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# The Role of the Hypothalamic-Pituitary-Gonadal (HPG) Axis in the Development of the Gut Microbiome

A Dissertation submitted in partial satisfaction of the requirements
for the degree Doctor of Philosophy

in

Biology

by

Laura Gilman Sisk-Hackworth

Committee in charge:

University of California San Diego

Professor Varykina G. Thackray, Co-Chair
Professor Joseph Pogliano

San Diego State University

Professor Scott T. Kelley, Chair
Professor Marina Kalyuzhnaya
Professor David Lipson

2024

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The Dissertation of Laura Gilman Sisk-Hackworth is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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_____
Co-Chair

_____
Chair

University of California San Diego

San Diego State University

2024

# DEDICATION

This dissertation is dedicated to my parents, Yvonne and Dave Sisk, who have been there for me throughout my schooling, from tutoring me in grade school to supporting me through college, to helping keep me sane during the stresses of going to graduate school during a pandemic. I don't know how I would have survived 2020 without you.

To my family members who have consistently shown up for me, whether in person or from afar. Thank you for always being there. To Katie, specifically, for believing in my particular magic.

To all the teachers who nurtured my curiosity and challenged me to dream bigger.

To my long-time friends who helped me get here. Specifically, to Dr. Letty Thottathil, who first encouraged me to pursue a Ph.D., and Siddharth Prabhu, who believed in me when I was unsure of myself. To Avery Delmaine, who commiserated with me as we wrote our graduate school applications together. To Joshua Nevin and Victor Vasquez, whose humor kept me in good spirits during the application process and beyond.

Finally, this dissertation is dedicated to my spouse, Mark Hackworth. You have been my biggest cheerleader since we were 16. Thank you for always having faith in me and telling me to go for it, even when you have no idea what I am doing. Thank you for being my calm harbor in the storm and for holding down the fort while I finish this journey.

EPIGRAPH

If you try and take a cat apart to see how it works,
the first thing you have on your hands is a nonworking cat.

Douglas Adams, *The Salmon of Doubt*

TABLE OF CONTENTS

DISSERTATION APPROVAL PAGE ..... iii

DEDICATION ..... iv

EPIGRAPH ..... v

TABLE OF CONTENTS ..... vi

LIST OF FIGURES ..... viii

LIST OF TABLES ..... ix

ACKNOWLEDGEMENTS ..... x

VITA ..... xiii

ABSTRACT OF THE DISSERTATION ..... xv

CHAPTER 1: Sex, Puberty, and the Gut Microbiome ..... 1

Abstract ..... 2

Introduction ..... 2

Sex differences and the gut microbiome ..... 3

Microbial modification of sex steroids ..... 6

Sex differences and bile acids ..... 7

Interplay between bile acids, the gut microbiome, and PCOS ..... 8

The immune system, sex, and the gut microbiome ..... 8

Conclusions and future perspectives ..... 8

Acknowledgements ..... 16

CHAPTER 2: Genetic Hypogonadal mouse model reveals niche-specific influence of reproductive axis and sex on intestinal microbial communities ..... 17

Abstract ..... 18

Introduction ..... 19

Methods ..... 21

Results ..... 22

Discussion ..... 28

References ..... 33

Acknowledgements ..... 37

CHAPTER 3: Influence of the Reproductive Axis on Gut Microbiome Maturation During Puberty ..... 38

Abstract ..... 38

Introduction ..... 38

Methods ..... 41

Results ..... 48

Supplemental Figures ..... 66

Supplemental Tables ..... 67

Discussion ..... 71

References ..... 84

DISSERTATION DISCUSSION ..... 85

References ..... 88

LIST OF FIGURES

Figure 1.1. Sex differences in the human gut microbiome arise during puberty ..... 4

Figure 1.2. Activation of the Hypothalamic-Pituitary-Gonadal axis ..... 5

Figure 1.3. Enterohepatic circulation of sex steroids and bile acids ..... 6

Figure 2.1. Experimental Design ..... 20

Figure 2.2. Intestinal environment, *hpg* status, and sex affect microbial composition ..... 23

Figure 2.3. Intestinal environment affects alpha diversity measures more than sex and *hpg* genotype ..... 24

Figure 2.4. Intestinal environment, sex, and *hpg* status affect family-level abundance ..... 26

Figure 2.5. Genotype and sex influence intestinal beta diversity in a niche-specific manner 27

Figure 2.6. Genus-level bacterial balance defines intestinal community differences by sex and genotype ..... 29

Figure 3.1. Experimental Design ..... 42

Figure 3.2. Age (week), hpg genotype, and sex influence gut microbial family abundances and beta diversity computed from 16S sequencing data ..... 50

Figure 3.3. Age-based genera balances differentiate adult mice by sex and hpg genotype. ... 52

Figure 3.4. Puberty alters fecal bacterial community composition and networks at the species level in metagenomes. ..... 57

Figure 3.5. HPG axis influences structure of adult fecal bacterial networks. ..... 58

Figure 3.6. Microbial genes and orthologous groups differ by age, genotype and sex. ..... 61

Figure 3.7. Gut lipid metabolites differ by age, sex, and genotype. ..... 63

Figure 3.8. Gut lipid metabolites differ by age, sex, and genotype. ..... 65

Supplemental Figure 3.1. Network statistics box and whisker plots for week 3 and week 10 networks. ..... 66

Supplemental Figure 3.2. Network visualizations for week 3 networks computed on 5000 bootstrap replicates of MAG species assignments. ..... 67

LIST OF TABLES

Table 2.1. Sex and *hpg* genotype affect alpha diversity in the cecum only ..... 25

Table 2.2. p Values and R2 from mixed-effect PERMANOVA for Euclidean distances..... 25

Table 2.3. Families showing an effect of section, sample type, sex, or *hpg* genotype..... 26

Table 3.1. Results of linear mixed-effect model analysis with top 10 most abundant bacterial families. ..... 49

Table 3.2. PERMANOVA results for Euclidean distances computed for MAG bacterial species. P-values less than 0.05 are bolded. ..... 54

Table 3.3. MAG species network statistics for week 3 and week 10 (5000 bootstrap replicates) and Binomial tests p-values comparing week 3 and week 10 networks. ..... 55

Table 3.4. PERMANOVA results for Euclidean distances computed for Gene and Ortholog abundances. P-values less than 0.05 are bolded. ..... 60

Table 3.5. PERMANOVA results for Euclidean distances computed for metabolite datasets. P-values less than 0.05 are bolded. ..... 61

Table 3.6. Out-of bag error and accuracy for Random Forest models on sex differences for pre-pubertal (week 3) or post-pubertal (week 10) wild-type or mutant mice ..... 62

Table 3.7. Table 7. T-test results (p-values) for lipid class sex differences in adult mice within each genotype. P-values are FDR-corrected with values < 0.05 in boldface. ..... 63

Table 3.8. FDR corrected t-test results (p-values) for amine class sex differences in adult mice within each genotype. P-values <0.05 are in boldface. ..... 65

Supplemental Table 3.1. List of the species indicated by each label in the network plots ..... 68

Supplemental Table 3.2. Taxonomic identities of the 20 most-connected nodes (species) for each week 3 network. E = Edges ..... 69

Supplemental Table 3.3. Taxonomic identities of the 20 most-connected nodes (species) for each week 10 network. E = Edges ..... 70

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Chapter 3, in full, in part is currently being prepared for submission for publication of the material by Sisk-Hackworth L, Akhavan, S, Krutkin, D, Kelley, ST, and Thackray, VG. The dissertation author was the primary researcher and author of this material.

VITA

| | |
|---|---|
| 2016 | Bachelor of Science in Biological Sciences, California Polytechnic State University San Luis Obispo |
| 2016-2018 | Research Associate, United States Environmental Protection Agency and Oak Ridge Associated Universities |
| 2018-2019 | Research Associate, Salk Institute for Biological Studies |
| 2019-2020 | Teaching Associate, San Diego State University |
| 2024 | Instructor of Record, San Diego State University |
| 2024 | Doctor of Philosophy in Biology, University of California San Diego and San Diego State University |

PUBLICATIONS

Aslam, S., Roach, D. Nikoloch, M., Biswas, B., Schooley, R., Bishop-Lilly, K., Rice, G., Cer, R., Hamilton, T., Henry, M., Luong, T., Salabarria, A., **Sisk-Hackworth, L**., Filippov, A., Lebreton, F., Hall, L., Paz, R.N., Onallah, H., Livni, G., Shostak, E., Wieder-Finesod, A., Yahav, D., Yerushalmy, O., Alkalay-Oren, S., Braunstein, R., Khalifa, L., Rimon, A., Gelman, D., Hazan, R. (2024*).* Pseudomonas aeruginosa ventricular assist device infections: Findings from ineffective phage therapies in five cases. *Antimicrobial Agents and Chemotherapy* 0:e01728-23. https://doi.org/10.1128/aac.01728-23

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ABSTRACT OF THE DISSERTATION

The Role of the Hypothalamic-Pituitary-Gonadal (HPG) Axis in the Development of the Gut Microbiome

by

Laura Gilman Sisk-Hackworth

Doctor of Philosophy in Biology

University of California San Diego, 2024
San Diego State University, 2024

Professor Scott T. Kelley, Chair
Professor Varykina G. Thackray, Co-Chair

The gut microbiome, a complex set of communities within the intestinal tract, interacts with the host to impact health and disease. Many sex-specific diseases, including reproductive, metabolic, and auto-immune diseases, have been linked to the composition of the gut microbiome. Studies have also shown that sex differences in the gut microbiome develop in adulthood. Given this, understanding how sex differences in the gut microbiome develop is necessary to understand how sex-specific, gut microbiome-linked diseases develop and can be treated. In Chapter 1, I describe what was previously known about the timing of when sex differences develop in the gut microbiome, the effect of sex steroids on the gut microbiome, and propose mechanisms through which sex steroids could affect gut microbiome composition. To investigate the role of reproductive development on sexual

maturation of the gut microbiota in this, I used a hypogonadal mouse model, in which the hypothalamic-pituitary-gonadal (HPG) axis is not activated, to determine the impact of the HPG axis on sexual maturation of the gut microbiota. In Chapter 2, I use 16S sequencing of intestinal samples to show that activation of the HPG axis during development leads to niche-specific sexual maturation of the intestinal microbiome. Results from Chapter 2 also indicate that sex chromosomes may cause sex differences in the gut microbiome in some parts of the intestine. In Chapter 3, I show that HPG axis activation during the pubertal period specifically is necessary for sexual differentiation of the gut microbiota, but that some aspects of gut microbiome maturation during puberty are independent of the HPG axis. Overall, these results indicate that HPG axis activation is critical for both general maturation and sexual differentiation of the gut microbiota, but that the HPG axis may not be the only factor contributing to this maturation. This suggests that microbiome-based therapeutics, especially for sex-specific disorders, should consider patient sex, age, and hormonal status.

# CHAPTER 1: Sex, Puberty, and the Gut Microbiome

Laura Sisk-Hackworth[1,2], Scott T Kelley[2] and Varykina G Thackray[1]

[1]Department of Obstetrics, Gynecology and Reproductive Sciences, University of California, San Diego, La Jolla, California, USA

[2]Department of Biology, San Diego State University, San Diego, California, USA;

REPRODUCTION
REVIEW

# Sex, puberty, and the gut microbiome

Laura Sisk-Hackworth[1], Scott T Kelley[1] and Varykina G Thackray[2]

[1]*Department of Biology, San Diego State University, San Diego, California, USA and* [2]*Department of Obstetrics, Gynecology and Reproductive Sciences, University of California, San Diego, La Jolla, California, USA*

*Correspondence should be addressed to V Thackray; Email: vthackray@ucsd.edu*

## Abstract

**In brief:** Sex differences in the gut microbiome may impact multiple aspects of human health and disease. In this study, we review the evidence for microbial sex differences in puberty and adulthood and discuss potential mechanisms driving differentiation of the sex-specific gut microbiome.

**Abstract:** In humans, the gut microbiome is strongly implicated in numerous sex-specific physiological processes and diseases. Given this, it is important to understand how sex differentiation of the gut microbiome occurs and how these differences contribute to host health and disease. While it is commonly believed that the gut microbiome stabilizes after 3 years of age, our review of the literature found considerable evidence that the gut microbiome continues to mature during and after puberty in a sex-dependent manner. We also review the intriguing, though sparse, literature on potential mechanisms by which host sex may influence the gut microbiome, and vice versa, via sex steroids, bile acids, and the immune system. We conclude that the evidence for the existence of a sex-specific gut microbiome is strong but that there is a dearth of research on how host–microbe interactions lead to this differentiation. Finally, we discuss the types of future studies needed to understand the processes driving the maturation of sex-specific microbial communities and the interplay between gut microbiota, host sex, and human health.

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## Introduction

Next-generation sequencing (NGS) has revolutionized our understanding of the human gut microbiome. The combination of NGS, multiplex-library construction, and bioinformatics has revealed gut microbial diversity to be temporally dynamic and highly variable among both populations and individuals. Moreover, the gut microbial community collectively contains two orders of magnitude of more genes than the human genome (Zhu *et al.* 2010, Huttenhower *et al.* 2012, Falony *et al.* 2016, Levy *et al.* 2020). Variation in abiotic (pH, oxygen levels, nutrition) and biotic factors (immune surveillance, signaling molecules) creates numerous niches along the alimentary tract that select for highly differentiated communities of microorganisms (Barlow *et al.* 2020). Conversely, the microbes in the mammalian gut influence a broad range of host physiological parameters ranging from metabolism (Baars *et al.* 2018, Acharya *et al.* 2019, Colldén *et al.* 2019) to immunity (Markle *et al.* 2014, Candon *et al.* 2015, Vatanen *et al.* 2016), to the nervous system (Desbonnet *et al.* 2015, Murray *et al.* 2020, Heinzel *et al.* 2021), and to the reproductive system (Acharya *et al.* 2019, Arroyo *et al.* 2019).

The human gut microbiome develops rapidly after birth, when the alimentary canal is seeded by the mother and environmental microbiomes (Breitbart *et al.* 2008, Bäckhed *et al.* 2015, Dominguez-Bello *et al.* 2016, Ferretti *et al.* 2018, Helve *et al.* 2019, Song *et al.* 2021). Studies showed that the infant gut microbiome rapidly increases in diversity and begins to resemble adult gut microbiome taxonomic composition and functional pathways by 2–3 years of age (Kurokawa *et al.* 2007, Koenig *et al.* 2011, Yatsunenko *et al.* 2012, Bokulich *et al.* 2016). Based on these initial studies, the gut microbiome of young children was thought to be fully mature by age 3. However, the gut microbiomes of children can be readily distinguished from adults (Agans *et al.* 2011, Yatsunenko *et al.* 2012, Ringel-Kulka *et al.* 2013, Dominianni *et al.* 2015, Hollister *et al.* 2015).

Many important and profound changes in human physiology occur during puberty, which may also be the case with the gut microbiome. In humans, sex-specific processes have been linked to the gut microbiome, including cognition and anxiety (Desbonnet *et al.* 2015, Murray *et al.* 2019), liver metabolism (van Keulen *et al.* 2020, Bhat *et al.* 2021), immunity (Shepherd *et al.* 2020, Wilharm *et al.* 2021), and menstrual regularity (Wang *et al.* 2020a). In addition, diseases which emerge during and after puberty have been linked to changes in gut microbial composition, often in a sex-specific manner. These include inflammatory bowel disease (IBD)

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(Ferguson & Sedgwick 1994, Franzosa *et al.* 2019), type I diabetes (T1D) (Markle *et al.* 2013, Yurkovetskiy *et al.* 2013), lupus (Young *et al.* 2014, He *et al.* 2016), obesity (Joriba *et al.* 2019), polycystic ovary syndrome (PCOS) (Torres *et al.* 2018, van Hooff *et al.* 1999, Jobira *et al.* 2020), and endometriosis (Yuan *et al.* 2018, Svensson *et al.* 2021). In addition to playing a role in the etiology and pathophysiology of various diseases, emerging differences in gut microbiota during puberty may lead to variable responses to drug treatments. Understanding the development of sex differences in the gut microbiome may be critical for designing and optimizing therapeutic approaches to treat gut microbiome-related diseases. Given the number of sex-specific diseases related to the gut and the potential for these sex differences to impact drug therapies, it is worth reviewing what is currently known about sex differentiation of the gut microbiome and the processes that drive maturation of the sex-specific gut microbial community.

## Sex differences and the gut microbiome

A survey of the literature investigating the relationship between sex and the gut microbiome strongly supports the hypothesis that human and rodent gut microbiomes differentiate during puberty and that this differentiation results in sex-specific communities by adulthood. While early data from the Human Microbiome Project did not detect sex differences in the gut microbiome (Huttenhower *et al.* 2012), subsequent studies have consistently identified distinct gut microbial communities between males and females. Numerous studies in both mice and humans have found sex differences in gut microbiome beta diversity (between-sample taxonomic composition) (Fig. 1) (Mueller *et al.* 2006, Li *et al.* 2008, Markle *et al.* 2013, Ding & Schloss 2014, Dominianni *et al.* 2015, Org *et al.* 2016, Falony *et al.* 2016, Sheng *et al.* 2017, Borgo *et al.* 2018, Gao *et al.* 2018, Santos-Marcos *et al.* 2018, Sinha *et al.* 2019, Takagi *et al.* 2019, Mayneris-Perxachs *et al.* 2020, Cui *et al.* 2021, Peters *et al.* 2022). Multiple studies have also shown that alpha diversity (within-sample taxonomic diversity) tends to be higher in adult females than adult males, though these differences are less pronounced in older adults (Falony *et al.* 2016, Borgo *et al.* 2018, Gao *et al.* 2018, Cuesta-Zuluaga *et al.* 2019, Sinha *et al.* 2019, Takagi *et al.* 2019, Peters *et al.* 2022). Sex was also among the ten factors that most explained variability in human gut microbial composition (Falony *et al.* 2016).

Since multiple studies support the idea that there are sex differences in the adult gut microbiome, it follows that there must be a point during development where the gut microbiome differentiates by sex. While initial studies reported that the microbial community in young children (1–3 years of age) resembled that of adults (Kurokawa *et al.* 2007, Yatsunenko *et al.* 2012), follow-up studies showed significant differentiation of beta diversity between child and adult microbiomes (Agans *et al.* 2011, Ringel-Kulka *et al.* 2013, Hollister *et al.* 2015). A study of human dizygotic twins found no differentiation in gut microbial beta diversity between male and female infant twins but did find differentiation between male and female pubertal twins (Yatsunenko *et al.* 2012). Similarly, another study revealed no sex differentiation in gut microbial beta diversity in pre-pubertal children but did show differentiation by sex in pubertal subjects (Yuan *et al.* 2020). Moreover, beta diversity of male and female microbiomes in late puberty were more similar to adult microbiomes of the same sex than in early puberty (Korpela *et al.* 2021). Similar patterns occurred in mouse studies: sex differences in alpha and beta diversity in mouse gut microbiomes were not observed before puberty but became evident after puberty (Markle *et al.* 2013, Yurkovetskiy *et al.* 2013). While some taxonomic differences between sexes in puberty were noted (Yuan *et al.* 2020, Korpela *et al.* 2021), there have been too few studies with small sample sizes to make strong conclusions about pubertal-specific taxonomic differences. Collectively, these studies support the idea that the mammalian gut microbiome differentiates during puberty, resulting in sex differences in adulthood.

Despite a consistent pattern of community-level sex differentiation in the gut microbiome, the specific taxa differentiated by sex varies among studies. For example, early work suggested that the ratio of the common gut phyla, Bacteroidetes and Firmicutes, differed between male and female gut microbiomes (Gomez *et al.* 2012, Huttenhower *et al.* 2012). However, follow-up studies found no difference in the Bacteroidetes to Firmicutes ratio, indicating that it is not a reliable indicator of sex differences (Singh & Manning 2016, Elderman *et al.* 2018, Santos-Marcos *et al.* 2018, Takagi *et al.* 2019). The abundances of specific bacterial genera or species have also not been reliable markers of sex differentiation. While individual studies identified bacterial taxa associated with microbiome sex differentiation, the same taxa did not change consistently in all studies. The reasons for the lack of consistency are unclear but may partly be due to external factors influencing the microbial gut composition in the various study populations (e.g. geography, age, and diet) (Kim *et al.* 2019, Jaggar *et al.* 2020, Valeri & Endres 2021). This inconsistency may also be a result of functional redundancy in the gut microbiome. So far, most studies of gut microbiome sex differentiation have employed 16S rRNA bacterial gene sequencing which cannot directly analyze gene function (Fig. 1). Future studies applying techniques such as metagenomics, transcriptomics, or metabolomics to study gut microbiome function could be useful in determining the precise nature of gut microbiome sex differentiation.

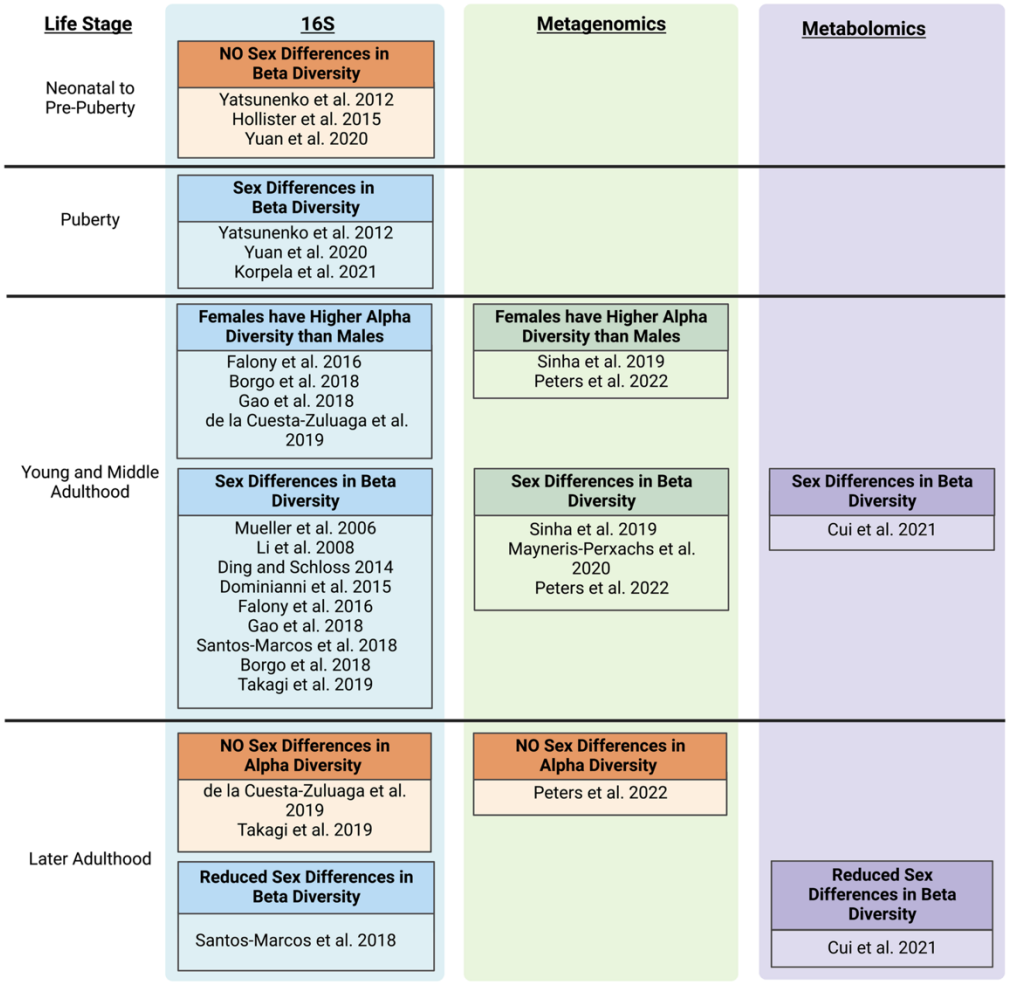


**Figure 1** Sex differences in the human gut microbiome arise during puberty. Studies providing evidence of sex differences are categorized by developmental stage, type of data analysis (e.g. 16S rRNA gene sequencing, metagenomics, or untargeted metabolomics) and observed effect on the gut microbiome. Created with BioRender.com.

## Sex steroids and the gut microbiome

Sex steroids are produced by the gonads during puberty in response to activation of the hypothalamic–pituitary–gonadal axis (Fig. 2). Observational studies in humans and studies in rodent models collectively suggest a linkage between sex steroids and the gut microbiome. For example, several studies manipulating sex steroid levels in mice indicated that estrogen and testosterone influenced gut microbial diversity and function. Gonadectomy of male and female mice shifted the gut community composition in both sexes and resulted in lower levels of sex differentiation as measured by beta diversity (Harada *et al.* 2016, Org *et al.* 2016, Choi *et al.* 2017, Kaliannan *et al.* 2018, Gao *et al.* 2021). Since gonad removal affects more than just the production of sex steroids, gonadectomy plus steroid replacement studies are necessary to discern whether sex steroids are responsible for a certain phenotype. One such study showed that treatment of ovariectomized female mice with estradiol shifted gut microbiome beta diversity to be more like intact females (Kaliannan *et al.* 2018). Another

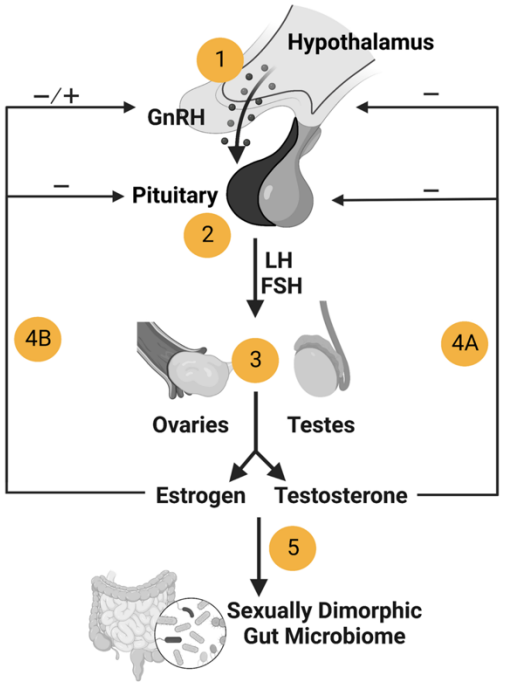


**Figure 2** Activation of the hypothalamic–pituitary–gonadal axis during puberty. (1) Gonadotropin-releasing hormone (GnRH) production is stimulated by kisspeptin secreted from neurons in the hypothalamus. (2) GnRH induces pituitary gonadotrope cells to produce and secrete the gonadotropins, follicle-stimulating hormone (FSH), and luteinizing hormone (LH). (3) In males, LH signaling in testicular Leydig cells results in testosterone production, while in females, LH stimulates testosterone production in ovarian theca cells, which is then converted to estrogen in granulosa cells by aromatase. (4A) Testosterone exerts negative feedback in the hypothalamus and pituitary, while (4B) estrogen exerts both negative and positive feedback. (5) Sex steroids may directly or indirectly modulate the gut microbiome to be sexually dimorphic. Created with BioRender.com.

study demonstrated that treating castrated male mice with dihydrotestosterone shifted the gut microbiome beta diversity to be more like intact males (Gao *et al.* 2021).

In addition to studies with gonadectomized mice, which results in steroid insufficiency, other studies have employed steroid excess to study the relationship between sex steroids and the gut microbiome. For example, studies have shown that treatment of pubertal female mice or rats with the aromatase inhibitor letrozole, which elevates testosterone and decreases estrogen (Kauffman *et al.* 2015), results in a shift in gut bacterial alpha and beta diversity (Guo *et al.* 2016, Kelley *et al.* 2016). Treatment of female rats with dihydrotestosterone (a non-aromatizable androgen) was also found to alter the gut microbiome (Zheng *et al.* 2021, Rodriguez Paris *et al.* 2022). Interestingly, letrozole treatment affected the gut microbiome and metabolic phenotype of pubertal females differently than adult females, indicating that puberty may be a sensitive period for effects of steroids on the gut microbiome as well as the host (Arroyo *et al.* 2019, Torres, Ho *et al.* 2019, Torres, Skarra *et al.* 2019). Moreover, cohousing letrozole- and placebo-treated mice, in which mice exchanged gut microbiota through coprophagy, reduced testosterone levels and ameliorated reproductive and metabolic phenotypes in letrozole-treated mice (Torres, Ho *et al.* 2019, Ho *et al.* 2021). This potential bidirectional relationship between host sex steroids and the gut microbiome was also suggested by another study which showed that maternal high-fat diet induced metabolic dysregulation, early puberty, and infertility could be partially reversed in female offspring cohoused with healthy females (Wang *et al.* 2020a).

In humans, the evidence for a relationship between sex steroids and the gut microbiome comes from various observational studies with postmenopausal women or women using oral contraceptives. Two gut microbiome studies of women using oral contraceptive pills (estrogen and progesterone) found they had lower alpha diversity, differentiated beta diversity, and distinct gut microbial metabolic pathways compared to women not using oral contraceptives (Mihajlovic *et al.* 2021, Hua *et al.* 2022). Other studies showed that urinary estrogens and their metabolites were negatively correlated with gut microbiota alpha diversity in postmenopausal females but not premenopausal females (Flores *et al.* 2012, Fuhrman *et al.* 2014). In postmenopausal women, estrone levels and the ratio of parent estrogens to estrogen metabolites were associated with an increased relative abundance of specific bacterial taxa (Flores *et al.* 2012, Fuhrman *et al.* 2014). A metagenomics study also found significant differences in the taxonomic and functional pathway beta diversity of postmenopausal women compared to premenopausal women (Zhao *et al.* 2019). Interestingly, the one study we found that investigated changes in the gut microbiome directly within the intestinal tract, namely the duodenum, also showed a relationship between sex steroids and the microbiome. Specifically, Leite *et al.* reported that the duodenal microbiome of postmenopausal women treated with hormone replacement therapy was more like premenopausal women than postmenopausal women not taking hormone replacement therapy (Leite *et al.* 2022).

Another link between sex steroids and the gut microbiome was identified in women with PCOS with elevated testosterone levels (hyperandrogenism). Studies of gut microbial diversity found that women and adolescent girls with PCOS had lower alpha diversity and differences in beta diversity compared to healthy women and adolescent girls (Lindheim *et al.* 2017, Liu *et al.* 2017, Torres *et al.* 2018, Qi *et al.* 2019, Jobira

*et al.* 2020, Garcia-Beltran *et al.* 2021). Testosterone levels in women with PCOS also correlated with alpha diversity and the abundance of specific members of the gut microbial community (reviewed in (Rizk & Thackray 2021)). Intriguingly, Qi *et al.* reported that female mice transplanted with stool from women with PCOS developed metabolic and reproductive dysregulation (Qi *et al.* 2019), suggesting that changes in the gut microbiome may be sufficient to induce PCOS-like phenotypes.

### Microbial modifications of sex steroids

Several key studies support the hypothesis that gut bacteria play a critical role in enterohepatic circulation of sex steroids (Fig. 3). Studies in humans and rats demonstrated that antibiotic treatment dramatically increased the ratio of conjugated to deconjugated estrogens excreted in urine and feces (Adlercreutz *et al.* 1984, Goldin & Gorbach 1984, Flores *et al.* 2012). Moreover, Colldén *et al.* found negligible deconjugation of testosterone glucuronide in the intestinal tract of germ-free male mice (Colldén *et al.* 2019). This study also showed that the concentration of androgen glucuronide in normal mice was high in the small intestine and low in the cecum and colon, indicating that microbial deconjugation of androgen glucuronide

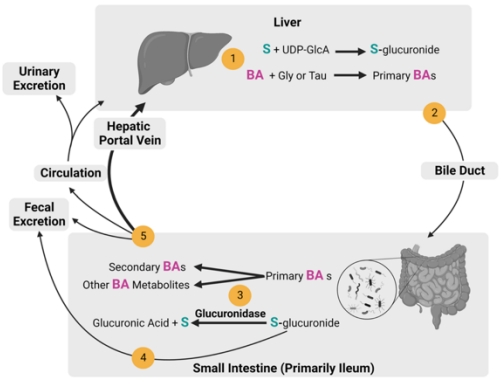


**Figure 3** Enterohepatic circulation of sex steroids and bile acids. (1) Sex steroids (S) are conjugated with glucuronide from uridine 5'-diphosphoglucuronic acid (UDP-GlcA) by liver enzymes. Bile acids (BAs) are synthesized and conjugated with glycine (Gly) or taurine (Tau) in the liver. (2) Sex steroid glucuronides (S-glucuronide) and primary BAs are transported to the gallbladder and excreted into the small intestine via the bile duct. (3) Gut bacteria deconjugate S-glucuronide and transform BAs into secondary BAs or other BA metabolites. (4) S-glucuronides that are not deconjugated are excreted through the feces. (5) Secondary BAs, other BAs metabolites, and deconjugated sex steroids are primarily reabsorbed through the hepatic portal vein to the liver. Alternatively, they are excreted through feces or returned to circulation, to be reabsorbed by the liver or excreted through urine. Created with BioRender.com.

(and presumably other steroids) occurs largely in the small intestine.

While gut bacteria facilitate enterohepatic cycling of sex steroids, the role of specific bacterial species in this process is not well understood. Studies have shown that a bacterial enzyme, β-glucuronidase, is primarily responsible for the deconjugation of steroid glucuronides (Pellock & Redinbo 2017, Sui *et al.* 2021) and that β-glucuronidase homologs are present and active in many common gut bacteria in mice and humans (Lombardi *et al.* 1978, Gadelle *et al.* 1985, Winter & Bokkenheuser 1987, Dabek *et al.* 2008, Gloux *et al.* 2011, McIntosh *et al.* 2012). β-glucuronidase homologs were found in members of many common gut bacterial families, including *Ruminococcaea, Lachnospiraceae, Clostridiaceae, Bacteroidaceae,* and *Tannerellaceae*. Many studies have demonstrated *Escherichia coli* deconjugation of various estrogen-glucuronides (Buehler *et al.* 1951, Graef *et al.* 1977, Lombardi *et al.* 1978, Legler *et al.* 2002), though studies on androgen glucuronides are lacking. Recently, Ervin *et al.* reported deconjugation of estrogen glucuronides by β-glucuronidases isolated from other gut bacteria, including *Faecalibacterium prausnitzii, Roseburia* species, and *Clostridium* species (Ervin *et al.* 2019). As β-glucuronidase genes are not present in all gut microbes, possession of this gene could provide a competitive advantage during puberty. Phylogenetic and structural diversity analyses of bacterial β-glucuronidase genes indicate that microbial β-glucuronidases are substrate-specific and fall into functional classes (Little *et al.* 2018, Biernat *et al.* 2019, Ervin *et al.* 2019). The fact that many common gut bacteria possess β-glucuronidase activity with different substrate specificity provides a potential mechanism by which sex steroids could differentiate the gut microbiome. In support of this idea, glucuronidase genes in gut metagenomes were reported to be higher in premenopausal women than in postmenopausal women and adult men (Peters *et al.* 2022).

Another mechanism by which sex steroids may impact bacterial communities is via bacterial metabolism of unconjugated (free) steroids. Metabolism of free androgens and estrogens, including redox reactions and partial cleavage of steroids, has been reported in human fecal cultures (Eriksson & Gustafsson 1971, Lombardi *et al.* 1978). These studies indicated that free steroids were not broken down completely by bacteria, so this activity may be for detoxifying steroids rather than for energy or anabolism. Indeed, free testosterone was shown to be toxic to gut bacteria such as *E. faecalis* and *E. coli*, suggesting that high concentrations of steroids may be toxic to some bacteria but not others (Plotkin *et al.* 2003). On the other hand, additional studies showed that estrogen and progesterone were completely degraded when added to fecal cultures (Li *et al.* 2018, Coombes *et al.* 2020). Collectively, these studies provide evidence that gut microbes metabolize sex steroids and

that this metabolism could potentially influence the composition of the gut microbial community in a sex-specific manner. However, the mechanisms by which this might occur are poorly understood and many more studies are needed to determine if and how these processes occur.

### Bile acids and the gut microbiome

While direct interaction of sex steroids with gut microbiota may lead to a sexually dimorphic gut microbiome, this is not the only possible mechanism. Sex steroids may also indirectly influence the gut microbial community by regulating host production and secretion of bile acids, which may then modulate the gut microbiota. Bile acids are important for emulsifying fats during digestion and as signaling molecules in glucose and lipid metabolism (Perino *et al.* 2021). In humans, the liver produces cholic acid (CA) and chenodeoxycholic acid (CDCA) from cholesterol (Russell 2003). Muricholic acid (MCA) is produced instead of CDCA in mice. Bile acids are conjugated with either taurine or glycine, then secreted as primary bile acids through the bile duct into the small intestine. Like sex steroids, bile acids circulate between the liver and the intestine through enterohepatic circulation (Fig. 3). Once secreted into the small intestine, microbial bile salt hydrolase (BSH) deconjugates bile acids with glycine or taurine. 70–90% of bile acids are reabsorbed from the ileum and transported to the liver through the hepatic portal vein (Angelin *et al.* 1982, Wahlström *et al.* 2016). The BSH gene is present in most major gut microbiome phyla (Firmicutes, Bacteroidetes, Actinobacteria, Fusobacteria, and Proteobacteria), and BSH enzymes in different phyla have different substrate specificity for glycine- or taurine-conjugated bile acids (Jones *et al.* 2008, Song *et al.* 2019). The widespread presence of BSH among members of the gut microbiota suggests that it could confer a competitive advantage by providing resistance to bile acid detergent toxicity (Jones *et al.* 2008). Another transformation of bile acids by gut microbiota is 7α-dehydroxylation, which produces the secondary bile acids deoxycholic acid (DCA) and lithocholic acid (MDCA in mice) (Lucas *et al.* 2021). 7α-dehydroxylase activity is less widespread among human gut bacteria than BSH activity, which may lead to niche specialization by species with this activity (Lucas *et al.* 2021). Less common transformations of bile acids include dehydroxylation, epimerization, isomerization, and oxidation (Eyssen *et al.* 1983, Degirolamo *et al.* 2014). As secondary bile acids have varying levels of toxicity to different microbes, producing more secondary bile acids may provide a competitive advantage for bacteria able to tolerate this toxicity and a mechanism for the host to influence the gut microbial community (Tian *et al.* 2020).

Multiple reviews indicate that microbial bile acid metabolism is widespread within the gut microbiota and that it creates a large diversity of bile acid-derived metabolites (Wahlström *et al.* 2016, Foley *et al.* 2019, Winston & Theriot 2020). However, apart from BSH activity, the prevalence of these functions within gut microbiota is unknown (Winston & Theriot 2020). While some effects of secondary bile acids on the host are known, such as anti-inflammatory properties, much remains to be discovered about how microbial transformation of bile acids affects host physiology (Ward *et al.* 2017, Lajczak-McGinley *et al.* 2020). A growing body of literature indicate that bile acids have a profound effect on host biology. For instance, both primary and secondary bile acids bind to host receptors such as farnesoid X receptor, G protein-coupled bile acid receptor 1, pregnane X receptor, vitamin D receptor, and retinoid-related orphan receptor and regulate metabolic and immune functions (Chiang 2013, Fiorucci *et al.* 2021). FXR, specifically, has been widely studied and implicated in glucose metabolism, lipid homeostasis, inflammation, and permeability of the intestinal barrier (Ding *et al.* 2015).

### Sex differences and bile acids

In humans, the bile acid profiles of newborn infants (infants less than 48 h old) were largely composed of primary bile acids and were markedly different from adult bile acid profiles (Wang *et al.* 2020*b*). In mice and rats, the gut microbiota and bile acid pool both diversify rapidly after birth. Interestingly, the diversity of secondary bile acids appears to increase until puberty after which time it stabilizes (Morris *et al.* 1983, van Best *et al.* 2020). During and after puberty, studies have shown that primary and secondary bile acid levels are higher in female than male rodents (Morris *et al.* 1983, Turley *et al.* 1998, Schwarz *et al.* 2001, Li-Hawkins *et al.* 2002, Jahnel *et al.* 2015, Baars *et al.* 2018). Ma *et al.* also identified differences in secondary bile acid levels between young and older adult mice and determined that these differences were reduced by cohousing, indicating that the gut microbiome influences bile acid levels (Ma *et al.* 2020).

There is also evidence for sex differences in bile acids in humans. Multiple studies have determined that total bile acid concentrations and levels of bile acid synthesis are higher in males than females (Bennion *et al.* 1978, Gälman *et al.* 2011, Steiner *et al.* 2012, Xiang *et al.* 2012, Frommherz *et al.* 2016). Since mice show the opposite pattern, this difference is important to consider when using mice as a model for the effects of bile acids on physiology. While there are consistent sex differences in the total bile acid pool in humans, the reported sex differences in terms of the types of primary and secondary acids vary among studies (Fisher & Yousef 1973, Bennion *et al.* 1978, Wang *et al.* 2020*b*). In addition, little is known about how age interacts with sex differences in primary and secondary bile

acid metabolism although one study indicated that sex steroids, which change in concentration throughout the lifespan, may indirectly affect secondary bile acid metabolism (Ridlon *et al.* 2010).

### Interplay between bile acids, the gut microbiome, and PCOS

Ho *et al.* found a relationship between gut microbiota and secondary bile acids in mice. In this study, letrozole treatment of female mice during puberty resulted in changes to secondary bile acid levels, gut microbiota abundances, metabolism, and the reproductive axis (Ho *et al.* 2021). In humans, women with PCOS were reported to have different levels of specific secondary bile acids compared to healthy controls and higher levels of BSH genes in their gut microbial metagenomes (Qi *et al.* 2019, Zhang *et al.* 2019, Yang *et al.* 2021*a*). Transplantation of stool from women with PCOS into adult female mice resulted in impaired fertility and glucose and insulin sensitivity, potentially modulated by the secondary bile acids tauroursodeoxycholic acid and glycoursodeoxycholic acid (Qi *et al.* 2019). Another study with fecal microbiome transplantation from women with PCOS to adult female mice showed decreased expression of the FXR receptor in the ileum and impaired insulin sensitivity (Yang *et al.* 2021*b*). These studies indicate a potential influence of the gut/liver axis on metabolic dysregulation and vice versa.

### The immune system, sex, and the gut microbiome

The immune system differentiates in a sex-specific manner during puberty and remains sexually dimorphic in adulthood (Hooper *et al.* 2012, Rizzetto *et al.* 2018, Fuhler 2020). Since both innate and adaptive immunity shapes many of the interactions in the gut microbiome and vice versa (Hooper *et al.* 2012, Foster *et al.* 2017, Shi *et al.* 2017, Wiertsema *et al.* 2021), the sexually dimorphic nature of the immune system creates another possible mechanism for generating a sex-specific gut microbiome (Whitacre *et al.* 1999, Lamason *et al.* 2006, Klein & Flanagan 2016, Sharma *et al.* 2018). The immune system is known to influence the gut microbiome through a number of mechanisms, such as preventing infiltration of the gut microbiota into host tissues (Gallo & Hooper 2012), secreting mucins and antimicrobial compounds into the intestine, and propagating microbial signals to other immune cells (Abreu 2010, Peterson & Artis 2014). Adaptive immune cells also surveil the gut microbiota and alter community composition through immunoglobulin secretion (Zhang *et al.* 2015, Fransen *et al.* 2015). In turn, the gut microbiome affects differentiation of T and B cells and pro- and anti-inflammatory cytokine levels (Fujimura *et al.* 2016, Sampson *et al.* 2016, Luu *et al.* 2018, Zhao & Elson 2018, Li *et al.* 2019, Lajczak-McGinley *et al.* 2020).

Given the importance of the immune system in regulating the gut microbiome, sex-specific differences in the immune system could shape the gut microbiome in a sex-specific manner. This idea is supported by studies showing an association between the gut microbiome and autoimmune disorders. Ulcerative colitis, a form of IBD, is diagnosed more frequently in females, while Chron's disease, another form of IBD, is diagnosed more often in males (Henderson *et al.* 2012, Hayter & Cook 2012, Herzog *et al.* 2014). Studies indicate that the gut microbiome is a major factor in IBD incidence and severity. Microbiome and metabolome composition in patients with IBD was different from healthy patients and associated with gut inflammation levels (Clooney *et al.* 2019, Dong *et al.* 2019, Franzosa *et al.* 2019). Gut microbiota may affect severity of IBD directly by activating immune cells through pattern recognition receptors or immunoglobulin binding or indirectly through bile acid signaling (Ding *et al.* 2015, Schirmer *et al.* 2019). Additionally, fecal microbiome transplantation is an effective treatment for many patients, though differences in effectiveness by donor and recipient are not well understood (Mańkowska-Wierzbicka *et al.* 2020, Cheng *et al.* 2021, Zhou *et al.* 2021). While differences in gut microbiome composition between patients with ulcerative colitis or Crohn's disease were reported, sex differences were not observed (Clooney *et al.* 2019, Dong *et al.* 2019). However, since sex differences in IBD patients or their microbiomes are often not analyzed or reported, it is possible that sex differentiation of the gut microbiome may contribute to differences in the development and pathology of the IBD subtypes and warrants future investigation. Given that sex-specific immune responses to bacterial metabolites are a candidate for how the gut microbiome contributes to sex differences in the incidence of IBD (Murray *et al.* 2019, Murray *et al.* 2020, Bhattarai *et al.* 2020, Spichak *et al.* 2021), it may also be important to determine if the sex of the donor and recipient needs to be considered in fecal microbiome transplantation for IBD treatment.

Another notable example of sex-specific differences in the immune system–gut microbiome relationship occurs in the non-obese diabetic (NOD) mouse model for the autoimmune disorder, T1D. In the NOD mouse model, T1D incidence after 30 weeks of age is ~80% in females and 20% in males, although this ratio varies depending on the mouse colony (Makino *et al.* 1980, Pozzilli *et al.* 1993, Yurkovetskiy *et al.* 2013). Intriguingly, castration of NOD males results in a T1D incidence similar to females, indicating that gonadal sex steroids may be important in protecting male mice from T1D (Makino *et al.* 1981). Additionally, Markle *et al.* found no sex differences in T1D in NOD mice raised in a germ-free environment, indicating that the gut microbiome was required for the sex differences in T1D incidence

(Markle *et al.* 2013). This and other studies also showed that the male microbiome was protective against T1D and that delivery of male gut microbiota to female mice via fecal transplantation was protective against T1D (Markle *et al.* 2013, Yurkovetskiy *et al.* 2013, Markle *et al.* 2014). Altogether, this evidence suggests that host–microbe interactions may play a key role in modulating sex differences in autoimmunity.

## Conclusions and future perspectives

Our review of the current literature on the relationship of sex to the gut microbiome in humans and in rodent models showed that a sex-specific gut microbiome develops during puberty and continues into adulthood. The majority of these studies relied on NGS of bacterial 16S rRNA gene amplicon libraries due to its cost-effectiveness and its ability to taxonomically identify both cultured and uncultured bacteria across all phyla. However, while the 16S gene provides useful information about gut bacterial diversity, it reveals little about the function of gut microbes. Metagenomic, metabolomic, and transcriptomic approaches can inform us about microbial gene content and expression and metabolite production and can identify changes in archaea, fungi, and viruses in addition to bacteria. Future studies employing these approaches will be needed to understand comprehensively how sex and sex steroids regulate the composition and function of gut microbial communities along the alimentary canal.

Understanding the role of gut microbiota in sex-specific human health and disease will require better knowledge of how sex steroids affect the gut microbiome, and vice versa. In humans, there is a lack of clinical studies investigating the relationship between sex steroids and the gut microbiome in men. Given that men commonly experience hypogonadism due to factors such as age, disease (e.g. obesity and type 2 diabetes), or drug treatment, for example, GnRH agonist for prostate cancer (Mittan *et al.* 2002, Zarotsky *et al.* 2014), future studies are needed to discern the relationship between sex steroids and the gut microbiome in men. Since gender-affirming care for transgender individuals often involves hormone therapy, study of the gut microbiome in individuals receiving cross sex steroid treatment is also important.

While many studies in humans and rodents have established correlations between sex differences and factors such as sex steroids, bile acids, and the immune system, few studies have investigated causal relationships and mechanisms. More studies are needed to investigate how microbiome manipulation affects sex steroid levels (e.g. via antibiotics, cohousing, or fecal transplants) and how sex steroid manipulation affects the gut microbiome (e.g. via gonadectomy and steroid replacement). Additionally, research should be undertaken to measure the levels of both conjugated and unconjugated steroids in males and females, especially when paired with gut microbiome manipulation. The use of germ-free or antibiotic-depleted mice will be key in establishing which sex-specific diseases require the presence of the gut microbiome and which disease phenotypes can be recapitulated by fecal microbiome transplantation.

Since there is growing evidence that there are sex differences in bile acid production in the liver, future research should investigate when sex differences in primary and secondary bile acid levels emerge in humans and rodents and how the gut microbiome contributes to these differences. Gut microbiota manipulation may provide insight into how the gut microbiome affects sex-specific bile acid metabolism and signaling. Altering host bile acid levels through host genetic knock-outs in the bile acid pathway or through diet supplementation could potentially be used to determine how sex-specific bile acid levels affect gut microbiota composition. Deciphering this relationship may be essential to understand how gut microbiota contributes to sex-specific metabolic disorders like PCOS and type 2 diabetes.

Finally, given the number of sex-specific autoimmune diseases, understanding the relationship between the gut microbiome and the immune system is likely to be important for developing microbial-based preventative measures and therapeutics for these disorders. Studies manipulating the immune system are needed to understand how the immune system shapes the sex-specific gut microbiome. Likewise, more studies are required to provide insight into how gut microbiota manipulation affects development of sex-specific autoimmune diseases like IBD and type I diabetes. Understanding how sex steroids, bile acids, the immune system, and the gut microbiome interact to create and maintain sex differences in microbiome-related diseases may have far-reaching implications especially since the gut microbiome is increasingly being considered a therapeutic target.

## Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.


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## Author contribution statement

L S-H, S T K and V G T conceived of the topic, created the figures, wrote, and edited the manuscript.

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# CHAPTER 2: Genetic hypogonadal mouse model reveals niche-specific influence of reproductive axis and sex on intestinal microbial communities

Laura Sisk-Hackworth[1,2], Jada Brown[1], Lillian Sau[1], Andrew A. Levine[2], Lai Ying Ivy Tam[1], Aishwarya Ramesh[1], Reeya S. Shah[1], Evelyn T. Kelley-Thackray[1], Sophia Wang[1], Anita Nguyen[1], Scott T Kelley[2] and Varykina G Thackray[1]

[1]Department of Obstetrics, Gynecology and Reproductive Sciences, University of California, San Diego, La Jolla, California, USA

[2]Department of Biology, San Diego State University, San Diego, California, USA

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# Genetic hypogonadal mouse model reveals niche-specific influence of reproductive axis and sex on intestinal microbial communities

Laura Sisk-Hackworth[1,2], Jada Brown[1], Lillian Sau[1], Andrew A. Levine[2], Lai Ying Ivy Tam[1], Aishwarya Ramesh[1], Reeya S. Shah[1], Evelyn T. Kelley-Thackray[1], Sophia Wang[1], Anita Nguyen[1], Scott T. Kelley[2] and Varykina G. Thackray[1*]

## Abstract

**Background** The gut microbiome has been linked to many diseases with sex bias including autoimmune, metabolic, neurological, and reproductive disorders. While numerous studies report sex differences in fecal microbial communities, the role of the reproductive axis in this differentiation is unclear and it is unknown how sex differentiation affects microbial diversity in specific regions of the small and large intestine.

**Methods** We used a genetic hypogonadal mouse model that does not produce sex steroids or go through puberty to investigate how sex and the reproductive axis impact bacterial diversity within the intestine. Using 16S rRNA gene sequencing, we analyzed alpha and beta diversity and taxonomic composition of fecal and intestinal communities from the lumen and mucosa of the duodenum, ileum, and cecum from adult female ($n = 20$) and male ($n = 20$) wild-type mice and female ($n = 17$) and male ($n = 20$) hypogonadal mice.

**Results** Both sex and reproductive axis inactivation altered bacterial composition in an intestinal section and niche-specific manner. Hypogonadism was significantly associated with bacteria from the *Bacteroidaceae, Eggerthellaceae, Muribaculaceae*, and *Rikenellaceae* families, which have genes for bile acid metabolism and mucin degradation. Microbial balances between males and females and between hypogonadal and wild-type mice were also intestinal section-specific. In addition, we identified 3 bacterial genera (*Escherichia Shigella*, *Lachnoclostridium*, and *Eggerthellaceae genus*) with higher abundance in wild-type female mice throughout the intestinal tract compared to both wild-type male and hypogonadal female mice, indicating that activation of the reproductive axis leads to female-specific differentiation of the gut microbiome. Our results also implicated factors independent of the reproductive axis (i.e., sex chromosomes) in shaping sex differences in intestinal communities. Additionally, our detailed profile of intestinal communities showed that fecal samples do not reflect bacterial diversity in the small intestine.

**Conclusions** Our results indicate that sex differences in the gut microbiome are intestinal niche-specific and that sampling feces or the large intestine may miss significant sex effects in the small intestine. These results strongly support the need to consider both sex and reproductive status when studying the gut microbiome and while developing microbial-based therapies.

*Correspondence:
Varykina G. Thackray
vthackray@health.ucsd.edu
Full list of author information is available at the end of the article

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**Highlights**

- Genetic inactivation of the reproductive axis impacts sex differences in the adult gut microbiome.
- Sex differences in the gut microbiome are specific to intestinal section (duodenum, ileum, and cecum) and to the lumen or mucosa.
- Reproductive axis-independent effects, e.g., sex chromosome factors may also regulate sex differences within the gut microbiome.
- Studies only sampling feces may miss sex differences in the small intestine and mucosal environments.

**Plain language summary**

Microbial communities in the intestinal tract, known as the gut microbiome, regulate many critical aspects of host physiology. Previous studies have shown that the diversity of the gut microbiome differs between the sexes. There are also many diseases with a sex bias linked to the gut microbiome, including autoimmune, metabolic, neurological, and reproductive disorders. The gut microbiome differentiates during puberty, but it is unknown if the reproductive axis, the system responsible for sexual maturation and production of gonadal sex hormones, is critical for this process. Furthermore, since most studies use feces to examine the gut microbiome, it is unknown how sex influences the microbial communities within different segments of the small and large intestine. To address this gap in knowledge, we used DNA-based molecular methods to compare the intestinal-specific microbiomes of a mouse model with a genetically inactivated reproductive axis to that of wild-type mice. We found that both sex and the reproductive axis impacted gut microbial diversity in an intestinal section-specific manner. We also detected significant differences in intestinal microbial diversity between male and female mutant mice, suggesting that sex chromosome factors also affect the gut microbiome. We also showed that fecal samples were dissimilar to small intestine microbial communities, indicating that studies only sampling feces likely miss sex differences specific to the small intestine. Our results strongly support the need to consider both sex and reproductive status when studying the gut microbiome and while developing microbial-based therapies.

## Introduction

Biological sex and the reproductive axis are fundamental to mammalian development and physiology. In addition to controlling sexual differentiation, fertility, and sex steroid production, the reproductive axis influences the nervous, musculoskeletal, cardiovascular, immune, hepatic, and gastrointestinal systems [1–7]. Additionally, many diseases have a sex bias, including autoimmune disorders such as lupus, Crohn's disease, and ulcerative colitis [8, 9], or are influenced by sex steroids, such as polycystic ovary syndrome and cardiovascular disease [5, 10].

Recently, sex and the reproductive axis have been implicated in regulating another critical aspect of mammalian biology, namely the gut microbiome. Numerous studies indicate that the human gut microbiome differentiates during puberty in a sex-dependent manner, coinciding with activation of the reproductive axis. Sex differences in the taxonomic composition of the gut microbiome (i.e., beta diversity) emerge during puberty, become more distinct in adulthood, and then diminish in older adults [11–26]. A similar pattern occurs with alpha diversity, i.e., within-sample biodiversity, where sex differences are most pronounced in young and middle-aged adults but disappear in later adulthood [19, 20, 22, 23, 25, 27, 28]. Sex differences in alpha and beta diversity have similarly been observed in rodents, indicating that mice make a good model for studying the connection between gut microbiota and sex-specific host physiology and diseases [29–32].

While there is growing evidence of a relationship between host sex and the composition and diversity of the gut microbiome, the precise nature of this interaction is unclear. Thus far, published studies detecting sex differences in the gut microbiome have used fecal samples as a proxy for intestinal microbial communities. However, the intestinal tract has distinct physiological sections with unique abiotic and biotic factors that create highly selective microhabitats for microbial communities, the diversity of which may not be captured by fecal samples. For example, low pH and high levels of bile acids and oxygen in the duodenum reduce microbial biomass and select for acid-tolerant, bile acid-transforming and oxygen-tolerant species, while in the cecum higher pH and lower oxygen levels select for anaerobic bacteria that ferment carbohydrates [33–36]. Furthermore, intestinal epithelial cells secrete mucins and antimicrobials, creating distinct mucosal niches for mucin-degrading and mucin-adherent bacteria along the intestinal tract [37–42]; for

example, paneth cells produce a gradient of antimicrobial peptides that decreases from the proximal to distal small intestine [33, 43]. The mucosal barrier structure also shifts from the small to large intestine, with a thin mucus layer in the small intestine and a thick, two-layer mucosa in the cecum [33]. The small intestine also has lower bacterial biomass and faster transit times than the large intestine [33]. While studies in humans, mice and other animals indicate that these physiological differences select for distinct microbiomes that differ in microbial composition and biomass both lengthwise and cross-sectionally within the intestinal tract [36, 39, 44–53], there is a lack of comprehensive studies that characterize both luminal and mucosal communities within different intestinal segments, particularly the duodenum.

There is also evidence that the physiology of the intestinal tract is influenced by sex. For instance, one study showed that testosterone levels were high in the intestinal lumen of male mice, while estrogen levels were high in females, and these levels differed by intestinal site [54]. Total bile acid levels within the intestine have also been shown to vary by sex in humans and mice [55–59]. Moreover, intestinal immune surveillance differs by sex. Schwerdtfeger and Tobet showed that mast cells were larger and more prevalent in female mice and paneth cells were more reactive in males when exposed to bacterial lipopolysaccharide [60]. Collectively, these studies suggest that sex and the reproductive axis may play an important role in shaping the intestinal microbiome in a site-specific manner.

Despite the potential influence of sex on site-specific intestinal microbiota, many studies have either not reported sex, only assayed one sex [44, 47, 52, 53, 61–66], or not tested for sex differences if both sexes were included [34, 39, 42, 49, 67, 68]. Recently, a study reported sex differences in the intestinal metabolome, but did not study the microbiota [69] and two studies tested for sex differences in the lumen of an intestinal section, but not the mucosa [70, 71]. No studies have investigated sex differences within both luminal and mucosal communities of the small intestine or compared them to large intestinal communities. Given the intense interest in the development of personalized microbial therapies for various human diseases [72–74], it is important to understand the influence of the reproductive axis and sex on microbial communities in both the small and large intestine.

In this study, we used the hypogonadal Gnrh1$^{hpg}$ mouse model to test the hypothesis that sex and the reproductive axis shape niche-specific bacterial diversity of the gut microbiome. Hypogonadism in the *hpg* mouse stems from a large truncation of the *Gnrh1* gene, leading to a lack of gonadal development [75, 76]. In homozygous mutants, the reproductive axis is inactive, meaning that male and female *hpg*$^{+/+}$ mice do not produce sex steroid hormones or go through puberty (Fig. 1A). In contrast to interventional methods such as gonadectomy, this model facilitates study of the effect of hypogonadism *starting in embryonic development* on maturation of the gut microbiome and isolates differentiation due to the hypothalamic–pituitary–gonadal (HPG) axis from other sex differences. To investigate how ablation of the HPG axis alters sexual development of the intestinal microbiome, we assayed intestinal microbial communities from adult male and female wild-type and male and female *hpg* mutant mice (Fig. 1B). Since sex effects likely vary both radially and lengthwise along the intestinal tract and small intestine samples, particularly mucosa, have high host and low microbial biomass [34], we performed 16S rRNA amplicon sequencing with universal bacterial primers on luminal and mucosal samples from the duodenum, ileum, and cecum, as well as the feces of each mouse (Fig. 1C). Our results provide an unprecedented view into the impact of the reproductive axis on the mammalian gut microbiome, showing that the HPG axis drives development of intestinal sex differences and that the effect of sex and the HPG axis is specific to each intestinal niche.

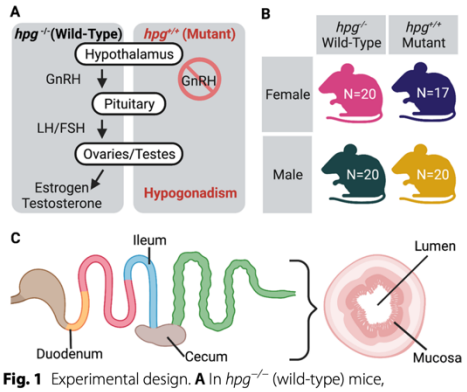


**Fig. 1** Experimental design. **A** In *hpg*$^{-/-}$ (wild-type) mice, the hypothalamic–pituitary–gonadal axis is functional. Gonadotropin-releasing hormone (GnRH) is secreted from the hypothalamus and stimulates production of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from gonadotrope cells in the anterior pituitary. LH and FSH then regulate steroidogenesis and gametogenesis in the ovaries and testes which results in sexual development and reproductive competence. In *hpg*$^{+/+}$ mutant mice, GnRH is not produced due to a truncation in the *Gnrh1* gene, resulting in hypogonadism. **B** The study included four experimental groups: female and male wild-type and mutant mice. *N* = number of mice per group. **C** Sampling of intestinal microbial communities was done in the duodenum, ileum, and cecum, both in the lumen and mucosa, resulting in 539 samples including fecal samples. Created with BioRender.com

## Methods

### *Hpg* mouse model

Hypogonadal (*hpg*) heterozygote mice were purchased from Jackson Labs (RRID:IMSR_JAX:000804). The $Gnrh1^{hpg}$ mutation is a ~ 33.5-kb deletion that removes two exons that encode most of the protein. Since *hpg* homozygous mice are infertile, *hpg* heterozygous male and female mice were crossed with each other and the offspring were genotyped to identify $hpg^{+/+}$ (mutant) or $hpg^{-/-}$ (wild-type) homozygous males and females. Two cohorts of breeding mice were generated with the aim of producing 20 mice per genotype and sex. In the final tally, cohort A consisted of 8 female mutants, 10 female wild types, 11 male mutants, and 11 male wild types, while cohort B consisted of 9 female mutants, 10 female wild types, 9 male mutants, and 9 male wild types. In total, 77 wild-type and mutant mice were produced from 31 litters. Mice were housed in a vivarium with a 12-h light (6:00 AM to 6:00 PM) and 12-h dark cycle and had access to water and food (Teklad Global 18% Protein Extruded Diet; Envigo, Indianapolis, IN) ad libitum. After weaning at 21 days of age, mice were single housed to prevent coprophagy from influencing the gut microbiome. The University of California, San Diego Institutional Animal Care and Use Committee approved all animal procedures used in this study (Protocol Number S14011).

### Sample collection and DNA isolation

When mice were 10 weeks of age, mice were weighed and fecal samples were collected, then mice were killed using 2.5% isoflurane and cervical dislocation. The duodenum (first 7 cm of the intestine), ileum (last 7 cm before the cecum), and cecum were excised from the intestine. Luminal contents were collected by squeezing the contents of each section into a tube. Once contents were collected, the intestinal mucosa were gently shaken in three washes of PBS to remove remaining lumen material. Samples were frozen immediately after collection (luminal contents) or washing (lining mucosa) and stored at − 80 °C. To minimize potential variability induced by the estrus cycle, female wild-type samples were collected during estrus which was determined by vaginal epithelial cell smears. Bacterial DNA was extracted from the fecal and intestinal samples and from two negative extraction controls and two ZymoBIOMICS Microbial Community Standard positive extraction controls (D6300 Zymo Research) with the DNeasy PowerSoil Pro Kit (Qiagen) according to the manufacturer's instructions, and stored at − 80 °C.

### 16S rRNA amplicon sequencing

The V4 hypervariable region of the 16S rRNA gene was PCR amplified using "universal" bacterial primers 515F and 806R, with the 806R primers barcoded with unique 12-bp Golay barcodes [77]. Amplifications were performed separately for each environment (feces, cecum, ileum, and duodenum), with each set including two negative extraction controls, two negative PCR controls, two DNA extraction positive controls (ZymoBIOMICS Microbial Community Standard), and two PCR positive controls (ZymoBIOMICS Microbial Community DNA Standard). The feces and cecum samples were amplified using the following steps: an initial denaturation temperature of 94 °C for 3 min, then 25 cycles of 45 s denaturation, 60 s of 50 °C annealing, 90 s of 72 °C extension, then a 72 °C final extension for ten minutes. For the ileum and duodenum samples, the number of cycles was increased to 35 due to the low bacterial biomass and high levels of host DNA, the annealing temperature was increased to 59 °C to decrease potential amplification of host mitochondrial ribosomal RNA, and the extension time was decreased to 30 s to reduce amplification of spurious bands greater than 300 base pairs in length (the size of the bacterial V4 region).

Amplicon sequencing libraries were prepared by The Scripps Research Institute Next Generation Sequencing Core. Four sets of amplicons were used in separate library construction and sequencing runs with: (1) 77 fecal samples, (2) 154 combined cecum lumen and mucosa samples, (3) 154 ileum combined lumen and mucosa samples, and (4) 154 duodenum combined lumen and mucosa samples. Each run also included two positive and two negative extraction controls, and two positive and two negative PCR controls. Amplicon products were cleaned with Zymo DNA Clean & Concentrator™-25 columns, quantified using a Qubit Flourometer (Life Technologies), and pooled. Sequencing libraries were prepared with the recommended Illumina protocol involving end-repair, A-tailing and adapter ligation. For the ileum and duodenum, the prepared DNA library was size-selected on a 2% agarose gel (410–470 bp) using the Agencourt SPRI system to reduce mitochondrial contamination (Beckman Coulter, Inc.). After library prep, libraries were PCR amplified with HiFi Polymerase (Kapa Biosystems) for 12 cycles. Quantitation, denaturation in 0.1 M NaOH, then dilution to 5 pM of libraries preceded loading libraries onto an Illumina single read flow-cell for sequencing on the Illumina MiSeq.

### 16S rRNA gene sequence quality control and QIIME2

Raw sequences (forward reads only) were processed with QIIME 2 (version 2022.8) [78]. Reads and metadata were

imported with qiime tools import. Reads were demultiplexed using the QIIME 2 cutadapt plugin [79]. This resulted in 8.58 million total reads with 34,616 average reads per sample for the fecal run, 8.64 million total reads with an average 51,439 reads per sample for the cecum run, 8.10 million total reads with an average 48,279 reads per sample for the ileum run, and 6.71 million total reads with an average 39,946 reads per sample for the duodenum run. Observed sequence variants (SVs) were produced with QIIME 2 plugin dada2, truncating at 240 base pairs based on quality scores [80].

SV taxonomy was assigned with QIIME 2's feature-classifier plugin using a pretrained naïve Bayes classifier trained on reference database Silva 138 [81–83]. Zymo positive sequencing controls were evaluated for expected and unexpected sequences. The percent genus abundance matched closely to expected genera abundance, while unexpected sequences (potential contaminants), comprised less than 1% of total SVs in the positive controls. Negative controls had low sequence counts and had between 39 and 672 times fewer counts than positive controls, and the composition of negative controls differed greatly from experimental samples. SVs belonging to non-gut bacteria families that were likely environmental contaminants (e.g., Deinococcaceae, Thermaceae, Thermoanaerobacterales Family III, Geodermatophilaceae, and Chitinophagaceae) were removed using qiime taxa filter-table. These potential contaminant families were not present in the cecal or fecal experimental samples and were only present at 0.099% and 0.012% of total SVs in the duodenum and ileum experimental samples, respectively. Contamination of the duodenum and ileum was likely due the increased PCR cycles necessary for 16S rRNA gene amplification of these low microbial biomass samples, which increases the likelihood of amplifying contaminants present in the environment or the reagents used in DNA extraction and PCR [84, 85]. Sequences classified as mitochondria were also removed with qiime taxa filter-table. Additional mitochondrial SVs were identified by blasting taxonomically "unassigned" SV sequences against the *Mus musculus* mitochondrial 12S sequence and removed from the SV table.

Removal of likely contaminants resulted in 741 SVs in the fecal samples, 606 SVs in the cecum samples, 832 SVs in ileal samples, and 1554 SVs in duodenum samples. These SVs were then zero-filtered to remove very low-abundance SVs using the CurvCut heuristic approach[86], which suggested feature removal of SVs present in 3 or fewer samples, resulting in a final count of 333 fecal SVs, 309 cecum SVs, 286 ileal SVs, and 281 duodenum SVs.

Filtered ASV taxonomy was collapsed to the species, genus, and family levels using the QIIME2 plugin qiime taxa collapse. A rooted tree was constructed from the SVs using QIIME 2 plugin phylogeny align-to-tree-mafft-fasttree [87, 88]. Shannon Index [89], Faith's Phylogenetic Diversity (PD) [90], and UniFrac distances [91, 92], were calculated using command qiime diversity core-metrics-phylogenetic. Rarefaction depth for alpha diversity and UniFrac metrics was based on where alpha diversity metrics leveled off (Supplemental Fig. 1), excluding 2 low-sequence count samples (mouse 461 duodenum mucosa and mouse 414 cecal mucosa).

#### Statistical analysis

Analysis of SVs and diversity metrics were performed in R version 4.2.1. For family relative abundance bar plots, SVs were summed by family and family counts were made relative to the total sample counts. All graphs were generated using ggplot2 3.4.0. [93]. SV, genus, and family count tables were transformed with the centered-log ratio (clr) to account for the compositional nature of the data [94, 95]. Euclidean distances for SV and family data were computed using clr-transformed feature tables with R vegan package 2.6.4 [96, 97]. NMDS ordination and PERMANOVA of Euclidean distances and unweighted UniFrac distances were also performed using vegan. Linear mixed-effect models were performed with package nlme version 3.1.161 [98] and FDR corrected for multiple comparison. Fixed effects were multiplied and random effects were mouse nested within cohort.

Microbial balances, coefficients, and AUC values were calculated with coda4microbiome [99]. The coda4microbiome approach, an update to the selbal program [100], identifies the most parsimonious microbial balances defining differences between microbial communities using penalized logistic regression (99). coda4microbiome outputs a log-contrast model for the balances between two different microbial communities; positive microbial coefficients indicate that the microbe contributes to the community with a higher balance, while negative microbial coefficients indicate that the microbe contributes to the community with a lower balance. The absolute value of the coefficient indicates the relative amount that the specific microbe contributes to the balance model. Microbial source tracking of fecal samples was done with SourceTracker 2 using the diagnostics function on clr-transformed SV count tables [101, 102]. Confidence intervals were calculated in Python version 3.10. Samples were not pooled for any analyses.

## Results

#### Bacterial composition is driven by intestinal microhabitat, *hpg* genotype and sex

Beta diversity (between-sample diversity) analysis of all samples combined found the strongest correlation of

bacterial community composition with intestinal section (duodenum, ileum, cecum) or feces, followed by sample type (lumen vs. mucosa). NMDS ordination showed a clear separation between small intestine samples (duodenum, ileum) versus large intestine (cecum) and feces (Fig. 2A), a pattern confirmed by mixed-effects PERMANOVA tests (Section: $p=0.0001$, $R^2=0.195$; Additional file 1: Table S1). These results support the idea that each section harbors a unique microbial composition resulting from differential abiotic and biotic factors [33, 103]. Our data also detected significant differentiation between lumen and mucosa communities, supporting a role of the intestinal epithelium in shaping intestinal gut communities (Sample Type: $p=0.0001$, $R^2=0.046$; Additional file 1: Table S1). In addition, PERMANOVA identified an effect of both genotype and sex on microbial community composition, with significant interactions between sex and genotype, and genotype and section (Additional file 1: Table S1). Many of these distinctions were readily observed in the NMDS plots, such as the differentiation of ileum lumen and mucosa communities in female mutants that was not observed in female wild-type samples (Fig. 2A).

#### Fecal samples are not a good proxy for small intestinal communities

In all four experimental groups of mice, fecal samples consistently clustered with cecum samples and separately from small intestine samples (duodenum and ileum) (Fig. 2A). SourceTracker analysis also showed that the cecum lumen was the dominant source for the fecal communities (sink) and contributed 45% while cecum mucosa contributed 9.9% (Fig. 2B; Additional file 1: Fig. S2). Surprisingly, this was followed by the duodenum lumen, which contributed 28.5% while the duodenum mucosa contributed 9%. The ileum lumen contributed less than 10% and the ileum mucosa contributed negligibly to the fecal microbiome (Fig. 2B; Additional file 1: Fig. S2). Given that microbial composition in the small intestine was not represented well in feces, we analyzed the effects of location, *hpg* genotype and sex on intestinal samples for the remainder of this study.

#### Luminal and mucosal habitats influence alpha and beta diversity in a section-specific manner

Both intestinal section (duodenum, ileum, or cecum) and sample type (lumen or mucosa) had a strong effect on alpha diversity (within-sample biodiversity) (Fig. 3A, B, Table 1). The duodenum and ileum both had lower biodiversity at the sequence variant (SV) level (Fig. 3A, Table 1), while overall Faith's PD (phylogenetic diversity) was lowest in the duodenum and highest in the cecum (Fig. 3B, Table 1). Interestingly, in the duodenum and ileum, both Shannon and phylogenetic diversity were higher in mucosa than lumen, while the opposite was true in the cecum (Fig. 3A, B). There was no correlation between alpha diversity and sex or *hpg* genotype in the duodenum or ileum (Table 1). However, there was an interaction between alpha diversity, sex, and *hpg* genotype in the cecum (Table 1).

Differences in SV beta diversity between the lumen and mucosa were more apparent in the duodenum and ileum than in the cecum (Additional file 1: Fig. S3) although PERMANOVA analysis showed significant differences between lumen and mucosa samples in all three sections for both Euclidean and UniFrac distances (Table 2, Additional file 1: Table S2). In the duodenum, NMDS ordination of both abundance-based Euclidean

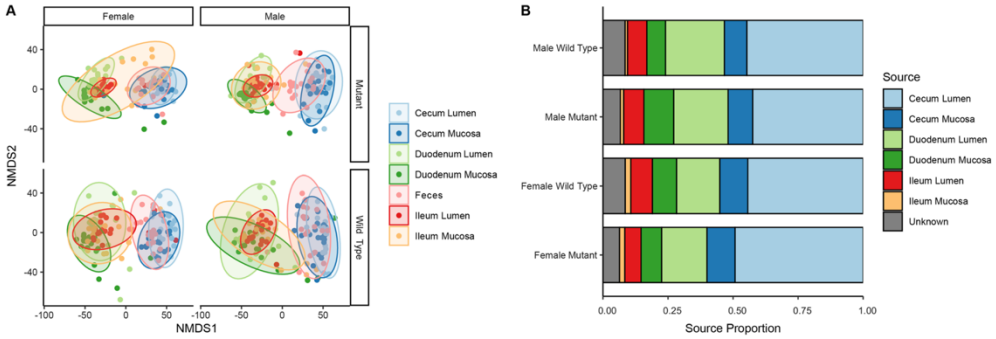


**Fig. 2** Intestinal environment, sex, and *hpg* status affect microbial composition. **A** NMDS ordination of Euclidean distances determined from 16S sequence variant (SV) counts of samples collected from mouse intestinal environments (lumen and mucosa of duodenum, ileum, and cecum) and feces. Samples are colored by sample type and NMDS ordinations were split by sex and *hpg* genotype (wild type and mutant). **B** Results of SourceTracker analysis using feces as sink and intestinal environments as sources. Values shown are means per group

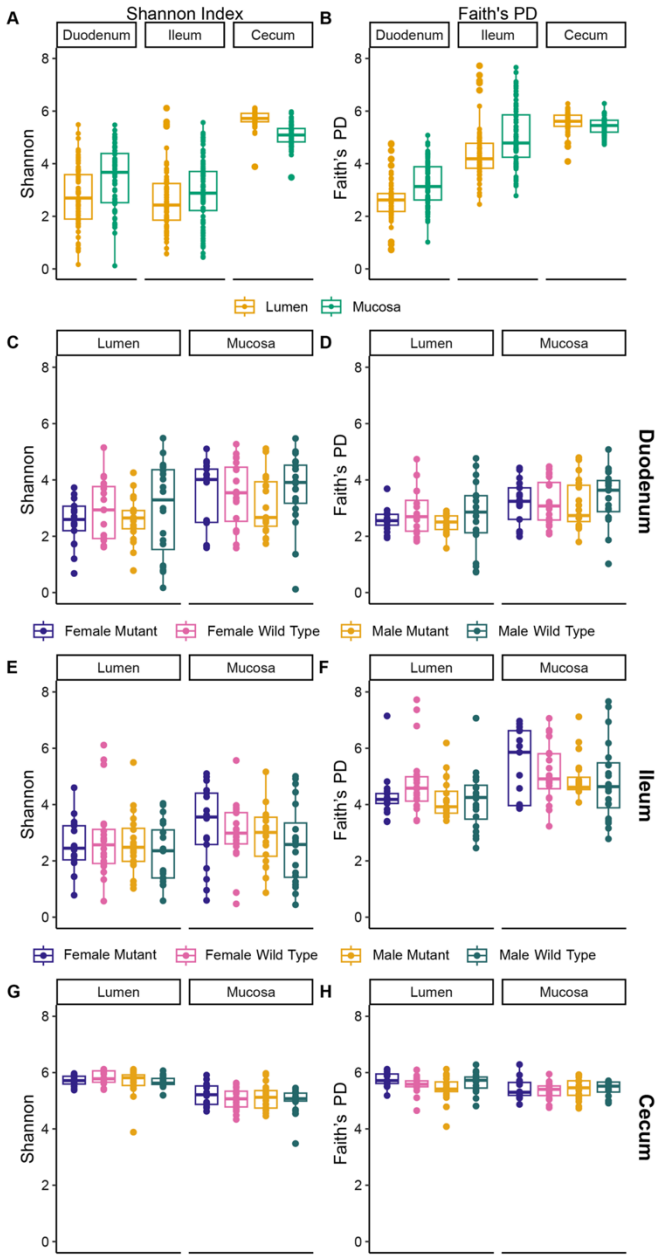


**Fig. 3** Intestinal environment affects alpha diversity measures more than sex and *hpg* genotype. **A** Shannon Index and **B** Faith's phylogenetic diversity (PD) for the lumen and mucosa of duodenum, ileum, and cecum samples for all groups combined. Shannon Index and Faith's PD by sex and *hpg* genotype in the duodenum (**C**, **D**), ileum (**E**, **F**), and cecum (**G**, **H**)

**Table 1** Sex and hpg genotype affect alpha diversity in the cecum only

| Fixed effect | Shannon index | | | Faith's PD | | |
|---|---|---|---|---|---|---|
| | Duodenum *p* | Ileum *p* | Cecum *p* | Duodenum *p* | Ileum *p* | Cecum *p* |
| Sample type | **6.63E−08** | **0.0173** | **2.20E−39** | **1.43E−09** | **7.90E−08** | **6.71E−07** |
| Sex | 0.9971 | 0.1686 | 0.1891 | 0.8487 | 0.0507 | 0.5933 |
| Genotype | 0.1288 | 0.4723 | 0.5406 | 0.1600 | 0.8488 | 0.9787 |
| Sex*Genotype | 0.6619 | 0.5185 | 0.7816 | 0.6576 | 0.6193 | **0.0488** |
| Sex*Sample type | 0.5894 | 0.5485 | 0.8357 | 0.3540 | 0.9291 | **0.0387** |
| Genotype*Sample type | 0.8476 | 0.3929 | 0.0537 | 0.4951 | 0.1120 | 0.3934 |
| Sex*Genotype*Sample type | 0.2929 | 0.3647 | 0.5487 | 0.4467 | 0.1322 | **0.0170** |

*p* value shown for linear mixed-effect model to determine effects of sex, *hpg* genotype, and sample type (lumen vs. mucosa). *P* values < 0.05 shown in bold

**Table 2** *p* Values and R2 from mixed-effect PERMANOVA for Euclidean distances

| Fixed effect | Duodenum | | Ileum | | Cecum | |
|---|---|---|---|---|---|---|
| | *p* value | $R^2$ | *p* value | $R^2$ | *p* value | $R^2$ |
| Sample type | **0.0001** | 0.0571 | **0.0001** | 0.0260 | **0.0001** | 0.0147 |
| Sex | 0.1953 | 0.0070 | **0.0274** | 0.0098 | **0.0015** | 0.0103 |
| Genotype | **0.0047** | 0.0122 | **0.0007** | 0.0151 | **0.0001** | 0.0152 |
| Sex*Genotype | 0.1238 | 0.0076 | **0.0168** | 0.0102 | **0.0001** | 0.0119 |
| Sex*Sample type | 0.0746 | 0.0083 | 0.9195 | 0.0047 | 1 | 0.0032 |
| Genotype*Sample type | 0.8671 | 0.0048 | 0.3920 | 0.0063 | 1 | 0.0031 |
| Sex*Genotype*Sample type | **0.0392** | 0.0091 | 0.2402 | 0.0070 | 0.9999 | 0.0032 |

*P* values < 0.05 shown in bold

and phylogenetic-based unweighted UniFrac distances showed a clear separation of lumen and mucosa samples (Additional file 1: Fig. S3). There was a greater difference in dispersion (inter-sample distances) between ileum lumen and mucosa samples when accounting for abundances (Additional file 1: Fig. S3A), while the clustering of ileum lumen and mucosa samples was more distinct when accounting for phylogeny (Additional file 1: Fig. S3B). By contrast, in the cecum, NMDS ordination of Euclidean distances showed minimal differences between lumen and mucosa, and unweighted UniFrac distances showed mucosal samples were only slightly more dispersed than lumen samples (Additional file 1: Fig. S3).

#### Intestinal environment drives microbial composition at the bacterial family level

Beta diversity analysis at a higher taxonomic level showed clear differences in the community composition of the three intestinal sections at the family level, with the duodenum having the highest compositional variability while the cecum had the least (Additional file 1: Fig. S4). Furthermore, the duodenum and ileum communities were more alike than either were to cecum communities and, of the three sections, the ileum had the strongest differentiation between lumen and mucosa (Additional file 1: Fig. S4). Abundances of the top ten families in the intestine, *Lactobacillaceae, Lachnospiraceae, Muribaculaceae, Deferribacteraceae, Clostridiaceae, Bacteroidaceae, Oscillospiraceae, Eggerthellaceae, Ruminococcaceae,* and *Rikenellaceae* differed by section or sample type (lumen vs mucosa) or had a significant interaction between section and sample type (Additional file 1: Table S3).

Like family-level beta diversity, bacterial family relative abundance showed that duodenum and ileum communities were more similar to each other than to cecum communities. The lumen of both the duodenum and ileum had higher proportions of *Eggerthellaceae* and *Lactobacillaceae* compared to the cecum (Fig. 4, Additional file 1: Fig. S5). However, the high proportion of *Clostridiaceae* in the ileum differentiated it from both the duodenum and cecum communities, and *Clostridiaceae* was higher in the ileum mucosa than the lumen (Fig. 4B). The duodenum was dominated by *Lactobacillaceae, Muribaculaceae, and Lachnospiraceae,* though *Lactobacillaceae* and *Muribaculaceae* were more abundant in the lumen, while *Lachnospiraceae* was more abundant in the mucosa (Fig. 4A). *Lachnospiraceae* dominated the

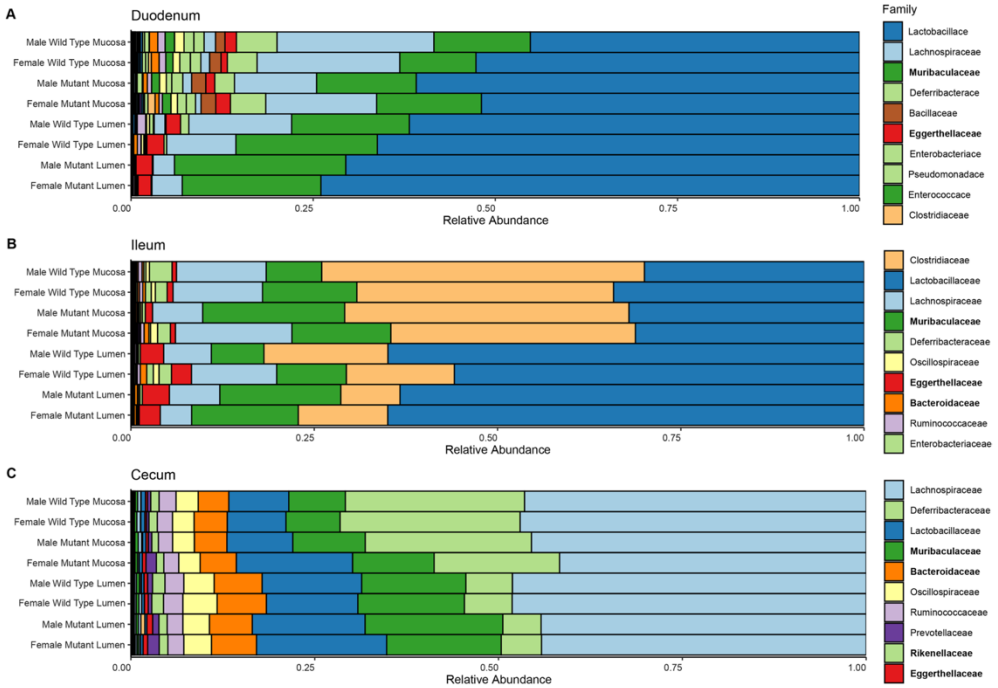


**Fig. 4** Intestinal environment, sex, and *hpg* status affect family-level abundance. Bacterial family-level taxonomic bar plots for **A** duodenum, **B** ileum, and **C** cecum samples. Proportions shown are means for each group. Bolded family names indicate a family's clr-abundance was significantly different by sex or *hpg* genotype, either alone or through interaction with another variable (Table 3)

cecum samples, which also had high relative abundances of several families that were in low abundance in the small intestine, including *Deferribacteraceae, Bacteroidaceae, Oscillospiraceae,* and *Ruminococcaceae* (Fig. 4C). Differences between lumen and mucosal communities in the cecum were also less distinct at the family level than in the small intestine sections (Fig. 4C). However, *Deferribacteraceae* was more relatively abundant in the cecum mucosa, while *Muribaculaceae, Lactobacillaceae, Bacteroidaceae, Oscillospiraceae, and Eggerthellaceae* were more abundant in the cecum lumen (Fig. 4C). Differences in family-level diversity due to *hpg* genotype and sex were also apparent: *Bacteroidaceae, Eggerthellaceae, Muribaculaceae,* and *Rikenellaceae* were significantly

**Table 3** Families showing an effect of section, sample type, sex, or *hpg* genotype

| Fixed effect | Bacteroidaceae | Eggerthellaceae | Rikenellaceae | Muribaculaceae |
|---|---|---|---|---|
| Sample type | **1.158E−04** | **1.447E−25** | **1.695E−03** | **8.330E−30** |
| Section | **1.068E−79** | **1.279E−22** | **9.833E−114** | **5.422E−22** |
| Genotype | 0.7974 | **0.0033** | 0.5142 | **1.627E−05** |
| Sex*Genotype | 0.7119 | 0.7119 | 0.7119 | **0.0401** |
| Genotype*Section | **0.0056** | 0.3226 | **0.0056** | **0.0056** |
| Sample type*Section | **1.267E−03** | **2.005E−08** | **1.480E−02** | **8.596E−15** |
| Sex*Genotype*Sample type*Section | 0.4384 | 0.0712 | 0.2403 | **0.0124** |

FDR-corrected *p* values shown for linear mixed-effect model. Fixed effects were section*sample type*sex**hpg*. Effects and interactions with no significant ($P < 0.05$) interactions were removed from the table. *P* values < 0.05 shown in bold

different between wild-type and *hpg* mutant mice and *Muribaculaceae* was significantly different between sexes (Table 3).

### Reproductive axis and sex niche-specifically impact intestinal community composition

Having detected relationships between microbial composition and sex or *hpg* genotype in the overall intestine (Additional file 1: Table S1), we performed a section-specific analysis of the impacts of these factors on the beta diversity of the duodenum, ileum, and cecum. Statistically robust effects of *hpg* genotype on beta diversity were detectable in all sections with both Euclidean and unweighted UniFrac distances (Table 2; Additional file 1: Table S2). Given the significant effect of sample type, we performed NMDS ordination analysis separately for lumen and mucosa for each intestinal section. These analyses showed that *hpg* genotype altered community composition in both sexes within the lumen and mucosa of duodenum (Fig. 5A), ileum (Fig. 5C), and cecum (Fig. 5E), with *hpg* mutants consistently displaying less intra-sample variation than wild type in the lumen. Interestingly, samples from ileum mucosa showed the opposite pattern: NMDS ordination showed a higher degree of inter-sample variation in female *hpg* mutants than wild-type samples (Fig. 5C).

A statistically significant effect of sex on beta diversity was also detectable in the ileum and cecum (Table 2;

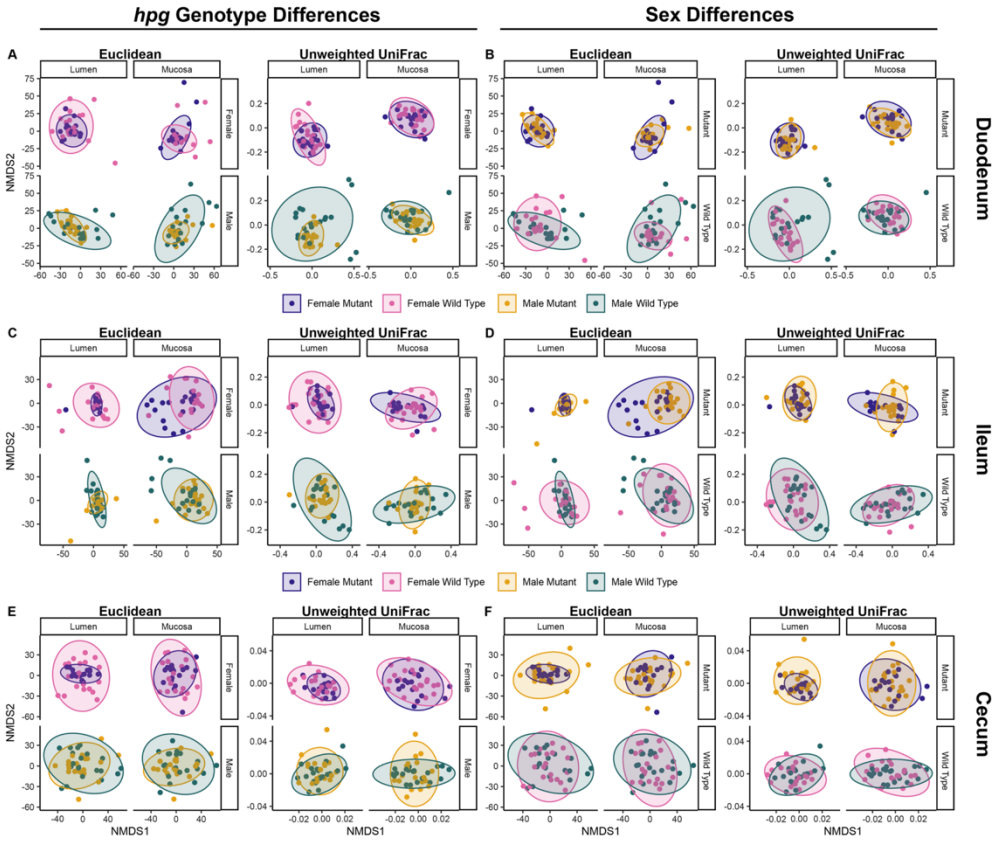


**Fig. 5** Genotype and sex influence intestinal beta diversity in a niche-specific manner. Euclidean (clr-transformed SV abundance) and unweighted UniFrac (phylogenetic) distances revealed *hpg* genotype and sex differences in microbial communities of the small intestine and cecum. Genotype differences within wild-type and *hpg* mutant mice for **A** duodenal, **C** ileum and **E** cecal samples. Sex differences within male and female mice for **B** duodenal, **D** ileum and **F** cecum samples

Additional file 1: Table S2). While sex alone did not affect beta diversity in the duodenum, there was a three-way interaction of sex, *hpg* genotype and sample type (Table 2). Differences between sexes in the duodenum were present in wild-type mice but not mutant mice and the dispersion differed by lumen and mucosa (Fig. 5B). In the ileum and cecum, sex correlated with microbial composition and there was an interaction between sex and genotype in the cecum (Table 2). In the ileum lumen, there were sex differences in wild-type but not *hpg* mutants, while in the mucosa, there were sex differences in both wild-type and mutant mice (Fig. 5D), indicating that sex chromosome effects independent of the reproductive axis may also play a role in development of sex differences in the ileum. NMDS ordination also illustrated an increase in inter-sample variation of female mutants compared with male mutants in the ileum mucosa (Fig. 5D). In the cecum, sex differences in composition were less pronounced than in the ileum (Fig. 5F). Additionally, the effect of the HPG axis in the cecum was similar for males and females, with NMDS ordination showing greater inter-sample variation among wild-type compared to mutant mice (Fig. 5F).

#### Niche-specific differentiation of bacterial abundances by sex and *hpg* genotype

Given that the HPG axis and sex had distinct effects on overall beta diversity in the duodenum, ileum, and cecum, we then used the compositional algorithm coda4microbiome to determine groups of genera whose abundances relative to each other collectively differentiate samples in each intestinal section microbiome by sex and genotype. Microbial balances that defined sex differences in both wild-type and mutant mice in addition to *hpg* genotype differences in female and male mice in the duodenum, ileum, and cecum are shown for genera contributing most strongly to the balance (Fig. 6) or for all genera in the balance (Additional file 1: Fig. S6). We found that the wild-type sex difference balance model performed better (had a higher area-under-the-curve (AUC) score) compared to the mutant model in the ileum (Additional file 1: Fig. S6). In addition, the wild-type sex difference balance model was more parsimonious (indicating a stronger sex-specific signal) compared to mutant mice in the duodenum and cecum (Fig. 6; Additional file 1: Fig. S6). We also found that balance models comparing wild-type and mutant mice performed better (had a higher AUC value) for female mice than for male mice, indicating that the differences between wild-type and mutant females were more consistent (Additional file 1: Fig. S6). The models also showed that distinct bacterial genera contributed to the balances that defined sex differences in wild-type mice versus mutant mice and that some of these genera also contributed to genotype difference in females or males (Fig. 6D). For example, *Lachnoclostridium* in the duodenum contributed to the balance defining females versus males in wild-type mice and also contributed to the balance defining wild-type versus mutants in females (Fig. 6 A, D). Since the lumen and mucosa samples were combined for the balance analysis, heatmaps showed that the relative abundance of genera identified in the balance analysis differed in wild-type males and females between lumen and mucosa, particularly in the duodenum and ileum (Additional file 1: Fig. S7A–C).

## Discussion

Studies in humans and rodent models have shown that the fecal microbiomes of biological males and females are similar prior to puberty but then diverge in a sex-dependent manner during puberty [11, 12, 30, 104]. This pattern strongly suggests that activation of the HPG axis is responsible for sex differences in the gut microbiome but how this axis affects the microbial communities along the intestinal tract is unknown. In humans, it is very difficult to investigate the influence of the reproductive axis per se on the gut microbiome or to distinguish its effects from other factors including sex chromosomes. Furthermore, direct sampling of microflora along the human intestinal tract is a considerable challenge as is controlling for diet, which is a major confounding factor in gut microbiome studies. Additionally, while gonadectomy and sex steroid replacement studies indicate that sex steroid action in adult mice can regulate gut microbiota [30, 31, 105–108], the role of the reproductive axis in sex differentiation of the gut microbiome is unclear. Thus, our use of a genetic mouse model that knocks out *Gnrh1* gene expression in embryonic development and prevents activation of the HPG axis provides a powerful means to isolate the effects of the reproductive axis and readily examine its impacts on the microbiomes in different intestinal sections (both lumen and mucosa), while controlling for the effects of diet. In this study, we showed for the first time that the reproductive axis and sex exert niche-specific effects on intestinal microbial communities, with pronounced effects on overall bacterial community composition (beta diversity) and abundances of specific bacteria at the family, genus, and SV level.

In comparing mouse intestinal and fecal microbiomes, we found that fecal samples poorly represented the bacterial composition of the small intestine (duodenum and ileum), though the cecum was relatively well represented. Our study confirmed results from previous studies that found that fecal samples were more like samples from the large intestine than the small intestine [36, 46, 47, 53, 109], although our study was the first to compare fecal samples with samples from both the lumen and mucosa

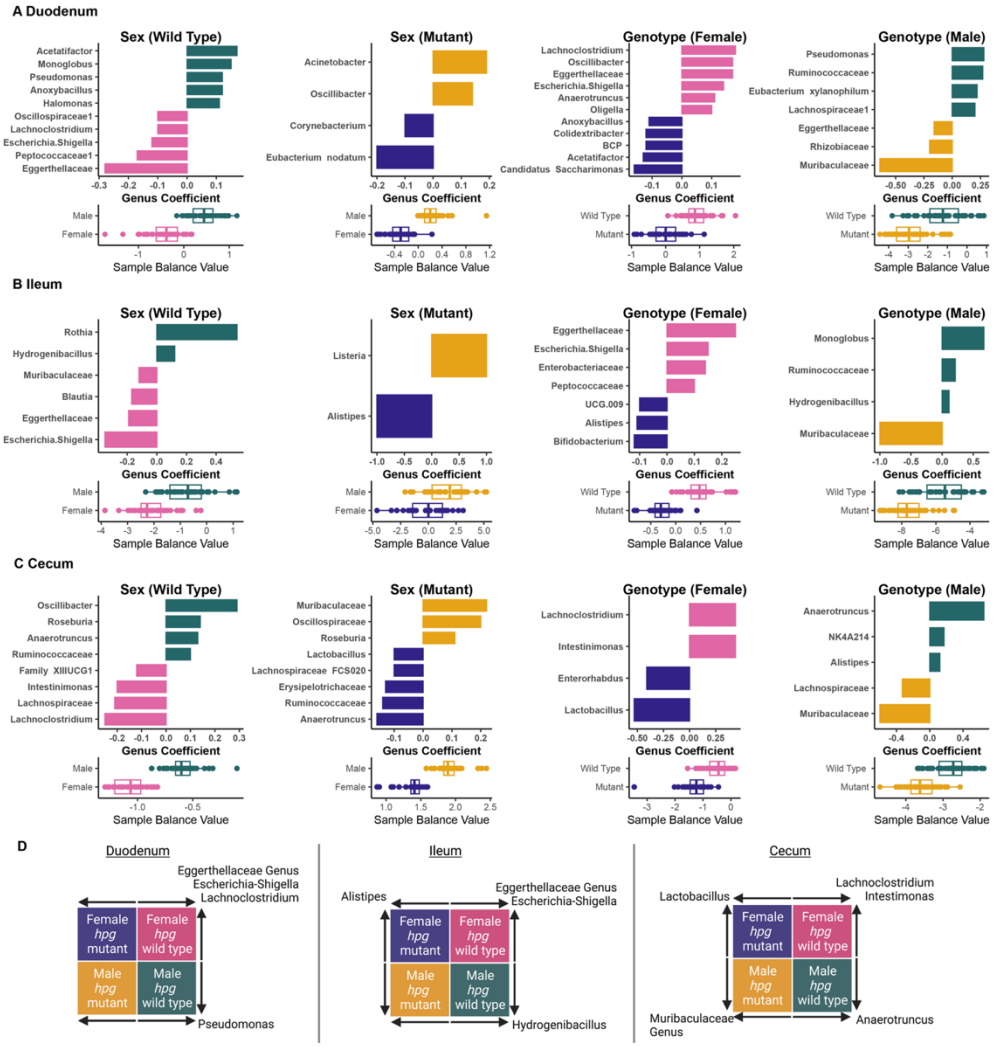


**Fig. 6** Genus-level bacterial balances define intestinal community differences by sex and genotype. Genus-level microbial balances defining wild-type sex differences, mutant sex differences, female *hpg* genotype differences, and male *hpg* genotype differences are shown for **A** duodenum, **B** ileum, and **C** cecum. For each balance, coefficients of genera contributing to the balance are shown with bar graphs. The length of the bar (Genus Coefficient) indicates the proportion that each genus contributed to the balance; genera contributing an absolute balance of at least 0.05 are shown. The direction of the bar indicates whether the genus contributed positively or negatively to the balance, and indicates in which group (male, female, mutant or wild-type) the genus was more abundant. Balance values for each sample in the specified groups are shown below each coefficient graph. For instance, in A, *Acetatifactor* contributed positively to the balance, indicating *Acetatifactor* had a higher relative abundance in male than female wild-type samples. **D** Schematic showing genera with a coefficient of at least 0.05 that contributed to both sex and genotype differences, with the arrow indicating the group in which the genus was relatively more abundant within each balance. BCP: *Burkholderia/Caballeronia/Paraburkholderia* genus group

of the duodenum, ileum and cecum. SourceTracker analysis also found that the strongest, most consistent source for fecal microbes was the cecum and, while there was a signal from the duodenum, the ileum was barely represented. Thus, if the impacts of a condition or treatment on the microbiome are most pronounced in the small intestine, they may be difficult or impossible to detect in fecal samples. Differential taxonomic abundances in the duodenum could easily be masked or even contradicted by data from cecum communities which contain orders of magnitude more bacteria. This could explain why fecal studies have not identified sex differences in members of the *Eggerthellaceae* family while our study found sex differentiation in the relative abundances of *Eggerthellaceae* in the small intestine. It is not clear why SourceTracker indicated that duodenum contributes more to fecal samples than the ileum given that the ileum is more proximal to the feces. However, the ileum has a higher relative abundance of *Clostridiaceae* than the cecum and fecal samples, suggesting that these bacteria do not grow as well in these other environments. In contrast, a higher proportion of duodenum-associated bacteria are found in cecum and feces.

Our comprehensive taxonomic mapping of mucosal and luminal communities in the duodenum, ileum, and cecum found an extremely strong relationship between intestinal environment and microbial community diversity and composition. In agreement with prior studies [46, 47], we found that the small intestine had lower alpha diversity than the cecum, with the lowest alpha diversity in the duodenum. This may be a consequence of the duodenum's higher oxygenation, lower pH, and high transit times compared with other intestinal sections, resulting in greater environmental stochasticity. Greater environmental stochasticity tends to reduce community alpha diversity over time [110]; however, stochastic events in an environment can create temporal niches that allow low-abundance taxa or otherwise poor competitors to survive [110, 111]. For example, the presence of *Halomonas,* which are aerobic or facultatively anaerobic, in the duodenum may be due to sudden increases in the oxygen level after eating due to the stomach depositing oxygenated contents into the duodenum or due to increased blood flow to the epithelial cells after food consumption [112, 113]. High environmental stochasticity may also explain why the duodenum had the greatest inter-individual community variability at the family level and the highest number of low-abundance SVs. In the small intestine, mucosal communities had higher levels of alpha diversity than luminal communities, while in the cecum the differences in alpha diversity between mucosa and lumen were small or nonexistent. This suggests that the mucosa may protect against the low pH and bile acids in the duodenum and ileum that make survival challenging to many taxa [114, 115].

Differences in intestinal environments were also reflected in bacterial family-level community composition. In agreement with prior studies, members of the acid-tolerant, bile acid-transforming *Lactobacillaceae* [114, 116] were a much higher proportion of small intestine communities than the cecum, and were also a higher proportion of luminal than mucosal communities in the small intestine [34, 47, 53, 117]. In addition to *Lactobacillaceae*, other abundant families in the duodenum included *Muribaculaceae*, *Lachnospiraceae,* and *Deferribacteraceae*. *Muribaculaceae* (previously named S24-7) has been positively associated with intestinal short-chain fatty acid (SCFA) levels and possesses genes for bile acid deconjugation [118–120]. *Lachnospiraceae* and *Deferribacteraceae* species have been identified to have host mucin-degrading activity and activate immune signaling [40, 121]. The duodenal mucosa also had a higher relative abundance of *Bacillaceae, Enterobacteriaceae, Pseudomonadaceae, Staphylococcaceae, Enterococcaceae,* and *Oscillospiraceae* compared to the lumen, all of which have previously been associated with intestinal mucosa communities or mucin-degrading activity [122–124]. Only two studies have compared the duodenal lumen to the duodenal mucosa in mice [44, 125], so these findings add to the limited prior knowledge about the mucosal community in the duodenum.

While *Muribaculaceae* and *Lachnospiraceae* were also relatively abundant in the ileum, the most striking finding in the ileum was the high proportion of *Clostridiaceae.* The most abundant member of *Clostridiaceae* in the ileum was *Candidatus Arthromitus*, an uncultured segmented filamentous bacteria known to attach primarily to ileal epithelial cells [126, 127]. Another interesting finding was the relatively high proportion of *Eggerthellaceae* in the small intestine. Although commonly isolated from human and animal feces [123, 128, 129], little is known about the role of *Eggerthellaceae* species in the gut, though they have genes for host mucin degradation [123]. Additionally, our findings agreed with another study[39] that *Lachnospiraceae* dominate the cecum and that the cecum mucosa has a higher relative abundance of *Deferribacteraceae* compared to the lumen; however, we also present new evidence that the cecum lumen maintains higher relative abundances of *Muribaculaceae* and *Lachnospiraceae* compared to the mucosa.

Using the *hpg* mouse model, we also identified several bacterial families whose relative abundances were influenced by the reproductive axis. The relative abundances of *Bacteroidaceae, Eggerthellaceae, Muribaculaceae,* and *Rikenellaceae* were significantly different between *hpg* and wild-type mice, and this effect varied

between luminal and mucosal communities and by section. Since members of these families are known mucin degraders [123, 130, 131], one possibility is that differences between *hpg* mutant and wild-type host mucin production resulted in altered abundances of these families. Additionally, members of *Eggerthellaceae* and *Bacteroidaceae* have genes for bile acid metabolism [132] and members of *Bacteroides* and *Rikenellaceae* have putative glucuronidase activity [133], so bile acid levels and sex steroid levels may influence the competitive advantages of these taxa within the intestinal tract.

The effects of the HPG axis and sex were even more noticeable at the SV level. We showed that the effect of the reproductive axis and sex was more pronounced on overall composition (beta diversity) of intestinal microbial communities than biodiversity (alpha diversity). Beta diversity was strongly influenced by *hpg* genotype in each of the three intestinal sections, though the degree of differentiation was section- and site- (lumen vs. mucosa) specific. For example, genotype differences between wild-type and *hpg* mutant beta diversity in the duodenum were more pronounced in the lumen than the mucosa. Sex differences were also detectable in each intestinal section, though again the effects were section- and site-specific. For example, only wild-type mice showed sex differences in the duodenum, while in the ileum both wild-type and mutant *hpg* mice showed sex differences, though the later was only apparent in ileum mucosa samples. Collectively, these results strongly support the hypothesis that activation of the reproductive axis in wild-type mice due to *Gnrh1* gene expression results in a differentiation of intestinal microbial communities in adults that does not occur in *hpg* mutant mice. These results also indicate that the effects of sex and the reproductive axis are highly differential within the intestinal tract.

One of the most consistent patterns across all intestinal sections was a difference in SV-level community composition between wild-type mice and *hpg* mice, with NMDS plots showing greater dispersion in wild-type mice of both sexes compared to *hpg* mice. This result indicates that a principal effect of the HPG axis on the microbiome is an increase in inter-individual bacterial diversity. This agrees with prior findings showing greater inter-individual diversity in fecal samples between adult humans than between pre-pubertal children [134]. It is possible that this occurs because activation of the reproductive axis increases habitat complexity within the intestinal tract due to its effects on the host (e.g., sex steroids, bile acids, immune system [44, 135–139]). Habitat complexity tends to increase beta diversity and niche differentiation [140–142]. This finding also suggests that intestinal microbial communities in *hpg* mutant mice may be constrained to pre-pubertal-like levels of habitat complexity, resulting in lower levels of inter-individual variability.

In addition to overall bacterial composition, we found that the microbial balances defining sex and *hpg* genotype differences were niche-specific. Interestingly, some genera characteristic of differences between wild-type male and females also differentiated wild-type mice from mutants of the same sex. For example, in the duodenum *Lachnoclostridium* and *Escherichia Shigella* were more abundant in the balances differentiating wild-type females from wild-type males *and* wild-type females from mutant females. Similar patterns were observed in the ileum and cecum although the bacteria did not completely overlap. This pattern is interesting, as a metabolic feature common to members of *Lachnoclostridium* and *Escherichia Shigella* is the ability to deconjugate bile acids, which are at higher levels in female mice [55, 143, 144]. Some of the other genera that were part of microbial balances for wild-type sex differences also have putative glucuronidase or BSH activity. For example, *Anaerotruncus* (glucuronidase and BSH activity*)* and *Pseudomonas* (BSH activity) drove part of the balance for wild-type males in the duodenum and cecum, respectively, and *Lachnoclostridium* (BSH activity), *Blautia* (glucuronidase and BSH activity*),* and *Escherichia Shigella* (glucuronidase and BSH activity*)* tipped the microbial balance towards wild-type females in different parts of intestine [133, 145–147]. These findings highlight the potential for bile acids and sex steroids to regulate intestinal microbial communities and the need for future mechanistic studies to understand how these biotic factors mediate sex differences in intestinal communities. Given that the HPG axis is also active during late gestation and the early postnatal period, these other developmental periods may contribute to adult sex differences in the gut microbiome in addition to puberty. To address this, future studies should investigate whether any sex differences in the gut microbiome, particularly the small intestine, develop prior to puberty. Furthermore, to understand how sex influences microbial metabolism in intestinal communities, methodologies need to be developed for metagenomics or transcriptomics that account for the high host and low microbial biomass in these environments.

Since the reproductive axis was embryonically inactivated in *hpg* mice, we were also able to show that host sex differences not related to gonadal sex steroids also shaped the gut microbiome in a section-specific manner. For instance, the sex differences we observed in SV-level community composition between *hpg* mutant male and females in the ileum mucosa and in genus-level balances cannot be a result of sex steroids because *hpg* mutant mice do not produce gonadal steroid hormones. This indicates that another mechanism, like sex chromosome

effects, led to sex differences in these instances. This finding opens a fundamental new area of investigation focused on genetic effects of sex chromosomes not mediated by the HPG axis on the gut microbiome. These effects might include the *SRY* gene or copy number of X chromosomes, which have been shown to influence physiology independently of sex steroids [148]. This could be investigated using the Four Core Genotypes mouse model, which isolates the effect of sex chromosomes from gonadal hormones [149].

### Perspectives and significance

While studies have compared luminal and mucosal communities of the small intestine or cecum in mice and humans, few have compared the duodenum lumen and mucosa [47, 125], none have explored sex differences in both small intestine lumen and mucosa, and none have investigated the influence of the reproductive axis on differentiation of intestinal microbial communities. Our study indicates the importance of including both sexes in gut microbiome studies, since disease or treatment effects in males may not correspond to effects in females and vice versa. We also demonstrate the importance of employing intestinal samples when studying sex differences and emphasize that simply because a sex difference is not observed in fecal samples it cannot be assumed that there are no sex differences in intestinal microbial communities. In addition, the effects of gonadal steroid insufficiency/excess and treatments such as hormone replacement therapy on gut health should be further investigated. Moreover, for diseases with a sex bias that have a gut microbiome connection, sex differences in the gut microbiome should be explored as a mechanism for the sexual dimorphism. Furthermore, sex and hormonal status may need to be considered for personalized microbial-based therapies and fecal microbiota transplants. Finally, future studies are needed to investigate the mechanisms through which the HPG axis regulates differentiation of intestinal microbiomes and how these mechanisms relate to human health and disease.

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13293-023-00564-1.

**Additional file 1: Table S1.** Location, sex and *hpg* genotype affect compositional differences between samples. P values, degrees of freedom (DF), sum of squares (SumOFSqs), R2, and F statistics from mixed-effect model PERMANOVA for effects of section, sample type (lumen vs. mucosa), sex, and *hpg* genotype. P values < 0.05 shown in bold. **Table S2.** R2 and p values from mixed-effect PERMANOVAs for unweighted UniFrac distances. P values < 0.05 shown in bold. **Table S3.** Effect of section, sample type, sex, or *hpg* genotype on top 10 most abundant bacterial families clr-transformed abundances. FDR-corrected p values shown for linear mixed-effect model. Fixed effects were section*sample type*sex**hpg*. Non-significant interactions were removed. P values < 0.05 shown in bold. **Figure S1.** Rarefaction curves by intestinal section. A Duodenum, B Ileum, and C Cecum. **Figure S2.** Source contribution to feces by *hpg* genotype and sex. 95% confidence intervals shown for SourceTracker source proportions of feces (sink) samples by sex and *hpg* genotype. **Figure S3.** Beta diversity differences between lumen and mucosa. A NMDS orientation plots of Euclidean distances comparing lumen and mucosa in the duodenum, ileum, and cecum. B NMDS plots of unweighted UniFrac distances for the duodenum, ileum, and cecum. The clr-transformed counts of genera were fit to each ordination and arrows are the vector average of the genus. Genera shown had the top 10 $R^2$ values of significant genera fit to the ordination (FDR-corrected p values < 0.05 determined by permutation test). **Figure S4.** NMDS ordination of Euclidean distances based on bacterial family abundances for each intestinal environment. **Figure S5.** Family relative abundance for each individual sample. Relative abundance of each family for A duodenum, B ileum, and C cecum. Each bar represents a mouse intestinal sample. **Figure S6.** Strength and complexity of genus-level balance models. Sex differences in wild-type and mutant mice and *hpg* genotype differences in female and male mice in the A duodenum, B ileum, and C cecum. AUC (area under the curve) indicates the model strength (AUC of 1 indicates a model with perfect predictive value). Abbreviations: ANPR genus: *Allorhizobium /Neorhizobium/Pararhizobium/Rhizobium* genus group. BCP genus: *Burkholderia/Caballeronia/Paraburkholderi* genus group. **Figure S7.** Relative abundance of genera in *hpg* wild-type balance differs by sample type. Heatmaps comparing genera abundances between wild-type sexes separating lumen and mucosa for the A duodenum, B ileum, and C cecum.


#### Acknowledgements

Not applicable.


#### Author contributions

L.S-H., S.T.K., and V.G.T. conceived and designed the study. L.S-H., J.B., L.S., L.T., A.R., R.S.S., E.T.K-T., S.W., and A.N. performed the experiments. L.S-H., A.A.L., S.T.K., and V.G.T analyzed and interpreted the data. L.S-H., S.T.K., and V.G.T. wrote the paper.


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#### Availability of data and materials

Sequence reads from this study were deposited into SRA under BioProject PRJNA983444. The QIIME 2, SourceTracker, Python, and R code used to analyze the data, as well as the taxonomic features tables (SV-, family-, and genus-level) and metadata with barcodes are available at (https://github.com/laurasisk/hpg_axis_intestinal_microbiome).

## Declarations

#### Ethics approval and consent to participate

The University of California, San Diego Institutional Animal Care and Use Committee approved all animal procedures used in this study (Protocol Number S14011).

#### Consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.


#### Author details

[1]University of California San Diego, La Jolla, CA, USA. [2]San Diego State University, San Diego, CA, USA.



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# CHAPTER 3: Influence of the Reproductive Axis on Gut Microbiome Maturation During Puberty


## Abstract

The gut microbiome plays a key role in human health and gut dysbiosis and is linked to many sex-specific diseases including auto-immune, metabolic, and neurological disorders. Activation of the hypothalamic-pituitary-gonadal (HPG) axis during puberty leads to sexual maturation and development of sex differences through the action of gonadal sex steroids. While the gut microbiome also undergoes sex differentiation, the mechanisms involved remain poorly understood. Using a genetic hypogonadal (hpg) mouse model, we sampled the fecal microbiome of male and female wild-type and hpg mutant mice before and after puberty to determine how microbial taxonomy and function are influenced by age, sex, and the HPG axis. We showed that HPG axis activation during puberty is required for sexual maturation of the gut microbiota composition, community structure, and metabolic functions. We also show that some sex differences in taxonomic composition and amine metabolism developed independently of the HPG axis, indicating that sex chromosomes are sufficient to cause certain sex differences in the gut microbiome. We also showed that age, independent of HPG axis activation, led to some aspects of pubertal maturation of the gut microbiota community composition and putative functions. These results have implications for microbiome-based treatments, indicating that sex, hormonal status, and age should be considered when designing microbiome-based therapeutics.


## Introduction

Studying sex differences is important for understanding and developing treatments for diseases with a sex bias. Many diseases are sex-biased in their incidence, prevalence, and/or

severity, including numerous autoimmune[1], cardiovascular [2], and metabolic diseases [3]. Additionally, women are more likely to experience adverse drug reactions, due to both the historical exclusion of women from clinical trials and sex differences in drug pharmacokinetics [4-6].

Sex differences in mammals arise from sex chromosome genes and gonadal sex steroids. Differences in genetic content and gene doses between XX and XY individuals lead to sex differences in gene expression [7]. Though most X genes are inactivated, about 15% of genes escape inactivation, resulting in double gene dosage for those genes in females compared to males [8]. X chromosome gene dosage has been shown to impact immunity, inflammation, and adiposity and is associated with rates of stroke severity and the autoimmune disorder, systemic lupus erythematosus; two disorders with sex bias [1, 9-11]. The Y chromosome contains fewer genes than the X, but still contributes to development of sex differences beyond the SRY gene that leads to formation of the testes during gestation. Y chromosome genes are primarily expressed in the testes or are housekeeping genes homologous to X chromosome genes [12]. Sex differences in expression of X/Y chromosome homologous genes have been observed in nonreproductive tissues, and Y chromosome genes are implicated in sex differences in hypertension and cholesterol levels [2].

It has long been appreciated that gonadal sex steroids are crucial for sex differences. During gestation, ovaries develop unless the SRY gene is expressed, which leads to testes development. Activation of the hypothalamic-pituitary-gonadal (HPG) axis results in the production of kisspeptin in the hypothalamus which stimulates the release of gonadotropin-releasing hormone (GnRH). GnRH then regulates the production of follicle-stimulating hormone and luteizing hormone, which then stimulate the production of testosterone in the

gonads which is converted to estrogen by the aromatase enzyme. (Figure 3.1 A). Different levels of sex steroids lead to sexual development by differentially regulating gene expression throughout the body. The HPG axis is activated briefly during the neonatal period (mini-puberty) and then again at the start of puberty, when the majority of sexual maturation occurs [13].

Like the mammalian host, the gut microbiome also undergoes sexual maturation. Understanding how this process occur is important because the gut microbiome is linked to many diseases that have a sex bias or perturbation of sex steroid levels, including inflammatory bowel syndrome [14], polycystic ovary syndrome [15], and type I diabetes [16]. Prior to puberty, studies observed no sex differences in the gut microbiome in either humans or mouse models [17-19], though studies comparing sex differences in the pre-pubescent gut microbiome are few. During puberty, sex differences in the composition of the gut microbiome develop and are maintained in reproductive-aged adults [20-23]. The cause of this sexual differentiation is not well understood, though it has been shown that gonadectomy and sex steroid replacement can shift gut microbiome composition in mice to resemble the microbiome of intact mice [24-27]. Since the timing of sexual differentiation of the gut microbiome mirrors sexual development of the host, this provided a rationale to investigate if the HPG axis is required for sex differences in the gut microbiome and if so, when was this effect exerted e.g. during mini-puberty or puberty.

To understand how the HPG axis in the host influences maturation of gut microbial taxonomic composition and function, we investigated changes in the gut microbiome using feces as a proxy before and after puberty using a hypogonadal (hpg) mouse model (Figure 3.1 A). In this model, the HPG axis is not activated in homozygous mutant mice (hpg+/+) but is

activated in wild-type mice (hpg -/-). By comparing how the microbiome developed in mutant and wild-type male and female mice, we showed that the HPG axis exerts a strong effect on the taxonomic composition of the gut microbiome during puberty. We also showed that HPG axis activation caused sex differences in bacterial species network structure and connectivity. Moreover, the HPG axis was required for sex differences in microbial gene beta diversity, and microbial lipid metabolism. However, some sex differences in taxonomy and amine metabolism were also observed in mutant mice, indicating that an HPG-independent effect, e.g. sex chromosomes, may contribute to some sex differences in the gut microbiome.

**Methods**

Hpg Mouse Model

Heterozygote hypogonadal (*hpg*) mice with a *Gnrh1*$^{hpg}$ mutation were purchased from Jackson Labs (RRID:IMSR_JAX:000804). The *Gnrh1*$^{hpg}$ mutation is a ~33.5 kb deletion that removes two exons that encode most of the protein. *hpg* heterozygous (*hpg*$^{+/-}$) male and female mice were crossed and the offspring were genotyped to identify *hpg*$^{+/+}$ (mutant) or *hpg*$^{-/-}$ (wild-type) homozygous males and females. Two cohorts of breeding mice were generated with the aim of producing 20 mice per genotype and sex (Figure 3.1 B). Cohort A consisted of 8 female mutants, 10 female wild types, 11 male mutants, and 11 male wild types, while cohort B consisted of 9 female mutants, 10 female wild types, 9 male mutants, and 9 male wild types. In total, 77 wild-type and mutant mice were produced from 31 litters. Mice were housed in a vivarium with a 12-hour light (6:00 AM to 6:00 PM) and 12-hour dark cycle and had access to water and food (Teklad Global 18% Protein Extruded Diet; Envigo, Indianapolis, IN) *ad libitum*. After weaning at 21 days of age, mice were single housed to prevent coprophagy from influencing the gut microbiome. The University of California, San

Diego Institutional Animal Care and Use Committee approved all animal procedures used in this study (Protocol Number S14011).

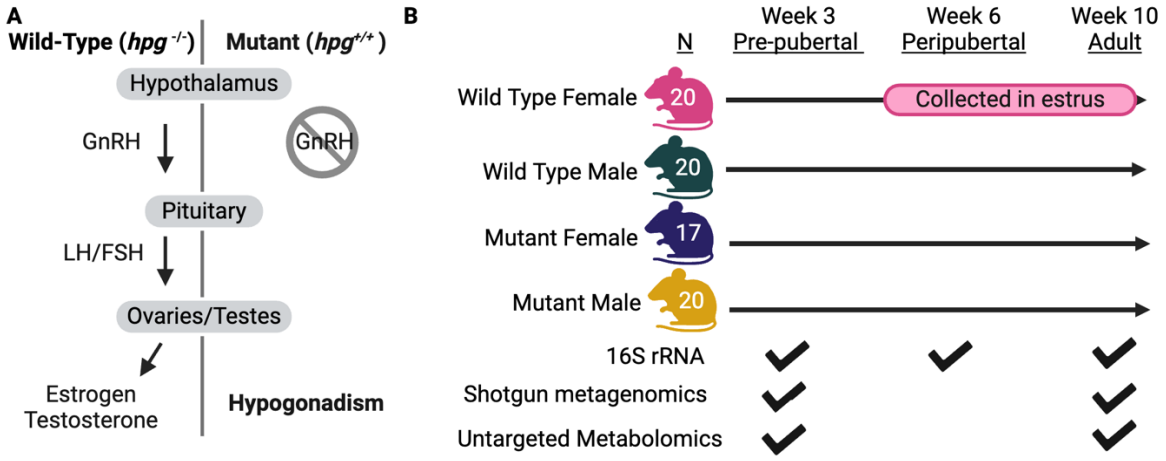



Figure 3.1. Experimental Design.
(A) When the hypothalamic-pituitary-gonadal (HPG) axis is activated in wild-type mice (*hpg*$^{-/-}$), the hypothalamus produces gonadotropin-releasing hormone (GnRH) that stimulates the anterior pituitary to secrete gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Gonadotropins regulate steroidogenesis and gametogenesis in the gonads (ovaries or teste), which results in secretion of sex steroids, sexual development, and reproductive competence. In mutant (*hpg*$^{+/+}$) mice, the *Gnrh1* gene is truncated, so the GnRH peptide hormone is not produced, leading to an inactive HPG axis and hypogonadism. (B) Four experimental groups were included in the study: female and male wild-type mice and female and male mutant mice. N = mice per group. Fecal samples were collected at weeks 3 (pre-pubertal), 6 (peripubertal), and 10 (post-pubertal). For wild-type mice, fecal samples were only collected in the estrus phase of the estrus cycle in weeks 6 and 10. 16S rRNA amplicon sequencing was conducted on fecal samples at all weeks, and shotgun metagenomic sequencing and untargeted metabolomics were performed on fecal samples at weeks 3 and 10. Created with BioRender.com.

Sample Collection and DNA Isolation

Fecal samples were collected and mice were weighed at 3, 6, and 10 weeks of age (Figure 3.1 B). Samples were frozen immediately after collection and stored at −80°C. To minimize potential variability induced by the estrus cycle, female wild-type samples were collected during estrus (determined by vaginal epithelial cell smears) at 6 and 10 weeks of age. Bacterial DNA was extracted from fecal samples and from two negative extraction

controls and two ZymoBIOMICS Microbial Community Standard positive extraction controls (D6300 Zymo Research) with the DNeasy PowerSoil Pro Kit (Qiagen) according to the manufacturer's instructions, and stored at −80°C.

16S rRNA Amplicon Sequencing

The 16S rRNA gene in all fecal samples and controls was PCR amplified using "universal" bacterial primers 515F and 806R for the V4 hypervariable region, with the 806R primers barcoded with unique 12-base pair (bp) Golay barcodes [28]. 16S rRNA amplification was also performed on two negative extraction controls, two negative PCR controls, two DNA extraction positive controls (ZymoBIOMICS Microbial Community Standard), and two PCR positive controls (ZymoBIOMICS Microbial Community DNA Standard). Amplification was performed using the following steps: an initial denaturation temperature of 94°C for 3 minutes, then 25 cycles of 45 seconds denaturation, 60 seconds of 50°C annealing, 90 seconds of 72°C extension, then a 72°C final extension for ten minutes.

Amplicon sequencing libraries were prepared for the 231 experimental samples and 8 controls by The Scripps Research Institute Next Generation Sequencing Core. Amplicon products were cleaned with Zymo DNA Clean & Concentrator™-25 columns, quantified using a Qubit Flourometer (Life Technologies), and pooled. Sequencing libraries were prepared with the recommended Illumina protocol involving end-repair, A-tailing and adapter ligation. After library prep, libraries were PCR amplified with HiFi Polymerase (Kapa Biosystems) for 12 cycles. Quantitation, denaturation in 0.1 N NaOH, then dilution to 5pM of libraries preceded loading libraries onto an Illumina single read flow-cell for sequencing on the Illumina MiSeq.

16S rRNA sequence quality control and QIIME2

Forward read raw sequences were processed with QIIME 2 (version 2022.8) [29]. Reads and metadata were imported with qiime tools import. Reads were demultiplexed using QIIME 2 cutadapt [30]. This resulted in 8.58 million total reads with 34,616 average reads per sample. Sequence variants (SVs) were determined with dada2, truncating at 240 base pairs based on quality scores [31]. QIIME 2's feature classifier plugin was used to assign taxonomy to SVs using a pretrained naïve Bayes classifier trained on reference database Silva 138 [32, 33]. Negative sequencing and DNA extraction controls and Zymo positive community sequencing and DNA extraction controls were evaluated for expected and unexpected sequences. Positive control community composition closely matched the expected genera abundances, while unexpected sequences in the positive controls were less than 1% of SVs. Negative controls had low sequence counts, with 18 to 880 times fewer counts than the lowest-count positive control. The composition of the negative controls greatly differed from the experimental samples. Sequences classified as mitochondria were removed with qiime taxa filter-table, leaving 741 SVs in the feature table. To deal with sparse features, SVs not present in at least five samples were removed using CurvCut [34], resulting in a final count of 333 SVs.

Shotgun Metagenomic Sequencing

Shotgun metagenomic sequencing was performed on cohort B week 3 and 10 fecal sample DNA by the UCSD Institute for Genomic Medicine. Libraries were prepared by fragmenting 500 ng of genomic DNA from each sample by sonicating with Adaptive Focused Acoustics (E220 Focused Ultrasonicator, Covaris, Woburn, Massachusetts). This produced an average fragment size of 500 base pairs. The KAPA Hyper Prep Kit (KAPA Biosystems,

Wilmington, MA, USA) was used to generate sequencing libraries following manufacturer's instructions using 4 cycles of amplification. Library quality was assessed using High Sensitivity D1000 kit on a 4200 TapeStation instrument (Agilent Technologies, Santa Clara, CA, USA). The NovaSeq Sequencing System (Illumina, San Diego, CA, USA) was used to generate 150 bp paired-end reads to obtain ~35 million reads per sample. An average of 76.4 million reads per sample were produced.

Shotgun Metagenomics Sequence Quality Control

Paired-end reads were filtered and trimmed based on quality score and read length and adaptors and poly(G) tails were detected and removed by Fastp version 0.23.2 [35]. This decreased the average read length from 151 base pairs to 147 base pairs. The samples averaged 97 percent of bases with a Q20 or higher quality score, and 92 percent of bases with a Q30 or higher quality score. Quality-filtered and trimmed reads were then mapped to the Mus Musculus genome (Assembly GRCm39, Refseq GCF_000001635.27 downloaded from Ensembl) using Bowtie2 version 2.3.4.1[36]. Unmapped (non-host) reads were reconstructed to paired-end reads using SAMtools version 1.5 [37]. Singletons in forward and reverse read files for each samples were removed using fastq-pair version [38]. An average of 6.2 percent of reads were classified as host reads.

Shotgun Metagenome Genome Assemblies and Annotation

Metagenome-assembled genomes (MAGs) were assembled with MEGAHIT version 1.2.9 [39]. Assembly was evaluated with QUAST version 4.4 [40]. Contigs over 250 bp were kept, with 685,960 total contigs, of which 90,918 contigs were over 1,000 bp and 889 contigs were over 100,000 bp. Contigs were uploaded to KBase [41] and binned with MaxBin 2.0 v2.2.4 [42]. MAG quality was evaluated with CheckM v1.0.18 on KBase [43]. GTDB-Tk

version 2.4.0 was used to annotate bins to the species level with the Gene Taxonomy Database [44, 45]. Salmon was used to determine relative counts of bins, and counts were summed for bins of the same species [46]. Prodigal version 2.6.3 was used to predict coding sequences (genes) from contigs [47]. Eggnog mapper and the Eggnog database version 5 were used to annotate gene orthologous groups [48, 49]. Salmon was used to generate a count table of coding sequences (genes) for each sample [47]. Gene features not present in at least four samples were removed using CurvCut [34].

Metabolomics Mass Spectrometry and Annotation

West Coast Metabolomics performed metabolomic profiling on cohort B week 3 and 10 fecal samples. Metabolites were extracted using methyl-tert-butyl ether extraction and prepared as described by Maytash et. al [50]. The top layer (methanol fraction) was used for the lipidomics, while the bottom layer was used for analysis of biogenic amines. After extraction, sample fractions were reconstituted via vortexing for ten seconds and sonicating for 5 minutes, then centrifuged for 2 minutes at 16,100 x g. Spectra for lipids were produced with UHPLC ESI QTOF MS/MS (Agilent 6530(pos) 6546(neg) Santa Clara, CA ), spectra for primary metabolites were produced with ALEX-CIS GCTOF MS (LECO Pegasus St. Joseph, MI), and spectra for biogenic amines and other small metabolites were produced using HILIC-ESI QTOF MS/MS (Sciex 6600 Framingham, MA).

Metabolites were annotated with a West Coast Metabolomics in-house mzRT library and MS/MS spectral matching with NIST/MoNA (National Institute of Standards and Technology/MassBank of North America) libraries. 17.6% of lipid metabolites and 9.1% of amine metabolites were able to be matched to a known compound. For each platform, peak heights were normalized to internal standards to account for within-series drifts of instrument

sensitivity by dividing the raw metabolite (i) peak height within a sample (j) by the sum of peak heights (iTIC) for all internal standards within a sample (j), and multiplying that value by the average of all internal standards for all samples:

$$\text{metabolite } ij\text{,normalized} = (\text{metabolite } ij\text{, raw} / \text{iTIC } j\ ) * \text{iTIC average}$$

Statistical Analyses

Statistical analyses and graphing were performed in R version 4.2.1. All graphs, with the exception of network analyses, were generated using ggplot2 3.4.0. To compute family relative abundances, SVs were summed by family and family counts were made relative to the total sample counts. Family and gene feature tables were transformed using the centered-log-ratio for statistical analyses [51]. Linear mixed-effect model analysis on family clr-transformed counts was performed with R package nlme [52]. Euclidean distances of clr-transformed counts, NMDS ordination of Euclidean distances, and PERMANOVA analysis of Euclidean distances were computed with Vegan [53]. Genera-level balances, coefficients, and AUC values were computed with coda4microbiome [54]. Random forest was performed on clr-transformed transformed metabolite abundances using a 70/30 train/test split using package randomForest [55]. For top important metabolites, normality of the data was determined with the Shapiro-Wilk test and t tests were performed on normalized peak heights. Within each analysis, p-values for multiple comparisons were corrected with the false discovery rate (FDR) method.

Network Analyses

Network analysis was performed in python version 3.1 using matplotlib version 3.6.3 [56], network version 3.0 [57], numpy version 1.23.5 [58], pandas version 2.0.2 [59], and

scipy version 1.13.0 [60]. 5000 bootstrapped replicates of the MAG species clr-transformed abundance dataset were created, where each bootstrap was randomly resampled with replacement to create new datasets. For each mouse group at weeks 3 and 10, Spearman correlations between species were calculated for each of the 5000 datasets. Networks were drawn and network statistics were calculated from the average of the species correlations on each of the 5000 datasets for edges with a correlation coefficient of 0.8 or higher. Network statistics were compared with binomial tests.

## Results

Broad taxonomic changes in murine gut microbiota are largely complete by 6 weeks of age

First, I profiled the family-level relative abundance of samples at each age using 16S data. A statistical analysis of the effect of age, hpg genotype, and sex on the ten most abundant bacterial families found that the relative abundance of these families was significantly different by sex, genotype, age, or a combination of these effects (Table 3.1). Overall family-level relative abundances at week 6 (peripubertal) were more similar to week 10 (adult) samples than week 3 (pre-pubertal) samples (Figure 3.1A). Compared pre-pubertal samples, peripubertal and adult samples showed a greater relative abundance of *Muribaculaceae, Lactobacillaceae*, and *Oscillospiraceae*, and a diminished relative abundance of *Bacteroidaceae* and *Prevotellaceae* (Figure 3.1A). This pattern was consistent with beta diversity analyses at the sequence variant (SV) level. Non-metric multidimensional scaling (NMDS) of Euclidean distances showed that peripubertal and adult samples overlapped for all mouse experimental groups, while the pre-puberty cluster was distinct (Figure 3.1B).

HPG axis and sex influence family-level relative abundances

There was a strong effect of genotype on the relative abundances of 7 bacterial families, especially when age was taken into account (Table 3.1). There was a significant effect of genotype alone on *Muribaculaceae* and *Eggerthellaceae* abundances, while *Muribaculaceae, Eggerthellaceae, Lactobacillaceae, Lachnospiraceae, Bacteroidaceae, Prevotellaceae,* and *Oscillospiraceae* abundances were affected by a combination of age and genotype (Table 3.1). The combined effects of sex and genotype, or sex and age, also affected relative abundances of *Lachnospiraceae, Bacteroidaceae, Muribacilaceae, Oscillospiraceae, Deferribacteraceae, Ruminococcaceae,* and *Eggerthellaceae* (Table 3.1). *Bacteroidaceae* was also different by the combined effect of sex and age, and the FDR-corrected p values for this effect on *Lachnospiraceae, Muribaculaceae, Oscillospiraceae, Rikenellaceae*, and *Eggerthellaceae* were only slightly larger than a cutoff of 0.5, indicating that these families also may be affected by sex in an age-dependent manner.

Table 3.1. Results (p-values*) of linear mixed-effect model analysis with top 10 most abundant bacterial families.

| **Family** | **Sex** | **Genotype** | **Age** | **Sex: Genotype** | **Sex: Age** | **Genotype: Age** |
|---|---|---|---|---|---|---|
| Lactobacillaceae | 0.8327 | 0.1529 | **2.50E-07** | 0.0901 | 0.2841 | **0.0092** |
| Lachnospiraceae | 0.9054 | 0.3094 | **5.43E-06** | **0.0011** | 0.0537 | **0.0032** |
| Bacteroidaceae | 0.3803 | 0.7363 | **8.29E-12** | **0.0021** | **0.0451** | **0.0011** |
| Muribaculaceae | 0.3803 | **0.0172** | **7.91E-13** | **3.0E-5** | 0.0599 | **3.04E-08** |
| Prevotellaceae | 0.9054 | 0.7363 | **0.00031** | 0.3311 | 0.5930 | **3.71E-06** |
| Oscillospiraceae | 0.8327 | 0.1529 | **7.10E-10** | **0.0130** | 0.0595 | **0.0104** |
| Deferribacteraceae | 0.9141 | 0.1577 | **0.0158** | **0.0101** | 0.6978 | 0.2595 |
| Ruminococcaceae | 0.9054 | 0.1529 | **0.04507** | **0.0136** | 0.1118 | 0.0944 |
| Rikenellaceae | 0.3803 | 0.4024 | **1.17E-09** | 0.9062 | 0.0595 | 0.9788 |
| Eggerthellaceae | 0.9141 | **0.0202** | **1.93E-12** | **0.0192** | 0.0595 | **0.0016** |

Genotype was associated with clear differences in specific family-level bacterial abundances pre- versus post-puberty. *Prevotellaceae* abundances were higher in wild type mice pre-puberty, but not in adults. Members from the *Muribaculaceae* family were more

abundant in mutant mice at peripuberty and adulthood, and *Lachnospiraceae* were more relatively abundant in wild type mice at peripuberty and adulthood (Figure 3.1A).

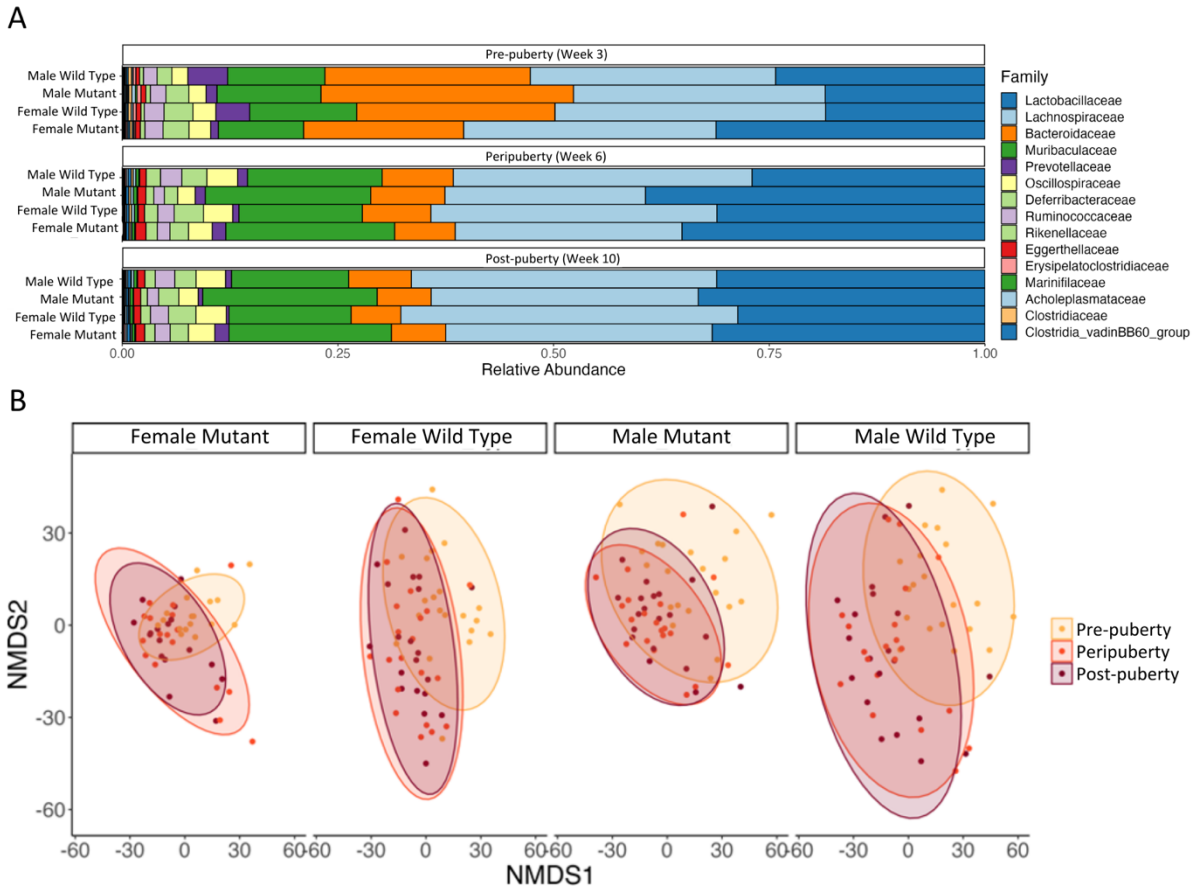



Figure 3.2. Age (week), hpg genotype, and sex influence gut microbial family abundances and beta diversity computed from 16S sequencing data.
(A) Bacterial family relative abundances of each mouse group (male wild type, male mutant, female wild type, and female mutant mice) at age 3 (pre-puberty), 6 (peri-puberty), and 10 weeks (post-puberty). NMDS ordinations by mouse group for (B) Euclidean distances identified by different colors by age.

Genera balances differentiate post-pubertal gut microbial communities by sex and genotype

To determine how age and reproductive development influence microbial community composition at a lower taxonomic level, I modeled microbial balances over time for each mouse group (wild-type female, wild-type male, mutant female, and mutant male). Genera

coefficients indicate the relative proportion that the bacterial genus abundance contributed to the balance. The 16S dataset was used because the sample size was double that of the metagenomic dataset, and genera-level models were used because they had considerably higher AUC scores (better fit to the data) than SV-level models. Balance analysis using genera abundances differentiated microbial communities by age, genotype, and sex. With regards to age, *Candidatus arthromitus* and *Bacteroides* were identified as being more abundant in week 3 (pre-puberty) balances for all mouse groups compared to weeks 6 and 10 (Figure 3.3 A-D) while *Anaerotrunctus* was more abundant in week 3 balances for *hpg* mutants and female wild-type mice (Figure 3.3 A-C). For wild type, but not mutant, mice the week 3 genera coefficients also included *Alloprevotella* (Figure 3.3 C-D). While no genera were more abundant in week 10 balances across all four groups, some week 10 genera coefficients were conserved by genotype: *Alistipes, Lactobacillus,* and *Intestimonas* were more abundant in week 10 wild type balances and *Muribaculaceae* was more abundant in week 10 balances of *hpg* mutant mice, regardless of sex (Figure 3.3 E).

Week 10 genera coefficients also differed by sex for both wild-type and mutant mice, with *Entoerorhabdus* and a *Lachnospiraceae* genus more relatively abundant in adult mutant females, while *Harryflintia,* an *Eggerthellaceae* genus, *Colidextribacter, Butrycoccus, RF39, Muribaculum, Intestimonas, and A2* were more relatively abundant in adult mutant male (Figure 3.3 A-B). *A2, Muribaculum, Enterorhabdus, RF39, Harryflintia, the Eubacterium*

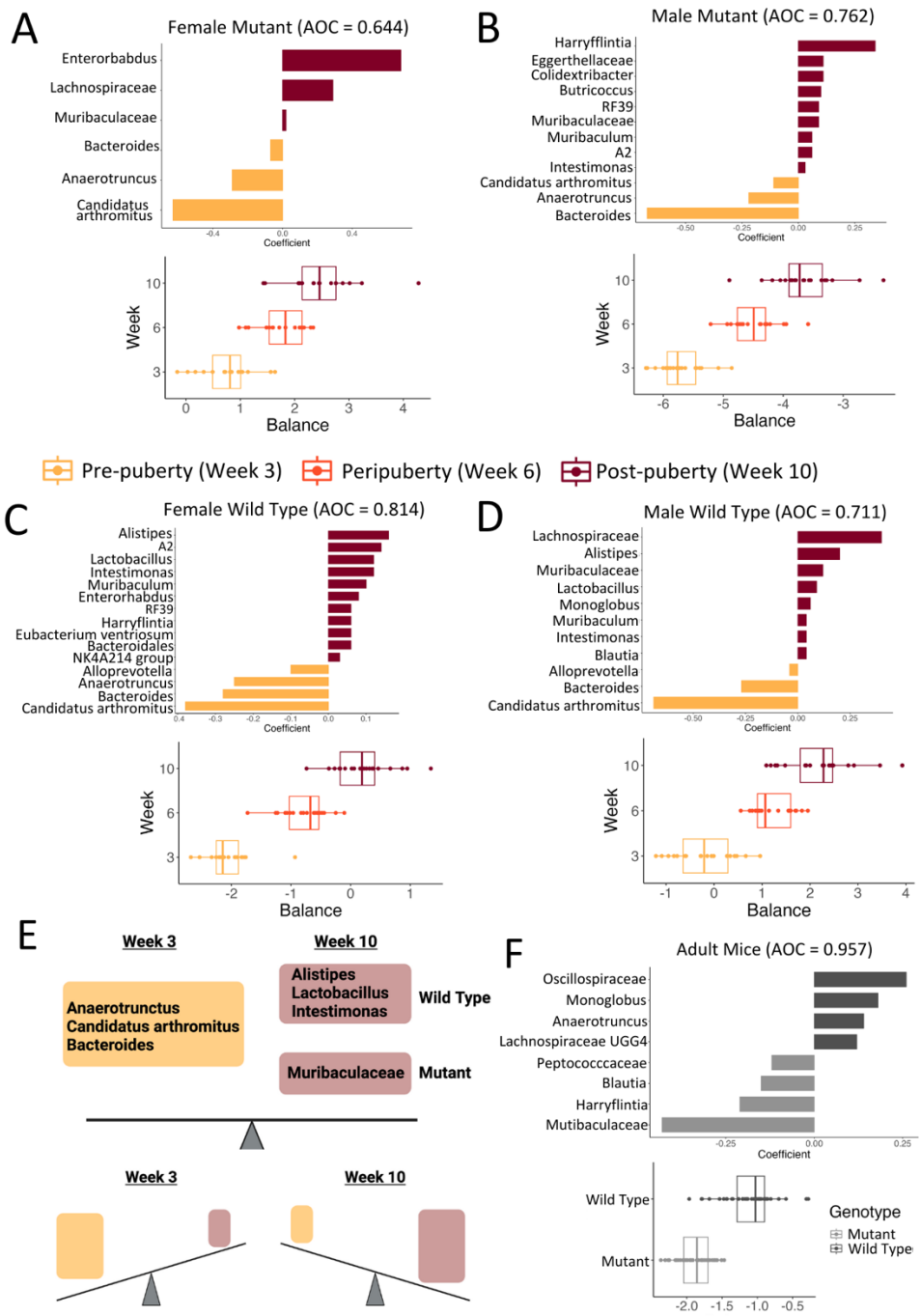



Figure 3.3. Age-based genera balances differentiate adult mice by sex and hpg genotype. Genera coefficient values and balance scores for balances defining age-based community differences (A) female mutant, (B) male mutant, (C) female wild-type, and (D) male wild-type mice 3, 6 and 10 weeks of age. (E) Summary schematic of common genera defining longitudinal balances at week 3 and at week 10 based on hpg genotype. (F) Genera balance coefficient values and balance distribution for mutant vs wild-type mice at week 10.

*ventriosium* group, and a *Bacteroidales* genus were more relatively abundant in adult female wild-type balances, while *Lachnospiraceae* genus, a *Muribaculaceae* genus, *Monoglobus,* and *Blautia* were more relatively abundant in the adult male wild-type balances (Figure 3.3 C-D).

Given that age-based balances for each group showed conserved differences between *hpg* mutant and wild-type mice regardless of sex, we modeled balances differentiating *hpg* mutant and wild-type communities for both sexes in adulthood at 10 weeks of age. The AUC of this model was higher than the age comparison models, indicating the strength of this method at discriminating samples based on genotype in adult mice. Similar to the results of the age-based balances, *Muribaculaceae* was more relatively abundant in mutant mice in adulthood, while *Alistipes* was more relatively abundant in wild-type mice. The conserved difference between wild-type and mutant mice, in both the age-based and adult *hpg*-based balances, indicates a strong effect of the HPG axis on development of the gut microbiome during the pubertal period.

HPG axis activation affects sex differences in beta diversity at the species level

Since the 16S dataset did not annotate most SVs to the species level, I used metagenome-assembled genomes (MAGs) to identify bacterial species and determine how age and the HPG axis altered bacterial community composition. Species-level beta diversity analysis using Euclidean distances between samples identified significant effects of age, genotype, and sex, with age having the strongest effect followed by sex and genotype (Table 3.2). NMDS ordination of Euclidean distances on bacterial species showed that slight sex differences in beta diversity were apparent before puberty in both wild type and mutant mice (Figure 3.4A). Post-puberty, dispersion in mutant mice increased for both males and females compared to pre-puberty and sex differences beta diversity were absent (Figure 3.4 A). For

wild-type mice, adult male wild-type beta dispersion was much smaller compared to pre-pubertal samples, resulting in a sex difference in wild-type mice post-puberty (Figure 3.4 A).

Table 3.2. PERMANOVA results for Euclidean distances computed for MAG bacterial species. P-values less than 0.05 are bolded.

| | Species | |
|---|---|---|
| | R2 | Pr(>F) |
| **Sex** | 0.0336 | **0.0012** |
| **Genotype** | 0.0224 | **0.0280** |
| **Week** | 0.1013 | **0.0001** |
| **Sex:Genotype** | 0.0120 | 0.4283 |
| **Sex:Week** | 0.0103 | 0.6113 |
| **Genotype:Week** | 0.0106 | 0.5621 |
| **Sex:Genotype:Week** | 0.0084 | 0.8186 |

HPG axis activation during puberty in females results in lower network connectivity

To further investigate how age and the HPG axis modulated community composition at the species level, we used a bootstrap approach (5000 replicates) to construct correlation networks based on MAG-derived bacterial species counts for each mouse group at weeks 3 and 10 (before and after puberty). Network structure and connectivity metrics were similar for all 4 groups of mice before puberty (Supplemental Figures 3.1 and 3.2). In contrast, many network metrics including number of edges (connections), degree centrality (connections per species), transitivity (degree to which nodes cluster together), and density (ratio of actual to potential edges) increased significantly from pre-to-post puberty with the exception of metrics for female wild-type mice which decreased (Table 3.3, Supplemental Figure 3.1). As visualized, a reduction in edges also occurred in wild-type females post-puberty compared to pre-puberty (Figure 3.4 B), which contrasted sharply to the increase in number of edges that occurred in other groups, especially compared to female mutant mice (Figure 3.4 C).

The clustering structure of female wild-type network also differed from the other networks after puberty. Before puberty, overall network structure was visually similar in all 4

groups (Supplemental Figure 3.2). However, in adult networks, mutant networks appeared strikingly different to wild-type networks, with more overall edges (Figure 3- 5A-D, Table 3.3), and all adult networks except the wild-type female network contained two distinct clusters of positively correlated species (Figure 3.5A-D).

Table 3.3. MAG species network statistics for week 3 and week 10 (5000 bootstrap replicates) and Binomial tests p-values comparing week 3 and week 10 networks.
P values are FDR corrected. P values listed as 0 are not actually zero, but lower than the program's precision. P values < 0.05 are bolded.

| | | Number of Edges | Number of Nodes | Average Degree Centrality | Transitivity | Closeness Centrality | Density |
|---|---|---|---|---|---|---|---|
| Male Wild-Type | Week 3 | 1875.32 | 176.634 | 0.121 | 0.615 | 0.317 | 0.121 |
| | Week 10 | 2027.82 | 176.484 | 0.131 | 0.628 | 0.324 | 2027.82 |
| | p-value | **1.44E-19** | **0** | **1.17E-21** | **6.69E-12** | **4.36E-10** | **0.0011** |
| Female Wild-Type | Week 3 | 1762.696 | 175.875 | 0.114 | 0.628 | 0.33 | 0.114 |
| | Week 10 | 1600.781 | 175.709 | 0.104 | 0.6 | 0.319 | 0.104 |
| | p-value | **4.72E-26** | **3.36E-178** | **6.40E-26** | **3.23E-66** | **2.54E-22** | **2.86E-58** |
| Male Mutant | Week 3 | 1950.793 | 176.624 | 0.125 | 0.624 | 0.319 | 0.125 |
| | Week 10 | 2193.015 | 176.476 | 0.141 | 0.655 | 0.328 | 0.141 |
| | p-value | **3.83E-31** | **0** | **1.40E-34** | **7.64E-46** | **7.51E-14** | **0.0078** |
| Female Mutant | Week 3 | 2005.21 | 176.523 | 0.129 | 0.643 | 0.322 | 0.129 |
| | Week 10 | 2409.349 | 176.311 | 0.155 | 0.669 | 0.337 | 0.155 |
| | p-value | **4.37E-80** | **1.61E-306** | **2.04E-88** | **2.56E-29** | **1.13E-35** | 0.7664 |

Pre-pubertal networks display HPG-independent sex differences in clustering patterns

To investigate how age, HPG axis, and sex influenced which species were most connected in each network, we determined the twenty-most connected nodes in each network. The majority of these species in both pre- and post-pubertal networks belonged to *Prevotellaceae, Muribaculaceae*, and *Lachnospira*ceae families (Supplemental Tables 3.2 and 3.3). The specific species that were most connected varied between networks, though members of the *Duncaniella* and *Paramuribaculum* genera (family *Muribaculaceae*) were among the most connected nodes across networks (Supplemental Tables 3.2 and 3.3).

While overall network metrics were similar between the 4 groups of mice before puberty, we did observe network structure-related sex differences in both wild-type and

mutant mice with regards to highly connected bacterial species. In pre-pubertal communities, highly connected nodes in males were dispersed through the networks and did not display strong clustering, though two nodes, an unclassified bin (labeled 168) and an unidentified *Lachnospiraceae* species UBA3282 (labeled 154), clustered together in both networks (Figure 3.4 D, E). Prepubertal female networks showed stronger clustering of networks than in males, with most of the highly connected nodes clustering together, containing species predominantly in *Prevotellaceae, Muribaculaceae*, and *Lachnospiraceae* (Figure 3.4 F, G).

Sex-specific species clustering in adult networks is driven by HPG axis activation in females

In adult wild-type networks, clustering of species was sex-specific in wild-type mice but not mutant mice, indicating an HPG-dependent sex difference that developed during puberty (Figure 3.5). In adults, most highly connected species fell into positively connected clusters that were dominated by specific families; one cluster type consisted primarily of *Muribaculaceae* and *Prevotellaceae* species (Cluster 1) and the other cluster type consisted primarily of *Lachnospiraceae* species (Cluster 2) (Figure 3.5). The female wild-type network was the only adult network that lacked Cluster 2, and the only adult network where *Eggerthellaceae* species were also part of Cluster 1. Comparison of the highly connected species among the adult networks found that *Evtepia* species (labeled 88 and 89, unclassified *Eubacteriales*) and *Coprobacter secundus* (labeled 61, family *Barnesiellaceae*), in Cluster 2, were among the most-connected nodes for all networks with the notable exception of wild-type females. In contrast, only the female wild-type network included *Mucispirillum schaedleri* (labeled 128, family *Muribaculaceae*) and *Lawsonibacter* species (labeled 112, family *Oscillospiraceae*), which were not in a cluster (Figure 3.5C).

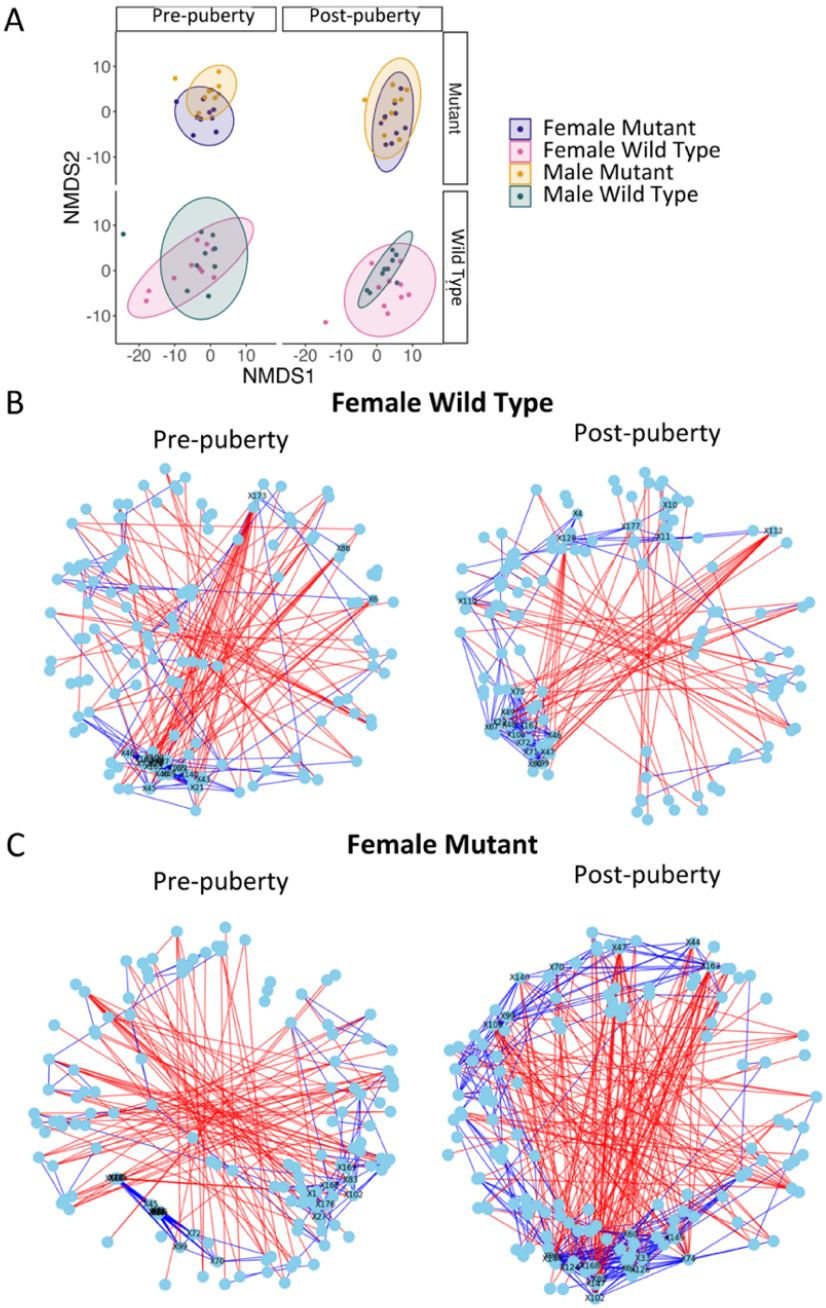



Figure 3.4. Puberty alters fecal bacterial community composition and networks at the species level in metagenomes.
(A) NMDS ordination of Euclidian distances for species abundances between samples demonstrate sex differences before puberty (wild type and mutants) and after puberty (wild-type). Visualizations of pre-puberty and post-puberty for female wild-type (B) and female mutant (C) mice. Blue lines are positive correlation and red lines are negative correlation. Nodes are colored in light blue and represent species. The top 20 species with the most nodes are labeled with numbers corresponding to species listed in Supplemental Table 1.

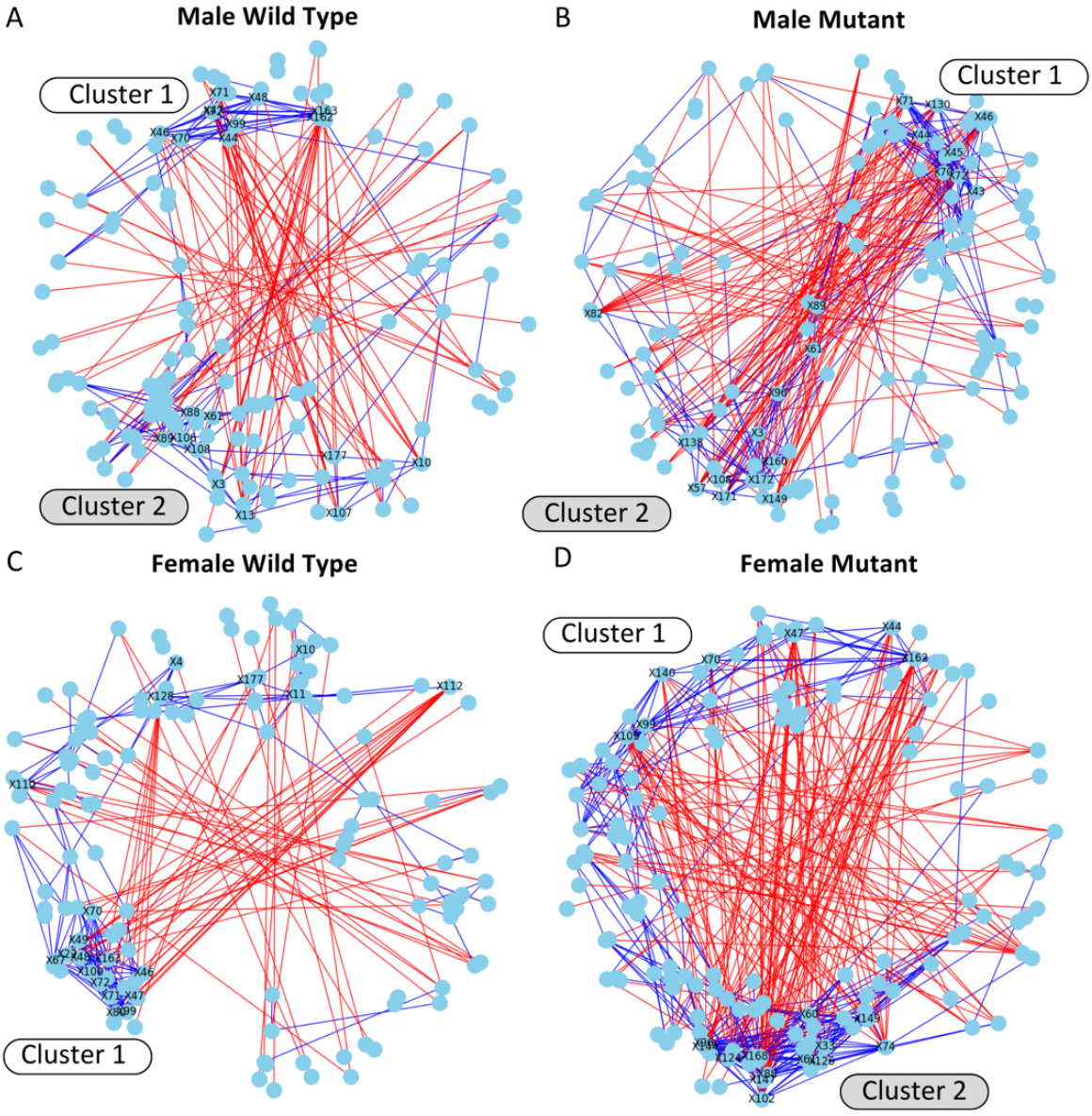



Figure 3.5. HPG axis influences structure of adult fecal bacterial networks.

Visualization of week 10 networks for (A) male wild-type, (B) male mutant, (C) female wild-type, and (D) female mutant mice. Blue lines indicate positive correlations and red lines negative. Nodes are colored in light blue and represent species. The 20 most connected nodes in each network are labeled with numbers corresponding to species listed in Supplemental Table 1. Cluster 1 consists primarily of *Muribaculaceae* and *Prevotellaceae* species while Cluster 2 consists primarily of *Lachnospiraceae* species.

Sex-specific species clustering in adult networks is driven by HPG axis activation in females

In adult wild-type networks, clustering of species was sex-specific in wild-type mice but not mutant mice, indicating an HPG-dependent sex difference that developed during puberty (Figure 3.5). In adults, most highly connected species fell into positively connected clusters that were dominated by specific families; one cluster type consisted primarily of *Muribaculaceae* and *Prevotellaceae* species (Cluster 1) and the other cluster type consisted primarily of *Lachnospiraceae* species (Cluster 2) (Figure 3.5). The female wild-type network was the only adult network that lacked Cluster 2, and the only adult network where *Eggerthellaceae* species were also part of Cluster 1. Comparison of the highly connected species among the adult networks found that *Evtepia* species (labeled 88 and 89, Unclassified *Eubacteriales)* and *Coprobacter secundus* (labeled 61, family *Barnesiellaceae*), in Cluster 2, were among the most-connected nodes for all networks with the notable exception of wild-type females. In contrast, only the female wild-type network included *Mucispirillum schaedleri* (labeled 128, family *Muribaculaceae*) and *Lawsonibacter* species (labeled 112, family *Oscillospiraceae*), which were not in a cluster (Figure 3.5C).

Activation of the HPG axis during puberty leads to greater beta diversity of bacterial genes

After determining how sex and genotype influenced microbial taxonomic composition, we then investigated how putative bacterial functions differed before and after puberty. We examined differences in the overall set of predicted bacterial genes and orthologous groups of genes. PERMANOVA tests on Euclidean distances of gene and ortholog abundances between samples revealed a significant effect of sex, genotype, and age (pre- vs. post-puberty) on beta diversity, with the strongest effect due to age (Table 3.3). The effect of genotype was distinct for microbial genes and orthologs. For genes, the beta-

diversity of wild-type and mutant samples in males and females was similar before puberty, while after puberty, wild-type samples had greater dispersion than mutant samples, reflecting an effect of activation of the HPG axis (Figure 3.6 A). In contrast, beta dispersion for all groups was reduced post-puberty for orthologs (Figure 3.6 A), indicating that the hpg effect on bacterial genes is due to changes in the abundance of specific genes instead of gene categories. Sex had less of an effect on the beta diversity for genes and orthologs (Figure 3.6 B).

Table 3.4. PERMANOVA results for Euclidean distances computed for Gene and Ortholog abundances. P-values less than 0.05 are bolded.

| | **Genes** | | **Orthologs** | |
|---|---|---|---|---|
| | R2 | P | R2 | P |
| **Sex** | 0.0210 | **0.0012** | 0.0222 | **0.0015** |
| **Genotype** | 0.0230 | **0.0002** | 0.0226 | **0.0010** |
| **Age** | 0.0554 | **0.0001** | 0.0671 | **0.0001** |
| **Sex:Genotype** | 0.0159 | 0.0592 | 0.0157 | 0.0781 |
| **Sex:Age** | 0.0110 | 0.8736 | 0.0109 | 0.8396 |
| **Genotype:Age** | 0.0143 | 0.1838 | 0.0141 | 0.1973 |
| **Sex:Genotype:Age** | 0.0103 | 0.9664 | 0.0098 | 0.9750 |

Activation of HPG axis during puberty leads to sex differences in lipid metabolites

Since we observed a strong effect of the HPG axis on the beta diversity of bacterial genes in mice after puberty, we then investigated the influence of age, sex and genotype on metabolites involved in lipid metabolism. PERMANOVA analysis indicated that beta diversity of lipid metabolites was different by age (pre- vs post-puberty) and by the combined effect of sex, genotype, and age (Table 3.5). NMDS ordination indicated that for both mutant and wild-type mice, sex differences were not apparent before puberty, but sex differences emerged in wild-type mice after puberty (Figure 3.7 A).

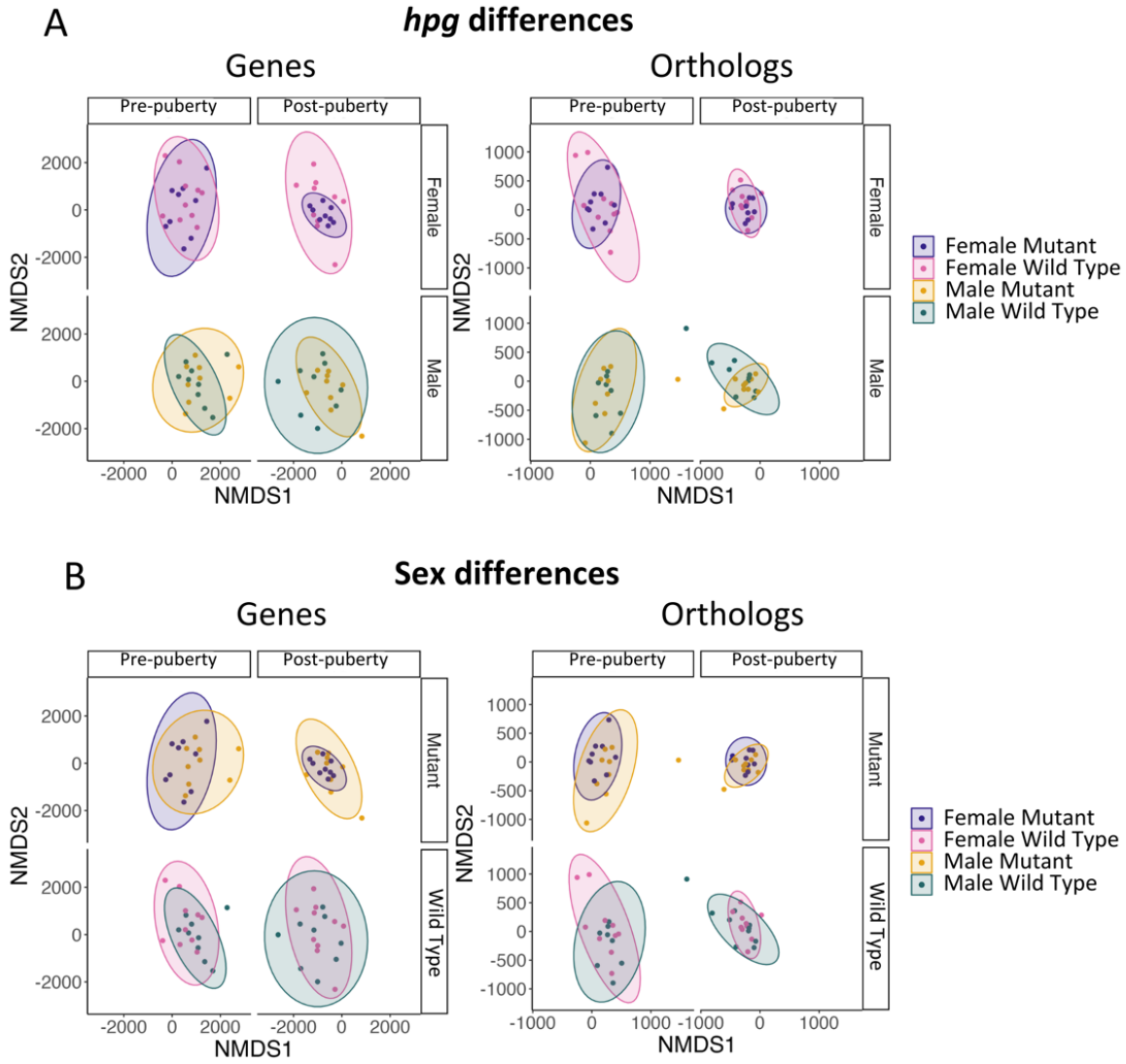



Figure 3.6. Microbial genes and orthologous groups differ by age, genotype and sex. NMDS ordination of Euclidian distances comparing (A) genotype and (B) sex differences for genes and ortholog abundances between samples.

Table 3.5. PERMANOVA results for Euclidean distances computed for metabolite datasets. P-values less than 0.05 are bolded.

| | Lipids | | Amines | |
|---|---|---|---|---|
| | R2 | P | R2 | P |
| **Sex** | 0.02128 | 0.0982 | 0.02698 | **0.0436** |
| **Genotype** | 0.01287 | 0.3129 | 0.01104 | 0.4527 |
| **Age** | 0.12143 | **0.0001** | 0.05328 | **0.0014** |
| **Sex:Genotype** | 0.01768 | 0.1637 | 0.02444 | 0.0650 |
| **Sex:Age** | 0.01501 | 0.2409 | 0.04057 | **0.0078** |
| **Genotype:Age** | 0.01585 | 0.2166 | 0.01039 | 0.4862 |
| **Sex:Genotype:Age** | 0.04389 | **0.0045** | 0.03033 | **0.0256** |

Table 3.6. Out-of bag error and accuracy for Random Forest models on sex differences for pre-pubertal (week 3) or post-pubertal (week 10) wild-type or mutant mice

| Dataset | Age | Week/Genotype | Out-of-bag error | Accuracy on test set |
|---|---|---|---|---|
| Amines | Pre-Puberty | Mutants | 42% | 42% |
| Amines | Post-Puberty | Mutants | 25% | 25% |
| Amines | Pre-Puberty | Wild Type | 39% | 33% |
| Amines | Post-Puberty | Wild Type | 30% | 50% |
| Lipids | Pre-Puberty | Mutants | 33% | 67% |
| Lipids | Post-Puberty | Mutants | 50% | 50% |
| Lipids | Pre-Puberty | Wild Type | 54% | 16% |
| Lipids | Post-Puberty | Wild Type | 15% | 67% |

We also used Random Forest to determine which lipid classes were predictive in classifying sex differences before or after puberty (Figure 3.7 B). Interestingly, the model for adult wild-type mice had a significantly lower out-of-bag (OOB) error (15%) indicating a better fit to the data, while the other models had OOB errors of <30% (Table 6). For the 5 most important lipid classes differentiating wild-type males and females, all but phosphatidylethanolamines were significantly different by sex for adult wild-type mice, and none were significantly different by sex for adult mutant mice (Table 3.7). Fatty acids and N-acyl-lyphophosphatidylethanolamines were more abundant in wild-type males than females, and phosphatidylethanolamine alkenyls and acylcarnitines (fatty acid metabolites) were more abundant in wild-type females than males (Figure 3.7C). Taken together, these results indicate that activation of HPG axis during puberty drives sexual differentiation of gut lipid metabolism.

Sex differences in amine metabolites are driven by HPG-dependent and -independent effects

In addition to age, PERMANOVA analysis of fecal amine metabolites showed that beta diversity was significantly different by sex, the combined effect of sex and age, and the combined effect of sex, genotype, and age (Table 3.5). NMDS ordination showed that, while

slight sex differences in amine beta diversity occurred before puberty, sex differences were readily apparent after puberty in both wild-type and mutant mice (Figure 3.8 A).

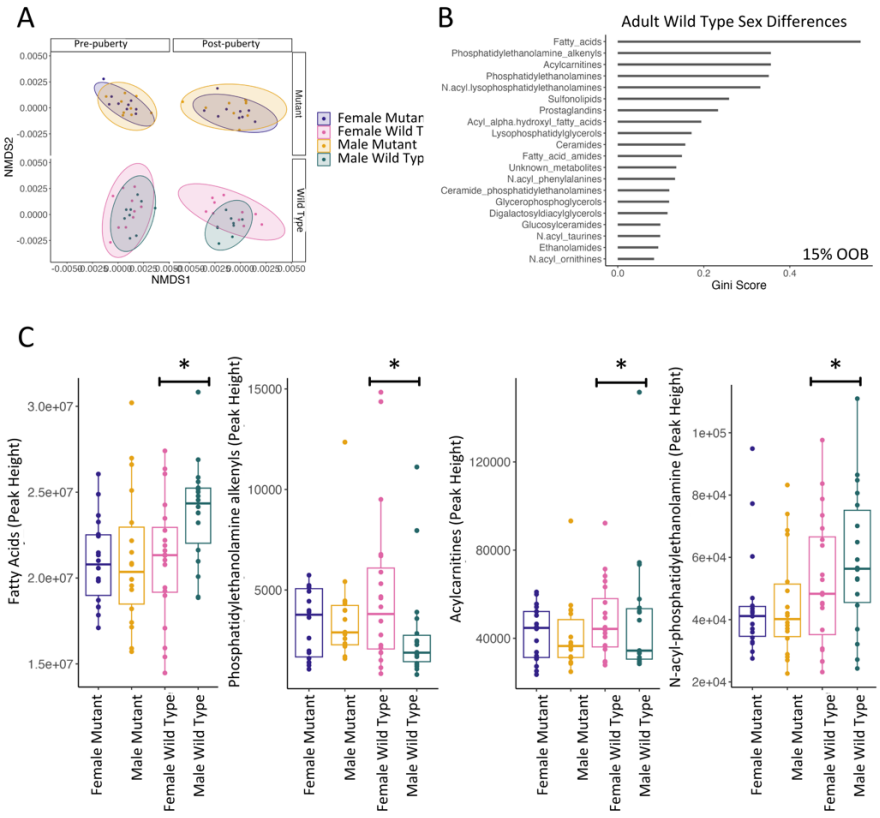



Figure 3.7. Gut lipid metabolites differ by age, sex, and genotype.

(A) NMDS ordination of Euclidian distances showing sex differences within each genotype pre- and post-puberty. (B) Gini score for the most important lipid classes differentiating male and female wild-type mice. (C) Abundances of lipid classes that were significantly different by sex. OOB stands for out-of-bag error.

Table 3.7. T-test results (p-values) for lipid class sex differences in adult mice within each genotype. P-values are FDR-corrected with values < 0.05 in boldface.

| | Wild Type | Mutant |
|---|---|---|
| Acylcarnitines | **0.0257** | 0.8636 |
| Fatty acids | **0.0044** | 0.3539 |
| N-acyl-lysophosphatidylethanolamines | **0.0360** | 0.5963 |
| Phosphatidylethanolamine alkenyls | **0.0257** | 0.5965 |
| Phosphatidylethanolamines | 0.1417 | 0.3606 |

To determine which amines differentiated mice by sex before and after puberty, we employed Random Forest for both wild-type and mutant mice. For pre-pubertal mice, model out-of-bag (OOB) error was high while for post-pubertal mice, the model OOB error was lower but model accuracy on the test set was quite low (25% and 50%, respectively; Table 6). To investigate whether the levels of metabolites in specific amine classes identified in the Random Forest models as differentiating adult males and females were different between the sexes, we compared the abundances of the top 5 most-important amine classes for each model and found that three of the top amine classes were significantly different between males and females (Table 3.8, Figure 3.8 B-C). Interestingly, carboxylic acids were different between mutant males and females, while quaternary ammonium compounds and xanthurenates were different between wild-type males and females (Figure 3.8 D). These results indicate that adult sex differences in gut amine metabolites are due to both HPG-dependent and -independent effects.

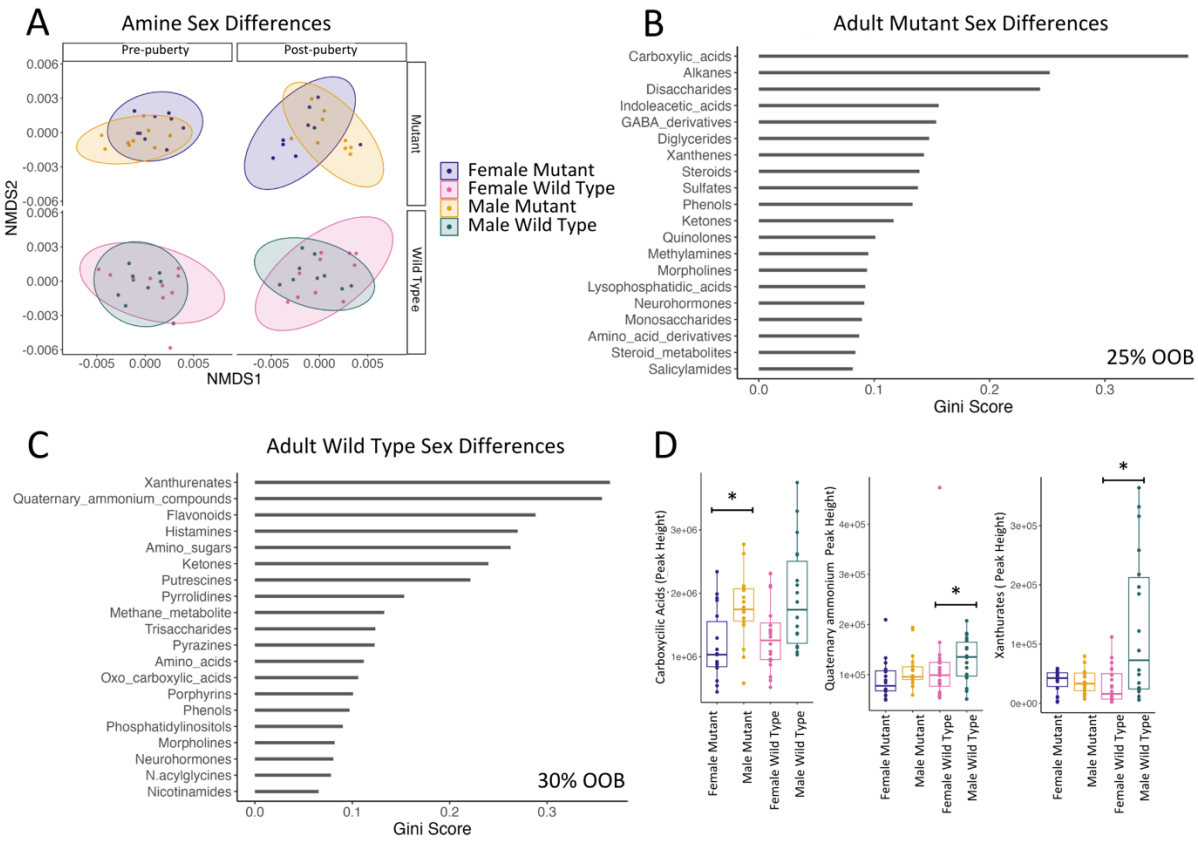
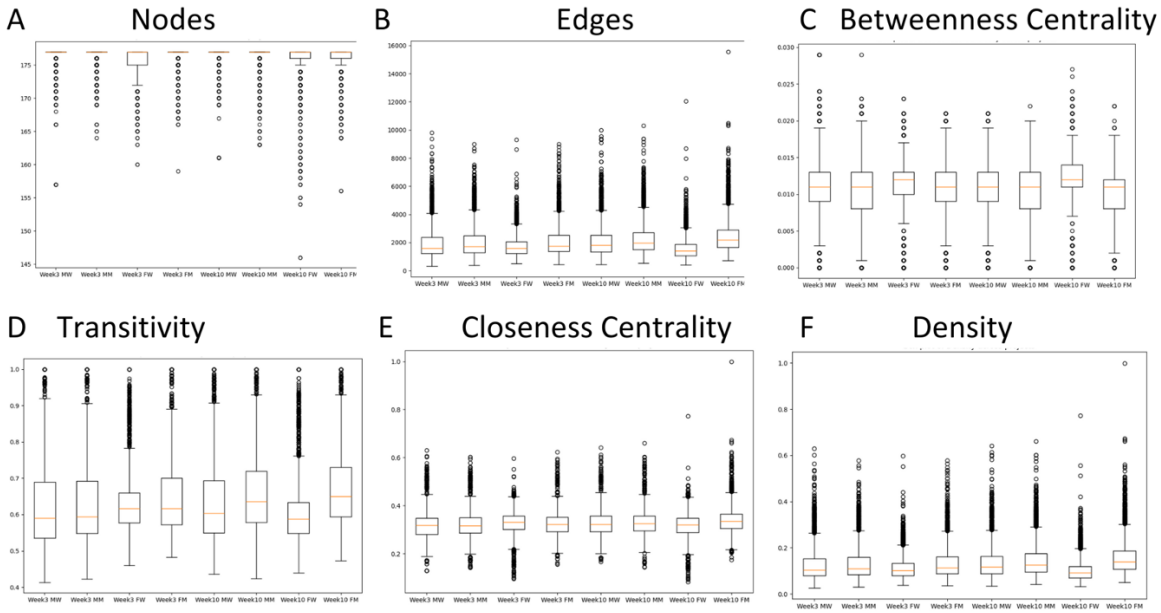



Figure 3.8. Gut lipid metabolites differ by age, sex, and genotype.
(A) NMDS ordination of Euclidian distances showing sex differences within each genotype pre- and post-puberty. (B) Gini score for the most important lipid classes differentiating male and female wild-type mice. (C) Abundances of lipid classes that were significantly different by sex. OOB stands for out-of-bag error.

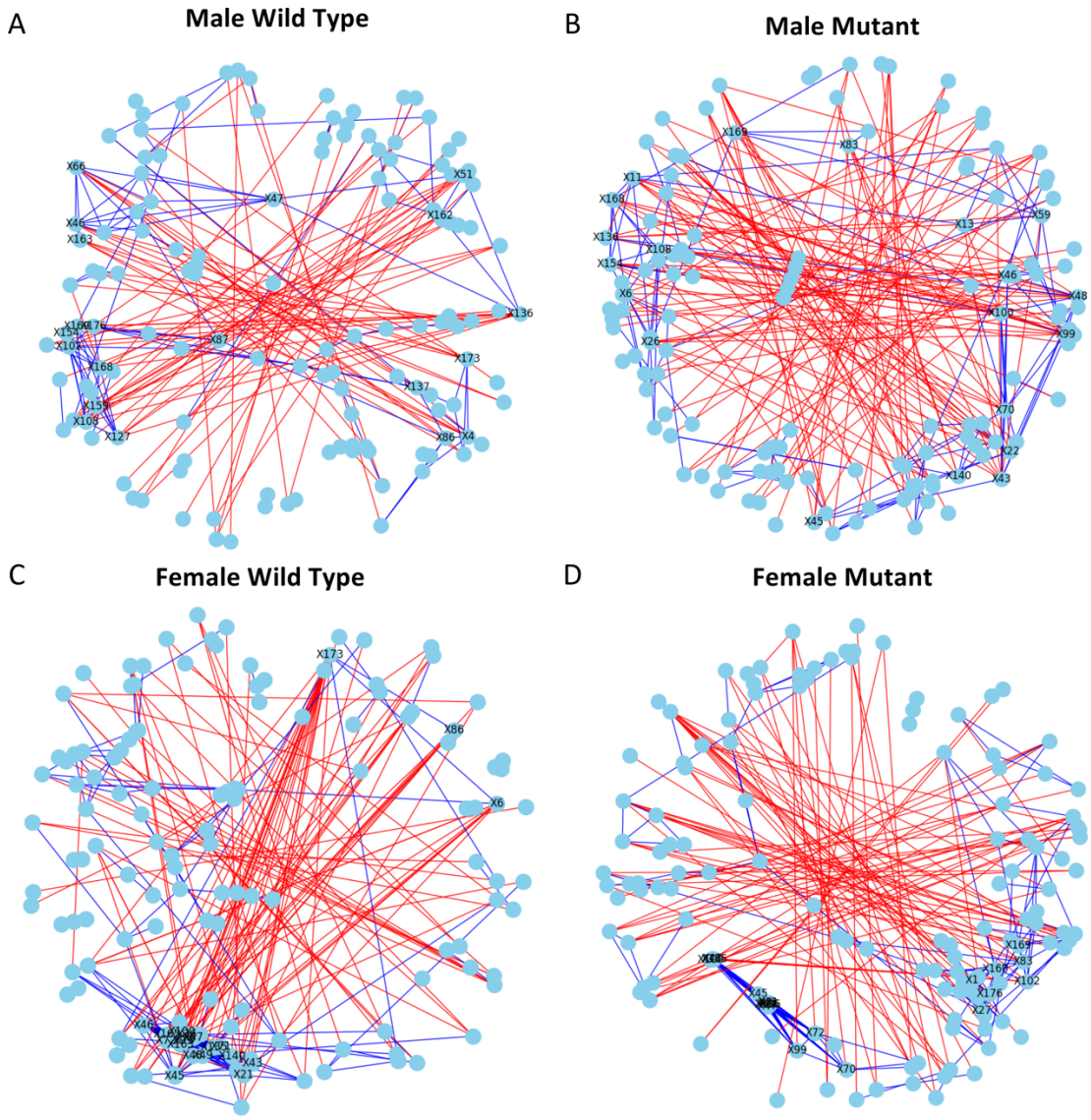
Table 3.8. FDR corrected t-test results (p-values) for amine class sex differences in adult mice within each genotype. P-values <0.05 are in boldface.

| | Wild-type Mice | Mutant Mice |
|---|---|---|
| Alkanes | 0.1204 | 0.9811 |
| Amino sugars | 0.0525 | 0.7692 |
| Carboxylic acids | 0.0525 | **0.0345** |
| Disaccharides | 0.2168 | 0.2712 |
| Flavonoids | 0.0525 | 0.1752 |
| GABA derivatives | 0.6987 | 0.5274 |
| Histamines | 0.0525 | 0.5274 |
| Indoleacetic acids | 0.1057 | 0.1680 |
| Quaternary ammonium compounds | **0.0085** | 0.2002 |
| Xanthurenates | **0.0119** | 0.2712 |

## Supplemental Figures




Supplemental Figure 3.1. Network statistics box and whisker plots for week 3 and week 10 networks.
Networks were computed on 5000 bootstrap replicates of MAG species assignments. (abbreviations: MW = male wild type, MM = male mutant, FW = female wild type, FM = female mutant).




Supplemental Figure 3.2. Network visualizations for week 3 networks computed on 5000 bootstrap replicates of MAG species assignments.

Networks for (A) male wild-type (B) male mutant (C) female wild-type, and (D) female mutant mice. Blue lines are positive correlation and red lines are negative correlation. Nodes are colored in light blue and represent species. The top 20 species with the most nodes are labeled with numbers corresponding to species listed in Supplemental Table 1.

**Supplemental Tables**

Supplemental Table 3.1. List of the species indicated by each label in the network plots

| | Species | | Species | | Species |
|---|---|---|---|---|---|
| **X1** | 14-2_sp | **X61** | Coprobacter_secundus | **X120** | MGBC163016_sp910589135 |
| **X2** | 14-2_sp000403315 | **X62** | Coproplasma_sp | **X121** | MGBC163490_sp910588075 |
| **X3** | 14-2_sp004793545 | **X63** | Coproplasma_sp013316055 | **X122** | MGBC164599_sp |
| **X4** | 14-2_sp910576385 | **X64** | Coproplasma_sp910577555 | **X123** | MGBC164599_sp910575625 |
| **X5** | 14-2_sp910577785 | **X65** | Coproplasma_sp910578145 | **X124** | MGBC165282_sp910589265 |
| **X6** | 14-2_sp910580015 | **X66** | Coproplasma_sp910586065 | **X125** | Massilicoli_timonensis |
| **X7** | 1XD42-69_sp | **X67** | D16-34_sp910575165 | **X126** | Merdisoma_sp |
| **X8** | 1XD42-69_sp009911505 | **X68** | D16-63_sp | **X127** | Merdisoma_sp910574475 |
| **X9** | 1XD42-69_sp011959925 | **X69** | Duncaniella_dubosii | **X128** | Mucispirillum_schaedleri |
| **X10** | 1XD8-76_sp910573755 | **X70** | Duncaniella_freteri | **X129** | Muribaculum_gordoncarteri |
| **X11** | Acetatifactor_sp | **X71** | Duncaniella_muris | **X130** | Muribaculum_intestinale |
| **X12** | Acetatifactor_sp011959105 | **X72** | Duncaniella_sp | **X131** | Nanosyncoccus_sp |
| **X13** | Acetatifactor_sp910585425 | **X73** | Dysosmobacter_sp | **X132** | Odoribacter_sp910589025 |
| **X14** | Acetatifactor_sp910585615 | **X74** | Dysosmobacter_sp022797935 | **X133** | Oscillospiraceae_sp |
| **X15** | Acetatifactor_sp910585665 | **X75** | Dysosmobacter_sp910584995 | **X134** | Paralachnospira_sp022792335 |
| **X16** | Acetatifactor_sp910589035 | **X76** | Dysosmobacter_sp910588175 | **X135** | Paramuribaculum_intestinale |
| **X17** | Acutalibacter_sp | **X77** | Enterenecus_sp | **X136** | Paramuribaculum_sp001689565 |
| **X18** | Acutalibacter_sp910580045 | **X78** | Enterenecus_sp910585265 | **X137** | Pelethenecus_sp910577745 |
| **X19** | Acutalibacter_sp910587995 | **X79** | Enterococcus_faecalis | **X138** | Pelethomonas_sp |
| **X20** | Adlercreutzia_mucosicola | **X80** | Enterorhabdus_sp009911695 | **X139** | Pelethomonas_sp910579965 |
| **X21** | Adlercreutzia_muris | **X81** | Eubacterium_F_sp | **X140** | Phocaeicola_barnesiae |
| **X22** | Adlercreutzia_sp | **X82** | Eubacterium_G_sp910574895 | **X141** | Pseudobutyricicoccus_sp910579575 |
| **X23** | Alistipes_sp910575495 | **X83** | Eubacterium_J_sp | **X142** | Pumilibacter_sp |
| **X24** | Alloprevotella_sp002933955 | **X84** | Eubacterium_J_sp910574915 | **X143** | RGIG4057_sp910577675 |
| **X25** | Anaerocaecibacter_sp910577995 | **X85** | Eubacterium_J_sp910579075 | **X144** | RGIG7193_sp |
| **X26** | Anaerotignum_sp910576545 | **X86** | Eubacterium_J_sp910586445 | **X145** | RGIG7193_sp022791755 |
| **X27** | Anaerotruncus_colihominis | **X87** | Eubacterium_plexicaudatum | **X146** | RGIG8607_sp910588535 |
| **X28** | Anaerotruncus_sp | **X88** | Evtepia_sp | **X147** | Roseburia_sp910587865 |
| **X29** | Anaerotruncus_sp000403395 | **X89** | Evtepia_sp910584805 | **X148** | Ruminiclostridium_E_sp910585505 |
| **X30** | Anaerotruncus_sp003612625 | **X90** | Faecivivens_sp022793675 | **X149** | Schaedlerella_sp |
| **X31** | Anaerotruncus_sp910586875 | **X91** | Fimisoma_sp | **X150** | Sporofaciens_sp |
| **X32** | Angelakisella_sp | **X92** | Flavonifractor_plautii | **X151** | Sporofaciens_sp910575835 |
| **X33** | Angelakisella_sp022795575 | **X93** | Gallimonas_sp910586825 | **X152** | UBA11774_sp022790905 |
| **X34** | Angelakisella_sp910585535 | **X94** | HGM11386_sp022791055 | **X153** | UBA1381_sp |
| **X35** | Avidehalobacter_sp022797295 | **X95** | HGM13010_sp022800755 | **X154** | UBA3282_sp |
| **X36** | Bacteroides_acidifaciens | **X96** | JAAVYD01_sp022790955 | **X155** | UBA3282_sp003611805 |
| **X37** | Bacteroides_sp002491635 | **X97** | JAAVYR01_sp022791255 | **X156** | UBA3282_sp009774655 |
| **X38** | Bacteroides_uniformis | **X98** | JAAWBF01_sp | **X157** | UBA3282_sp910575245 |
| **X39** | Butyribacter_sp009774235 | **X99** | JAFLTL01_sp | **X158** | UBA3282_sp910577735 |
| **X40** | Butyricicoccaceae_sp | **X100** | JAFLTL01_sp910578395 | **X159** | UBA3282_sp910578115 |
| **X41** | Butyricimonas_virosa | **X101** | JANFXS01_sp022791155 | **X160** | UBA3402_sp022792815 |
| **X42** | C-19_sp | **X102** | Kineothri_sp | **X161** | UBA7109_sp910575605 |
| **X43** | CAG-485_sp | **X103** | Kineothrix_sp011958945 | **X162** | UBA7173_sp |
| **X44** | CAG-485_sp002361215 | **X104** | Kineothrix_sp910588855 | **X163** | UBA7173_sp001689485 |
| **X45** | CAG-485_sp002362485 | **X105** | Lachnoclostridium_B_sp | **X164** | UMGS1071_sp022801595 |
| **X46** | CAG-873_sp | **X106** | Lachnoclostridium_pacaense | **X165** | Unclassified_BinA |
| **X47** | CAG-873_sp002490635 | **X107** | Lachnoclostridium_phocaeense | **X166** | Unclassified_BinB |
| **X48** | CAG-873_sp009775265 | **X108** | Lachnospiraceae_sp | **X167** | Unclassified_BinC |
| **X49** | CAG-873_sp011959565 | **X109** | Lactobacillus_amylovorus | **X168** | Unclassified_BinD |
| **X50** | CAG-95_sp000403495 | **X110** | Lactobacillus_johnsonii | **X169** | Unclassified_BinE |
| **X51** | CAJFPI01_sp022801715 | **X111** | Lactobacillus_ultunensis | **X170** | Unclassified_BinF |
| **X52** | CAJTFG01_sp910575705 | **X112** | Lawsonibacter_sp | **X171** | Unclassified_BinG |
| **X53** | COE1_sp | **X113** | Lawsonibacter_sp022801435 | **X172** | Unclassified_BinH |
| **X54** | COE1_sp000403215 | **X114** | Ligilactobacillus_murinus | **X173** | Ventrimonas_sp |
| **X55** | COE1_sp003513705 | **X115** | MD308_sp910584615 | **X174** | Ventrimonas_sp910577765 |
| **X56** | COE1_sp910585765 | **X116** | MGBC131033_sp910585655 | **X175** | Zhenpiania_sp |
| **X57** | Caccovicinus_sp910575565 | **X117** | MGBC136627_sp910585935 | **X176** | [Clostridium]_clostridioforme |
| **X58** | Choladocola_sp | **X118** | MGBC163016_sp | **X177** | [Clostridium]_innocuum |
| **X59** | Choladocola_sp009774145 | **X119** | MGBC163016_sp910588905 | | |
| **X60** | Clostridium_Q_sp | **X120** | MGBC163016_sp910589135 | | |

Supplemental Table 3.2. Taxonomic identities of the 20 most-connected nodes (species) for each week 3 network. E = Edges

| Male Wild-Type Week 3 | | Male Mutant Week 3 | | Female Wild Type Week 3 | | Female Mutant Week 3 | |
|---|---|---|---|---|---|---|---|
| **Species** | | **Species** | | **Species** | **E** | **Species** | **E** |
| Coproplasma_ sp910586065 (Borkfalkiaceae) | 11 | CAG-873_sp009775265 (Provetellaceae ) | 14 | CAG-873_ sp009775265 (Provetellaceae ) | 21 | Duncaniella_sp (Muribaculaceae) | 15 |
| Paramuribaculum sp001689565 (Muribaculaceae) | 11 | UBA3282_sp (Lachnospiraceae) | 13 | CAG-873_ sp011959565 (Provetellaceae ) | 21 | JAFLTL01_sp (Muribaculaceae) | 13 |
| Unclassified_BinD | 11 | CAG-485_sp (Provetellaceae) | 13 | CAG-873_sp (Provetellaceae ) | 20 | [Clostridium] clostridioforme (Lachnospiraceae) | 12 |
| CAG-873_sp (Provetellaceae ) | 10 | Acetatifactor_sp (Lachnospiraceae) | 12 | Ventrimonas_sp (Lachnospiraceae) | 19 | Anaerotruncus_ colihominis (Oscillospiraceae) | 12 |
| Kineothrix_sp (Lachnospiraceae) | 10 | JAFLTL01_sp910578395 (Muribaculaceae) | 12 | Adlercreutzia_muris (Eggerthellaceae) | 19 | Duncaniella_freteri (Muribaculaceae) | 12 |
| Unclassified_BinE | 10 | Adlercreutzia_sp (Eggerthellaceae) | 11 | CAG-873_sp002490635 ( Provetellaceae ) | 19 | CAG-485_sp ( Provetellaceae ) | 12 |
| Lachnospiraceae_sp | 10 | Duncaniella_freteri (Muribaculaceae) | 11 | JAFLTL01_sp (Muribaculaceae) | 19 | JAFLTL01_sp (Muribaculaceae) | 12 |
| Merdisoma_ sp910574475 (Lachnospiraceae) | 10 | JAFLTL01_sp (Muribaculaceae) | 11 | UBA7173 _sp001689485 (Muribaculaceae) | 19 | Paramuribaculum_ intestinale | 12 |
| [Clostridium]_ clostridioforme (Lachnospiraceae) | 9 | Anaerotignum_ sp910576545 (Anaerotignaceae) | 10 | JAFLTL01_sp (Muribaculaceae) | 19 | Paramuribaculum_ sp001689565 | 12 |
| CAJFPI01_ sp022801715 (Oscillospiraceae) | 9 | Unclassified_BinD | 9 | Duncaniella_sp (Muribaculaceae) | 18 | CAG-873_sp (Provetellaceae ) | 12 |
| Pelethenecus_ sp910577745 | 8 | CAG-873_sp ( Provetellaceae ) | 9 | Unclassified_BinA | 17 | CAG-873_sp002490635 ( Provetellaceae ) | 12 |
| CAG-873_ sp002490635 ( Provetellaceae ) | 8 | Choladocola_sp009774145 (Bartonellaceae) | 8 | Phocaeicola_barnesiae (Bacteroidaceae) | 16 | CAG-873_sp009775265 ( Provetellaceae ) | 12 |
| UBA3282_ sp910578115 (Unidentified Lachnospiraceae) | 8 | 14-2_sp910580015 ( Lachnospiraceae) | 8 | Duncaniella_muris (Muribaculaceae) | 16 | CAG-873_sp011959565 ( Provetellaceae ) | 12 |
| 14-2_ sp910576385 ( Lachnospiraceae) | 7 | Lachnospiraceae_sp | 8 | Paramuribaculum Intestinale (Muribaculaceae) | 14 | Duncaniella_muris ( Muribaculaceae) | 12 |
| Ventrimonas_sp (Lachnospiraceae) | 7 | Unclassified_BinE | 8 | Duncaniella_freteri (Muribaculaceae) | 14 | Eubacterium_J_sp ( Eubacteriaceae) | 11 |
| UBA7173_sp ( Muribaculaceae) | 7 | Acetatifactor_ sp910585425 | 7 | CAG-485_sp ( Provetellaceae ) | 13 | Unclassified_BinE | 11 |
| UBA3282_sp (Unidentified Lachnospiraceae) | 7 | Paramuribaculum_ sp001689565 (Muribaculaceae) | 7 | CAG-485_sp002362485 ( Provetellaceae ) | 12 | CAG-485_sp002362485 ( Provetellaceae ) | 11 |
| UBA7173_ sp001689485 ( Muribaculaceae) | 6 | Phocaeicola_barnesiae (Bacteroidaceae) | 7 | 14-2_sp910580015 ( Lachnospiraceae) | 10 | Kineothrix_sp (Lachnospiraceae) | 10 |
| Eubacterium_J_ sp910586445 (Lachnospiraceae) | 6 | CAG-485_sp002362485 ( Provetellaceae ) | 7 | Bacteroides_acidifaciens (Bacteroidaceae) | 9 | 14-2_sp (Lachnospiraceae) | 9 |
| Eubacterium_ plexicaudatum (Eubacteriaceae) | 6 | Eubacterium_J_sp ( Lachnospiraceae) | 7 | Eubacterium_Jsp910586445 ( Lachnospiraceae)) | 9 | UBA3402_sp022792815 (Unidentified Lachnospiraceae) | 9 |

Supplemental Table 3.3. Taxonomic identities of the 20 most-connected nodes (species) for each week 10 network. E = Edges

| Male Wild-Type Week 10 | | Male Mutant Week 10 | | Female Wild Type Week 10 | | Female Mutant Week 10 | |
|---|---|---|---|---|---|---|---|
| **Species** | **E** | **Species** | **E** | **Species** | **E** | **Species** | **E** |
| UBA7173_sp001689485 ( Muribaculaceae) | 17 | CAG-873_sp (Provetellaceae ) | 17 | JAFLTL01_sp (Muribaculaceae) | 18 | Kineothrix_sp (Lachnospiraceae) | 24 |
| CAG-873_sp (Provetellaceae ) | 14 | Duncaniella_muris (Unidentified Muribaculaceae) | 17 | Enterorhabdus_ sp009911695 (Eggerthellaceae) | 16 | UBA7173_sp (Muribaculaceae) | 23 |
| Duncaniella_freteri (Muribaculaceae) | 14 | Duncaniella_sp (Unidentified Muribaculaceae) | 17 | Adlercreutzia_sp (Eggerthellaceae) | 15 | CAG-873_sp002490635 (Provetellaceae ) | 22 |
| CAG-485_sp002361215 (Provetellaceae ) | 13 | Muribaculum_intestinale (Muribaculaceae) | 16 | CAG-873_sp002490635 (Provetellaceae ) | 15 | Evtepia_sp (Eubacteriales) | 22 |
| CAG-873_sp002490635 ( Provetellaceae ) | 12 | CAG-485_sp002361215 ( Provetellaceae ) | 16 | CAG-873_sp011959565 ( Provetellaceae ) | 14 | Dysosmobacter (Oscillospiraceae) | 22 |
| Duncaniella_sp (Muribaculaceae) | 12 | Caccovicinus_ sp910575565 (Lachnospiraceae) | 14 | CAG-873_sp (Provetellaceae ) | 14 | Coprobacter_secundus (Barnesiellaceae) | 20 |
| Duncaniella_muris (Muribaculaceae) | 11 | CAG-485_sp002362485 (Provetellaceae ) | 14 | CAG-873_sp009775265 (Provetellaceae ) | 14 | JAAVYD01_sp022790 955 (Anaerovoracaceae) | 17 |
| Lachnospiraceae_sp | 11 | JAAVYD01_sp0227909 55 (Anaerovoracaceae) | 13 | Duncaniella_muris (Unidentified Muribaculaceae) | 14 | Unclassified_BinD | 17 |
| Acetatifactor_sp9105854 25 (Lachnospiraceae) | 10 | Lachnospiraceae_sp | 13 | JAFLTL01_sp (Muribaculaceae) | 14 | Angelakisella_sp0227 95575 (Ruminococcaceae) | 17 |
| Evtepia_sp910584805 (Eubacteriales) | 10 | Unclassified_BinG | 13 | Lawsonibacter_sp (Oscillospiraceae) | 14 | Lactobacillus amylovorus (Lactobacillaceae) | 17 |
| UBA7173_sp ( Muribaculaceae) | 10 | Schaedlerella_sp (Lachnospiraceae) | 13 | Duncaniella_sp (Unidentified Muribaculaceae) | 12 | Merdisoma_sp (Lachnospiraceae) | 17 |
| 1XD8-76_sp910573755 ( Lachnospiraceae) | 10 | Coprobacter_secundus (Barnesiellaceae) | 12 | UBA7173_sp001689485 (Muribaculaceae) | 12 | RGIG7193_sp (Lachnospiraceae) | 17 |
| JAFLTL01_sp (Muribaculaceae) | 10 | CAG-485_sp (Provetellaceae ) | 12 | D16-34_sp910575165 (Eggerthellaceae) | 11 | Roseburia_sp9105878 65 (Lachnospiraceae) | 17 |
| CAG-873_sp009775265 (Provetellaceae ) | 10 | Eubacterium_G_ sp910574895 | 12 | Duncaniella_freteri (Unidentified Muribaculaceae) | 11 | MGBC165282_ sp910589265 (Lachnospiraceae) | 16 |
| Evtepia_sp ( Eubacteriales) | 10 | 14-2_sp004793545 (Lachnospiraceae) | 11 | Acetatifactor_sp (Lachnospiraceae) | 10 | CAG-485_sp002361215 (Provetellaceae ) | 16 |
| Lachnoclostridium_pacae nse (Lachnospiraceae) | 9 | Duncaniella_freteri (Muribaculaceae) | 11 | Mucispirillum schaedleri (Mucispirillaceae) | 10 | Phocaeicola_barnesiae (Bacteroidaceae) | 15 |
| [Clostridium]_innocuum (Coprobacillaceae) | 9 | Ventrimonas_sp9105777 65 (Lachnospiraceae) | 10 | 1XD8-76_sp910573755 (Lachnospiraceae) | 7 | JAFLTL01_sp (Muribaculaceae) | 15 |
| Lachnoclostridium_phoca eense (Lachnospiraceae) | 9 | Evtepia_sp910584805 (Eubacteriales) | 10 | Lactobacillus_johnsonii (Lactobacillaceae) | 7 | Duncaniella_freteri (Muribaculaceae) | 15 |
| 14-2_sp004793545 (Lachnospiraceae) | 8 | Lachnoclostridium_B_sp (Lachnospiraceae) | 10 | 14-2_sp910576385 (Lachnospiraceae) | 6 | Clostridium_Q_sp (Lachnospiraceae) | 14 |
| Coprobacter_secundus (Barnesiellaceae) | 8 | Pelethomonas_sp (Oscillospiraceae) | 10 | [Clostridium]_innocuum (Coprobacillaceae) | 6 | Pelethomonas_sp (Oscillospiraceae) | 14 |

## Discussion

Multi-omics analysis of the fecal microbiome using a hypogonadal mouse model revealed that activation of the HPG axis during puberty was required for sexual differentiation of the gut microbiome. In particular, the activation of the HPG axis during puberty was necessary for sexual differentiation of gut microbial composition and gut lipid metabolism. This study also provided evidence that the HPG axis is not the only factor involved in the maturation of the gut microbiome. Age had a strong effect on the gut microbiome in both wild-type and *hpg* mutant mice and some sex differences in the gut microbiome were detected in both wild-type and mutant mice before puberty, indicating that sex chromosome gene dosage may also influence specific sex differences in gut microbial taxa and function.

### Significant pubertal maturation of the gut microbiome was independent of the HPG axis

Analysis of gut microbiota taxa and putative functions showed that age, independent of HPG axis activation, strongly shaped the gut microbiome during the pubertal period. This is in contrast to a common postulate in the field that sexual development during puberty is the primary factor driving maturation of the gut microbiome during that period [61] and also contrasts with previous notions that gut microbiome maturation is complete by pre-puberty [62, 63]. However, my data shows that age, independent of sex or HPG axis activation, had the largest effect on broad changes in the gut microbiota composition and putative functions. For example, comparisons of pre-, peri-, and post-puberty showed age had the strongest influence on bacterial family abundance in both wild-type and mutant mice (Table 3.1) and that age had the strongest impact on SV-level clustering of samples (Table 3.2). Furthermore, balance analysis identified 3 genera that were more relatively abundant pre-puberty in both

wild-type and mutant mice, namely *Anaerotrunctus* (*Oscillospiraceae*), Bacteroides (*Bacteroidaceae*), and *Candidatus arthromitus* (*Clostridiaceae*) (Figure 3.3). A common set of bacteria that differentiated pre-pubertal mice from adult mice in all four groups indicated that there were factors independent of sexual development such as weaning and growth that resulted in reduced abundance of these genera in adult mice.

Analysis of orthologous groups of genes annotated from MAGs also indicated a HPG-independent effect of age during puberty: the beta dispersion of orthologous groups in all mouse groups collapsed post-puberty (Figure 3.6). Orthologs, groups of genes that have evolved from a common ancestor, represent a higher functional level than genes. For example, all bile salt hydrolases (BSHs), which include many different proteins with a related function (hydrolysis), belong to a single ortholog group. Our data therefore suggests that as the animals grow during puberty, the broad functions of the gut microbiota converge to a shared functional profile and that this process is not affected by the HPG axis.

Some age-based gut microbiome maturation during puberty was HPG axis dependent

Bacterial family level-analyses showed that the HPG axis was required for maturation of the gut microbiome during puberty and had similar effects in male and female mice. The *Muribaculaceae* family was more relatively abundant in mutant mice compared to wild-type in adulthood, and an unidentified *Muribaculaceae* genus was more abundant in mutant mice in the genus balance differentiating adult mutant and wild-type mice (Figure 3.3). Bacteria in the *Muribaculaceae* family are known for complex carbohydrate degradation, including degradation of host mucins, and have genes for bile acid transformation [64]. Previous work showed that *Muribaculaceae* was especially abundant in mutant adult mice in microbial communities of the small and large intestine [65]. In contrast, *Lachnospiraceae* was more

abundant in wild-type mice at peripuberty and adulthood compared to mutant mice, a result supported by a balance analysis that showed one specific genus of *Lachnospiraceae* genus to be more abundant in wild-type mice that mutant mice (Figures 2, 3). Sisk-Hackworth et al. (2023) also found higher abundances of *Lachnospiraceae* in the cecum lumen of wild-type mice compared to mutant mice. Studies have shown that *Lachnospiraceae* degrade host mucins and produce short-chain fatty acids that can affect host inflammation [66, 67]. Taken together, these results indicate that HPG axis activation during puberty suppresses abundance of bacteria in the *Muribaculaceae* family and promotes abundance of *Lachnospiraceae*.

Analysis of genes annotated from MAGs indicated that the HPG axis is also necessary for diversification of the bacterial community gene functional diversity in adults (Figure 3.6). Before puberty, all four groups of mice showed high beta dispersion in gene abundance, but post-puberty, beta dispersion collapsed in mutants while it increased in wild-type mice, indicating that activation of the HPG axis generates higher levels of inter-sample genetic differentiation in the gut microbiome. This is in contrast to dispersion of orthologous groups of genes, whose diversity collapses during puberty independent of HPG axis activation, as stated in the previous section. However, the beta diversity analysis at the gene level indicates that the abundance of the various genes within these orthologous groups are affected by the HPG axis (e.g., not all BSH genes are affected in the same manner).

Some gut microbiome sex differences require HPG axis activation during puberty

I also showed that HPG axis activation during puberty also led to some sex differences in the gut microbiome. An analysis of the metagenomic MAG-species abundances showed that in wild-type mice, sex differences in species beta dispersion developed during the pubertal period, while this was not true for mutant mice (Figure 3.4A). Furthermore, analysis

of species-level bacterial community networks also revealed HPG-dependent sex differences that were absent pre-puberty but became clear in adult wild-type mice. A comparison of numerous network metrics found no sex differences pre-puberty (Supplemental Figure 3.2, week 3; Table 3.3). Post-puberty, all networks besides the female wild-type network increased in metrics of connectivity (number of edges, degree centrality, closeness centrality, and transitivity; Table 3) and all had two clusters of co-occurring species (Figure 3.5, week 10; Table 3.3). In contrast, connectivity metrics decreased in the female wild-type network post-puberty and only one cluster of co-occurring species was present (Figure 3.5). In environmental microbiomes, environmental stress has been shown to destabilize community networks, for example resulting in lower connectivity, fewer edges, fewer nodes, and fewer co-occurrence clusters, similar to what was seen in the adult female wild-type mouse networks here [68] (Figure 3.4, Figure 3.5). It is possible that activation of the HPG axis in female mice during puberty results in greater environmental instability in the intestinal environment due to cyclic changes in hormone levels as a result of the estrous cycle, resulting in lower network connectivity and a disappearance of the *Lachnospiraceae* cluster. One potential explanation for this is that gastrointestinal transit time has been shown to be highly responsive to estrogen and progesterone levels and has been identified as an important variable in sex differences within the human gut microbiome [69, 70].

While the species within the co-occurrence clusters differed between adult mice, the clusters were dominated either by *Muribaculaceae* and *Prevotellaceae* species in Cluster 1, and *Lachnospiraceae* species in Cluster 2, and Cluster 2 was missing from female wild-type mice (Figure 3.5). While direct interactions between connected species in networks cannot be assumed from co-occurrence networks, organization of species networks into co-occurrence

clusters grouped to the family level likely indicates that the species in these clusters occupy similar niches within the gut environment or are mutually dependent and that different clusters will occupy different niches [71]. Network clustering into groups based on higher-level taxonomy have been seen in mouse models of Alzheimer's disease and exercise stress [72, 73]. While *Muribaculaceae, Prevotellaceae*, and *Lachnospiraceae* are all known to degrade carbohydrates in the gut [67, 74], *Lachnospiraceae* species are key butyrate producers and have also been shown to ferment amino acids [75-77]. That the adult wild-type female species networks were missing the *Lachnospiraceae* co-occurrence cluster may indicate that the species occupying this niche were not selected for in the intestinal environment induced by the female HPG axis.

Additionally, activation of the HPG axis during puberty was required for sexual differentiation of lipid metabolite beta diversity (Figure 3.7). Specifically, I found that fatty acid and phosphatidylethanolamine metabolites differentiated adult wild-type males and females but not adult mutant males and females (Table 3.7). Notably, these HPG-dependent sex differences in taxa and putative functions were absent pre-puberty, which ruled out the neonatal period (mini-puberty) as a contributing factor to sexual differentiation of the gut microbiome due the HPG axis.

Some sex differences in the gut microbiome developed due to sex chromosome effects

Notably, this study showed that some sex differences in the gut microbiome were independent of the HPG axis, indicating that gene dosage effects of sex chromosomes likely play a role in sexual differentiation of some aspects of the gut microbiome. For instance, slight sex differences in beta diversity were observed at 3 weeks of age in mutant mice, indicating that these sex differences were independent of activation of HPG axis during

puberty (Figure 3.4). While studies on pre-pubertal sex differences are few [19], some studies have shown sex differences in pre-pubescent mice in gene expression in the liver and intestine [78, 79]. Post-puberty, however, the sex differences observed in mutant mice beta diversity diminished, indicating that sex chromosome effects may be transient or masked in adulthood by other factors.

Additionally, metabolomic analyses indicated that adult sex differences in fecal amine metabolism were also HPG independent. Beta diversity differences in amines were most evident in adult mutant mice, indicating that sex differences resulting from gene dosage effects of sex chromosomes were largely masked by activation of the HPG axis during puberty in wild-type mice (Figure 3.8). A similar masking phenomenon wherein sex steroids were antagonistic to sex chromosome effects has been previously described in a mouse model with a sexually-dimorphic immune response [80]. For specific amine classes, there were both HPG-dependent and independent sex effects. Carboxylic acids differentiated male and female mutant mice, while quaternary ammonium compounds and xanthurates differentiated male and female wild-type mice. Altogether, these results reveal complex interactions between gonadal sex steroids and sex chromosomes in shaping functional sex differences in the gut microbiome.

Implications for microbiome-based therapeutics and future directions

These findings have important implications for treatment of diseases linked to the gut microbiome that have sex bias, alterations in sex steroid levels, and/or develop during specific life stages including childhood (pre-puberty), puberty, and adulthood (post-puberty). Given that the FDA approved two fecal microbiome products in 2023 for treatment of recurrent Clostridium difficile, there is considerable interest in developing novel pre- and postbiotic

therapies for many diseases. Given our results, patients may respond to microbiome-based therapeutics differently based on pubertal status, sex steroid levels (e.g. pre vs post-menopausal women, women with polycystic ovary syndrome), or medications the patient is taking (e.g. hormone replacement therapy, hormonal contraceptives, gonadotropin-releasing hormone agonist/antagonist). Additionally, given the HPG-dependent sex difference in microbial species diversity, community structure and metabolites between adult wild-type males and females, microbiome-based therapeutics for peri-pubertal and adult patients may need to target different microbial species in females and males. Our results showing lower connectiveness in adult female wild-type networks also prompts questions concerning gut microbiome community stability in adult women. Given the differences in length between the estrus cycle (~ 5 days) and the human menstrual cycle (~ 28 days), studies should investigate whether the menstrual cycle induces similar community instability in women, and whether a woman's cycle length impacts network stability. This could have implications for microbiome-linked conditions where women's cycle length is affected, such as PCOS and endometriosis [15, 81]. It should also be determined whether conditions resulting in static steroid levels, e.g. taking hormonal contraceptives, HRT, or menopause, mirror the hypogonadal state seen here, to determine whether the reduced microbial network connectiveness is related to the oscillating nature of sex steroid levels in the estrus cycle. This could also be tested mechanistically in mice using gonadectomy and steroid replacement studies.

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# DISSERTATION DISCUSSION

Many gut-microbiome linked diseases that are sex-specific or linked to changes in sex steroid levels develop during or after puberty [1-3]. Understanding how sex and puberty affect the gut microbiome is important for understanding how these diseases develop and may inform development of microbiome-based treatments. Furthermore, prior studies in humans and mouse models have shown that sex differences in the gut microbiome develop during puberty [4-7], prompting the question of how reproductive development during this period influences maturation of the gut microbiome. Studies on sex differences in the gut microbiota have also largely lacked inquiry into microbial communities within the small intestine and functional analysis of the gut microbiome. Therefore, I used the hpg mouse model to determine how adult intestinal microbiota were affected by the reproductive axis and how fecal microbial composition and functional characteristics were affected by HPG axis activation during puberty.

My data supports the commonly posited idea among gut microbial ecologists that gonadal sex steroids are necessary for sexual differentiation of the gut microbiome during puberty [8-10]. More specifically, my studies convincingly demonstrated that the HPG axis is required for sexual differentiation of microbial taxa in the intestine, and that these sex effects are intestinal niche-specific (Chapter 2) and that puberty is a critical period for sexual differentiation for both microbiome taxonomic composition and functions (Chapter 3). However, my findings departed from two other studies focused on sexual differentiation of gut microbiota in mice in that most sex differences in beta diversity I observed were due to beta dispersion, not to separate clustering (distinct centroids) of samples that was observed in two prior studies [6, 7]. One possibility is that this difference is due to a strong autoimmune

influence in the non-obese diabetic (NOD) mouse model, in which females are genetically more susceptible to type 1 diabetes. The lack of a strong sex difference in autoimmunity in the hpg mouse model, in addition to different genetic backgrounds between NOD (CTS) and hpg (BDF2), may explain the stark sex differences observed in the fecal microbiome of adult NOD mice. The wild-type sex differences I observed in the hpg mice were more similar to sex differences in adult humans, with more subtle differences in centroid or dispersion compared to the NOD mouse model studies [11, 12].

My identification of HPG-independent effects on maturation of the gut microbiome is a novel finding and had not previously been proposed in the field although there have been many previous studies that demonstrated gene dosage effects of sex chromosomes on host physiology. Given the abundance of immune genes on the X chromosome, immune action in the gut is a likely candidate for sexual differentiation of the gut microbiome due to sex chromosomes. Indeed, differential abundance of autoimmune genes in the small and large intestine have been observed between pre-pubertal female and male mice [13]. Another mechanism through which this could occur is differential expression of X/Y homologous genes: many of these genes exhibited differential expression between adult females and males in the liver, colon, and small intestine of mice [14].

However, given that most sex differences identified in my studies were dependent on activation of the HPG axis, most sexual maturation of the gut microbiome is likely due to direct or indirect action of gonadal sex steroids. As I reviewed more extensively in Chapter 1, the HPG axis may alter the gut microbiome directly, through action of sex steroids on the gut microbiome, or indirectly, through sex-specific host physiology that interacts with the gut microbiota. It is notable that the mechanisms that give rise to sex differences in the gut

microbiome remain almost completely unknown. To further tease out these mechanisms, future experiments should investigate the development of taxonomic and functional sex differences within intestinal microbial communities using metagenomics and metabolomics with the four core genotypes mouse model, which separates the effect of sex steroids and sex chromosomes on sexual maturation by translocation of the SRY gene to an autosomal chromosome combined with gonadectomy and steroid replacement [15, 16]. In vitro, the effect of sex steroids and bile acids on growth of individual gut microbes and composition of mock communities should be investigated to determine if these compounds are sufficient to alter community composition and function in a sex-dependent manner. Additionally, in vivo mouse models of innate or adaptive immune function could be used to determine the contribution of the immune system to sexual maturation of the gut microbiota.

Investigation of the maturation of taxonomic and functional sex differences in the small intestine is another frontier that should be explored. Some microbiome-linked diseases, like inflammatory bowel disease [17], are both sex specific and impact different parts of the intestine. My work in chapter 2 and 3 indicate that microbiome-based interventions for diseases like inflammatory bowel disease may need to take into account sex, hormonal status, and intestinal section. However, our understanding of taxonomic sex differences within the small intestine is severely lacking and currently depends on amplicon-based sequencing methods. To investigate intestinal sex differences in gut microbiome function within the small intestine, better methods to remove host DNA from sample extraction must be developed, as host contamination in the small intestine currently limits analysis of microbial metatranscriptomics and metagenomics. For example, a pilot study performed in our lab showed that over 99% of DNA sequenced with shotgun metagenomics in the duodenum and

ileum belonged to the host. Furthermore, metabolomic profiling of different intestinal microbial communities from male and female wild-type versus hpg mutant mice should be performed to understand how intestinal environment impacts microbial metabolite production. In addition, transcriptomic profiling of host tissues such as the liver and distal ileum in male and female wild-type versus mutant mice would provide insight into the effect of the reproductive axis and sex on the overall entero-hepatic system.

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