement studies in OVX and OVX+HFD mouse models showed that E2 treatment can attenuate weight gain and protect against insulin resistance[32–34] but only in the presence of ERα[35–37]. These findings further indicate that estrogen signaling via ERα is protective against metabolic dysregulation in females.

Androgens

Androgens are steroid hormones that play a critical role in the development of skeletal muscle[38–40], brain structure[41–43], and male reproductive organs and function[44,45]. Androgens primarily bind to androgen receptors (AR) and mediate their biological effects through both classical and nonclassical signaling pathways[46]. While androgens are synthesized in both males and females (in the testes, ovaries, and adrenal glands), androgen levels are significantly higher in males. The main circulating androgen in males and females is T, which can be converted into estrogen through aromatization (**Figure 2A**). Mutations in the aromatase enzyme or inhibition of aromatization result in disorders involving hyperandrogenemia[47,48]. Interestingly, excess levels of androgens in females have been associated with metabolic dysregulation including insulin resistance, high cholesterol, and high blood pressure[47,49]. Another pathway involving androgens is the reduction of T into a more potent androgen known as dihydrotestosterone (DHT) via the enzyme 5α-reductase[44]. Since DHT cannot be aromatized into an estrogen, it has been used in various mouse models to simulate states of hyperandrogenism.

Polycystic Ovary Syndrome

Polycystic ovary syndrome (PCOS) is a common endocrine and metabolic disorder that occurs in reproductive-age women and has a worldwide incidence of 5-15%[50]. According to the Rotterdam Consensus criteria (2003), a diagnosis of PCOS requires two of the following: (1) hyperandrogenemia, (2) oligomenorrhea or amenorrhea, and (3) polycystic ovaries[50]. Women with PCOS have a higher likelihood of experiencing reproductive issues such as infertility[51,52], miscarriage[53], and pregnancy complications[53]. Not only do women with PCOS present with reproductive phenotypes, but they also typically present with metabolic phenotypes such as

obesity and insulin resistance[52]. Additionally, it has been shown that women with PCOS have an increased risk for developing Type 2 diabetes[52,54], hypertension[55], and non-alcoholic fatty liver disease[56]. While the pathology of PCOS is currently unknown, twin studies have indicated that the cause of PCOS is likely to be a combination of genetic and environmental factors[57,58]. There are no known cures for PCOS, and treatments for PCOS address specific symptoms associated with the disorder.

Recently, a mouse model that recapitulates both reproductive and metabolic hallmarks of PCOS was developed[59]. This model involves the administration of letrozole (LET), a non-steroidal aromatase inhibitor, in a 3 mg, slow-releasing pellet that is implanted subcutaneously into mice[59]. In mammals, the aromatase enzyme converts T into E2 (**Figure 2A**). However, administration of LET inhibits the function of the aromatase enzyme, leading to an increased concentration of T and a lower concentration of E2 in the body (**Figure 2B**). LET treatment of pubertal female mice results in reproductive symptoms of PCOS including hyperandrogenism, elevated LH, irregular estrus cycles, and polycystic ovaries[59]. Additionally, pubertal LET mice exhibit metabolic symptoms such as weight gain, abdominal adiposity, elevated FBI and FBG, insulin resistance, and dyslipidemia[60].

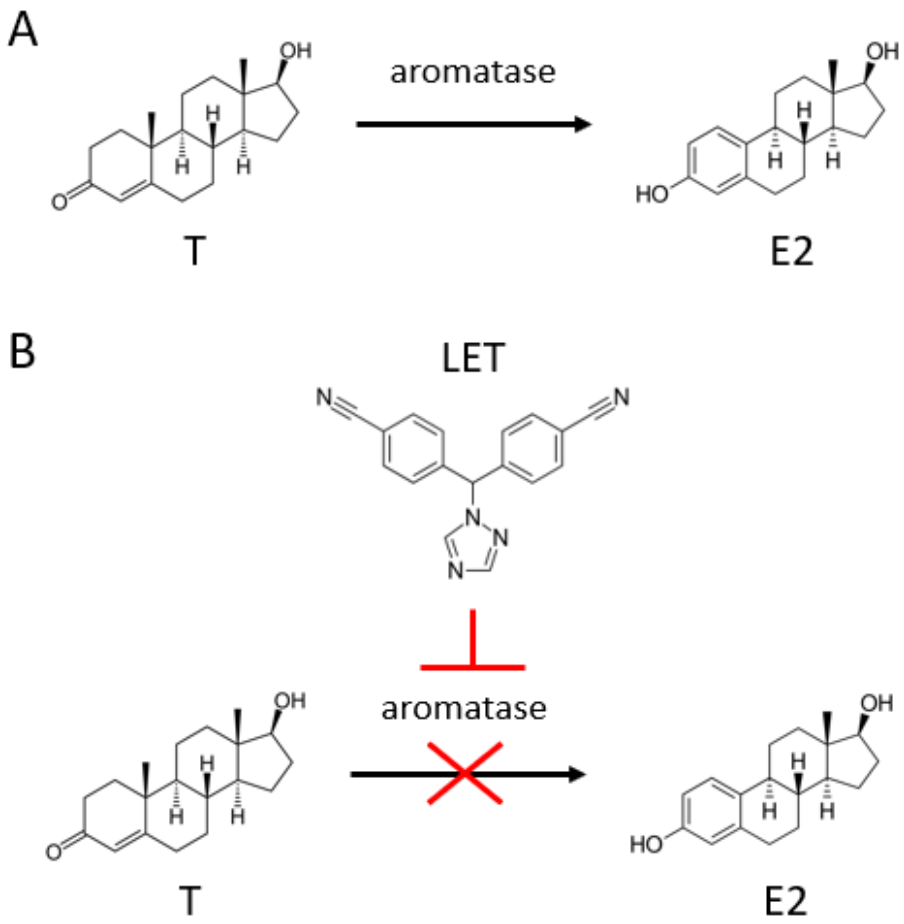


Figure 2: Letrozole inhibits the aromatization of testosterone to estradiol. (Panel A) Testosterone (T) is aromatized to estradiol (E2) in the ovary and other tissues that contain the aromatase enzyme. **(Panel B)** Administration of letrozole (LET), a non-steroidal aromatase inhibitor, blocks the aromatase enzyme from converting T to E2, leading to an increased concentration of T and a decreased concentration of E2.

Gut Microbiome

The gut microbiome, which consists of microbes in the intestinal tract and their metabolites, plays an important role in human health and disease[61,62] including complex interactions between host metabolism and gut microbes[63–66]. The biodiversity of the gut microbiome can be measured by using several different metrics including alpha diversity, or species richness within samples, and beta diversity, or the similarity of microbial compositions between samples.

Numerous studies have reported associations between changes in gut microbiota and metabolic disorders such as obesity, type 2 diabetes, and PCOS in humans and rodent models[67–72]. In addition, studies demonstrated that fecal microbiome transplantation from obese human donors into germ-free mice resulted in an obese phenotype[73,74], indicating that changes in the gut microbiome may be sufficient to induce metabolic dysregulation. Furthermore, studies showed that cohousing germ-free mice transplanted with stool from lean donors with mice transplanted with stool from obese donors resulted in a protective effect[71,73,74], suggesting that exchange of the gut microbiome between mice due to coprophagy was sufficient to protect the mice from developing obesity.

More recently, studies have explored the relationship between the gut microbiome and estrogen and have implicated the significance of the estrobolome, the collection of microbes that can metabolize estrogens and modulate their enterohepatic circulation[75,76]. While one study found that post-menopausal women had lower gut microbial alpha diversity compared to pre-menopausal women that was correlated with estrogen insufficiency[77], another study reported no differences between these two groups of women[78]. Similarly, in rodent models, there have been inconsistent findings regarding whether there is an association between the gut microbiome and

estrogen levels. One study found that OVX mice had lower gut microbial alpha diversity than sham-operated mice[79], but other studies indicated that there was no correlation between gut biodiversity and OVX[80–82]. It is interesting to note, however, that differences in gut microbial beta diversity were consistently found between sham-operated mice and OVX mice[33,79–81].

There is also evidence that OVX+HFD alters the gut microbiome in a unique way compared to OVX alone. It was reported that the gut microbiome of OVX+HFD mice had increased abundance of bacterial taxa from the phyla Verrucomicrobia and Proteobacteria as well as *Akkermansia* compared to that of OVX mice fed normal chow[79]. Moreover, the presence of *Bifidobacterium animalis* was only detected in OVX mice compared to OVX+HFD mice[79]. In an OVX+HFD and E2 replacement study, the beta diversity of OVX+HFD mice was shown to be different from that of HFD mice and OVX+HFD+E2 mice[33]. Interestingly, OVX+HFD+E2 mice had reduced abundance of *Proteobacteria* compared to OVX+HFD mice[33]. Findings from an OVX+HFD and E2 replacement study in leptin mutant (*ob/ob*) mice also indicated that E2 treatment was associated with increased abundance of S24-7 bacteria[83]. Altogether, these studies suggest that there may be important host/microbe interactions involved in the protective effect of E2 on OVX+HFD-induced metabolic dysregulation.

MATERIALS AND METHODS

Ovariectomy mouse model

Four-week-old and eight-week-old C57BL/6N female mice were purchased from Envigo (Indianapolis, IN). Mice were housed in a vivarium with a 12h:12h light/dark cycle (light period: 06.00-18.00). Mice were given ad libitum access to water and food (Teklad Global 18% Protein Extruded Diet, Envigo). All of the experiments were approved by the University of California San Diego Institutional Animal Care and Use Committee (Protocol S14011). At either four or eight weeks of age, all mice (n = 8/group for the first adult cohort with a total of 16 mice, n = 10/group for the second adult cohort with a total of 40 mice, and n = 8/group for the pubertal cohort with a total of 16 mice) underwent either a sham surgery or OVX. Mice were anesthetized with 2.5% isoflurane and placed in a prone position on a sterile field. A dorsal incision was made approximately 1 cm from the top of each leg. The ovary near the fat pad was exposed through an incision in the inner layer of skin and removed from the adherent tissue. Absorbable surgical suture (5-0, 18" Chromic Gut Absorbable Suture) was used to suture the abdominal cavity/muscle wall. The outer skin incision was closed with surgical wound clips. Mice that were sham-operated (SHAM) were subject to similar surgical procedures except for ovary removal. The effectiveness of the OVX procedure was assessed by determining estrous cycle stage from the predominant cell type in vaginal epithelial smears obtained during week 1 (one week after SHAM or OVX). Mice were weighed weekly throughout the experiment.

Insulin Tolerance Test

Mice were fasted for 5 hours and blood from the tail vein was collected to measure FBI. FBG was measured with a handheld glucometer (One Touch UltraMini, LifeScan, Inc., Milpitas, CA), and an intraperitoneal insulin tolerance test (ITT) was performed. FBG levels were

measured prior to time point 0. At time point 0, an intraperitoneal injection of insulin (0.75 U/kg in sterile saline; Humulin R U-100) was administered. Glucose was measured subsequently at 15, 30, 45, 60, 90, and 120 minutes post-administration of insulin.

Tissue collection

At the end of the experiment, the mice were euthanized with 2.5% isoflurane delivered with a precision vaporizer followed by a physical method. Terminal blood was collected through the inferior vena cava. Parametrial fat pads were dissected and weighed.

Hormone assays

Hormone levels were assessed at the end of the study (week 5). LH levels were measured using a radioimmunoassay (range 0.04 to 75 ng/mL) by the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core Facility. Serum insulin was measured with a mouse ELISA (ALPO) by the University of California, Davis Mouse Metabolic Phenotyping Center.

Statistical analyses

Data was expressed as the mean standard error of the mean for each group. Data residuals were checked for normality and data underwent Box Cox transformation if residuals were not normal. If transformation did not result in normality, a non-parametric test was used. The statistical package JMP 14 (SAS) was used to analyze differences between groups by Student t-test or two-way repeated measures ANOVA followed by post hoc comparisons of individual time points. Statistical significance was defined as $p < 0.05$.

Fecal sample collection and DNA isolation

Fecal samples were collected from one cohort of 8-week-old female mice (n = 10/group) prior to SHAM or OVX and once per week for 5 weeks. Fecal samples were frozen immediately after collection and stored at -80°C. Bacterial DNA was extracted from the samples with the DNeasy PowerSoil Kit (Qiagen) and stored at -80°C. The V4 hypervariable region of the 16S rRNA gene was PCR amplified with primers 515F and 806R[84]. The reverse primers contained unique 12-bp Golay barcodes that were incorporated into the PCR amplicons[84]. Amplicon sequence libraries were prepared at the Scripps Research Institute Next Generation Sequencing Core Facility, where the libraries were sequenced on an Illumina MiSeq as previously described[70].

16S rRNA gene sequence analysis

Raw sequences were imported into QIIME 2 (version 2019.10) with the q2-tools-import script, and sequences were demultiplexed with the q2-demux emp-single script. This procedure resulted in 3.4 million sequences, with an average of 28,000 sequences per sample. DADA2 software was used to obtain a set of observed sequence variants (SVs)[85]. Based on the quality scores, the forward reads were truncated at position 240 with the q2-dada2-denoise script. Taxonomy was assigned with a pretrained naive Bayes classifier (Greengenes 13_8 99% operational taxonomic units) and the q2-feature-classifier plugin[86]. Out of 120 samples, one was removed because of insufficient sequence coverage (OVX sample at week 2) resulting in 119 samples. In total, 1,126 SVs were identified from 119 fecal samples. The resulting SVs were then aligned in MAFFT[87], and a phylogenetic tree was built in FastTree[88]. Taxonomic distributions of the samples were calculated with the q2-taxa-barplot script. Alpha and beta diversity metrics were computed with the q2-diversity core-metrics script at a rarefied sampling depth of 9638. Two alpha diversity metrics, observed SVs and Faith phylogenetic diversity (PD),

were used to estimate microbial richness and phylogenetic biodiversity, respectively[89]. UniFrac was used to compare the similarity (beta diversity) between the microbial communities by calculating the shared PD between pairs of microbial communities[90,91].

Statistical analysis of 16S rRNA sequences

Statistical calculations were performed in the R statistical package (version 3.6.2) with the phyloseq (version 1.30.0)[92] and vegan package (version 2.5.6). Alpha diversity data were tested for normality via the Shapiro-Wilk test. A Linear Mixed-Effects model with repeated measures was used to analyze differences in alpha diversity using time and treatment as fixed effects. The model was fit by maximum-likelihood and was done with the nlme R package (version 3.1.144). P values were obtained using $\chi 2$ test performed using the car R package (version 3.0.7). Principal coordinate analysis (PCoA) plots[93] were constructed in the phyloseq R package. PCoA plots were used to represent the similarity of posttreatment (weeks 1 to 5) fecal microbiome samples based on multiple variables in the data set. Permutational multivariate analysis of variance (PERMANOVA) used post-treatment weighted and unweighted UniFrac distance measures to assess bacterial community compositional differences (999 permutations "vegan" package). DESeq2[94] (version 1.26.0) in the BiocManager package (version 1.30.10) was used to identify bacterial genera that were differentially abundant between OVX and SHAM mice. P values for the Differential Expression Analysis were obtained by Wald significance test, and false discovery rate correction was applied using a threshold of $p < 0.05$.

RESULTS

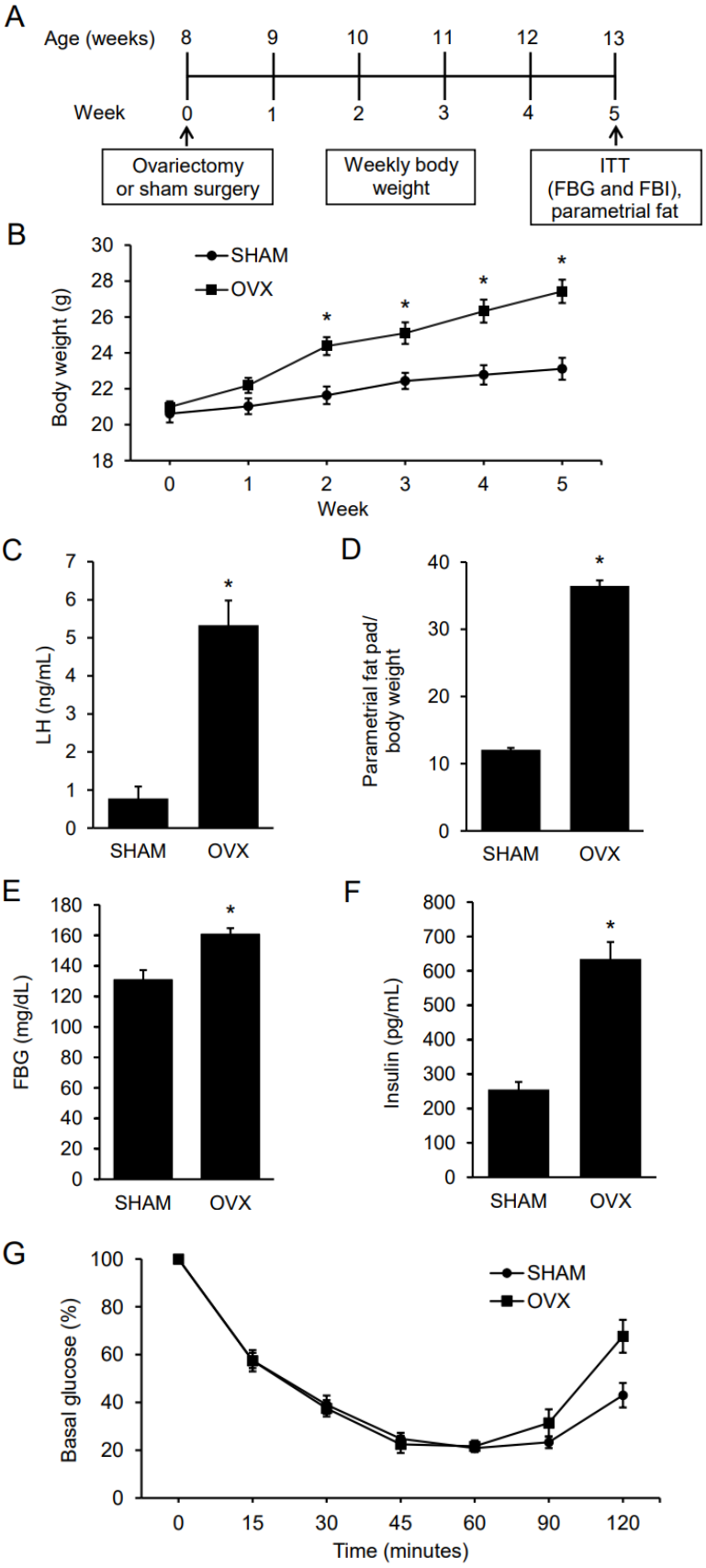
CHAPTER I: Minimal changes in gut microbiota are associated with post-ovariectomy weight gain in adult female mice

Since previous studies have suggested that gut microbes may play a role in the protective effect of estrogens on the long-term metabolic dysregulation that occurs in OVX females receiving HFD, we investigated whether the gut microbiome also influences the diet-independent weight gain observed shortly after OVX. As a complementary approach, we tested whether exposure to a healthy gut microbiome via a cohousing paradigm was protective against the development of metabolic phenotype associated with OVX.

Ovariectomy in mice results in metabolic dysregulation

Adult female mice were either SHAM or OVX at 8 weeks of age (n = 8 per group) (**Figure 3A**). Body weight was then assessed weekly for 5 weeks, and it was shown that OVX mice weighed significantly more than SHAM mice after 2 weeks (**Figure 3B**). Serum LH was measured after 5 weeks. As expected, OVX mice had elevated LH compared to SHAM mice (**Figure 3C**) due to the removal of E2-mediated negative feedback on the HPG axis (**Figure 1**). When parametrial fat was measured at the end of the experiment, OVX mice had increased parametrial fat relative to body weight (**Figure 3D**). An ITT was performed toward the end of the experiment and showed that OVX mice had elevated FBG and FBI compared to SHAM mice (**Figure 3E** and **Figure 3F**). Interestingly, OVX mice were not found to be insulin resistant (**Figure 3G**).

Figure 3: Ovariectomized mice develop distinct metabolic phenotype compared to sham-operated mice. (Panel A) Schematic of study design: female mice were either ovariectomized (OVX) or sham-operated (SHAM) at 8 weeks of age (n = 8 per group). Experimental procedures included weekly weight assessment, insulin tolerance test (ITT), and parametrial fat collection. **(Panels B-F)** Compared to SHAM mice, OVX mice had increased weight, luteinizing hormone (LH) levels, abdominal adiposity, fasting blood glucose (FBG) and fasting blood insulin (FBI) after 5 weeks. **(Panel G)** OVX mice had similar insulin sensitivity as SHAM mice after 5 weeks. Graph error bars represent standard error of the mean. Student *t*-test or repeated-measures ANOVA with post-hoc Student *t*-tests to compare OVX versus SHAM at specific time points were performed; * $p < 0.05$.


A
Age (weeks)
8
9
10
11
12
13
Week
0
1
2
3
4
5
Ovariectomy or sham surgery
Weekly body weight
ITT (FBG and FBI), parametrial fat
B
SHAM
OVX
Body weight (g)
Week
C
LH (ng/mL)
SHAM
OVX
D
Parametrial fat pad/ body weight
SHAM
OVX
E
FBG (mg/dL)
SHAM
OVX
F
Insulin (pg/mL)
SHAM
OVX
G
SHAM
OVX
Basal glucose (%)
Time (minutes)

Ovariectomy did not alter species richness (alpha diversity) of the gut microbiome

Within sample biodiversity (alpha) diversity in fecal samples from SHAM and OVX mice collected 5 weeks after surgery (n = 10 per group) was calculated using observed SVs as an estimate of species richness and Faith's PD as an estimate of species richness that accounts for phylogenetic relationships (**Figure 4**). There was no significant difference in the number of observed SVs between SHAM and OVX mice (**Figure 4A**). Moreover, Faith's PD was also similar between SHAM and OVX mice (**Figure 4B**), suggesting that the gut microbiome of SHAM and OVX mice 5 weeks after surgery was similar with regards to alpha diversity.

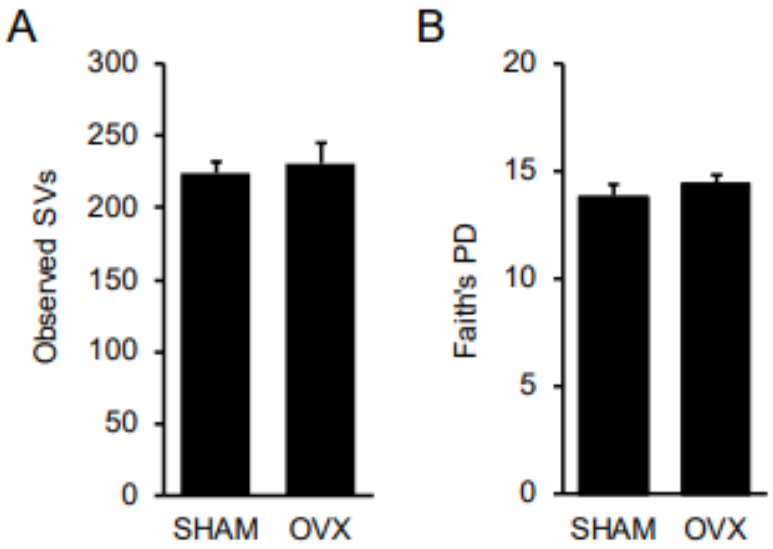


Figure 4: Ovariectomy was not associated with changes in alpha diversity of gut microbiome. Alpha diversity in fecal samples from SHAM and OVX mice (n = 10 per group) was calculated using **(Panel A)** the number of observed sequence variants (SVs) as an estimate of species richness and **(Panel B)** Faith phylogenetic diversity (PD) as an estimate of species richness that takes phylogenetic relationships into account. No difference in the number of observed SVs or Faith PD was observed between SHAM and OVX mice as determined by Student *t*-test. Graph error bars represent standard error of the mean.

Ovariectomy shifted the composition of the gut microbiome (beta diversity) as measured by unweighted but not weighted UniFrac

In addition to assessing alpha diversity, between sample (beta) diversity was estimated using weighted (takes SV abundance into account) and unweighted UniFrac analyses to compare the phylogenetic similarity of the gut microbial communities in samples obtained post-surgery in SHAM and OVX mice. Visualization of both weighted and unweighted UniFrac distances via PCoA did not reveal distinct clustering of samples by treatment (**Figure 5**). In addition, a PERMANOVA test (ADONIS) did not detect a significant effect of OVX on gut microbial composition for weighted UniFrac ($P = 0.179$) (**Figure 5A**). However, a PERMANOVA test for unweighted UniFrac indicated that there was an effect of OVX on gut microbial community structure when SV abundance was not taken into account ($P = 0.036$) (**Figure 5B**), suggesting that a few bacterial taxa may be driving differences in the gut microbiome after OVX.

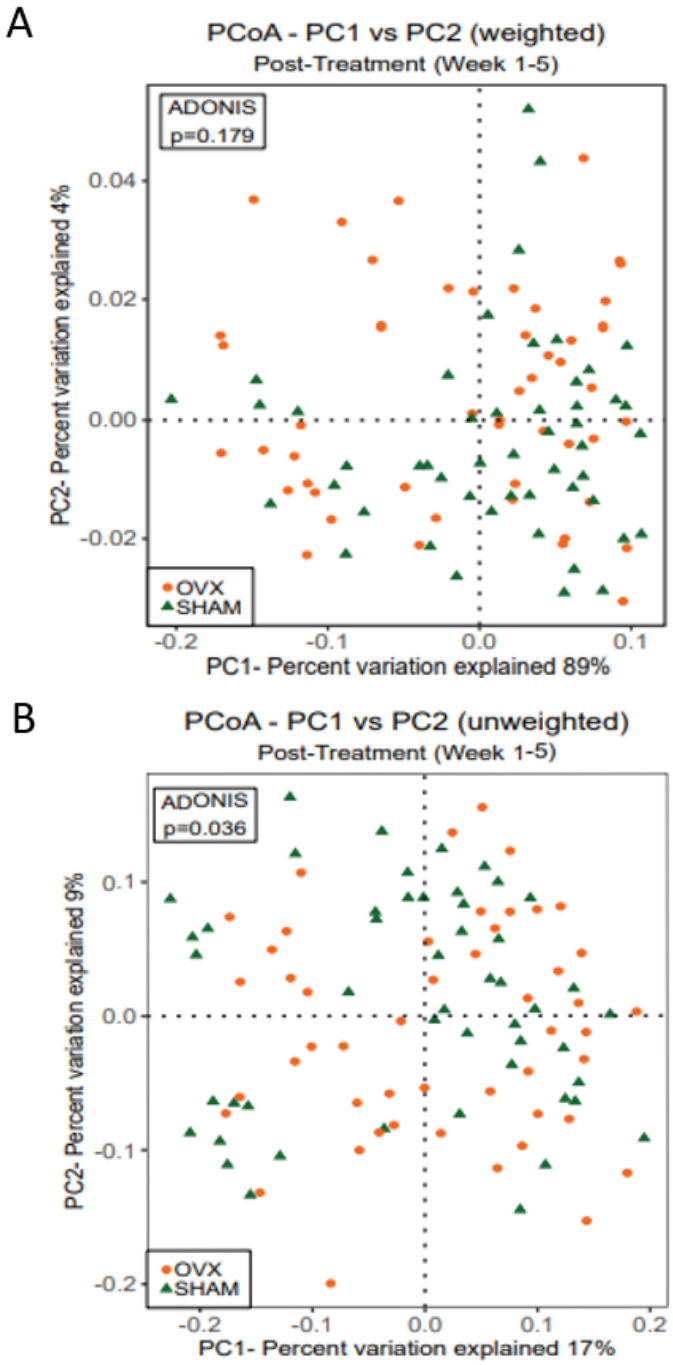


Figure 5: Ovariectomy was not associated with changes in beta diversity that takes taxa abundance into account. Beta diversity was measured using Principle Coordinate Analysis (PCoA) of **(Panel A)** weighted UniFrac and **(Panel B)** unweighted UniFrac for fecal samples from SHAM and OVX mice collected post-treatment (weeks 1-5). Proportion of variance explained by each principle coordinate axis is denoted in the corresponding axis labels. Results of permutational ANOVA (ADONIS) test are shown in box inset.

Ovariectomy is associated with changes in a few bacterial genera subsequent to weight gain

The differential abundance of gut bacteria in OVX versus SHAM mice was determined using DESeq2 (**Figure 6**). No significant differences in the relative abundance of bacterial taxa was found between SHAM and OVX mice at 1 or 2 weeks post-surgery despite a significant increase in weight at week 2. Two bacterial genera were identified with higher relative abundance (unidentified Lachnospiraceae and *Turicibacter*) and four bacterial genera with lower relative abundance (unidentified Clostridiales and *S24-7*) in OVX mice compared with SHAM mice three weeks after surgery (**Figure 6A**). Interestingly, an unidentified *Lachnospiraceae*, *Turicibacter* and S24-7 were still differentially abundant at week 4 while additional genera (*RF32*, an unidentified Bacteroides and Lachnospiraceae) were differentially abundant at this time point (**Figure 6B**). Additionally, DESeq2 identified a higher relative abundance of *Turicibacter* and lower relative abundance of an unidentified Clostridiales in OVX mice compared to SHAM mice five weeks after OVX (**Figure 6C**). These findings suggest that changes in gut bacterial abundances were symptomatic of OVX but did not cause metabolic dysregulation.

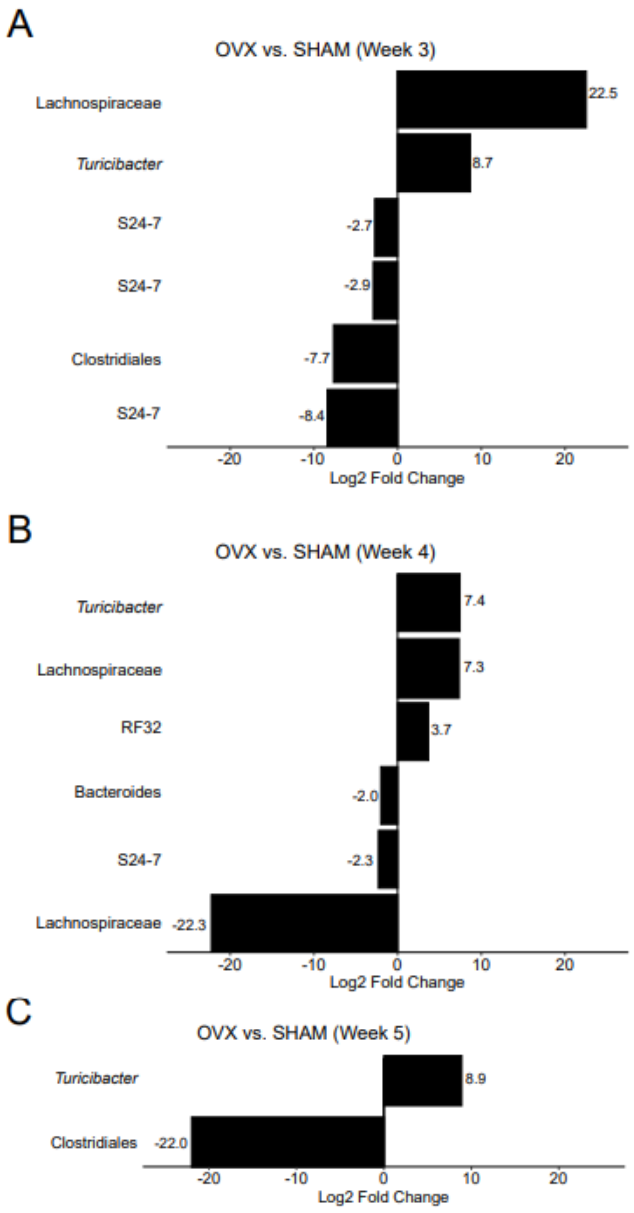


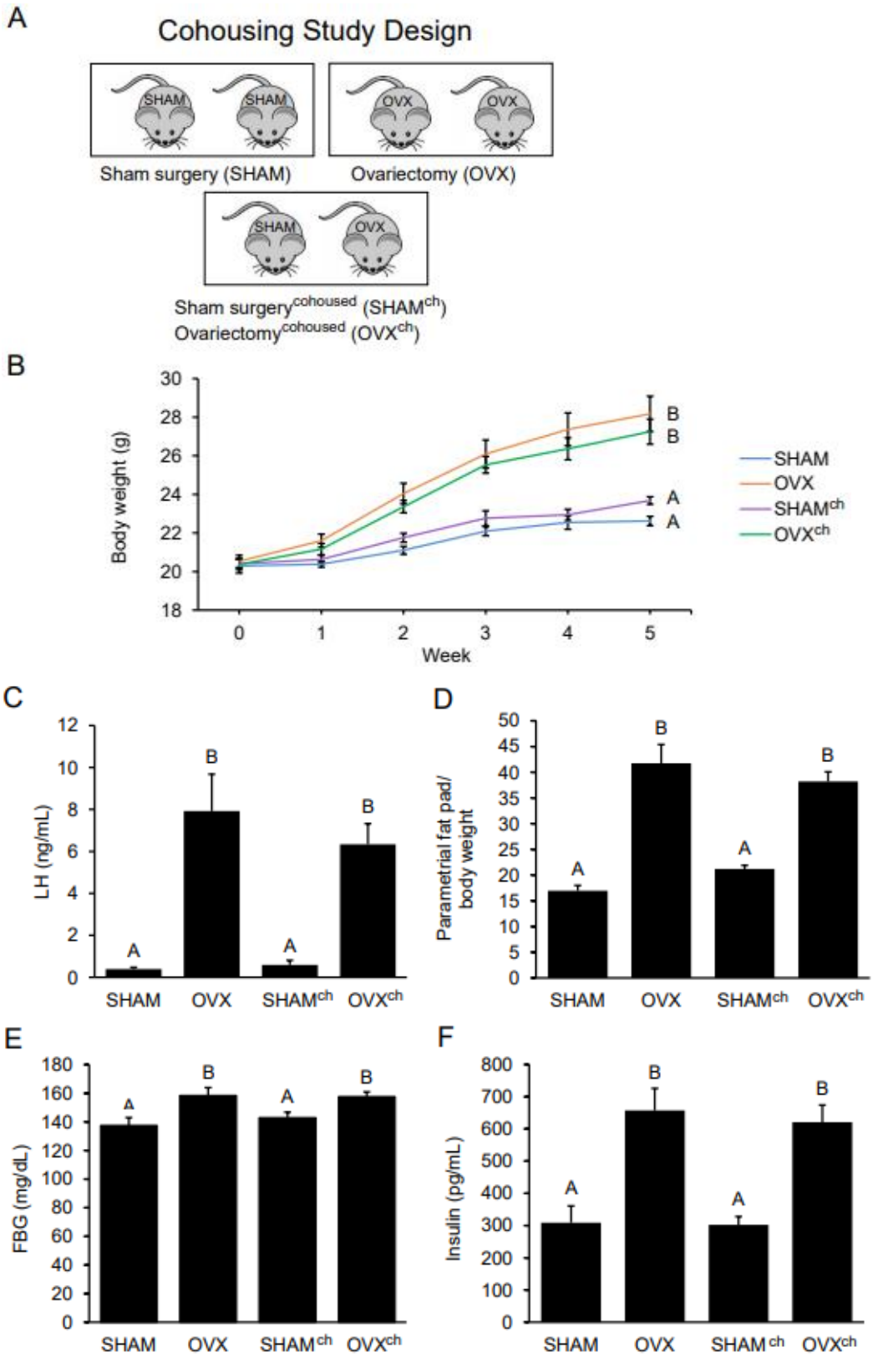
Figure 6: Changes in the relative abundance of S24-7, *Turicibacter*, unidentified Clostridiales and Lachnospiraceae occurred after ovariectomy-induced weight gain at 2 weeks. Results from DESeq2 differential abundance analysis were expressed as log2 fold change for SHAM versus OVX mice for weeks 3-5 after surgery (**Panels A-C**). Bacterial taxa that were significantly different between SHAM and OVX mice after multiple comparison correction and had a log2 fold effect of greater than 2.5 were displayed. Positive log2 fold changes represent bacterial genera increased in OVX mice relative to SHAM mice, and negative changes represent bacterial genera increased in SHAM mice relative to OVX mice. No significant differences in the relative abundance of bacterial taxa was found between SHAM and OVX mice at weeks 1-2 post-surgery.

Cohousing ovariectomized mice with sham-operated mice did not protect against metabolic dysregulation

To investigate whether exposure to a healthy gut microbiome can protect against metabolic dysregulation of OVX mice, a cohousing study was performed. Female mice were either OVX or SHAM at 8 weeks of age. The mice were housed two mice per cage in three different housing arrangements, resulting in four groups of mice (n = 10 per group): SHAM cohoused with SHAM, OVX cohoused with OVX, SHAM cohoused with OVX ($SHAM^{ch}$), or OVX cohoused with SHAM (OVX^{ch}) (**Figure 7A**). Body weight was assessed weekly for 5 weeks after surgery. OVX and OVX^{ch} mice had elevated weight compared to SHAM and $SHAM^{ch}$ mice after 5 weeks (**Figure 7B**). Serum LH levels were significantly elevated in OVX and OVX^{ch} mice but not in SHAM or $SHAM^{ch}$ mice (**Figure 7C**). Moreover, OVX and OVX^{ch} mice had greater abdominal adiposity compared to SHAM and $SHAM^{ch}$ mice when parametrial fat was measured relative to body weight at the end of the experiment (**Figure 7D**). Unlike SHAM and $SHAM^{ch}$ mice, OVX and OVX^{ch} mice also had increased FBG and FBI (**Figure 7E** and **Figure 7F**). These results indicate that cohousing did not protect against the development of the metabolic phenotype in OVX mice.

Chapter 1, in part, is a reprint of the material as it appears in Alterations in Gut Microbiota Do Not Play a Causal Role in Diet-independent Weight Gain Caused by Ovariectomy. *Journal of the Endocrine Society*. 2020. Lillian Sau, Christine M. Olmstead, Laura J. Cui, Annie Chen, Reeya S. Shah, Scott T. Kelley, and Varykina G. Thackray. The thesis author was the primary investigator and author of this chapter.

Figure 7: Cohousing ovariectomized mice with sham-operated mice did not protect against development of the ovariectomized metabolic phenotype. (**Panel A**) Design of cohousing study with adult female mice housed two per cage in three different housing arrangements that resulted in four groups of mice (n = 10 per group): SHAM, OVX, $SHAM^{ch}$, and OVX^{ch}. (**Panels B-F**) Compared to SHAM mice, OVX mice had increased weight, luteinizing hormone (LH) levels, abdominal adiposity, fasting blood glucose (FBG) and fasting blood insulin (FBI) after 5 weeks. (**Panels B-F**) Compared to OVX mice, OVX^{ch} mice had similar weight, LH levels, abdominal adiposity, FBG, and FBI. Graph error bars represent standard error of the mean. Different letters indicate significant differences in a one-way ANOVA or repeated-measures two-way ANOVA followed by post hoc comparisons with the Tukey-Kramer honestly significant difference test; $p < 0.05$.


A
Cohousing Study Design
SHAM
SHAM
OVX
OVX
Sham surgery (SHAM)
Ovariectomy (OVX)
SHAM
OVX
Sham surgery cohoused (SHAMch)
Ovariectomy cohoused (OVXch)
B
Body weight (g)
Week
SHAM
OVX
SHAMch
OVXch
C
LH (ng/mL)
D
Parametrial fat pad/ body weight
E
FBG (mg/dL)
F
Insulin (pg/mL)

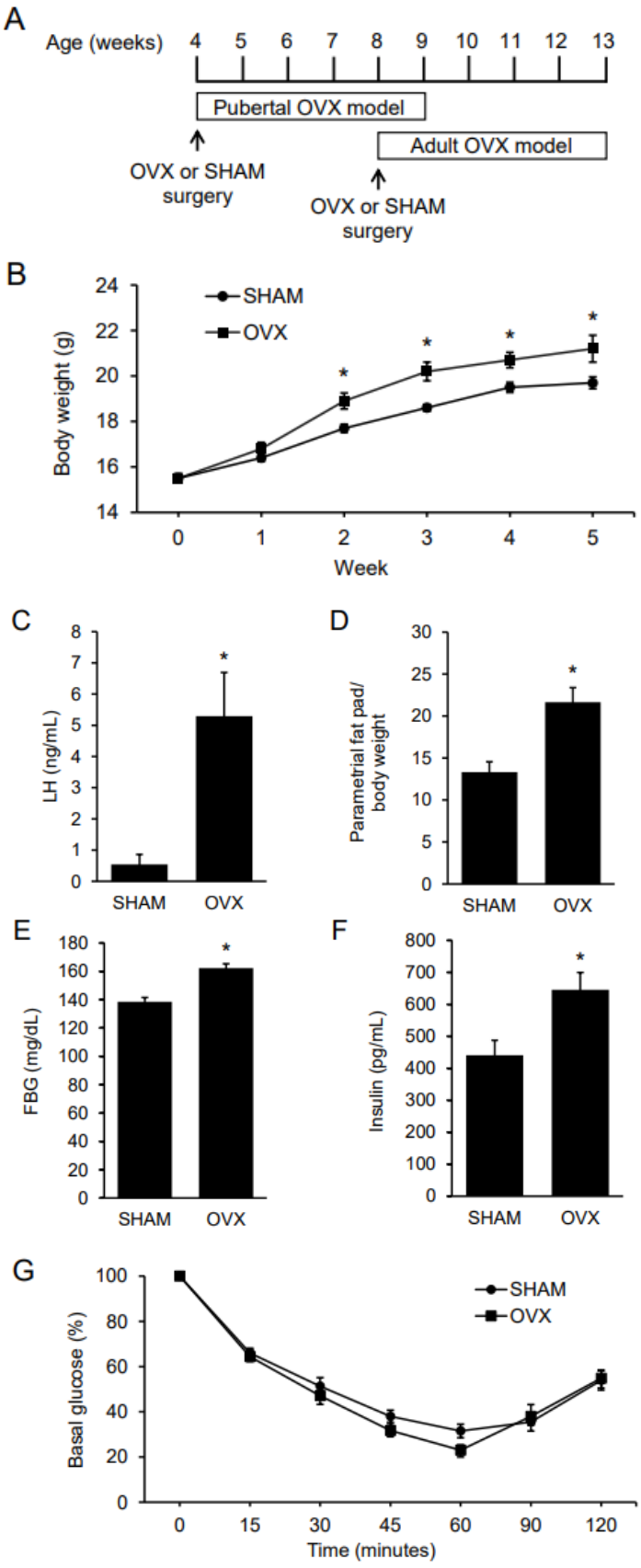
CHAPTER II: Comparing the metabolic phenotype of pubertal LET mouse model with pubertal OVX mouse model

Previously, we have utilized both pubertal and adult LET mouse models to investigate the role of the gut microbiome in PCOS[59,95,96]. Due to the strong metabolic phenotype observed in a pubertal LET mouse model of PCOS[59,95], we compared the metabolic phenotype of the pubertal LET model (high T; low E2) with a pubertal OVX model (low E2) to determine which phenotypes may be mediated by low E2. To account for the timing of treatment, reproductive and metabolic phenotypes from adult cohorts of OVX[97] and LET mice[96] were also used in the comparative analysis.

Ovariectomy in pubertal mice results in metabolic dysregulation

Pubertal female mice were either OVX or SHAM at 4 weeks of age (n = 8 per group) **(Figure 8A)**. Body weight was then assessed weekly for 5 weeks, and it was shown that OVX mice weighed significantly more than SHAM mice after 2 weeks (**Figure 8B**). Serum LH was measured after 5 weeks, and data showed that OVX mice had elevated LH compared to SHAM mice (**Figure 8C**). When parametrial fat was measured at the end of the experiment, OVX mice had increased parametrial fat relative to body weight (**Figure 8D**). An ITT performed toward the end of the experiment demonstrated that OVX mice had elevated FBG and FBI compared to SHAM mice (**Figure 8E-F**) and were not found to be insulin resistant (**Figure 8G**).

Figure 8: Ovariectomized pubertal mice develop distinct metabolic phenotype compared to sham-operated mice. (**Panel A**) Schematic of study design comparing pubertal model with adult model: female mice were either OVX or SHAM at 4 weeks of age (n = 8 per group). Experimental procedures included weekly weight assessment, ITT, and parametrial fat collection. (**Panels B-F**) Compared to SHAM mice, OVX mice had increased weight, LH levels, abdominal adiposity, FBG and FBI after 5 weeks. (**Panel G**) OVX mice had similar insulin sensitivity as SHAM mice after 5 weeks. Graph error bars represent standard error of the mean. Student *t*-test or repeated-measures ANOVA with post-hoc Student *t*-tests to compare OVX versus SHAM at specific time points were performed; * $p < 0.05$.


A
Age (weeks)
4 5 6 7 8 9 10 11 12 13
Pubertal OVX model
Adult OVX model
OVX or SHAM surgery
OVX or SHAM surgery
B
Body weight (g)
SHAM
OVX
Week
C
LH (ng/mL)
SHAM
OVX
D
Parametrial fat pad/ body weight
SHAM
OVX
E
FBG (mg/dL)
SHAM
OVX
F
Insulin (pg/mL)
SHAM
OVX
G
Basal glucose (%)
SHAM
OVX
Time (minutes)

Ovariectomized and letrozole-treated mice share similar metabolic phenotypes except for insulin resistance

Both pubertal and adult cohorts of OVX and LET mice had elevated weight gain, FBG and FBI, and parametrial fat (**Table 1**). Interestingly, out of the four cohorts, only pubertal LET mice developed insulin resistance (**Table 1**). To further assess the degree of similarity of the shared metabolic features between OVX and LET mice, we quantified percent change for weight gain, parametrial fat, FBI, and FBG for each pubertal cohort (**Table 2**). The averages of individual data for parametrial fat, FBI, and FBG were calculated for the following groups: LET mice (n = at least 9), placebo mice (n = 8), OVX mice (n = 10), and SHAM mice (n = 10). Percent change for each metabolic phenotype was then calculated for LET versus placebo for the pubertal LET cohort and OVX versus SHAM for the pubertal OVX cohort. To analyze percent change of weight gain, individual week 0 weights were first subtracted from week 5 weights to obtain values for net change in weight for each mouse. The averages of net change of the four treatment groups were then calculated and used to perform percent change analysis of weight gain for LET versus placebo and OVX versus SHAM.

Percent change analysis showed that pubertal LET mice had a 163% increase in body weight, 156% increase in parametrial fat/body weight, 25.9% increase in FBG, and 179% increase in FBI relative to pubertal placebo mice after 5 weeks of treatment (**Table 2**). On the other hand, pubertal OVX mice did not develop as drastic changes in their metabolic phenotype compared to pubertal SHAM mice. Pubertal OVX mice had a 36.9% increase in body weight, 62.4% increase in parametrial fat/body weight, 17.1% increase in FBG, and 46.3% increase in FBI relative to pubertal SHAM mice 5 weeks post-surgery (**Table 2**).

Table 1: Metabolic phenotypes of pubertal and adult cohorts of ovariectomized and letrozole mouse models. Metabolic phenotypes that were assessed include weight gain, FBI and FBG, parametrial fat, and insulin resistance. Yes = presence of phenotype, X = absence of phenotype.

Metabolic Phenotype	Pubertal LET (Kauffman et al., 2015; Torres et al., 2019)	Adult LET (Torres et al., 2019)	Pubertal OVX (Sau et al., in preparation)	Adult OVX (Sau et al., 2020)
↑ Weight gain	Yes	Yes	Yes	Yes
↑ FBG/FBI	Yes	Yes	Yes	Yes
↑ Abdominal fat	Yes	Yes	Yes	Yes
Insulin resistance	Yes	X	X	X

Table 2: Comparison of percent change of metabolic phenotypes between pubertal ovariectomized mice and pubertal letrozole-treated mice. Metabolic phenotypes that were assessed include weight gain, parametrial fat, FBI, and FBG. For parametrial fat, FBI, and FBG, the averages were calculated for the following groups: LET mice (n = at least 9), placebo mice (n = 8), OVX mice (n = 10), and SHAM mice (n = 10). Percent change was then calculated for LET versus placebo for the pubertal LET cohort and OVX versus SHAM for the pubertal OVX cohort. For weight gain analysis, week 0 weights were subtracted from week 5 weights to obtain net change in weight for each mouse. The averages of net change of the four groups were then calculated and used to perform percent change analysis for LET versus placebo and OVX versus SHAM weight gain.

Metabolic Phenotype	Pubertal OVX	Pubertal LET
Weight gain	36.9%	163%
Parametrial fat/body weight	62.4%	156%
FBG	17.1%	25.9%
FBI	46.3%	179%

Estrogen deficiency alone is not sufficient for the development of insulin resistance in a pubertal mouse model

While both pubertal OVX mice and pubertal LET mice share similar reproductive phenotypes such as elevated LH and a disrupted estrus cycle, only pubertal LET mice have increased levels of T and polycystic ovaries (**Table 3**). We expected these findings since the LET model simulates a PCOS-like state whereas the OVX model simulates a state of estrogen deficiency. Neither pubertal or adult OVX mice developed insulin resistance (**Figure 3G** and **Figure 8G**), which is in contrast with the pubertal LET cohort that did develop insulin resistance (**Table 1**). This suggests that a combination of high T and low E2 during a pubertal state may be necessary for developing insulin resistance in a mouse model.

Table 3: Reproductive phenotypes of pubertal and adult cohorts of ovariectomized and letrozole mouse models. Reproductive phenotypes that were assessed include LH and T levels, polycystic ovaries, and estrus cyclicity. Yes = presence of phenotype, X = absence of phenotype.

Reproductive Phenotype	Pubertal LET (Kauffman et al., 2015; Torres et al., 2019)	Adult LET (Torres et al., 2019)	Pubertal OVX (Sau et al., in preparation)	Adult OVX (Sau et al., 2020)
↑ LH	Yes	Yes	Yes	Yes
↑ T	Yes	Yes	X	X
Polycystic ovaries	Yes	Yes	X	X
Regular estrus cycle	X	X	X	X

DISCUSSION

Ovariectomy in female mice results in metabolic dysregulation

Our results demonstrated that OVX had a significant effect on metabolism in adult and pubertal female mice. In contrast to many previous studies that assessed the effect of OVX on metabolic phenotypes 3 months or more post-surgery, we focused on the effect of E2 deficiency in the short term after surgery. By two weeks post-surgery, adult and pubertal OVX mice had increased weight gain compared to SHAM mice (**Figure 3B** and **Figure 8B**). At five weeks post-surgery, adult and pubertal OVX mice had increased weight, abdominal adiposity, FBG, and FBI compared to SHAM mice (**Figure 3B, D-F** and **Figure 8B, D-F**), indicating that OVX has rapid effects on female metabolism. In addition, we found that neither pubertal or adult OVX mice developed insulin resistance (**Figure 3G** and **Figure 8G**) despite an increase in weight, FBG, and FBI. This result contrasts with multiple OVX+HFD studies that reported insulin intolerance in OVX+HFD mice[32–34] and supports the idea that short-term E2 deficiency is not sufficient to cause insulin resistance in an OVX mouse model.

Ovariectomy did not alter gut microbial alpha diversity but shifted beta diversity when measured by unweighted UniFrac

Our study also demonstrated that OVX had a minimal effect on overall gut microbiome composition in terms of alpha diversity. According to 16S rRNA gene sequencing analysis, there was no difference in alpha diversity (overall species richness) of gut bacteria between adult OVX and SHAM mice 5 weeks after surgery (**Figure 4**). However, another study that investigated changes in the gut microbiome after OVX reported that OVX mice had lower alpha diversity (measured by the number of observed OTUs and Shannon diversity index) in their gut microbiomes 12 weeks after surgery[79]. One possible explanation for this inconsistency is that the duration of our experiment did not allow for sufficient time for the gut microbiome of OVX mice to become different from the microbiome of SHAM mice, although an adult OVX rat model showed that there was no difference in alpha diversity of the gut microbiome between OVX and SHAM rats 13 weeks after surgery[81]. More longitudinal studies will be needed to determine whether OVX alters gut microbial composition in rodents and how much time is required for changes in the gut microbiome to occur.

In contrast with alpha diversity, we demonstrated that there was a difference in beta diversity between SHAM and OVX mice as measured by unweighted UniFrac but not weighted UniFrac analysis (**Figure 5**). While unweighted UniFrac is based on the presence or absence of observed bacterial species, weighted UniFrac takes into account the abundance of the observed bacterial species. Thus, the significant difference in unweighted UniFrac between SHAM and OVX mice may be due to a few bacterial taxa driving the change in gut microbial composition. Although other studies reported differences in beta diversity between SHAM and OVX mice using weighted UniFrac analysis[79,98], the durations of these experiments were much longer than

that of our study. Interestingly, one study showed a difference in beta diversity between SHAM and OVX mice 10 weeks post-surgery but not 6 weeks post-surgery[98], which again suggests that the length of time post-surgery may be important with regards to observing differences in the gut microbiome of OVX rodents compared to SHAM controls.

Ovariectomy was associated with changes in the relative abundance of a few bacterial taxa subsequent to weight gain

According to differential abundance analysis, we demonstrated that OVX in adult mice resulted in a few changes in the relative abundances of certain gut microbiota. In particular, we found increased relative abundance of *Turicibacter* and unidentified Lachnospiraceae as well as decreased relative abundance of *S24-7* and unidentified Clostridiales in the gut microbiome of OVX mice compared to SHAM mice 3-5 weeks post-surgery (**Figure 6**). Similar to our findings, increased relative abundance of unidentified Lachnospiraceae and decreased relative abundance of unidentified S24-7 in the gut microbiome of OVX mice was previously reported[98]. Clostridiales species were found to be enriched in HFD-fed mice and decreased in OVX mice in a OVX+HFD study[79], which is also consistent with our findings. While *Turicibacter* has not been previously linked to OVX, lower abundance of *Turicibacter* was associated with increased body weight in a HFD rodent model[99]. It was also reported that *Turicibacter* may be involved in short chain fatty acid production[100]. At 4 weeks post-surgery, we found increased relative abundance of *RF32* and decreased relative abundance of *Bacteroides* in the gut microbiome of OVX mice compared to SHAM mice (**Figure 6B**). The lower relative abundance of *Bacteroides* in the gut microbiome of OVX mice is consistent with findings from an OVX+HFD study[79], and higher abundance of *RF32* in the gut microbiome has been linked to HFD in a few mouse models[101,102].

It is important to note that significant changes in the gut microbiota of OVX mice were not observed 1 or 2 weeks post-surgery but instead occurred at weeks 3-5 after significant weight gain at week 2. These results support the idea that these changes in gut microbes do not play a

causal role in the weight gain associated with OVX but rather, are another symptom of E2 deficiency.

Although we observed minimal changes in the gut microbiome that were associated with short-term E2 deficiency in an adult OVX mouse model, a future direction that might be worth investigating is to determine whether changes in gut microbiota are associated with E2 insufficiency in a pubertal OVX mouse model. Similar to our findings, LET treatment of pubertal and adult mice was not correlated with significant changes in overall gut alpha diversity[95,96]. However, it was observed that LET treatment of both pubertal and adult female mice resulted in distinct shifts in gut microbial beta diversity compared to placebo mice when measured by weighted and unweighted UniFrac[95,96]. We also previously found that there was a significant difference in the bacterial taxa driving the shift in beta diversity in the two LET mouse models[96]. Thus, it would be interesting to see whether there are unique changes that occur in the gut microbiome during a pubertal state combined with E2 insufficiency and to compare these findings to those from the LET models.

Another rationale for exploring the relationship between gut microbiota and E2 deficiency in a pubertal state is because maturation of the gut microbiome has been associated with puberty, a period of substantial hormonal and metabolic change. Studies have demonstrated that children and adolescents have distinct gut microbial profiles compared to adults[103,104], and it was also shown that pubertal mice have different gut microbiomes compared to adult mice[105,106]. A strong positive correlation between gut bacteria alpha diversity and age was also reported in humans and mice[107,108]. Therefore, determining whether there are specific changes in gut microbiota associated with E2 deficiency in a pubertal versus adult state would be a useful approach to further elucidate the relationship between sex steroids and the gut microbiome.

Cohousing ovariectomized mice with sham-operated mice did not protect against development of metabolic dysregulation

To further explore whether changes in the gut microbiome post-OVX play a causal role in weight gain, we utilized a cohousing paradigm to test whether exposure to a healthy gut microbiome was protective against development of a metabolic phenotype induced by OVX in adult mice (**Figure 7A**). Overall, we found that cohousing did not attenuate OVX metabolic dysregulation since OVX mice that were cohoused with SHAM mice had similar metabolic symptoms as OVX mice that were not cohoused with SHAM including weight gain, abdominal adiposity, FBG, and FBI (**Figure 7B, D-F**). These results are in contrast with other cohousing studies in which protection against metabolic phenotypes was conferred to experimental mice that were cohoused with healthy control mice. For instance, gut microbiota from identical twins discordant for obesity (lean or obese) was transplanted into germ-free mice and resulted in lean or obese mice, respectively[73]. Notably, cohousing the obese mice with lean mice prevented the increased body weight and adiposity observed in obese mice that were not cohoused[73]. In another mouse study, cohousing offspring of HFD-fed mothers with the offspring of mothers fed normal diet was protective against developing insulin resistance[109]. Cohousing was also shown to have a protective effect in a LET-induced PCOS pubertal mouse model[95]. Cohousing LET mice with placebo mice resulted in significantly improved metabolic phenotypes including less weight gain, abdominal adiposity, and insulin intolerance[95]. Altogether, these studies demonstrate that, while cohousing can be protective against development of metabolic dysregulation in other contexts, it was unable to alter the development of OVX-induced weight gain.

Ovariectomized mice developed less severe metabolic dysregulation than letrozole-treated mice

Our comparison of the metabolic phenotypes from the pubertal OVX and pubertal LET mouse models showed that both cohorts developed metabolic dysregulation including elevated weight gain, FBG and FBI, and parametrial fat (**Table 1**). However, percent change analysis of the metabolic phenotypes that were assessed suggest that pubertal LET mice developed a more extreme metabolic phenotype compared to pubertal OVX mice (**Table 2**). Additionally, we found that only pubertal LET mice had insulin resistance among pubertal and adult cohorts of OVX and LET mice (**Table 1**). One explanation for the milder metabolic phenotype of the pubertal OVX model is that E2 deficiency alone does not lead to the same degree of metabolic dysregulation as that caused by both high T and low E2, which is characteristic of the LET model. Interestingly, preliminary data from a pubertal DHT mouse model indicates that high androgens alone is not sufficient for the development of insulin resistance in pubertal mice. In the future, it would be interesting to perform an experiment using a pubertal OVX+DHT cohort to investigate whether having a combination of high T and low E2 is necessary to develop insulin resistance in a pubertal mouse model.

Conclusion

In summary, this study demonstrated that minimal changes in gut microbiota observed after OVX do not appear to play a causal role in OVX-induced metabolic dysregulation since they occur after weight gain. This finding contrasts with alterations in the gut microbiome associated with metabolic dysregulation in OVX+HFD models[33,79]. Additionally, cohousing OVX mice with SHAM mice did not improve the metabolic phenotype of OVX mice, which further supports the idea that changes in the gut microbiome do not cause the metabolic dysregulation observed in the short term after OVX. While our findings suggest that the gut microbiome does not play a mechanistic role in the weight gain that occurs rapidly after OVX, additional studies will be needed to understand the relationship between E2 levels and changes in the relative abundance of bacteria such as *Turicibacter*, *S24-7*, as well as unidentified species of Lachnospiraceae and Clostridiales that may have effects on the host.

From a translational standpoint, our findings indicate that therapies targeted toward altering the gut microbiome are not likely to be effective in attenuating weight gain associated with E2 deficiency. This may have significant implications in several contexts of E2 insufficiency including menopause, hyperprolactinemia, and chemotherapy. In contrast, there may be potential for therapies that target the gut microbiome in treating metabolic symptoms associated with PCOS since the gut microbiome was shown to be changed in pubertal and adult LET mouse models[95,96] and had a protective effect against metabolic dysregulation in a pubertal LET mouse model[95]. Overall, future studies will need to be performed to further understand the role of the gut microbiome in pathologies associated with changes in sex steroid levels and the relevant physiological and molecular mechanisms involved.

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