MICROBIAL MANIPULATION OF THE BRAIN AND REPRODUCTIVE SYSTEM IN INFLAMMATORY BOWEL DISEASE

by

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in Microbiology and Immunology

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Abstract

Crohn's disease and ulcerative colitis, collectively termed inflammatory bowel disease (IBD), are chronic and incurable diseases of the gastrointestinal tract. Beyond the gastrointestinal tract, IBD patients exhibit high rates of hormonally driven sexual, reproductive, and psychiatric disorders. For instance, delayed puberty is reported in up to 85% of pediatric IBD patients, and sexual dysfunction is reported in up to 60% of adult female patients and up to 94% of adult male patients. Further, psychiatric illness is two to three times as prevalent in IBD patients compared to the general population.

Previously, systemic sex steroid levels were thought to be exclusively controlled by the hypothalamic-pituitary-gonadal axis. However, recent research has identified gut microbes capable of degrading androgens and reactivating estrogens in a clinically significant manner. Based on these findings, we proposed that the gut microbiota could play a role in driving the high prevalence of neuroendocrine comorbidities observed in IBD. Specifically, we hypothesized that IBD-like alterations to the murine gut would induce sexual, reproductive, and psychiatric effects like those seen in IBD patients. To test this hypothesis, we used the well-known dextran sodium sulfate (DSS) model of IBD to disrupt the murine gut microbiota and induce inflammation at specific developmental time points, and measured the effect on the gut, systemic sex steroid levels, reproductive development, brain cell morphology and behavior.

Confirming our hypothesis, we found that DSS induced inflammation reshapes the composition of the gut microbiota, compromises the integrity of the gut epithelium, impedes the development of the seminal vesicles, and causes changes in sex-specific behavior. In contrast, DSS did not appear to disrupt systemic sex steroid levels, affect the timing of pubertal onset, cause damage to the reproductive organs or alter mating behavior. Taken together, these results suggest that DSS inflammation selectively impacts certain aspects of the gut-endocrine-brain axis in IBD while leaving others unaffected. This points to the need for further research that can take a multi-system approach to investigating the complex and nuanced interplay between the gut microbiota, immune system, endocrine system, reproductive organs, brain, and behavior in IBD.

Lay Summary

In addition to debilitating gastrointestinal disturbances, inflammatory bowel disease (IBD) patients experience delayed puberty, sexual dysfunction, and psychiatric comorbidities at a higher rate that the rest of the population. However, the precise role IBD plays in these comorbidities, and whether it is a result of the gut inflammatory response or changes IBD has on the microbiota has remained unexplored. We sought to remedy this by creating a mouse model that captures the full arc of the disorder, its sexual and endocrine comorbidities, and probes the question of whether the effects are time point specific or chronic in nature.

Preface

This dissertation is the original intellectual product of the author, Sophie Cotton. I was responsible for the identification of research questions, design of the research work, performance of the research, and analysis of the research results in this thesis unless otherwise indicated.

Chapter 1 includes an adapted version of work published by myself, Charlotte Clayton and Dr. Carolina Tropini:

> Cotton, S., Clayton, C. A., & Tropini, C. (2023). Microbial endocrinology: the mechanisms by which the microbiota influences host sex steroids. *Trends in microbiology*, *31*(11), 1131–1142. https://doi.org/10.1016/j.tim.2023.03.010

Dr. Carolina Tropini and I conceived of the rationale, conducted the literature search, wrote, and edited the manuscript. Charlotte Clayton created the images and assisted with editing the manuscript.

Chapters 2, 3 and 4 present part of a research project collaboration between the Tropini Lab (a microbiology lab) and the Ciernia Lab (a brain and behavior lab) at the University of British Columbia. As the project lead for the Tropini lab, I was responsible for the gut side of the project under the supervision of Dr. Carolina Tropini. Olivia Sullivan was the project lead for the Ciernia lab and was responsible for brain side of the project under the supervision of Dr. Annie Ciernia. Together, Olivia and I collaborated to lead the endocrine and reproductive side of the project under the joint supervision of Dr. Carolina Tropini and Dr. Annie Ciernia.

Prior work by Dr. Kat Ng, a former member of the Tropini lab, was critical in laying the foundation for this thesis. While performing DSS experiments Dr. Ng observed an abnormal reproductive phenotype in the mice. It was this observation that led me to explore the literature on the gut-endocrine-brain axis in IBD and instigated the pursuit of this project. This thesis incorporates and analyzes some of Dr. Ng's data (colon length measurements and 16s data that she sequenced) and is referenced in the relevant sections.

For the mouse experiments I was responsible for ordering the mice, pairing and breeding the mice, weaning the mice and in collaboration with Olivia Sullivan I organized the experimental set up and administration of data collection. Olivia Sullivan and I trained and mentored a team of undergraduate students, Cal Rosete, Jatin Choudhary, and Claire Sie to assist us with the experiments. This team assisted us with daily data collection throughout the experiment which included weighing the mice, stool collection, puberty checks, estrus checks and mouse husbandry. Members of the Tropini lab Charlotte Clayton and Hans Ghezzi also assisted with daily data collection during holiday periods. Olivia Sullivan and I were responsible for organizing, administrating, and performing euthanasia and had hands on practical support with tube labeling, tissue collection, transport, and storage on the day of sacrifice from Dr. Annie Ciernia and the team.

Histology slides were prepared by Dr. Olena Mayden at the BC Children's Hospital Research Institute Histology Core facility and Ingrid Barta at the University of British Columbia's Animal Care Services Diagnostic & Research Histology Laboratory. Gut histology scoring was performed with Dr. Ian Welch at the University of British Columbia's Animal Care Services Diagnostic & Research Histology Laboratory and reproductive organ histology scoring was performed by Dr. Nick Nation at Animal Pathology Services in Alberta. Serum sex steroid measurements were performed in conjunction with the Soma lab at the University of British Columbia and later led by Dr. Jordan Hamden in the Ciernia lab.

I performed the gut imaging and analysis, the sequencing data analysis, the puberty data analysis, as well as the endocrine and reproductive data analysis described in this thesis.

This project required animal ethics approval. All experiments were performed in accordance with recommendations from the Canadian Council for Animal Care and the Animal Care Committee of the University of British Columbia approved all protocols (A19-0078, A19-0033, A19-0122, A22-0270, A23-0086).

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List of Abbreviations

DAI - Disease Activity Index

DHEA - Dehydroepiandrosterone

DSS - Dextran Sodium Sulphate

FSH - Follicle stimulating hormone

FISH - Fluorescence In Situ Hybridization

GIT - Gastrointestinal tract

GUS - β-Glucuronidase enzymes

IBD - Inflammatory Bowel Disease

LH - Luteinizing hormone

MUC2 - Mucin 2 glycan protein

PCOS - Polycystic ovary syndrome

3β-HSD - 3β-hydroxysteroid dehydrogenase

Glossary

Anti-TNFα antibodies: a class of drug commonly used in IBD to inhibit the immune signaling molecule tumor necrosis factor alpha (TNFα).
Agonist: a molecule or chemical compound that activates a receptor.
Dehydroepiandrosterone - Steroid hormone made by the adrenal glands and kidneys that can be made into weak androgens.
Endocrinology: the study of the physiology of the endocrine system and hormones.
Entner–Doudoroff pathway: a pathway for glucose metabolism in bacteria.
Estrous cycle: the female reproductive cycle in most mammals (the mammalian equivalent of the menstrual cycle).
FISH Staining - Fluorescence In Situ Hybridization is a technique used to measure fluorescently labeled DNA or RNA in cells. It uses specifically designed probes that bind to target pieces of DNA so that the researcher can elect which parts of the sample to visualize.
Glucuronide: a molecule containing a glucuronic acid linked via a glycosidic bond. In this thesis estrogens and androgens are highlighted as glucuronidated steroids.
Glycosidic bond: a covalent bond joining a carbohydrate molecule and another chemical group.
Hematochezia: finding of blood in stool.
Homeostasis: a self-sustaining process in which biochemical and physical conditions are kept in equilibrium and within certain limits by a living organism.
Hyperestrogenism: the physiological state of excess estrogen.
Hypoestrogenism: estrogen deficiency.
Menses: stage of the menstrual cycle when endometrial lining is shed and bleeding occurs.
Menstrual cycle: the term that encompasses the entirety of four phases of the reproductive cycle in females: menstrual phase (menses), follicular phase, ovulation phase and luteal phase).
Osteopenia: loss of bone mineral density. .
Osteoporosis: a more severe form of osteopenia that leads to weakening of bones.
Ovariectomized: surgical removal of the ovaries.
Polycystic ovarian syndrome (PCOS): an endocrine disease in females characterized by excess androgen levels causing menstrual irregularity, hirsutism, and anovulatory infertility.
Portal vein: a major blood vessel which drains the blood from the gastrointestinal tract to the liver and therefore brings gut microbiota products to the systemic circulation.

Xenobiotics: chemical substances that are not naturally produced by an organism but are found within it (e.g., plant constituents or drugs in a human).

Acknowledgements

I would like to acknowledge Professor Mark Lyte for pioneering the field of microbial neuroendocrinology at a time when it was not received popularly. Thank you for boldly blazing ahead so that today people like me can study something so astounding.

I would also like to acknowledge the late Paul Ramsay and the Ramsay Foundation for the generous funding that has made my research possible. I hope I can live up to Paul Ramsay's legacy as I 'commit myself to acting with humility, and learning with curiosity, loyalty, respect'. I am also thankful to PEO International for their generous International Peace Prize.

Finally, I would like to thank the team that made this project possible. To my supervisor, Dr. Carolina Tropini, thank you for all the work you've done to be in a place to enable my labmates and I this opportunity to study something so wonderful. To Dr. Annie Ciernia, and the entire Ciernia lab, thank you! Working on a project like this has been a dream come true and I am so thankful to have done it with such a wonderful team.

Chapter 1: Introduction and Literature Review

1.1 Overview and Summary

Crohn's disease and ulcerative colitis, collectively termed inflammatory bowel disease (IBD) are chronic, incurable conditions of the gastrointestinal tract that affect patients in a relapsing and remitting manner across the lifetime. There are two divisions of IBD - Crohn's disease (CD) and ulcerative colitis (UC), and they are differentiated by the location and characteristics of intestinal inflammation. Crohn's disease exhibits patchy inflammation along the entirety of the gastrointestinal tract [15] ,whereas ulcerative colitis (UC) manifests as continuous (i.e. non patchy) inflammation that is isolated to the large intestine and rectum [16]. Despite these regional differences, both Crohn's and colitis share many intestinal and extraintestinal clinical symptoms. The hallmark intestinal symptoms of IBD are diarrhea, abdominal pain, gastrointestinal bleeding and malnutrition [17]. However, the effects of the inflammation are not confined to the gastrointestinal tract [18]. In fact, the extraintestinal manifestations of IBD extend to nearly every organ system in the body. This includes arthritis in the musculoskeletal system, sexual dysfunction and delayed puberty in the reproductive system, and psoriasis and other skin conditions in the integumentary system [18]. In this thesis I focus on the extraintestinal symptoms seen in the brain-endocrine axis - namely delayed puberty, disrupted steroid hormones, altered brain histology, and sex-specific behavioral abnormalities. Further, I address these topics through the lens of their relationship to the gut microbiota and intestinal inflammation in IBD. In Chapter one I summarize recent literature on endocrine comorbidities in IBD, how the microbiota could play a role in them, and how we can measure this. In Chapter two I describe methods I used and the mouse model our group developed to model the gut microbiota -endocrine-brain relationship. In Chapter three and four I document the results and provide a discussion, and in Chapter five I conclude with thoughts on future directions and unanswered questions.

1.2 Endocrine comorbidities in IBD

Inflammatory bowel disease is most often diagnosed between the ages of 15 and 30, coinciding with the peak reproductive years throughout the lifespan [19,20]. Despite that, the symptoms - known for their high burden on the quality of life - often persist across the lifetime [1,2]. Increasing evidence shows that on top of the gastrointestinal disruptions to quality of life, patients also face sexual and psychiatric disorders at a higher rate than the general population [4,21]. Delayed puberty has been reported in up to 85% of pediatric IBD patients [3,6], rates of sexual dysfunction range from up to 60% in women and 94% of men [7], and endocrine-

associated psychiatric illness presents in IBD patients two to three times as often as the general population [8].

1.2.1 Delayed Puberty

Delayed puberty has been reported in both human [3,22] and murine [23] studies on IBD, and in both cases is thought to be out of proportion with the expected impacts of IBD nutritional deficits [11,24]. There are many complex factors that regulate the timing of pubertal onset, however the two dominant factors studied in IBD – microbiota composition and inflammation – have each been independently explored for their influence on the timing of pubertal onset [25]. Our mouse model (described in Chapter 2) combines IBD-like inflammation, disruption to the microbiota composition, and early life stress, all of which may contribute to the disrupted puberty phenotype.

1.2.2 Sexual dysfunction

A multitude of studies robustly show that sexual function and satisfaction is significantly impaired in both male and female IBD patients [21]. A large internet study led by the Crohns and Colitis Foundation of America found that out of over 2000 patients with IBD, 80% of them found their symptoms affected their sexual satisfaction and that this correlated with disease severity [20].

Males

In healthy males, erection is the result of a coordinated event between the vascular system, brain, and endocrine system [26], the latter two of which are known to be affected by IBD. Congruently, the prevalence of erectile dysfunction (ED) in males with IBD is extremely high, in some studies reaching prevalence rates of 94%, significantly surpassing the rates among other chronic inflammatory diseases, and the reported rate of 31% in the general population [27]. This high prevalence persists for patients throughout the course of the disease, independent of flare-ups, and it remains significant even after accounting for age which is normally a factor in increasing rates of ED - 95% of male IBD patients over 40 experienced ED compared to 10% in the general population [27]. Beyond physiological concerns, 44% of males with IBD express feeling severely sexually compromised because of their IBD [28]. This is reflected in studies that show that of men that receive prescription medication for ED, males with IBD are more likely to follow through and purchase it [36]. The high prevalence of sexual satisfaction among males with IBD led to the recent creation of an IBD-specific sexual scale (the IBD-MSDS) [29].

Females

Compared to male IBD patients, there is more fluctuation in sexual dysfunction rates for female IBD patients depending on active disease severity and remission [30]. In one study, compared to 24% of healthy controls, 49% of IBD patients in remission experienced sexual dysfunction, compared to 62% of patients with mild disease, 74% of those with moderate disease and 88% of those with severe disease [30].

Despite these high rates of sexual dysfunction and dissatisfaction in both males and females with IBD, only 40% of IBD patients felt comfortable discussing these comorbidities with their doctor [26,31,32]. This has led clinicians to identify sexual dysfunction as a 'blind spot' in the field, highlighting the need for research that investigates which components of IBD could be responsible for this comorbidity [26,26,30,33]. Given that sexual and psychiatric dysfunctions are of significant concern to IBD patients, contributing to the already high burden of the disease, more research is warranted on this subject.

1.2.3 Endocrine-related neuropsychiatric conditions in IBD

The prevalence of neuropsychiatric disorders such as major depressive disorder and anxiety disorder is high in IBD patients [34,35]. Low systemic testosterone is a known driver of depression and has been reported in up to 40% of males with IBD [36]. Further, neuropsychiatric disorders can influence other aspects of endocrine health. For example, among patients with IBD, depression is a larger determinant of sexual dysfunction than disease severity [32,37]. Interestingly, administration of testosterone improves IBD outcomes and neuropsychiatric outcomes [13], and the common IBD treatment anti-TNFα therapy has also shown to rescue disruptions to testosterone levels [38]. While much remains to be explored, the co-occurrence of neuropsychiatric impairments and low testosterone levels in IBD, along with their amelioration through both IBD treatment and testosterone administration, indicates a need to dissect the relationships between the symptoms.

1.2.4 Sex related differences in IBD

It is well established that there are many sex-based differences in the presentation of IBD, of which the most fundamental is that males are more likely to develop UC and females more likely to develop CD [39]. The differing levels of sex steroids between males and females has been proposed as one of the potential drivers of these sex-based differences, and there is some

evidence to support that. For example, Cornish et al. showed that use of estrogen contraceptive pills can increase the risk of CD by nearly 50% [40], and Pasvol et al. showed that each additional month of contraceptive exposure per year of follow up increases the risk of CD by 6.4% and UC by 3.3% [41]. Other meta-analyses have shown that oral contraceptives increase the risk of CD by 24% and UC by 30% [42]. It is important for gastroenterologists to be aware of the neurologic complications in patients with IBD and to evaluate patients with a history of demyelinating diseases or with symptoms of polyneuropathy before initiation of anti-TNF-alpha; therapy. Interestingly, given that women are more likely to develop UC, postmenopausal estrogen therapy is a risk factor for UC, but not for CD and the risk increases with each month of exposure [43]. Somewhat contradictingly, pregnancy-onset IBD in women is more likely to be UC not CD which is surprising given that pregnancy is a period of elevated estrogen levels[44]. Finally, more than half of female patients with IBD report a worsening of symptoms during menstruation, which is a period of decreased estrogen levels [45,46].

The discrepancy between the apparent effects of estrogen points to the involvement of other factors. Emerging work suggests that tissue specific changes in the expression levels of estrogen receptors are involved in regulating estrogen signaling in a clinically significant manner [47,48] and both estrogen receptor α and β have been implicated in modulating intestinal inflammation [49–53].

While much remains unknown about disruptions to sex steroid levels in IBD, recent advances in microbial endocrinology could explain microbial contributions to the phenomena. This will be explored more thoroughly in Chapter 1.3.

1.2.5 Conclusion

This interplay between severity and timing of onset of IBD and its influence on comorbid sexual and psychiatric dysfunctions remains unexplored. In this thesis I seek to remedy that by exploring in my how the IBD microbiota (Chapter 1.3) and gut inflammation (Chapter 1.4 and Chapter 3) contribute to these comorbidities. In Chapter one I describe the ways in which the microbiota can alter systemic sex steroids. In chapter two I document work with our murine model that captures the full arc of the endocrine and psychiatric comorbidities associated with IBD. In Chapter 3 and 4 I describe the results of our work.

1.3 Contribution of the gut microbiota to systemic endocrine homeostasis

1.3.1 Background and Overview

In the 1990's when Mark Lyte first proposed the microbiota as a modulator of systemic sex steroids there was disbelief from the field of microbiology at large. Today, thirty years later, there is an abundant and every-growing body of evidence that the microbiota does indeed alter levels of systemic sex steroids in a clinically significant way. Recent progress has propelled the field from initially showing correlational links to now defining the mechanisms by which microbes influence systemic sex hormones. In fact, the interaction between the commensal gut microbiota and host-secreted hormones has been shown to be critical for healthy development and capable of mediating disease progression in hormone-associated disorders. This chapter investigates the bidirectional relationship between microbes and sex steroid hormones, with a focus on microbiota-driven hormonal modifications and their effect on host health. Specifically, I focus on the production, activation and degradation of estrogens and androgens by gut microbes, and the downstream effects this has on the host.

1.3.2 The connection between the microbiota and endocrinology

The gut microbiota is a complex community of bacteria, fungi and viruses that live within the digestive tract, and is well-established as an essential component of host health and physiology. While its impact on digestion, synthesis of bioactive molecules, and immune system function have long been appreciated [54], we are now learning that the microbiota also plays a critical role in the metabolism of host sex hormones[55] [2,3]. Specifically, bacteria in the microbiota naturally regulate the levels of sex hormones through production of endogenous hormones, conversion between active and inactive forms, or influence over their absorption and transport. Importantly, disruption to the microbiota membership and function can lead to the development or exacerbation of hormone-driven diseases [2,4], which we will further discuss here.

While microbiology and endocrinology (see Glossary) were historically thought of as two distinct fields, research over the past decade has unveiled their interconnections and increasingly proven their dependence on each other. Current evidence makes clear that the relationship between the microbiota and host hormone levels is inextricable and bidirectional: the microbiota composition is a modulator of hormone levels and, conversely, hormone levels also impact microbial composition.

In this thesis, I will explore the mechanisms through which the microbiota can affect the bioavailability of the sex steroid hormones, focusing on estrogens and androgens, as well as the potential effects on host health when these interactions are modified.

1.3.3 The estrogens-microbiota interplay

1.3.3.1 Estrogens and their importance in host homeostasis and development

The estrogens - estrone, estradiol and estriol - are the dominant female sex hormones and are responsible for the development and modulation of the female reproductive system [5]. Estrone is the weakest estrogen and is typically present in females after menopause. Conversely, estradiol is the most potent estrogen - it is present in both males and females and is most abundant in reproductive-aged females. Finally, estriol is the form of estrogen active in pregnancy [5]. Throughout the lifespan, estrogens drive reproductive development in females and play a role in bone remodeling, cardiovascular health [6], skin health [7], immune defense [8] and more [9]. Homeostatic maintenance of estrogen within specific ranges is crucial for health - estrogen deficiency is associated with osteoporosis and compromised wound healing [10], while excess estrogen is the strongest risk factor for breast cancer in post-menopausal women [11,12], and a major risk factor for endometrial cancer [13]. Estrogen is so vital in preserving health that women are said to lose their “estrogen protection” after menopause [14], as their risk for diseases protected by estrogen rises to a similar level as for men [15,16].

The estrogens are the final product of the steroidogenic pathway, which converts cholesterol into steroid hormones [17]. Within the ovary, endocrine cells produce androgens and then convert them to estrogens via the action of the enzyme aromatase [17,18]. Under normal physiological conditions, estrogens are released into blood circulation. Like all steroid hormones, estrogens diffuse freely across the cell membrane and once within the cell they can bind to estrogen receptors either in the cytoplasm or in the nucleus [19]. In the nucleus, estrogens act as transcription factors and regulate the expression of a variety of genes [20] ranging from modulation of fat storage to the triggering of ovulation [21]. Despite their potency [22], estrogens are vulnerable to rapid deactivation in the liver. This deactivation occurs via a conjugation reaction whereby either glucuronic acid, a methyl group, or sulfonic acid are added to the estrogen steroid structure. These deactivating hepatic modifications (respectively termed glucuronidation, methylation, or sulfonation) prevent estrogen interaction with the estrogen receptors, and thereby inhibit downstream action of estrogen [20]. Further, conjugation makes estrogens more polar and hydrophilic, thereby enabling their absorption into the bloodstream and their elimination through the urine or feces [23] (Figure 1).

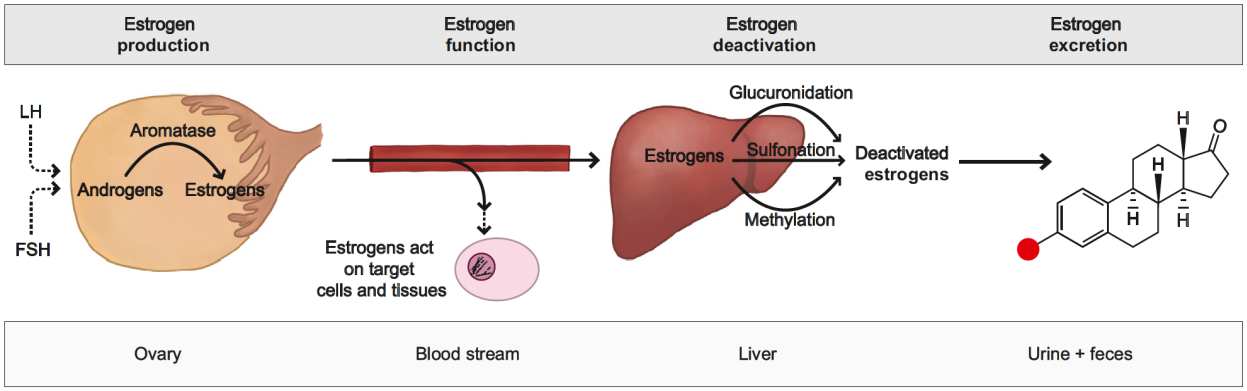

Figure 1: The life cycle of estrogen. Follicle stimulating hormone (FSH) and luteinizing hormone (LH) act on the ovary to induce the production of androgen which is then converted to estrogen. Estrogens are then transported in the blood, distributed to tissues, and deactivated in the liver before returning to the gut to either be reactivated or excreted.

1.3.3.2 Microbiota modification of estrogens

The microbiota regulates systemic estrogen levels in healthy humans [11] and has been shown to affect systemic active estrogen levels primarily through deglucuronidation, or the removal of glucuronides from steroids [24]. β-Glucuronidase (GUS) enzymes expressed by gut bacteria catalyze the cleavage of glycosidic bonds between glucuronic acid and estrogens (reviewed in [25]). Bacteria utilize deglucuronidation to release glucuronic acid, which is converted to pyruvate through the Entner-Doudoroff metabolic pathway [26]. The end product, pyruvate, can then enter the TCA cycle to produce energy for the cell [25]. On the other side, the re-activated estrogens may then enter the portal vein circulation and systemically activate downstream pathways affected by estrogens [25].

Many gut bacteria encode GUS, which have been identified both via sequencing [27] and from physiological studies of intestinal contents [28]. GUS have been found across all major gut bacteria phyla, including in prevalent microbiota genera of the Bacteroidota and Bacillota phyla, such *Bacteroides* and *Clostridium*, respectively [29]. At the subcellular level, genomic data from the Human Microbiome Project has shown that GUS can be targeted to specific parts of the cell. Bacillota members primarily maintain GUS intracellularly, while Bacteroidota species transport them across the inner bacterial membrane [27]. Beyond DNA annotations, several strains have been shown to exhibit GUS activity *in vitro* [25,30], although most have not been tested specifically with estrogen glucuronides as a substrate. In the case of *Escherichia coli* and *Lactobacillus gasseri*, bacterial cell lysates from these strains were shown to have GUS activity in the presence of estrogen glucuronides [30]. Finally, thirty-five human gut bacterial GUS enzymes were cloned in *E. coli* and were shown to reactivate both estrone and estradiol [24].

While not all purified GUS were found to be functional, almost half were able to cleave and reactivate estrone glucuronide, and just less than half estradiol glucuronide [24].
While most microbial studies have shown the importance of deglucuronidation in the modification of estrogens, human and murine gut bacteria have also been shown to encode sulfatases [31] that can remove sulfate esters from organic compounds [32] and could potentially be involved in estrogen modification and activation. Further *in vitro* studies showing the ability of live cells to modify estrogens are needed to identify the cellular processes and conditions required for these processes to take place.

Beyond activation of estrogens by deconjugation, bacteria have been shown to degrade steroid hormones. Estrogen degradation has been shown in environmental genera such as *Microbacterium*, *Sphingomonas* or *Pseudomonas* [33,34] and environmental *Escherichia* strains have been shown to convert estradiol to estrone [35]. While there is no direct evidence that these processes may be catalyzed by host-associated bacteria, further studies are required to identify whether pathways involved in the conversion or degradation of estrogens may occur in the intestinal tract.

Finally, evidence suggests that the gut microbiota can metabolize estrogen-like compounds known to activate estrogen receptors. Primarily in the context of soy-rich foods, gut bacteria have been shown to metabolize the isoflavone Daidzein to Equol; both are compounds which are structurally similar to estrogens and can exhibit agonistic activity towards estrogen receptors and alter gene expression [36].
Together, these studies show that diverse bacterial members can contribute to estrogen modification and activation **(Figure 2)**.

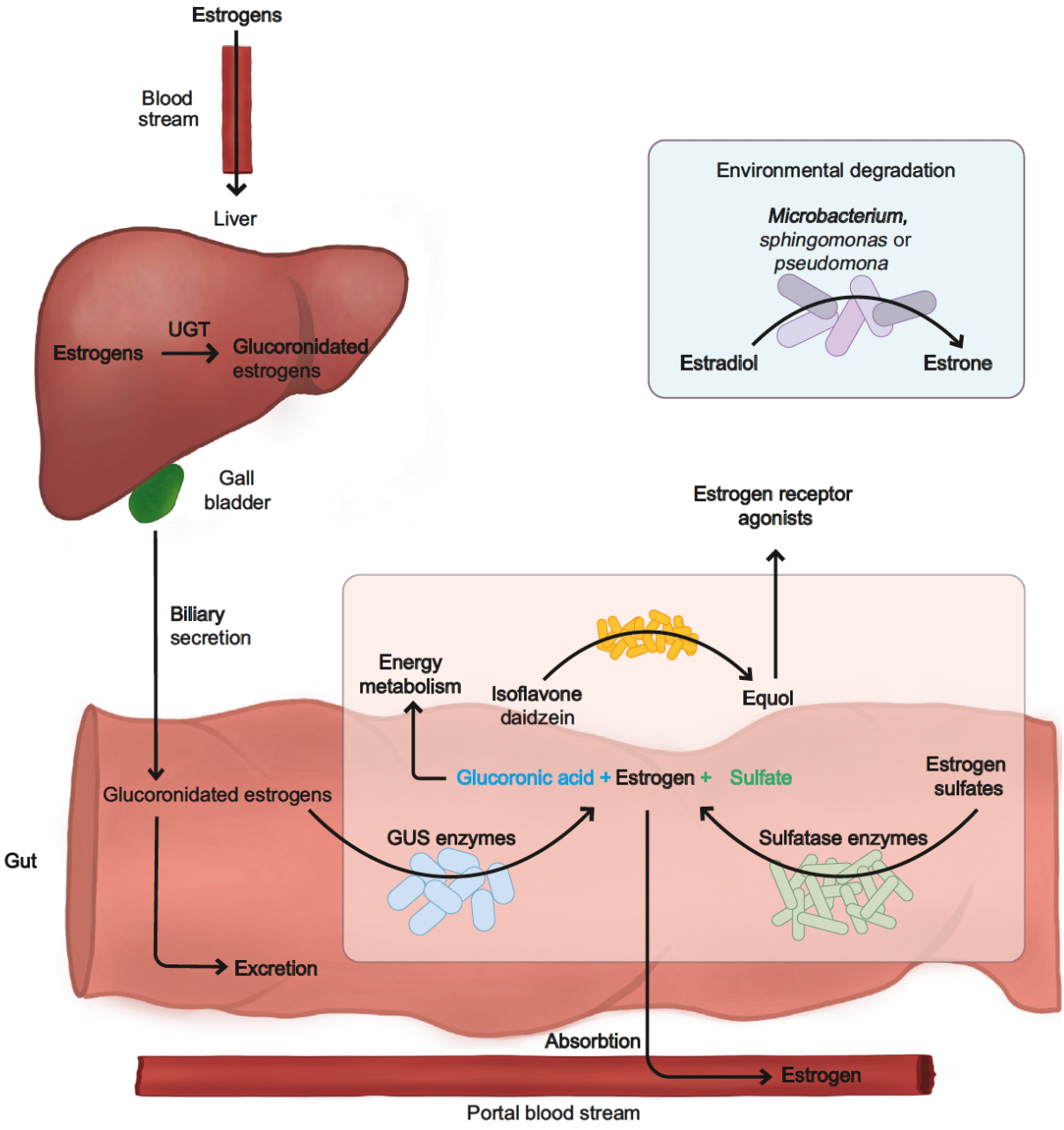

Figure 2: Estrogen transformation in the liver and gut. Estrogens are deactivated via conjugation in the liver, and some are reactivated by bacteria in the gut.

1.3.3.3 Changes to the gut microbiota impact host estrogen levels

Recent evidence highlighted above underscores gut bacterial members as important activators of estrogens. While *in vitro* studies on bacterial modification of estrogen are not necessarily predictive of *in vivo* effects, studies in animals devoid of a microbiota (germ-free) confirm the

essential role of gut bacteria in regulating systemic estrogen levels [37]. For instance, germ-free rats were shown to have undetectable levels of steroid hormones [38], a finding that was confirmed for estradiol specifically in germ-free mice [39]. Tellingly, the re-introduction of bacteria to germ-free mice due to contamination restored the estrous cycles and reproductive function in the mice [40]. This suggests that estrogen levels can be at least partially rescued by bacterial colonization [40].

Aside from the complete removal of the microbiota, smaller modifications to the bacterial membership have also been shown to affect estrogen levels. Antibiotics are well known to modify microbiota membership and diversity: their use has been shown to modify estrogen levels in humans and lead to contraceptive failure in women [41,42]. Furthermore, the use of antibiotics in pregnant women was shown to inhibit bacterial deconjugation of estriol and to increase its secretion in the feces and urine [43].

Finally, previous studies have shown that the transfer of a male murine microbiome into a female mouse removed the protective estrogenic effect against metabolic syndrome [44], indicating that sex-specific microbiome differences may play an important role in establishing physiological levels of these hormones in the host.

Importantly, several conditions associated with low microbiota diversity (e.g., inflammatory bowel disease and a variety of cancers [45]) have been linked to changes in estrogen levels. Moreover, estrogen reactivation by the gut microbiota has been proposed to have a role in malignancy (reviewed in [46]). Specifically, the gut microbiome has been implicated in a number of estrogen-associated conditions including endometriosis, polycystic ovarian syndrome, endometrial hyperplasia and breast cancer [25, 41]. Despite tantalizing correlations, causal links between estrogen produced by bacteria and estrogen-driven pathogenesis remain to be confirmed [48] and are an active area of research in the field.

Having explored the relationship between the gut microbiota and the bioavailability of estrogens, we next consider how androgen levels and activity are modulated by bacteria.

1.3.4 The androgens-microbiota interplay

1.3.4.1 Androgens and the regulation of host development

Androgens are the sex steroid primarily responsible for male characteristics [49]. They are derived from the hormone progesterone and exist in five main forms: dehydroepiandrosterone, androstenedione, androstenediol, testosterone and the most potent, dihydrotestosterone [50]. Healthy androgen levels are essential across the lifespan: from initiating male sex differentiation *in utero* [51], to inducing puberty in adolescence [52], to driving sexually dimorphic

characteristics in adulthood [53], they play a critical role in health. Conversely, androgen deficiency is associated with a wide range of disorders, some of which have been directly connected to the microbiota. Just as in the case of estrogens, androgens can be inactivated by conjugation (via sulfation or glucuronidation), and the relative levels of conjugated versus free androgens correlate with disease risk factors [54]. Mechanistically, bacterial enzymes produced by the gut microbiota are capable of both synthesizing [55] and degrading testosterone [56]. Studies also show that the gut microbiota can alter the systemic serum testosterone levels [56]. The following section will explore these findings in *in vitro* experiments, *in vivo* studies in mice, as well as in the clinical context **(Figure 3)**.

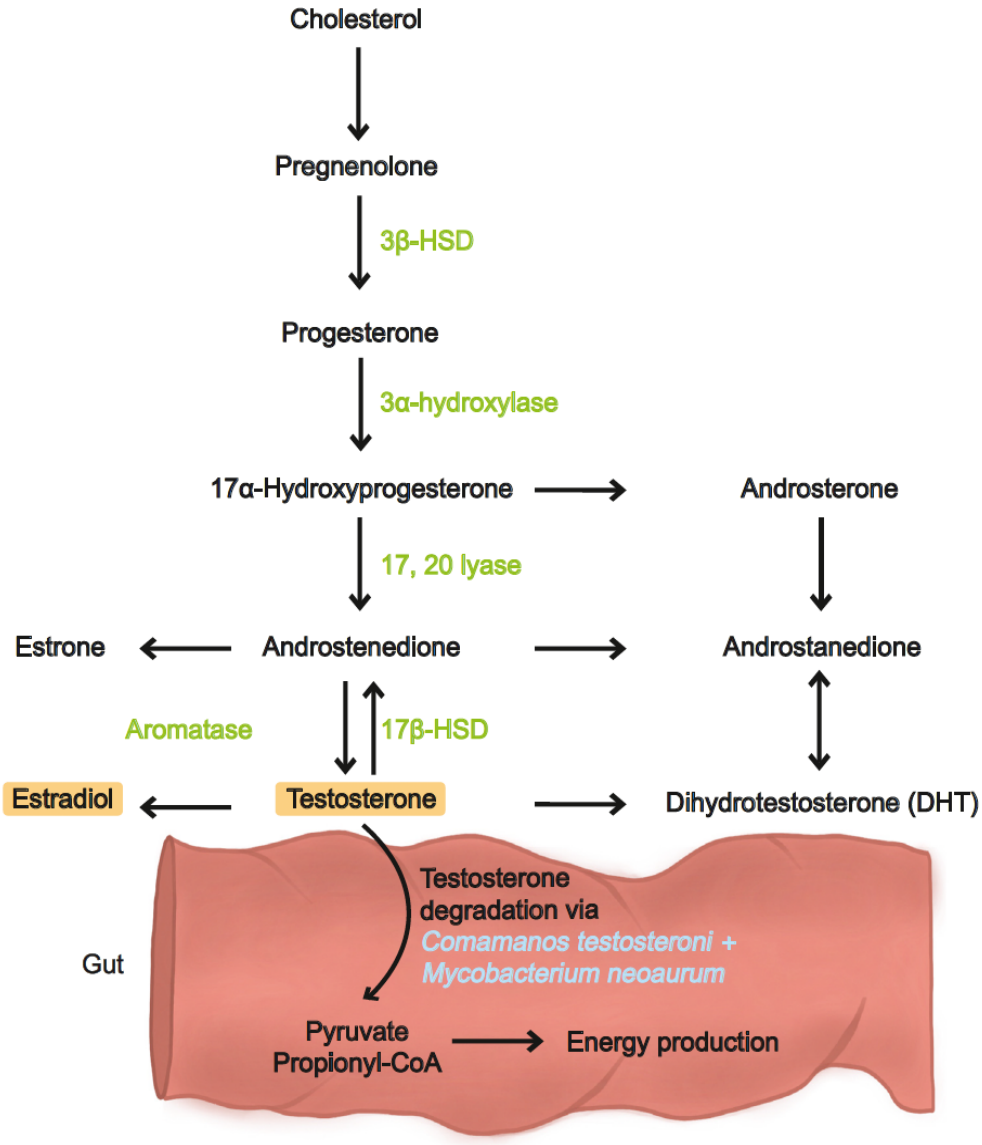

Figure 3: The life cycle of androgens. The biochemical pathway leading to the production of testosterone before it can be degraded by bacteria within the gut.

1.3.4.2 Bacterially driven degradation of testosterone

The first documentation of the role of bacteria in modulating testosterone was in the 1950's when Dodson and Muir [57,58] identified the degradation of testosterone by *Comamonas testosteroni* via a biochemical pathway now known as the Superpathway of Testosterone and Androsterone Degradation [59,60]. In this pathway, testosterone undergoes dehydrogenation and hydroxylation to create pyruvate and propionyl-CoA [60,61] which can be used in other cellular processes to produce energy.

Since the discovery of *C. testosteroni*'s ability to break down testosterone, multiple bacterial strains have been identified to be able to perform the same degradative processes. However, the majority have been studied in environmental contexts such as waste waters, soil, and marine environments, rather than in host-associated bacteria. For example, multiple *Pseudomonas* strains such as *Novosphingobium tardaugens* [62]*, Sphingomonas wittichii, Shewanella paeleana,* and Actinobacteria strains including *Thermomonospora curvata and Salinispora arenicola* found in sewage, soil, and aquatic environments have been characterized to degrade testosterone through multiple pathways [63].

In contrast with the many isolates identified in nature, only two human-associated isolates, *C. testosteroni* and *Mycobacterium neoaurum*, have been identified as testosterone degraders [56,64]. The former has been shown to be a rare human pathogen involved in bacteremia and sepsis [65]. The latter, *M. neoaurum* has been isolated from human fecal samples and has been associated with lower systemic testosterone levels in patients that harbor it [56].

1.3.4.3 Testosterone production by bacteria

Testosterone production by microbes has been well studied due to the pharmaceutical demand for effective methods to produce therapeutic testosterone in large quantities [55]. The genus *Mycobacterium* is one of the most extensively studied for testosterone production, and the biochemical processes underlying this chemical reaction have been clearly defined across multiple strains [55,66,67]. Beyond bacteria, soil and plant fungi such as *Aspergillus* and *Cochliobolus* have been shown to produce testosterone [68,69]. To date no host-associated fungi have been identified to produce testosterone in the gut. However, the host-associated bacterial species *Lactobacillus bulgaricus* was shown to have testosterone synthesis capability from cholesterol *in vitro* [70].

1.3.4.4 Changes to the gut microbiota impacts host testosterone levels

The previously discussed findings of gut microbiota members modifying testosterone levels and availability are supported by *in vivo* studies in complex microbiota settings. As previously described in the case of estrogens, the absence of a microbiota is sufficient to cause changes in sex hormone levels. Indeed, germ-free mice have lower serum testosterone levels than mice with a conventional microbiota [71]. Specifically, they have higher levels of testosterone and dihydrotestosterone glucuronides but very low levels of free dihydrotestosterone [72]. Furthermore, germ free mice were found to have lower intratesticular testosterone levels compared to specific pathogen free mice [73]. Unlike in the case of estrogens, there is limited direct evidence to support the deglucuronidation of androgens by gut bacteria, and further work is required to explore the ability of previously identified GUS enzymes to activate androgens. A recent study also compared the levels of testosterone and dihydrotestosterone glucuronides versus free dihydrotestosterone in the small intestine (which have lower bacterial diversity) to the distal intestine (higher bacterial diversity [72]). It was shown free testosterone levels were highest in the regions with greater bacterial diversity [72].

Beyond microbiota diversity and highlighting the importance of sex difference in microbiota investigations, a landmark study further demonstrated that colonizing young female mice with an adult male mouse microbiome altered the female endocrine profile, resulting in elevated testosterone levels [74]. Several studies have also demonstrated the androgen modulating effect of the microbiome in the context of polycystic ovary syndrome (PCOS) - a disease characterized by excess levels of androgens. Initially, it was found that the genera *Lactobacillus*, *Ruminococcus* and *Clostridium* were lower in a PCOS rat model [75]. The authors then treated the PCOS rats with either a fecal microbiota transplant from a healthy rat or with a *Lactobacillus* probiotic and showed that testosterone biosynthesis decreased in the treated rats [75]. Furthermore, another study colonized PCOS rats with *Lactobacillus* and *Bifidobacterium* probiotics and showed that the probiotics could rescue the disrupted testosterone levels more effectively than Diane-35, a clinical treatment for PCOS [76]. These results highlight the potential therapeutic effect of microbiota therapies in the context of hormonal dysregulation and the importance of the microbiota in regulation of hormonal host processes.

1.3.4.5 Systemic effects of testosterone modification via the microbiota

The examples presented above highlight the important relationship between the microbiota and androgens in the context of animal models. Recent studies have made major headway in demonstrating that those principles translate to humans. High serum testosterone levels have

been associated with presence of *Acinetobacter, Dorea, Ruminococcus* and *Megamonus* [77], while low serum testosterone levels have been associated with *Lachnoclostridium, Blautia* and *Bergeyella* [78] in humans. Clinically, disrupted androgen levels have been linked to a changed bacterial profile in type 2 diabetes [78], depression [56], PCOS [76] and prostate cancer patients [79]. Recently, studies have been able to move beyond correlation and have described the direct mechanisms by which specific bacteria modulate systemic androgen levels and impact disease.

In the case of prostate cancer, studies have shown that patients suffering from this disease had a microbiome enriched in metabolic pathways for androgen synthesis compared to healthy controls [80]. The current gold standard of treatment for prostate cancer is androgen deprivation, which is often achieved via castration [81]. However, while castration initially succeeds at inhibiting tumor growth by obstructing androgen production, the effects can be temporary, as over time some patients begin producing androgens again [81]. Tellingly, researchers showed that the microbiota was responsible for the creation of extra-gonadal androgen in castration-resistant prostate cancer patients, as well as in mice [79]. The study found that the microbiota of patients that produce androgens post-castration was enriched in *Ruminococcus* and *Bacteroides* [79]. To test whether these bacteria directly caused the production of testosterone, the researchers introduced deuterated pregnenolone, a precursor for steroid biosynthesis, into germ-free and *R. gnavus* and *B. acidifaciens*-colonized castrated mice. They then measured the conversion to testosterone in the serum and confirmed that *R. gnavus* and *B. acidifaciens* could convert deuterated pregnenolone to testosterone, as well as increase tumor growth and disease progression [79]. Promisingly, the researchers showed in mice that broad-spectrum antibiotics successfully inhibited microbiota production of androgens, causing reduced levels of prostate cancer cells, delayed tumor growth and increased survival [79]. These insights can lead to novel therapies addressing testosterone production by the gut microbiota.

Beyond prostate cancer, where elevated testosterone levels are detrimental to host health, low testosterone levels associated with depression were shown to be dependent on a changed microbiota [56]. A recent study causally linked *M. neoaurum* with depression and low testosterone levels [56]. The authors grew the gut microbiome of patients with depression on testosterone and most patients' gut microbiota was capable of degrading the testosterone to a weaker intermediate, androstenedione, and, in some cases, it could even further degrade the

androstenedione [56]. Importantly, they found that by introducing 3β-hydroxysteroid dehydrogenase (3β-HSD), the enzyme responsible for testosterone degradation, into *E. coli* and mono-colonizing germ-free rats with this strain, they reduced systemic testosterone levels and induced symptoms of depression [56]. Finally, they found that testosterone administration relieved the symptoms of depression in the rats [56].

1.3.5 The Future of Microbial Endocrinology

We have long known that alterations of steroid hormone levels in an animal host impact its physiology and health state; it has also been clear that bacteria and fungi can produce, degrade, and modify steroids hormones. Evolutionary analyses further suggest that many of the genes necessary for endocrine signaling may have originated in microbes and were acquired by humans through horizontal gene transfer [82,83], further highlighting the connection between microbiology and endocrinology. New research coming from multiple fields has now connected these two disciplines into a unique interdisciplinary field at the intersection of many other subjects, including neurobiology [84] and immunology [85]. As we learn more about the incredible repertoire of functions host-associated microbes have, there are sure to be more discoveries of the interplay between the endocrine system, the microbiota and host health. Excitingly, active research has identified the importance of gut microbiota function and steroid modification in diseases such as PCOS, prostate cancer and depression. Interestingly, other diseases associated with a changed microbiota also display altered sex steroid levels but have yet to be investigated in the context of microbial endocrinology.

For example, in the case of inflammatory bowel disease (IBD), some male IBD patients have decreased testosterone levels and experience growth failure, delayed puberty [86,87], erectile dysfunction [88], depression [89], and osteoporosis [90], all of which can be consequences of low testosterone levels. Excitingly, these comorbidities can be ameliorated via the administration of testosterone, which also improves clinical symptoms of IBD [91]. Importantly, these links are supported by correlational studies that have shown that serum testosterone levels can be associated with specific microbial patterns. For example, men with higher levels of serum testosterone have more diverse microbiomes, and a higher prevalence of *Acinetobacter*, *Dorea*, *Ruminococcus* and *Megamonus* when compared to men with lower serum testosterone levels [77]. Generally, the microbiome is now starting to be considered as a key factor in male infertility (reviewed in [92]). Further studies will be required to identify whether an altered microbiota is causative of these changes. As both systemic estrogen and testosterone levels are manipulable via the microbiota, mechanisms defining which species, genes and pathways

are responsible for changes to hormonal levels *in vitro* and in *vivo* are required to support the development of microbiota therapies and to better understand the causes of disease.

In conclusion, the field of microbial endocrinology is only getting started - understanding the mechanisms of bacterial interaction with steroid hormones has the potential to not only expand our knowledge on the connections between microbial function and host physiology, but also change the way many diseases are treated.

1.4 Architecture of the gut in IBD

1.4.1 Anatomy of the physical gastrointestinal tract in IBD

The surface area of the human gastrointestinal tract averages around $32m^2$, making it one of the largest and most vulnerable points of contact between the human body and the external environment [56]. Constantly facing mechanical, chemical and biological stress, a healthy gastrointestinal tract is well adapted and optimized at both the macroscopic and microscopic levels to protect the body from the "external" environment. In IBD these adaptations are compromised at both the macroscopic and microscopic levels, and while the precise etiology of IBD remains unclear, it is accepted that these deficits in the protective function of the gastrointestinal tract (GIT) are key drivers in the disease. While there are a number of similarities between CD and UC, the anatomy of their pathology varies both macroscopically and microscopically. Not only does the location of the inflammation differ in terms of the region along the longitudinal length of the gastrointestinal tract, but it also varies in the depth in which it pervades the gastrointestinal tract.

1.4.1.1 Macroscopic comparison of a healthy GIT and an IBD GIT

At a macroscopic level, a healthy gastrointestinal tract consists of 4 layers - the mucosa (the epithelium and lamina propria), the submucosa (thin layer of connective tissue beneath the mucosa that contains nerves, blood vessels, lymph vessels and glands), the muscularis propria (inner layers of both circular and longitudinal muscle) and the serosa (the outermost layer) [57]. In Crohn's disease, inflammation is transmural, meaning it affects all four layers of the mucosa, submucosa, muscularis propria and serosa. By contrast, inflammation in ulcerative colitis is confined to the mucosa and submucosa [58].

1.4.1.2 Microscopic comparison of a healthy GIT and IBD GIT

Zooming in beyond the four anatomical layers of the GIT, there are additional features at the microscopic level that are designed to act as protection from the external environment: the epithelial layer and mucosal layer. This tightly regulated composite barrier is believed to be what protects healthy individuals from developing an inflammatory response to the abundant bacteria within the GIT. There are five main epithelial cell types that are derived from the pluripotent stem cells that line the basement of crypts: (1) enterocytes representing >80% of all epithelial cells, (2) goblet cells responsible for mucin production for mucous, (3) Paneth cells responsible for secreting antimicrobial peptides, growth factors and digestive enzymes (4) enteroendocrine

cells which produce peptide hormones and (5) M cells specialized for antigen sampling [59,60]. The first two are of most relevance to inflammatory bowel disease.

Enterocytes:

Enterocytes, the most abundant epithelial cell in the GIT, are simple columnar epithelial cells that form a monolayer along the entirety of the small and large intestines. In each region of the GIT the monolayer is arranged to optimize the type of absorption at that site - in the small intestine the enterocytes project into the lumen forming finger like projections enabling enhancement of nutrient absorption, in the large the enterocytes invaginate away from the lumen to form crypts, allowing for the absorption of water [61,61]. In the small intestines, where nutrient absorption is a key function, surface area is further increased by the protrusion of finger-like projections, whereas in the large intestine, where water absorption and stool formation is a primary function, enterocytes have a flat luminal surface [62]. In healthy individuals enterocytes rejuvenate every four to five days, however in IBD there is an increased rate of epithelial cell death which can outpace the rate of enterocyte proliferation, leading to a compromised epithelial layer [63].

Goblet cells

Goblet cells, whose name is owed to their shape, are specialized epithelial cells that produce and secrete mucus and are renewed every 3-7 days [64]. In healthy individuals, goblet cells are found in both the crypts and villi of the small intestine, and solely the crypts in the large intestine [65]. As the GIT progresses longitudinally the abundance of goblet cells increases proportional to the abundance of bacteria. Goblet cells comprise 4% of epithelial cells in the duodenum, 6% of epithelial cells in the jejunum, 12% of epithelial cells in the ileum and 16% of epithelial cells in the distal colon [65]. These changes in abundance are believed to correspond to the increase in bacteria along the GIT [65]. In both types of IBD patients have a reduced quantity of goblet cells, and the ones they do have are defective [66]. While a lot remains unknown, it is believed that inflammation is responsible for this reduction and damage [67].

1.4.2. The Mucus layer

1.4.2.1 Introduction to the mucus layer

Once primarily appreciated for its lubricant properties, the mucus layer has been increasingly recognised for its crucial role in mediating the dynamic symbiotic relationship between host and the microbiota. In a healthy individual mucus is secreted by the goblet cells and forms a thick

and continuous biphasic barrier that physically, chemically and mechanically protects underlying epithelial cells from direct contact with bacteria and bolus [68] and thereby prevents inflammation [69]. The mucus consists predominantly of water (90-95%) and hydrophilic mucin glycoproteins (1-10%) that organize themselves into an extensive net-like structure of polymers [70,71].

As with goblet cell distribution, the organization and abundance of mucus changes longitudinally along the GIT, following the trend of increasing mucus thickness correlating with increasing microbial abundance. In the small intestine there is a single monolayer of mucus, and in the large intestine there is a biphasic inner and outer layer [72]. The inner layer is secreted by goblet cells and anchors tightly to the epithelium. It is dense, stratified, void of bacteria and completely renews every hour in humans and every 1-2 hours in mice [64,73–75]. As the inner layer turns over, it becomes less dense, unanchored, and has a less defined outer border that is easily shed, and can harbor bacteria [72].

1.4.2.2 MUC2 Molecular structure and function:

There are over 20 known mucin genes in the body but they collectively encode only four distinct mucin proteins - MUC2, MUC5AC, MUC5B, and MUC6 [76,77]. These mucins are highly glycosylated glycoproteins characterized by their high molecular weight, large size and high numbers of tandem repeats in their peptide sequences [78]. The primary mucin responsible for the intestinal mucus layer in both mice and humans is MUC2 [69,79]. The peptide sequence in MUC2 consists of the hydroxy amino acids proline, threonine, and serine [69]. The threonine and serine residues are heavily glycosylated with hydroxyl (-OH) linked oligosaccharides (O-glycans). The high degree of O-glycosylation contributes significantly to the mucus, accounting for up to 80% of the mucus' overall molecular mass and driving a number of its protective properties [69,80,81]. For example, O-glycans are attached to up to 90% of the amino acid residues and therefore act as a physical shield restricting protease access to the peptide core of mucins and therefore making the mucus protease resistant [82]. Additionally, the oligosaccharides are the component of mucins that bind to water - giving mucus its gel-like properties that enable it to act both as a lubricant and a robust physical barrier [83]. Finally, O-glycans have also been shown to have potential antimicrobial properties [69,78]. Together, these three points demonstrated the importance of glycosylation in conferring on mucus its protective properties.

1.4.2.3 Comparison of mucus layer in healthy GIT and IBD GIT

There are a number of differences in the quantity and quality of the mucus between healthy individuals and IBD patients and it is well established that these mucus differences are a key driver of IBD pathogenesis [84]. In regards to quantity, IBD patients not only have fewer goblet cells as mentioned above, but their goblet cells also exhibit a downregulation of the MUC2 gene, leading to decreased production of mucus [85]. These decreases are further exacerbated during active inflammatory flare ups [86]. Beyond decreased production of mucus, IBD patients also exhibit signs of increased deterioration of mucus. In contrast to the stable and uniform levels of mucin O-glycosylation in healthy individuals [83,87], mucins in IBD patients exhibit significant decreases in the proportion of serine and threonine molecules that are glycosylated and the glycan molecules are smaller [88]. Interestingly, the magnitude of the disruption to glycosylation corresponds with disease severity [88], suggesting that the decrease in glycosylation compromises the protective properties of the mucus [89,90]. The resistance to proteases is one example of this - the reduction in size and number of glycans makes the protein core of mucins spatially more vulnerable to proteases and mucolytic bacteria.

1.5 Modeling the gut-endocrine-brain axis in IBD

1.5.1 Models of Inflammatory Bowel Disease

The worldwide increase in IBD prevalence has catalyzed interest and concern among healthcare professionals and scientific communities, leading to a surge in research efforts to understand the disease. IBD models range from clinical studies that use endoscopy or ex vivo samples taken during surgery to organoid studies that enable study of the epithelium and microbiota [91]. However, due to the multifactorial and multi-system nature of IBD, mouse models remain one of the best methods for modeling IBD and its systemic effects [92,93]. As such, there are currently over 50 mouse models of inflammatory bowel disease (IBD) [94]. These mouse models can be broadly divided into four groups: (1) genetically engineered, (2) chemically induced, (3) immunologically induced and (4) spontaneous [93]. Of these, the most widely used mouse model of colitis is a chemical model called the dextran sodium sulfate (DSS) model [95].

1.5.2 The Dextran Sodium Sulfate Model

The dextran sodium sulfate model was developed in Sweden in 1991 and has since become the most widely used model of colitis in over 10,000 published scientific articles, with an average of 2.5 articles published using it each month so far in 2023 [96,97]. The model that induces colitis-like symptoms such as weight loss, rectal bleeding, diarrhea, and blood in stool is popular due to its simplicity and relative reproducibility. It is administered by supplementing mouse water with DSS in concentrations between 0-7% w/v and onset of colitis-like symptoms is seen within 3-5 days [98].

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What molecular structure is responsible for such multifaceted effects that so closely resemble colitis? DSS is a sulfated polysaccharide that consists of 1,6-linked α-glucose units with two sodium sulfate groups (-OSO3Na) attached to each α-glucose unit making a helix structure [99]. The molecule is water soluble, negatively charged and varies greatly in size from 5 to 1400 kDa with optimal effectiveness between 40-50 kDa [95]. Interestingly, the inflammatory effects of DSS are only seen in vivo, not in vitro, indicating that a component of the living system is required to interact with DSS to produce the inflammatory response [100]. There is further evidence to cement this hypothesis - DSS-associated molecules, such as sodium sulfate or the parent molecule dextran are not capable of inducing in vitro or in vivo inflammation either, indicating that DSS itself is required to form a complex with something in the intestinal lumen [100]. It has now been confirmed that this is indeed the case, DSS induces colitis by binding to

long and medium-chain-length fatty acids in the lumen of the colon, and forming nanometer complexes that are capable of fusing with the epithelial cell membranes and entering into the cytoplasm of the cells [100]. It appears that the DSS-fatty acids complexes form through electrostatic interactions between the sulfate group of DSS and the carboxylate groups of the medium or long chain fatty acids [100]. Once within the epithelial cells, it is believed that the dextran group is responsible for triggering the cells inflammation cascade [100], indicating that together each of the components of the DSS molecule are involved in its efficacy in triggering colitis-like symptoms.

One of the strengths of the DSS model is that the symptoms it creates are easily definable and scale in terms of disease severity. For instance - higher concentrations of DSS or factors that cause increased susceptibility to DSS, cause a measurable increase in markers of disease severity such as the severity and number of lesions in the mucosa, the number of neutrophils and lymphocytes present. This makes the DSS model an ideal candidate for investigating the degree to which colonic DSS inflammation is responsible for extraintestinal comorbidities. Put differently, it allows investigation into whether more pronounced inflammation corresponds to increased severity in other extraintestinal symptoms.

For this reason, we sought to explore whether varying degrees of DSS severity corresponded to changes in extraintestinal endocrine comorbidities. We sought to determine whether there were differences between acute and chronic administration of DSS.

1.5.3 The DSS model and extraintestinal endocrine comorbidities

Multiple studies have explored the influence of DSS on the endocrine comorbidities seen in patients with IBD. One group, Deboer et al., have published extensively on the impact of DSS on puberty. They have shown that DSS causes pubertal delays out of proportion to leptin and corticosterone in female mice [11], and out of proportion to food intake and body weight in male mice [11]. Further, they showed that injections of anti-TNFα antibodies were capable of partially rescuing DSS induced pubertal delays [101]. Another group, Chen et al., showed that the testes down regulate androgen biosynthesis genes and decrease androgen production in response to DSS inflammation [102]. Given the foundation for endocrine comorbidities in the DSS model, we determined that it would be an ideal model to explore the gut microbiota-endocrine-brain axis.

1.6 Goal and hypotheses for this study

Objective 1: The first goal was to develop a model that could be used to investigate the full arc of the gut-endocrine-brain relationship. I hypothesized that the DSS model could be used to capture the full arc of the gut-endocrine-brain comorbidities of IBD and that it could help determine if there were critical developmental time points when the endocrine system and brain were more susceptible to DSS inflammation.

Objective 2: The second goal was to identify critical periods in which a healthy gut environment and microbiota is required for normal endocrine and reproductive function. I hypothesized that using the DSS model we could determine if there were critical developmental time points when the endocrine system and brain were more susceptible to IBD-like inflammation.

Objective 3: The third goal was to determine to what extent the severity of inflammation corresponded with severity of endocrine and brain comorbidities. I hypothesized that the endocrine alterations would vary in severity in proportion to the fluctuations in intensity of inflammation within the gut.

Chapter 2: Materials and Methods

2.1 Mice

All mice experiments were performed in strict accordance with the University of British Columbia Animal Care Committee protocols and took place on site at the University of British Columbia animal facility. C57BL/6 (strain #000664 [103]) wild-type mice from Jackson Laboratories were bred and the first generation of offspring were used for experiments. Mice were weaned at postnatal day 19 and housed by sex with 4-5 animals per cage for the duration of the experiments. All mice were ear notched and tailed marked for identification with their markings corresponding to experimental identification numbers. The mice were kept in a temperature (22ºC) and humidity (40-60%) controlled environment on a 12 hour light cycle and were fed JL Rat and Mouse Auto 6F 5K67 Lab diet *ad libitum* throughout the experiment. Other than when being treated with DSS treatment mice were given *ad libitum* access to autoclaved distilled water. All experiments were designed to use the minimum number of mice necessary and minimize their suffering where possible.

2.2 Experimental design

Colitis was induced by administering 3% w/v Dextran Sodium Sulfate 36,000 - 50,000kDa (MP Biomedicals [97]) into the mouse water *ad libitum* for either an acute period (study 1) or a chronic period (study 2). The control mice received normal drinking water throughout the entirety of the experiment. The experimental timeline is shown in Figure 4.

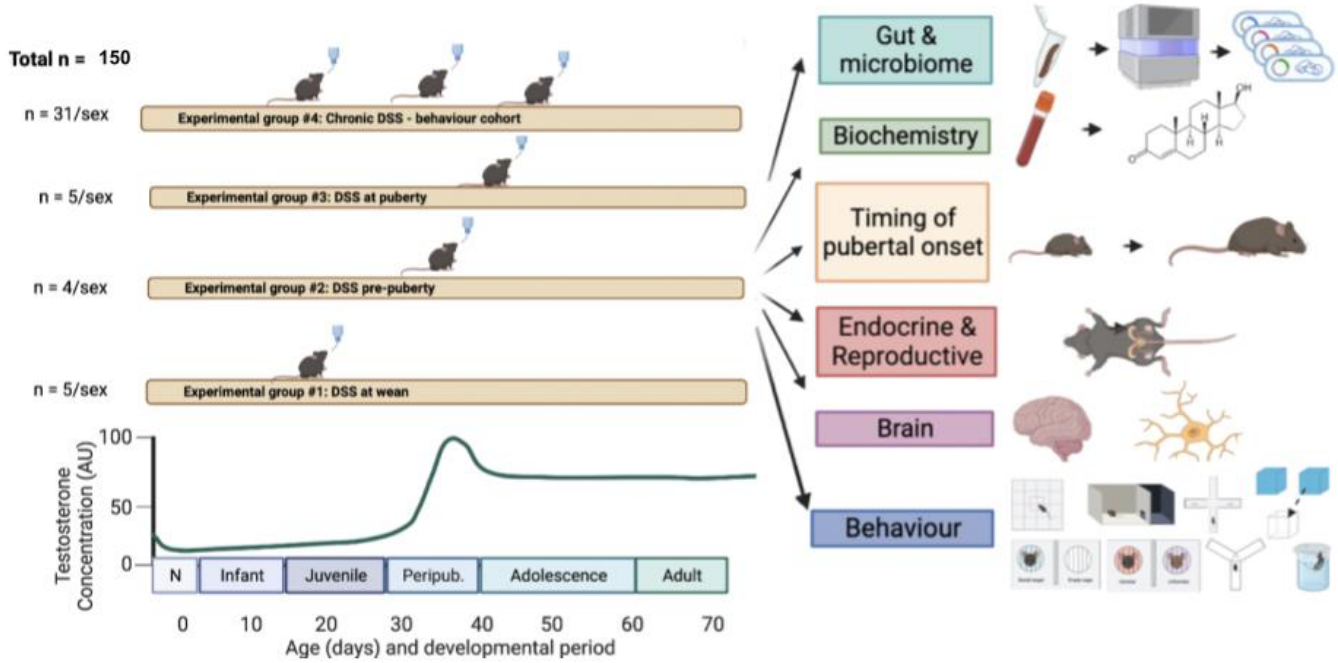


Figure 4: Experimental design for the DSS experiments. Administration of DSS corresponds to developmental time

points throughout the juvenile period and adolescence. Group 1: DSS acutely administered at wean, Group 2: DSS acutely administered pre-puberty, Group 3: DSS administered at puberty and Group 4: DSS administered chronically from the juvenile period until adulthood. These experiments led to analysis of DSS severity, stool, serum, pubertal markers, the brain and behavior.

2.2.1 Study one: Acute induction of DSS colitis at specific time points

We sought to investigate whether DSS administered before, during or after these reproductive milestones could trigger changes in reproductive development. In healthy male mice, androgens have been shown to remain at steady and low levels until there is an increase between postnatal day 30 and day 50 (see **Figure 5** from Bell, 2018) [104,105]. It is thought that this shift in testosterone levels accompanies the onset of puberty [105]. In healthy female mice, estrogen levels begin to rise gradually from postnatal day 28 into adulthood accompanying the onset of fertility (see **Figure 5** adapted from Bell, 2018) [104,105]. Accordingly, we induced DSS colitis for 5 days at specific time points that correspond with these known pubertal developmental milestones: pre-puberty (postnatal day 21-26), puberty (post natal day 34-39) and post puberty (post natal day 43-49). Due to human error the last group was on DSS for 5.5 days as the DSS was switched to water the next morning. The pre-puberty and puberty mice were sacrificed at postnatal day 40 and the post puberty mice were sacrificed at postnatal day 50.

2.2.2 Study two: Chronic and intermittent induction of DSS colitis across the lifespan

In this study we chronically induced DSS colitis from the juvenile period until adulthood. The first 5 day administration of DSS was from postnatal day 21-26, the second administration was from postnatal day 43 to 48 and the final administration was from postnatal day 65 to 70.

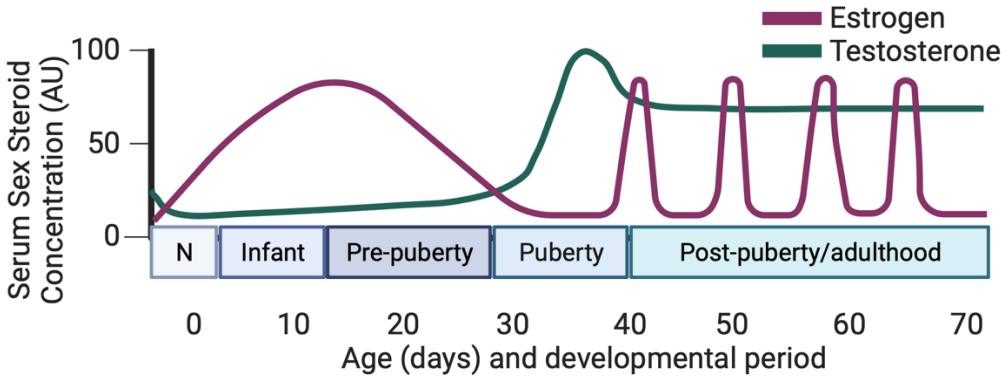


Figure 5. Fluctuation of estrogen and testosterone in murine serum throughout development. Figure created in BioRender and based on the work of Bell MR (2018).

2.3 Experimental procedures

2.3.1 Assessments during experimental period

2.3.1.1 Stool sampling

Fresh stool samples were collected daily during DSS administration periods and 3-4 times per week during recovery periods. Sampling was performed during a consistent time window each day (16:00-21:00) to minimize microbiota variability associated with diurnal rhythms. Samples were collected either by scruffing one mouse at a time, or, when DSS disease severity worsened and caused delay in defecation, the mice were temporarily placed in a single container until a sample was produced. Upon collection, samples were immediately placed on ice until all samples were collected and then they were transported for storage at −80 °C until analysis was completed.

2.3.1.2 Weight measurements

Weight loss has been determined to be sufficient as a single marker in demonstrating that DSS has efficiently induced colitis [106]. Therefore, mice were weighed daily during treatment periods, and 3-4 times per week during recovery periods. In accordance with IACUC and the University of British Columbia Animal Care Committee protocols animals were euthanized if their body weight dropped more than 20-30% [107].

2.3.1.3 Reproductive markers

Male puberty onset identification

Murine balano-preputial skin separation (BPS) is the cleavage of the prepuce from the glans penis and can be used as a marker of the onset of puberty in male mice [108–110]. A group of our mice were specifically assigned to this experiment to avoid stress effects for the mice that were used for other experimental measures. The mice designated to the puberty check group (DSS-treated n = 23, Control group n = 10) were examined daily for BPS from postnatal day 21 to postnatal day 40. This was done by daily attempts to manually extrude the prepuce with gentle pressure until the day it would easily separate from the penis. Scoring was performed on a three point scale: 1 = does not extrude, 2 = beginning to extrude, 3 = completely extrudes. Once mice demonstrated complete prepuce separation and scored as level 3 they were considered to have reached puberty and ceased undergoing daily BPS examination. Photos were taken daily using a tripod and light ring to ensure consistency in image quality.

Female puberty onset and estrous stage identification

The onset of puberty in mice can be measured by determining the timing of the vaginal opening and the onset of estrous cycles (the murine equivalent of the human female menstrual cycle). These two events coincide alongside each other and typically occur around postnatal day 26 in C57bl7 mice [111,112]. Unlike the human menstrual cycle which lasts approximately 28 days and involves shedding of blood, the murine estrus cycle lasts 4-5 days and does not involve the shedding of blood [111,112]. Instead, each stage is marked by a change in the predominant cell type present in the vaginal canal - either nucleated epithelial cells, cornified epithelial cells, or leukocytes [112,113].

Estimation of timing of vaginal opening

A group of female mice were assigned to be in a puberty check group to avoid the potential of stress effects confounding other measurements in our study (DSS group n = 5, Control group n = 5). We examined the mice assigned to the puberty group for the onset of vaginal opening from postnatal day 21 until full vaginal opening or post natal day 50. To perform the vaginal opening check mice were placed with their front paws resting on the cage while they were gently elevated by holding the tail, enabling physical examination of the vagina. Similar to the male assessment, a three point scoring system was used to classify the stage of vaginal opening: 1 = completely closed, 2 = beginning to open, 3 - completely open. Photos were taken daily using a tripod and light ring to ensure consistency in image quality.

Estrus cycle identification

Estrus cycle identification was performed according to the protocol described by Byers et al. [113]. This was performed immediately after vaginal opening observation during the juvenile period, adolescence and, immediately prior to euthanasia on the days mice were sacrificed. A vaginal cell sample was collected by gently pipetting PBS or saline solution up and down within the vaginal canal and then pipetting the vagina-exposed liquid onto a microscope slide. The slides were air dried, transported to the laboratory, and stained according to the methods described by McLean et al. [114]. Specifically, a droplet of 0.1% crystal violet stain was pipetted onto the vaginal smear and left to stain for one minute and then washed with ddH2O [114]. After drying, cytology was observed at 200x magnification with a Brightfield microscope and mice were assessed as experiencing one of the following stages: proestrus (measured by presence of nucleated epithelial cells), estrus (anucleated cornified cells), metestrus (presence of mixture of cells), diestrus (presence of predominantly leukocytes) [111].

2.3.2 Euthanasia:

Mice were anesthetized with isoflurane and then euthanized via cardiac puncture and perfused with phosphate-buffered saline (PBS). Mice were sacrificed either immediately after DSS treatment or after sexual and behavioral testing. All euthanasia procedures commenced at 08:00 and effort was made to finish by 14:00 to minimize effects of diurnal rhythm on changes to microbiota and blood samples. Mice were sacrificed at the ages displayed in the following table:

Experimental group	Sample size for DSS treated mice	Approximate age by postnatal day
DSS at wean	n= 5/sex	Postnatal day 70
DSS pre-puberty	n= 5/sex	Postnatal day 70
DSS at puberty	n = 4 males	Postnatal day 70
Sacrifice immediately after 3 rounds of chronic DSS administration	n = 3 females, 1 male	Postnatal day 71
Sacrifice after sexual behavioral testing	n = 5 females, 5 males	Postnatal day 112-115
Sacrifice after multiple rounds of behavior testing	n = 8/sex	Postnatal day 130-134

Table 1: Age of experimental groups at time of sacrifice.

2.3.3 Tissue Collection:

Blood

Immediately after euthanasia with isoflurane, and prior to perfusion with PBS, blood was taken via cardiac puncture with a 21G needle and 1 ml syringe. Blood was transferred into BD Microtainer™ Serum Separator Tubes (Becton Dickinson [115]) and allowed to coagulate at room temperature for at least 30 minutes and up to 5 hours. After coagulation, serum was centrifuged at 3000 rpm for 20 minutes at 4°C, and subsequently pipetted into Eppendorf tubes and stored at -70°C.

Brain

Following perfusion with PBS, the brain was rapidly extracted and dissected into regions before being placed on dry ice. As previously mentioned, this project was a collaboration with the Ciernia lab, and a masters student in the lab, Olivia Sullivan was responsible for the brain and behavioral assessments.

Liver

Following removal of the brain, the liver was rapidly removed to minimize tissue and DNA degradation. After weighing a portion of the liver was transferred to an Eppendorf tube and placed on dry ice until storage at -80 °C.

Reproductive organs

Subsequently, the reproductive organs (seminal vesicles and testes for males, uterus and ovaries for females) were dissected, weighed, and then placed into 10% neutral buffered formalin for 48 hours. Following this fixation period, the reproductive organs were washed twice in 70% ethanol prior to being transferred to 70% ethanol for storage at room temperature until processing. These organs were sectioned and stained with hematoxylin and eosin by Ingrid Barta at the University of British Columbia's Animal Care Services Diagnostic & Research Histology Laboratory. Following staining, the reproductive organ slides were sent to Alberta for scoring by Dr. Nick Nation of Animal Pathology Services.

2.3.4 Serum Steroid Analysis

Sex steroids were quantified either by a multi spot assay or liquid chromatography with tandem mas spectrometry (LC MS-MS). The multisport assay system was from Meso Scale Discovery, MSD, Rockville, MD, USA [116] and was used to measure testosterone, DHEA, estradiol and progesterone. The assay was performed according to the manufacturer's instructions [116]. Liquid chromatography with tandem mass spectrometry (LC MS-MS) was used to measure progesterone, estradiol, testosterone, androstenedione and dihydrotestosterone.

2.3.5 Colon Measurements and Imaging

Upon euthanasia mouse colons were immediately measured for length in cm using a standard ruler. Some experimental groups were assigned to be analyzed via imaging and some were assigned to be analyzed for their intestinal contents. The group assigned to analysis of intestinal contents was dissected using a butterfly cut to slice down the center of the intestinal wall, and the contents were extracted by and placed without fixative into Eppendorf tubes and onto dry ice before being stored at -80 °C until analysis by Claire Sie, a member of the Tropini lab.

The colons in the groups assigned to analysis by imaging were gently coiled into a volute and placed in a Tissue-Loc HistoScreen cassette. The colons were then immersed in a solution of

methanol-Carnoy's fixative (60% methanol, 30% chloroform and 10% glacial acetic acid) where they were fixed at room temperature for up to two weeks. Following the fixation period, the colons were paraffin embedded, sliced to 4 µm sections, stained with either H&E or immunofluorescent staining, and imaged.

H&E Staining

H&E staining was performed by Ingrid Barta at the University of British Columbia's Animal Care Services Diagnostic & Research Histology Laboratory, or Claire Sie, a member of our lab. H&E slides were scored with the help of Ian Welch at the University of British Columbia's Animal Care Services Diagnostic & Research Histology Laboratory.

Fluorescence In Situ Hybridization Staining

Fluorescence In Situ Hybridization (FISH) staining (see glossary) was performed as described in a recent paper from our lab by Ng & Tropini [117]. Specifically, nucleated cells (host epithelial cells and microbiota bacterial cells) were stained with DAPI, the mucus layer was counter stained with Rhodamine-labeled Ulex Europaeus Agglutinin I (UEA-1) (Vector Laboratories), and the bacteria were counterstained with Eub338-FITC (ThermoFisher).

Staining procedures followed the above-mentioned protocol from Ng & Tropini 2021 [117]. Briefly, slides were deparaffinized by heating at 60°C for 10 minutes, and then two 10 minute incubations in using pre-warmed xylene, followed by one quick wash in 99.5% ethanol for 5 minutes. Samples were then incubated in a hybridization solution containing 10 µg/ml EUB338 at 50°C for 3 hours. After the FISH staining), slides were incubated for 10 minutes at 50°C in a pre-warmed FISH washing buffer and washed in PBS. After washing, the slides were counterstained with UEA-1 and DAPI and for 45 minutes at 4°C and then washed quickly in PBS three times. Finally, slides were mounted using Vectashield and coverslips were secured in place using clear nail polish. Slides were allowed to dry completely and then were stored in a dark box in a 4°C fridge for up to two weeks before imaging.

Imaging was performed within two weeks of staining with the Zeiss LSM 700 confocal microscope using Zen (blue edition) software [118]. Images were annotated and cropped using Fiji [119]. Quantification of the mucus thickness was calculated using BacSpace in MATLAB 2023 (Mathworks) [120].

2.3.7 Statistical analysis

Data were analyzed and plotted using RStudio and the packages listed below in **Table 2**. Statistical significance was determined using the conventional threshold of $p<0.05$ and only observations that met this threshold were subjected to further analysis. Therefore, due to low sample sizes and humane endpoint deaths a number of the measurements were not able to be analyzed in a statistically robust way. In these instances, trends are visually presented, and acknowledgment is made regarding the lack of statistical significance.

2.3.8 Key resources

Key Resources	Source	Identifier
Mice		
C57BL/6J Mice	The Jackson Laboratory [103]	000664
Chemicals		
Dextran sulfate sodium salt, colitis grade (36,000 - 50,000kDa)	MP Biomedicals [97]	Catalog number: 160110 Lot number: S4140 CAS number: 9011-18-1
Stains		
DAPI D9542	Sigma-Aldrich	D9542-5MG
Fluorescein (FITC) Rhodamine red-labeled Ulex Europaeus Agglutinin I (UEA-1)	Thermo (Invitrogen)	VECTFL10612
Eub338-FITC stain (targeting 5'-GCTGCCTCCCGTAGGAGT-3')	ThermoFisher	10336022
Crystal violet	Sigma Aldrich	C0775-25G
Software		
MATLAB 2023b	Mathworks Inc. [121]	N/A
BacSpace	Earle et al., 2015 [120]	N/A
R version 4.1.2 (2021-11-01)	R Core Team (2021) [122]	N/A
RStudio: Integrated Development for R	RStudio Team (2020) [123]	N/A
dplyr v1.1.4	Wickham et al. [124]	N/A
edgeR v 3.1.8	Chen et al. (2016) [125]	N/A
ggplot2 v.3.4.1	Wickham H [126]	N/A
ggraph v.2.1.0	Pedersen TL [127]	N/A
tidyverse v.2.0.0	Wickham et al. [128]	N/A
Pheatmap v 1.0.12	Kolde (2019) [129]	
phloseq 1.22.3	McMurdie and Holmes (2013) [130]	N/A
rstatix v.0.7.2	Kassambara A [131]	N/A
Vegan v2.5.7	Oksanen K (2020) [132]	N/A

Table 2. Key resources.

Chapter 3: Gut Results and Discussion

This study investigated the time-dependency of extraintestinal endocrine and reproductive comorbidities in DSS colitis, focusing on two aspects: (1) specific developmental windows and (2) chronic DSS administration. DSS-colitis was induced in mice at different developmental time points (pre-puberty, (D0-D5), puberty (D13-D18) and post-puberty (D22-27) (see **Figure 6A** and **Figure 7A** for details). The time points were chosen based on the work of Bell (2018) and the methodology behind the chronic DSS administration was inspired by Wirtz et al. (2017) (see **Methods 2.2)** [104,133].

3.1 Results

3.1.1 DSS administration induces colitis in mice.

Acute and chronic DSS administration induced expected colitis-like symptoms such as weight loss (illustrated in **Figure 6A, 7A** and **8A**), reduced colon length (shown in **Figure 8D**), damage to the colonic epithelium and a deterioration of the mucus layer (as seen in **Figure 9**). These findings were accompanied by signs of rectal bleeding and hematochezia (see glossary). Independent of age and sex-based differences (discussed below in **sections 3.2** and **3.3**), the DSS treated mice exhibited the well-established finding that in the initial days of DSS treatment mice continue to gain weight before they enter a period of weight loss [98]. With the exception of the females that were acutely treated with DSS pre-puberty, DSS treated mice never fully recovered to the weight of healthy age-matched controls, even after two months of recovery time (**Figure 8B** and **C)**. Finally, as shown in **Figures 9,** the DSS treated mice exhibited signs of colonic epithelial damage with crypt damage and mucus degradation.

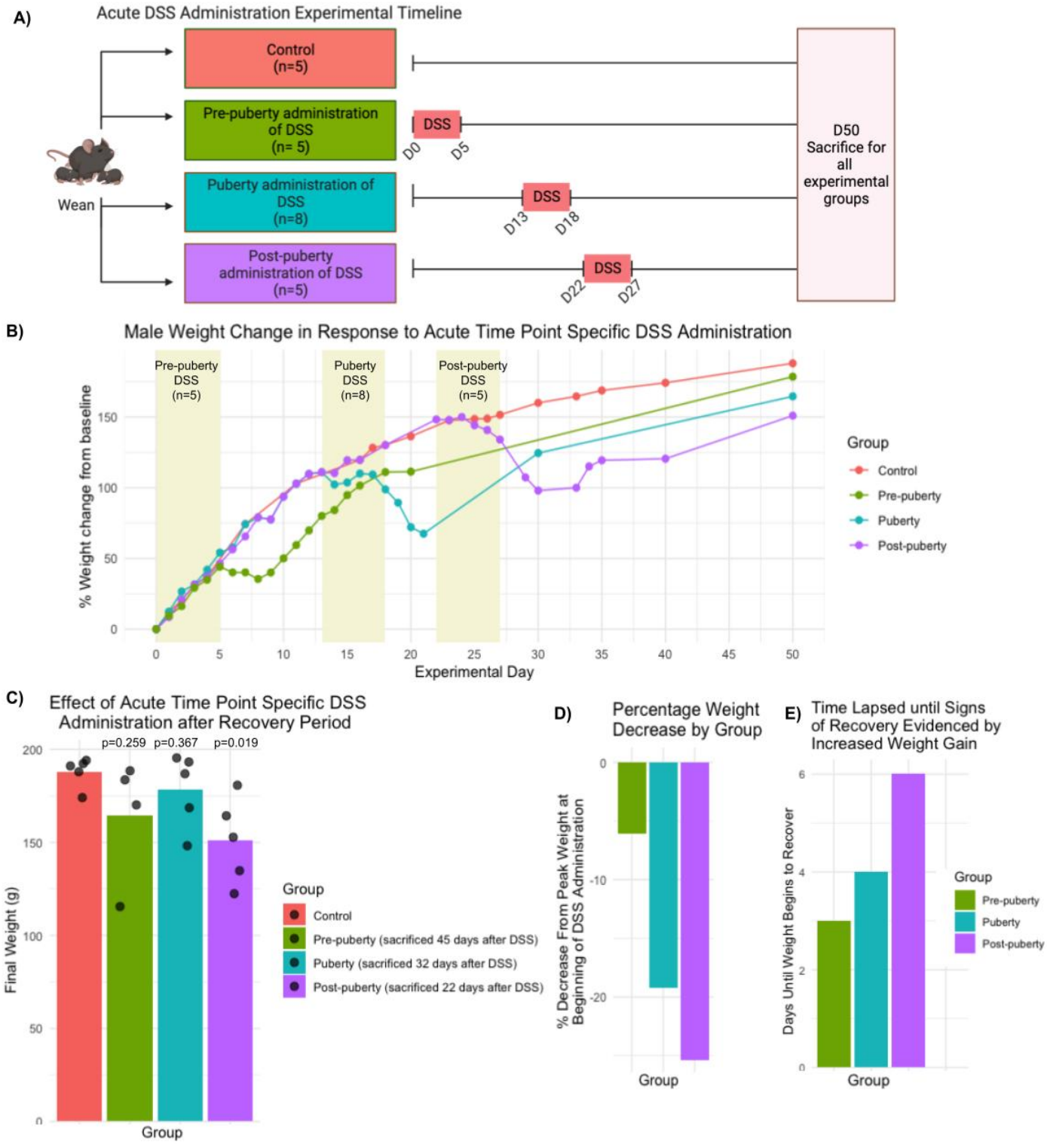


Figure 6. Impact of acute time point specific DSS administration in males A) Experimental timeline: DSS was administered acutely for 5 day periods that corresponded to specific and potentially sensitive developmental windows: pre-puberty (D0-D5), puberty (D13-18) and post-puberty (D22-27). **B)** The age-matched control group (represented in red) continuously gains weight throughout the experiment. The group treated pre-puberty (represented in green) gains weight during the initial days of DSS treatment and then rapidly loses weight for a 3 day period. The group treated during puberty (represented in teal) fluctuated in weight during the period of DSS administration and then rapidly lost weight for another 5 days. The group treated post-puberty maintained a steady

weight for the first 3 days of treatment and then rapidly lost weight over the next 5 days. Due to team logistics all of the experimental groups were sacrificed on the same day, meaning there were varying time intervals between the day of last DSS administration and the day of sacrifice on day 50. By day 50, it had been 45 days since the pre-puberty group received DSS, 32 days since the puberty group received DSS and 22 days since the post-puberty group received DSS. **C)** shows the mean final weight of each of the experimental groups compared to the healthy age-matched controls. As would be expected the group most recently exposed to DSS, the post-puberty showed the greatest weight reduction at time of sacrifice, however this result was not significant (ANOVA and Tukey HSD). Interestingly, **D)** shows that independent of time proximity relative to sacrifice, the post-puberty mice also experienced the greatest reduction in weight when measured as a percentage of their peak weight immediately prior to the beginning of DSS administration. A non-parametric Jonckheere-Terpstra test was run to test for significance in correlation between increasing age and more significant weight loss and yielded a JT = 2 and a corresponding p-value of 1. Therefore, statistical significance could not be established due to the low sample size. **E)** The average number of days it took each group to re-commence gaining weight after DSS treatment. A trend can be seen where older mice take longer to initiate recovery; however a Jonckheere-Terpstra test yielded a JT = 3 and a corresponding non-significant p-value of 0.3333 (JT = 3).

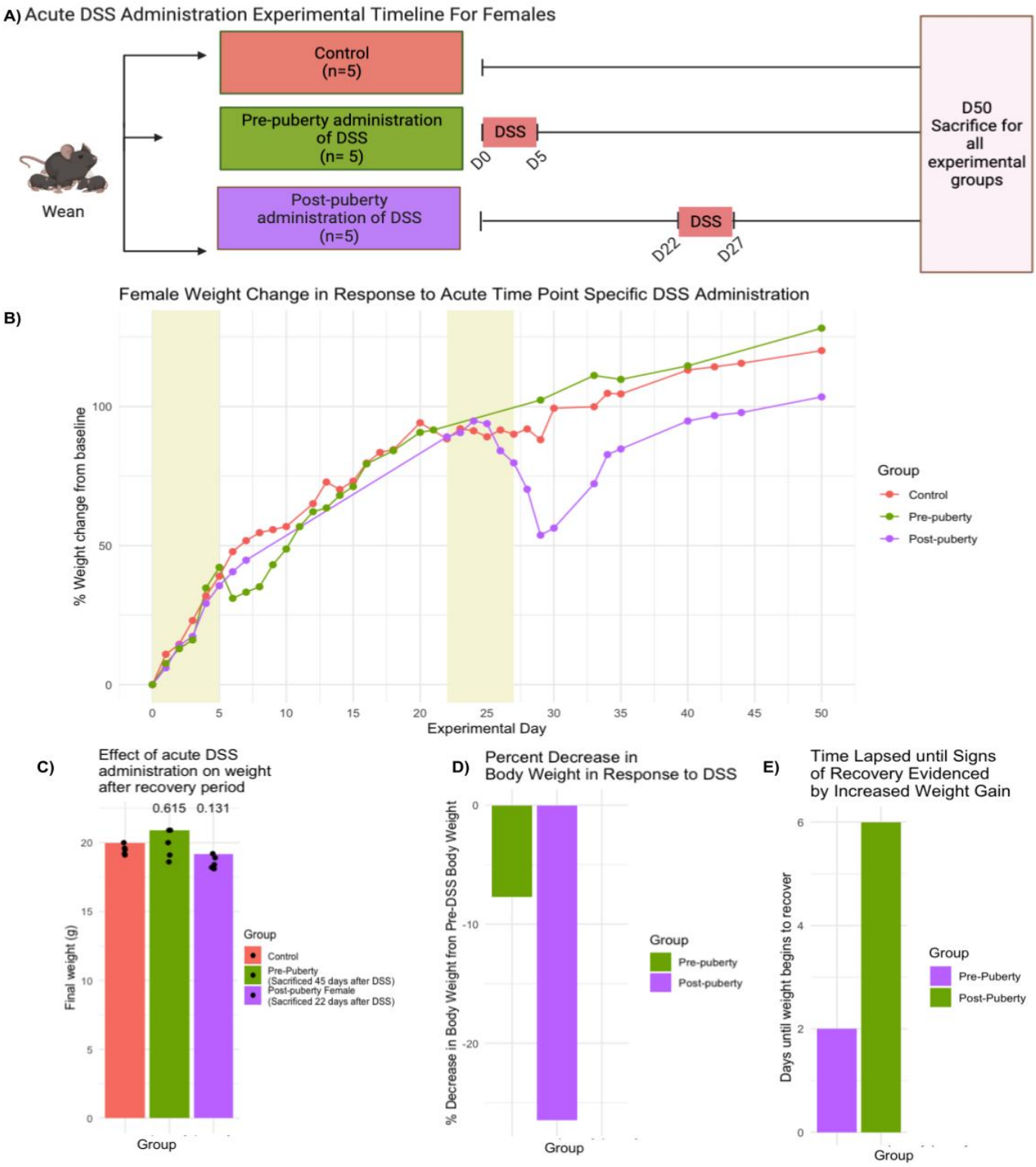


Figure 7. Impact of acute time point specific DSS administration in females A) Experimental timeline for Acute DSS treatment in females: DSS was administered acutely for 5 day periods at pre-puberty (D0-D5) and post-puberty (D22-27). **B)** The age-matched control group (represented in red) continuously gained weight throughout the experiment. The group treated pre-puberty (represented in green) gained weight during the initial days of DSS treatment and then rapidly lost weight for a 3 day period. The group treated post-puberty maintained a steady weight

for the first 3 days of treatment and then rapidly lost weight over the next 6 days. Due to team logistics all of the experimental groups were sacrificed on the same day, meaning there were varying time intervals between the day of last DSS administration and the day of sacrifice on day 50. By day 50, it had been 45 days since the pre-puberty group received DSS, and 22 days since the post-puberty group received DSS. **C)** shows the mean final weight of each of the experimental groups compared to the healthy age-matched controls at the time of sacrifice. Surprisingly, the pre-puberty group treated with DSS recovered their weight and
exceeded that of the healthy controls, however this result was not significant (ANOVA and Tukey HSD). Interestingly, **D)** shows that independent of time proximity relative to sacrifice, the post-puberty mice also experienced the greatest reduction in weight when measured as a percentage of their peak weight immediately prior to the beginning of DSS administration. Statistical significance could not be established due to the low sample size. **E)** The average number of days it took each group to re-commence gaining weight after DSS treatment. A trend can be seen where older mice take longer to initiate recovery; however, the Jonckheere-Terpstra test yielded a non-significant p-value of 0.3333 (JT = 3).

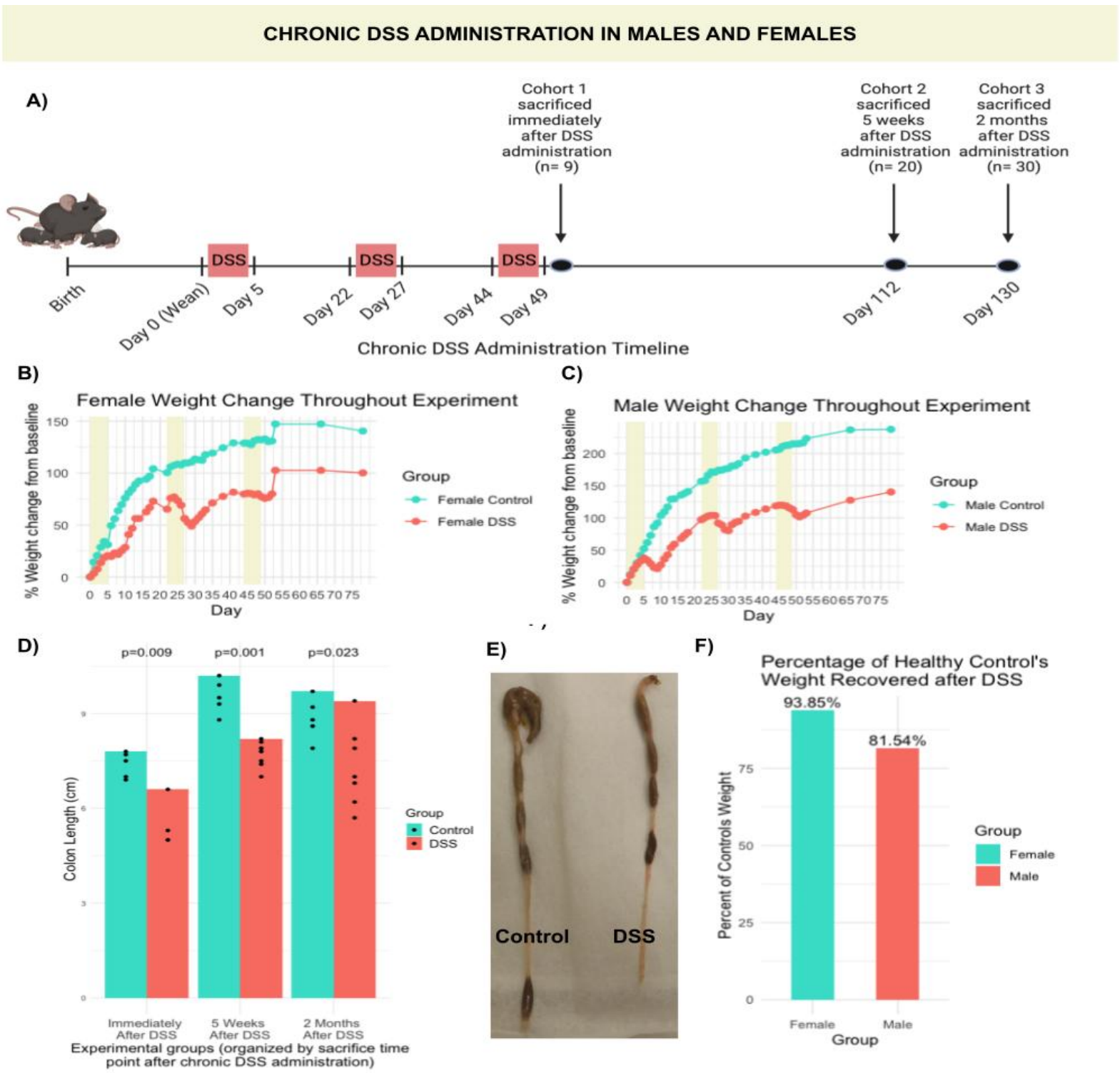


Figure 8: Impact of chronic DSS administration in males and females. Chronic DSS administration from infancy and through puberty leads to prolonged colitis-like symptoms that persist in adulthood. **A)** Experimental timeline for chronic infancy-adolescence study. 3.5% DSS was administered 3 times for 5 days. The first administration commenced immediately after weaning at postnatal day 21 (day 0 of the experiment), the second administration of DSS commenced at day 22 of the experiment, and the third at day 44 of the experiment. Mice were sacrificed at three separate time points: n=9 were sacrificed immediately after the third DSS administration, n=20 were sacrificed 5 weeks after the last DSS administration, and n=30 were sacrificed 2 months after the last DSS administration. **B)** Male and **C)** Female mice treated with DSS experienced a period of weight loss after each DSS treatment with the most extreme weight loss occurring after the first round (pre-puberty) and second round (puberty) of DSS administration. Although DSS treated mice gained weight after the periods of post-DSS weight loss they never fully recovered to the weights of healthy age-matched controls even two months after final treatment. **D)** Colon length is significantly decreased by DSS administration and this decrease is sustained even 2 months after final dose. Statistical significance was assessed using independent t-tests to compare the control and DSS groups at each time point. **E)** Photo of DSS treated and control colon. **F)** DSS treated females recover to weights closer to those of healthy controls than males do.

IMPACT OF CHRONIC DSS ADMINISTRATION ON MUCUS LAYER AND EPITHELIUM OF DISTAL COLON

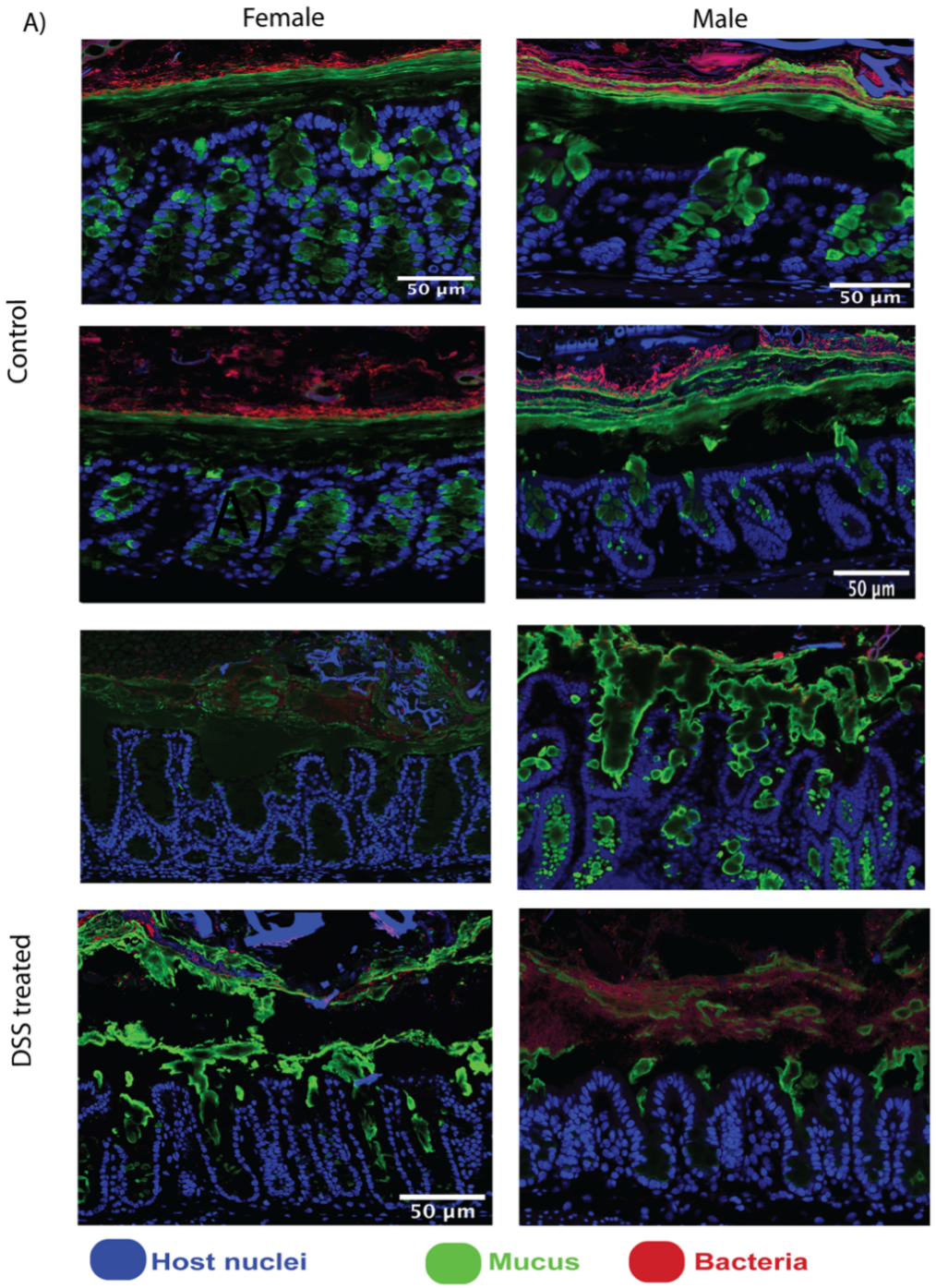


Figure 9. Impact of chronic DSS administration on mucus layer and epithelium of distal colon. A) Confocal images of control and DSS distal colon reveal changes to the quantity of goblet cell mucus secretion, the mucus architecture once secreted, and the localization of bacteria and their proximity to the epithelial cells**.** Mice were sacrificed on post-natal day 71 after 3 rounds of DSS. Sections were stained with DAPI (blue) to visualize epithelial and bacterial cells, UEA-1 (green) to show mucus and the Eub338 FISH probe (red) to better visualize bacteria. Images were taken at 100X magnification.

3.1.2 Age of exposure may be a determinant of DSS colitis severity

DSS treated mice exhibited signs of colitis at all time points, however the effects of colitis became more pronounced as the age of acute DSS administration increased. The oldest group of mice (the post-puberty group) showed the greatest signs of colitis evidenced by the most significant weight loss as percentage of body mass **(Figure 6D** and **7D)**, prolonged duration of flare up period and a delayed commencement of the recovery period **(Figure 6E and 7E)**. Conversely, the youngest group of DSS treated mice (pre-puberty) experienced the least severe weight loss and commenced recovery the fastest **(Figure 6D-E** and **7D-E)**. Validation of this trend was difficult given the low sample numbers and the fact that it is not possible to compare to age-matched controls since non-treated mice would not experience weight loss. I did however observe a partial continuation of this trend in the chronically treated mice - **Figure 10A and 10C** show that as mice aged, they exhibited more severe colon shrinkage and diminished ability to recover weight. However, these results cannot be solely attributed to age due to the compounding effects of repeated DSS exposure.

Despite the above-mentioned limitations, the correlation between increased age and DSS severity has been shown in prior research. Specifically, Park, et al. (2015) showed that 9 week old mice responded more to DSS than 5 week old mice, however their sample number was not high enough to ensure statistical significance [134]. Liu et al. (2020) showed that DSS severity is intensified in 14-18 month mice (human equivalent of 56-69 years) when compared to 6-8 week old mice (human equivalent of 12-20 years old) [135]. Albert & Marshall 2008 similarly showed that mice > 14 months old responded to DSS more intensely than 8-12 week old mice[136] . Beyond the DSS model, other studies particularly with the Muc2(-/-) model have compared IBD progression pre and post weaning[137] . However, to our knowledge this is the first attempt at providing further granularity in terms of age stratification when observing the effect of age on DSS severity.

To further understand the role of age in modulating DSS severity I sought to rule out other factors that could contribute to the variability. These factors include the concentration of DSS [138], influence of sex [139], and the microbiota composition [140,141] . Of these, the former can be ruled out as the mice were administered the same concentration of DSS from the same batch, and the latter two, sex-based differences and initial microbiota composition, will be addressed below in sections 3.1.3 and 3.1.4 respectively

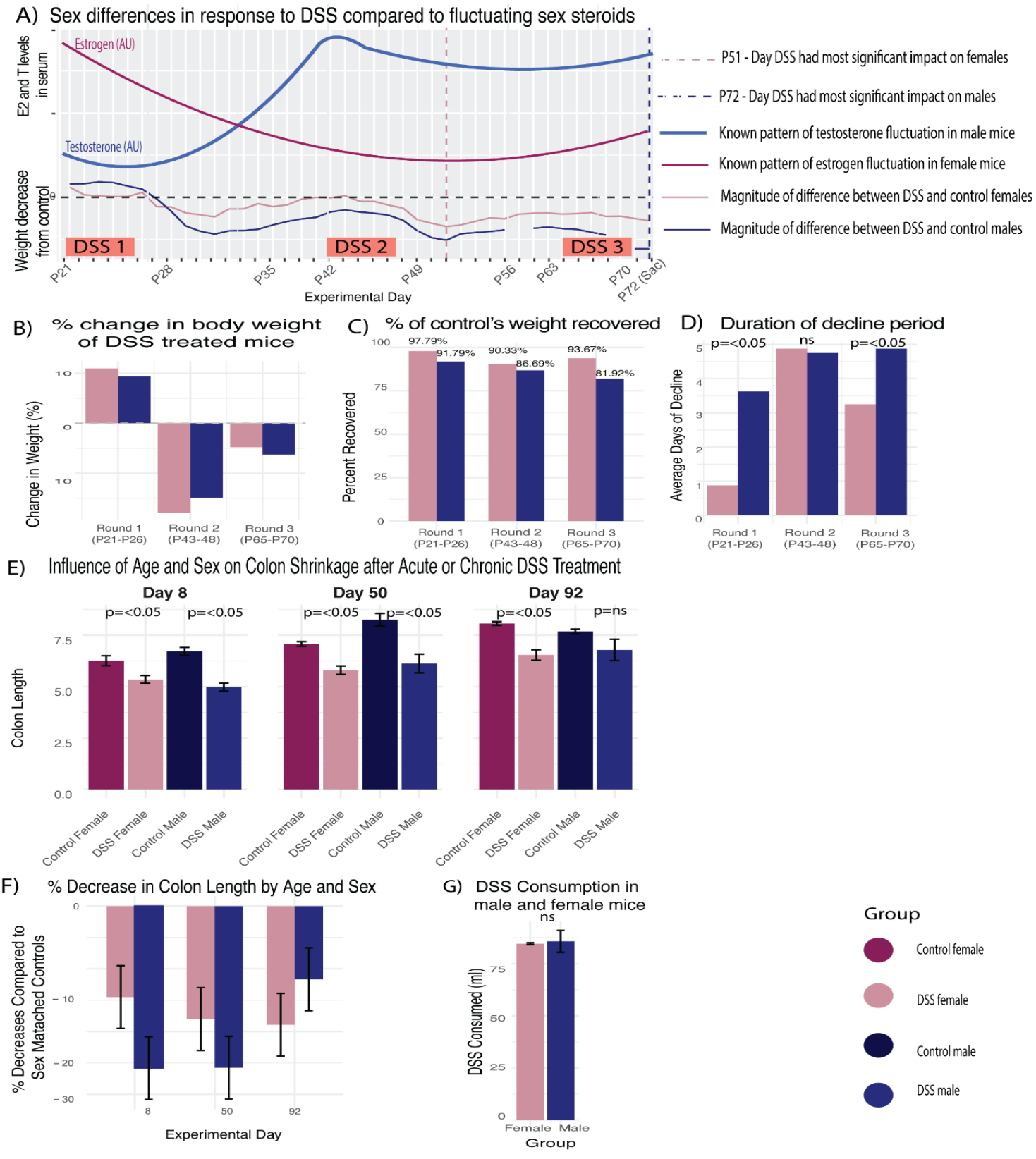


Figure 10. Age differences in DSS severity are driven by sex. A) Weight varied by age and by sex in response to 3 and 3.5% DSS treatment. The graph shows the difference between the weight of the DSS treated group and the weight of the controls by sex over time. The pink dotted line shows the P52 time point at which the female weight

differed most from the control weight, corresponding to one of the lower points in circulating estrogen levels. The blue dotted line shows the P72 time point at which the male DSS group differed the most from the control male weight. **B)** The bar graph shows the change in body weight as a percentage of the starting body weight for the DSS treated males and females for each DSS administration in the chronic DSS experiment. **C)** The bar plot shows the amount of weight recovered by each of the treatment groups when compared to age matched controls after each DSS administration period. **D)** The bar plot shows the duration of the DSS induced decline period. The decline period was counted as days on which individual mouse weights were lower than their weight the day prior. An independent T test was then used to compare the means between the male and female groups. For round 1 and round 3 the differences were significant ($p>0.05$) whereas for round 2 no significant difference was observed. **E)** The impact of DSS on colon length varied by age and sex, reflecting the same trend seen in the weight differences. The Shapiro-Wilk test was used to test for normality (>0.05) and a t test was performed to compare the difference in length between the DSS and control groups for each sex and the p-value is listed on the graph. **F)** The bar plot compares the percentage that the DSS treated mice shrunk when compared to age and sex matched controls. The female mice that were treated with DSS pre-puberty when estrogen levels were high experienced protection from DSS severity compared to their male counterparts. As the females aged this protective effect decreased and at post-puberty the male colon lengths were less affected than the females. (Day 8: control female n= 7, DSS female n = 6, control male n = 8, DSS male n = 9. Day 50: control female n = 4, DSS female n = 4, control male n = 4, DSS male n = 4, Day 92: control female n = 3, DSS female n = 4, control male n = 4, DSS male n = 4). **G)** Male and Female mice consume similar levels of DSS throughout the experiment.

3.1.3 Sex-based differences may drive variation in age-dependent DSS severity

Sex-based differences may drive faster and more complete recovery in females compared to males after DSS-colitis in an age dependent manner. In both acute and chronic DSS colitis there were observable sex based differences in the age driven changes in DSS susceptibility. For example females initiated recovery faster (**Figure 6E** and **Figure 7E)**, and showed greater ability to recover their weight (**Figure 6B** and **Figure 7B),** and colon length (**Figure 8F).** Due to low sample sizes, I was unable to determine the significance of these observations. To add statistical validity to the inquiry, I combined my data with data from a preliminary iteration of this experiment conducted by a lab member Dr. Kat Ng. The experimental set up was identical with the exception of the DSS concentration being 3.0% w/v instead of 3.5% w/v and from a different batch from the same manufacturer. Combining the data sets showed there are statistically significant differences in how sex mediates age driven severity. **Figure 10A** shows that the age of peak susceptibility to DSS is different for males than females. The magnitude of difference in weights between the DSS treated females and the control females is greatest at P51, after the second DSS treatment when estrogen levels are low. Males are most affected compared to controls in older age after the third DSS treatment when testosterone levels are at their peak (**Figure 10A)**. Further, at all time points males experience longer periods of DSS-induced weight decline (**Figure 10D),** slower recovery times (measured by days until commencement of sustained weight gain) and a less complete recovery when compared to the weight of healthy age and sex matched controls (**Figure 10C)**. Further, males experience greater colon shrinkage (**Figure 10E)**. When mice are sacrificed after a period of recovery female colon lengths are 18% shorter than age matched controls while male colon lengths are over 25% shorter than healthy controls (**Figure 10D).**

Given that the severity of DSS is tightly linked to the exposure to DSS, I sought to test if variability in DSS consumption could be a dependent variable. I measured the amount of DSS each cage drank each day and found that there was no statistically significant difference in DSS consumption between the male and female mice during the chronic DSS experiment (**Figure 10G).**

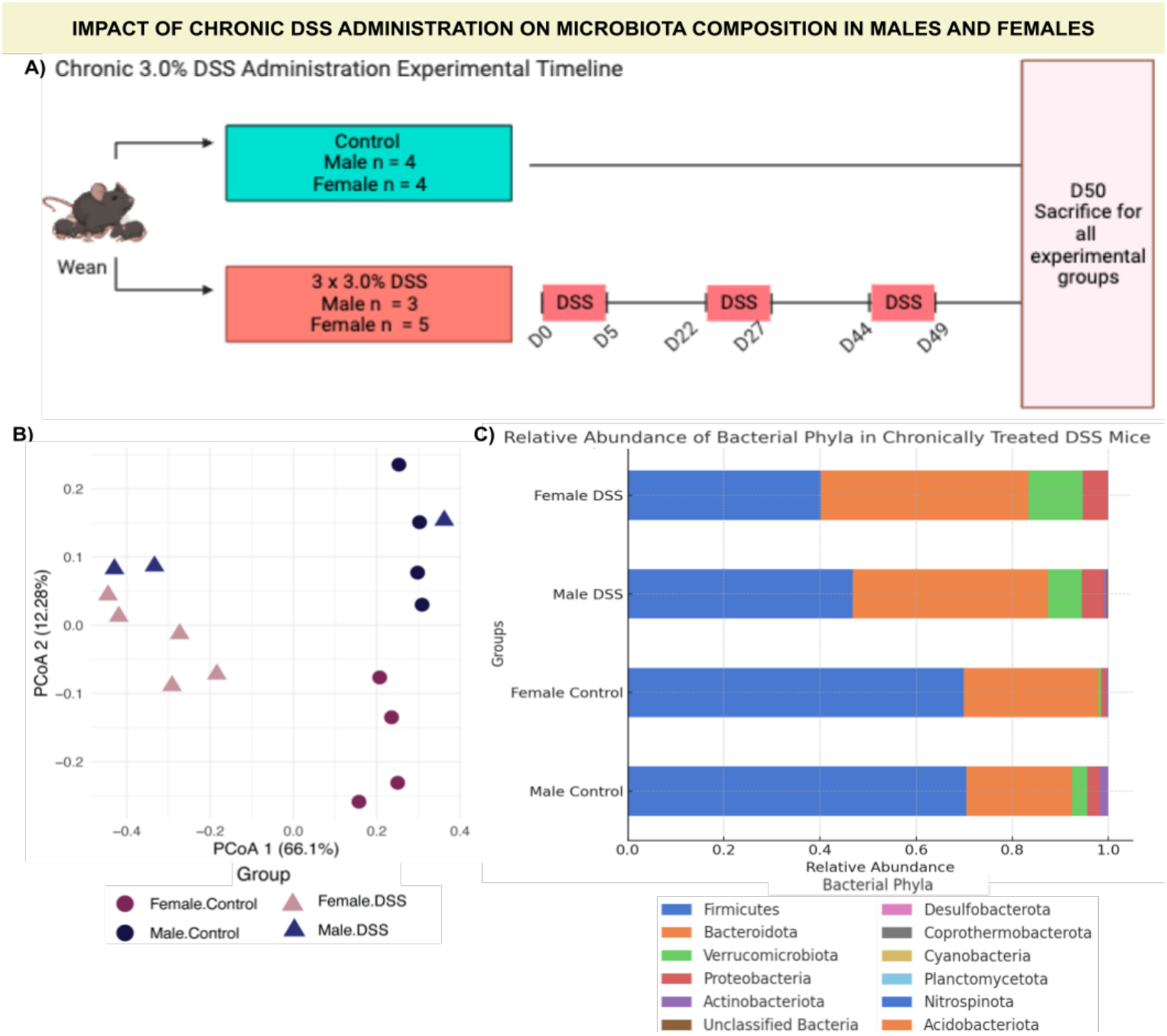


Figure 11. Impact of chronic DSS administration on microbiota composition in males and females. A) Experimental timeline for 3.0% weight/volume DSS experiment (n=4 per sex for the control group, 3 males and 5 females in the DSS treated group). This experiment was performed by Dr. Kat Ng and provided 16s rRNA data. **B)** PCoA plot showing beta diversity using Bray Curtis dissimilarity. The DSS samples cluster together and the control samples cluster together showing that DSS is driving a difference between the two groups. **C)** Relative abundance of 10 most abundant bacterial phyla in male and female DSS and control groups.

3.1.4 Microbiota composition is similar in males and female DSS treated mice

To try and further understand what could be driving the age and sex related variability in DSS severity I sought to investigate the potential contribution of the microbiota. An expanding body of research has highlighted the role of the microbiota in modulating DSS severity [140–142], and

work by Song et. al has alluded to the role of estrogen in shaping the microbiota and altering DSS susceptibility [143,144]. Due to practical limitations, I was unable to perform 16s rRNA sequencing on the stool collected in my acute and chronic experiments however I was able to analyze the aforementioned data set generated by Dr. Kat Ng. As shown in **Figure 11,** Dr. Ng's experiment mice were subjected to 3 rounds of 3.0% weight/volume DSS during the same developmental periods as the mice in my chronic DSS experiments. Mice were sacrificed at postnatal day 72 and day 50 of the experiment and cecal contents were collected for sequencing.

Figure 11b shows that DSS did drive changes in the microbiota of treated mice**.** Figure 11c shows that at the phylum level there was a shift in the Bacteroides/Firmicutes ratio in both the male and female mice treated with DSS. In both sexes, though more pronounced in the females, there was an increase in the relative abundance of Verrucomicrobiota. Multiple species within the Verrucomicrobiota phylum are known to be upregulated in response to estrogen, which is known to have a protective role against DSS (see discussion) [143]. However, when these shifts were tested for statistical significance with Mann-Whitney U tests they all returned p-values > 0.05.

3.2 Discussion

These results contribute to the emerging evidence that age and sex play a role in modulating the severity of DSS-induced colitis, particularly in the context of hormonally sensitive critical windows and sex-based differences that change throughout the lifetime. My results highlight that both age and biological sex alter the complex environment in which colitis acts and therefore warrant further investigation in terms of how they contribute to the experience of human patients with IBD.

Impact of developmental stages on DSS colitis severity

The observation that DSS colitis increases in severity with the age at the time of DSS administration highlights the importance of understanding the impact of age-related changes in inflammatory bowel disease. In some ways, this finding is surprising: colonic crypts in young mice are known to be fragile and easily broken [145] and the expression of CD44, a key protein known to play a role in protecting crypts from DSS damage [146], is at its lowest in the juvenile period [147,148]. Despite that, our results showing that younger mice (pre-puberty) exhibit greater resilience to DSS-induced colitis, experience less severe symptoms, and recover faster

match similar findings in the literature [135]. While the precise mechanism driving this phenomenon remains undefined, there is a lot of evidence in the literature pointing to potential explanations. I will confine this discussion to the spatially adjacent potential contributors (the epithelial lining, the mucus layer, and the microbiota) as their role in IBD, as well as their response to DSS, is well characterized and they all change across the lifetime. Further, I will highlight the sex-specific differences in their aging behavior and how they could potentially explain my results.

Age and the robustness of host epithelium

Previous studies in other organisms have repeatedly shown that the integrity of cell junctions deteriorates with age and as a result leads to compromise in the integrity of the intestinal barrier [149,150]. In fact, the relationship between intestinal barrier dysfunction and aging is so well conserved across species that it has been described as an evolutionary hallmark of aging [151]. It is thought that as an animal ages there is an increase in the number of intestinal epithelial cells produced but that simultaneously their individual quality declines [152,153]. Interestingly, sex differences have been reported in the gut epithelium age related decline - females experience more widespread structural damage to the epithelium but have a higher epithelial cell replacement rate, and males have a lower cell turn over but are less robust in responding to challenges [154]. This means that while the female gut is young it is more equipped to respond to insult and infection, but in old age is more prone to tumors [154]. Conversely, the male gut succumbs to challenges the female gut is able to overcome in early life, but when unopposed by damage, is structurally more intact in later life [154]. In addition to differences in the enterocytes, the abundance of T cells (CD3+) also changes with age and is influenced by sex - old mice have a higher percentage of T cells compared to younger mice and males have a higher percentage compared to females [155]. Finally, sex hormones are also known to have a role in modulating the integrity of the mucosal layer, and as a result there are sex differences in the integrity of the mucosal layer across the lifespan [156].

Mucus

In the same way the gut epithelial layer changes with age, the mucous layer also deteriorates with age [155,155,157,157,158]. Given that the mucous layer is critical in protecting against IBD [159], its temporal decline could provide another explanation for the age-related differences in DSS severity that are described in the literature[155]. Unlike the epithelial layer, the age-related decline of the mucus layer is not sex dependent. Although older mice have a thinner mucous

layer than younger mice, and females have a thicker mucus layer than male mice, there is not an interaction between these effects [155]. However, in the case of DSS, it is believed that eventual decreased availability of mucus is not due to the direct assault of DSS, but rather the waning availability of goblet cells capable of producing more mucus [160]. Consequently, although decline in mucus production is not normally sex biased, in the case of DSS, the slower epithelial turnover in males could decrease their resilience, providing another potential explanation for their heightened susceptibility, and highlighting a nuanced interaction of age, sex and disease severity.

Age, Sex and the Microbiota

Finally, the microbiota is increasingly recognized for the changes it undergoes and creates in relation to age and sex. One way that age could affect both DSS susceptibility in mice and IBD severity in humans is the natural age-related changes in the microbiota that are driven by changes in sex steroid hormones [161–164]. Estradiol is a key hormone that undergoes major fluctuations across the lifetime, and has been identified driving shifts in the gut microbiota that alter susceptibility to DSS [165]. Although estradiol has previously been shown to alter the microbiota composition in a direction that increases susceptibility to DSS [143], there are signs that it plays a protective role against DSS colitis and IBD. For example, one study found that ovariectomized mice experienced more severe DSS colitis but this effect was reversed when they were supplemented with estradiol [166]. Beyond the DSS model, estradiol is known to mediate weight changes and IBD severity in a microbiota-influenced manner [167,168]. This will be discussed in the next chapter.

Conclusion

In summary, these findings demonstrate the complex interplay between age, sex and DSS severity. Although this work is in mice, it highlights the necessity for a nuanced approach in understanding IBD in human patients, taking into account the complex interplay between age, sex, and biological changes in the gut. As the prevalence of IBD in younger populations continues to rise, furthering our understanding of these factors is vital. The author hopes that this study will contribute to paving the way for future research to further unravel the complexities of IBD, aiming for more targeted and effective treatment strategies that consider the patient's age and sex, thereby enhancing the quality of care and outcomes for individuals suffering from this condition.

Chapter 4: Endocrine results and discussion

It is known both clinically in humans and experimentally in mice that IBD induces extraintestinal disruptions to the endocrine and reproductive systems. Preliminary experiments performed by members of the Tropini lab and Ciernia lab at UBC hinted at DSS colitis disrupting sexual development in juvenile mice. Mice that were administered DSS in the juvenile period showed signs of reproductive underdevelopment at time of sacrifice in the form of immature sex organs. These findings were corroborated by others in the literature who also found that DSS treated can halt reproductive development [24,101]. This led me to explore whether these changes are time dependent based on critical developmental windows, or time dependent based on duration of exposure to DSS. Further, I sought to evaluate the degree of colitis severity in the gut corresponded with the severity of endocrine and reproductive abnormalities.

4.1 Results

IMPACT OF ACUTE AND CHRONIC DSS ADMINISTRATION ON REPRODUCTIVE ORGAN WEIGHT

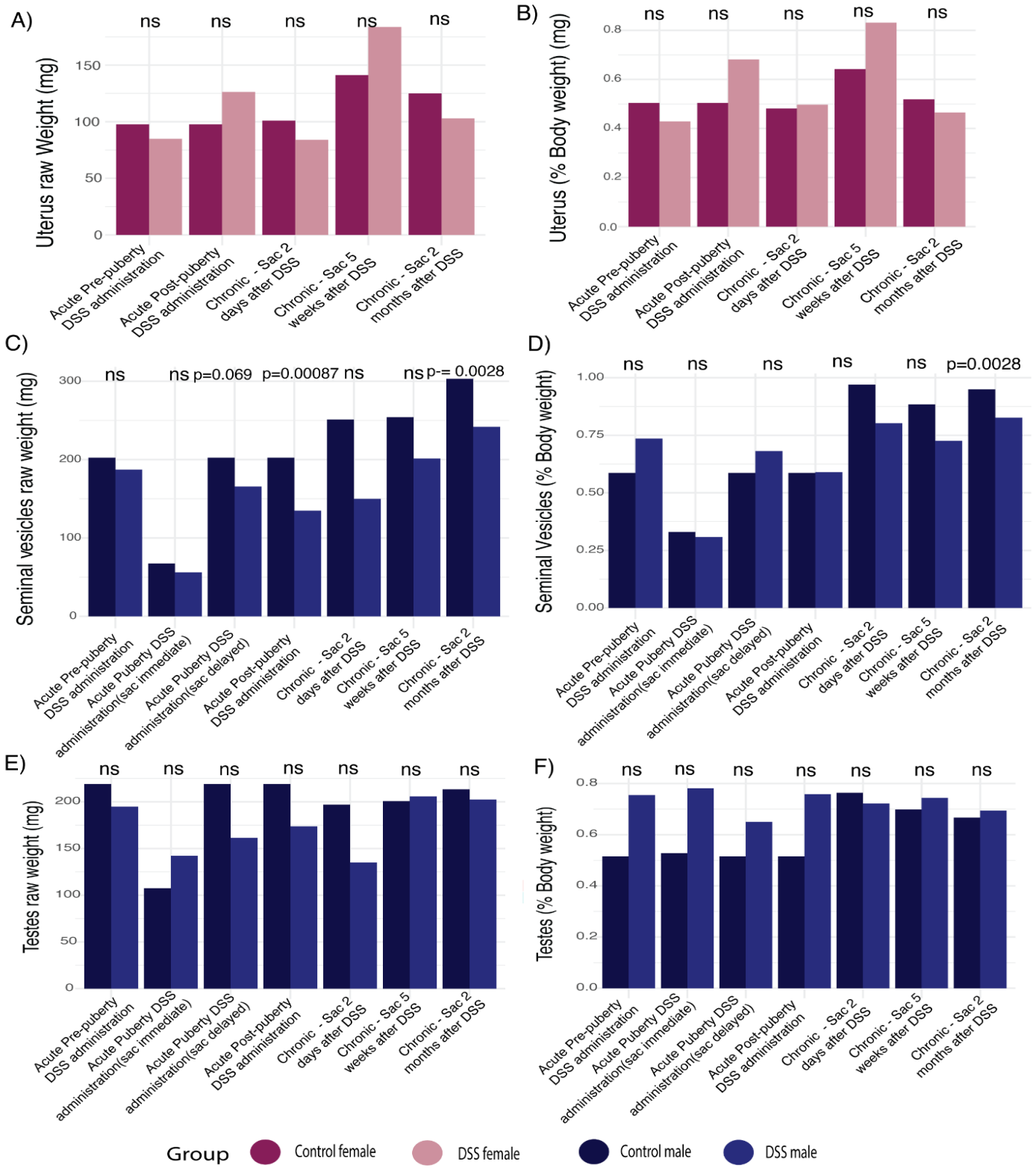


Figure 12: Comparative analysis of acute and chronic DSS treatments on murine reproductive organs. Sample sizes for each group are as follows - Female mice: Acute pre-puberty (DSS n=5, control n=4), Acute post-puberty (DSS n=5, control n=4), Chronic - sac 2 days after DSS (DSS n=3, control n=2), Chronic - 5 week sac (DSS

n=5, control n=5), Chronic 2 month (DSS n=8, control n=7). For the males: Acute pre-puberty (DSS n=5, control n=5), Acute puberty immediate (DSS n=4, control n=5), Acute puberty delayed (DSS n=4, control n=3), Acute post-puberty (DSS n=5, control n=5), Chronic - sac 2 days after DSS (DSS n=1, control n=3), Chronic - 5 week sac (DSS n=5, control n=5), Chronic 2 month (DSS n=8, control n=7). Shapiro-wilk tests were used to test for normality and then independent t-tests were performed using Welch's correction to account for variance inequality. Bars represent the mean organ weight for each group and p-values are labeled where relevant.

4.1.1 Chronic DSS administration causes changes to reproductive organ weights

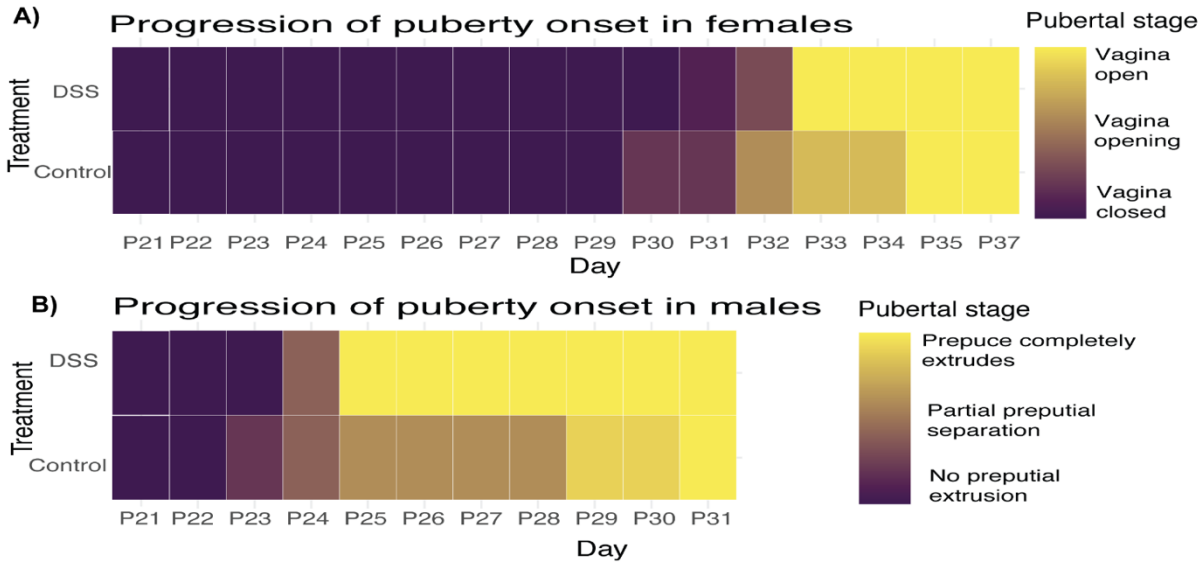
Reproductive organ weights have been found to be statistically powerful and physiologically sensitive endpoints to look at when determining reproductive damage [169]. Further, changes in male reproductive organ weights have been found to reflect changes in inflammatory status [170]. Therefore, measuring reproductive organ weights provided a method to quantitatively explore the preliminary observations of the reproductive underdevelopment made by Dr. Kat Ng and members of the Ciernia lab.

Figure 12 shows that chronic exposure to DSS causes a significant difference in weight to the seminal vesicles 2 months after chronic DSS treatments, but not 5 weeks after DSS treatments. Beyond the seminal vesicles, there were no significant differences in the weights of the reproductive organs after acute and chronic administration of DSS. However, there was a decrease, albeit insignificant, in the weight of the testes after acute DSS treatment in the juvenile, pre-puberty and pubertal stage (**Figure 12E**). Similarly, there were no significant differences in the weights of the seminal vesicles but there was an apparent insignificant decrease for the DSS treated groups (**Figure 12E**). There was no significant difference in the uterine weights between groups but there was insignificant variability among groups. This could potentially be due to the varying stages of the murine estrous cycle that the mice were in (see **Figure 16**).

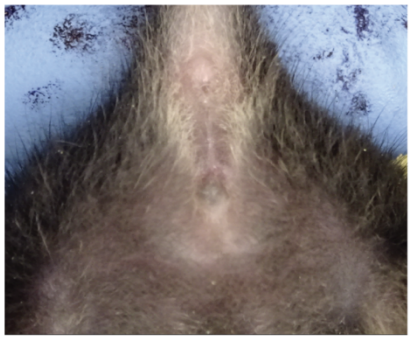
4.1.2 Chronic DSS treatment does not appear to cause signs of gonadal histopathology

In order to further investigate the macroscopic signs of reproductive organ underdevelopment we consulted with Dr. P Nation of Animal Pathology Services to assess whether there were accompanying microscopic changes to the reproductive organs. Specifically, in the males we sought to investigate whether there were changes to the mucosa of the seminal vesicles [171] or changes to the diameter of the epithelium of the seminiferous tubules [171]. In the females we sought to investigate whether there were changes to the endometrium and the oviducts [172]. Three sets of control male and female organs and 2 female and one male DSS treated set of organs were sent to Dr. Nation (due to premature death in the DSS group).

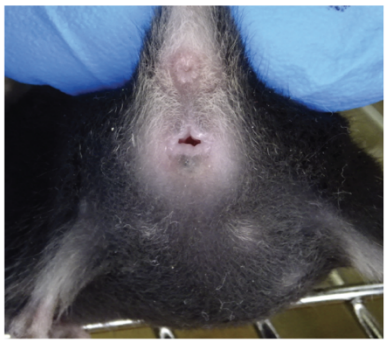
Upon investigation Dr. Nation found that in the females the ovaries, oviducts, uterus, cervix, and vaginal wall were all microscopically normal. In the male there was slightly reduced thickness of the mucosal epithelium of the seminal vesicles but the content of the seminal vesicles was microscopically normal.



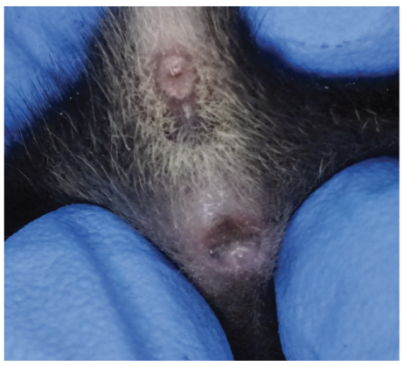
C) Pre-pubertal closed vaginal orifice



D) Post-pubertal opening of vaginal orifice



E) Pre-pubertal absence of preputial separation



F) Post-puberty preputial extrusion

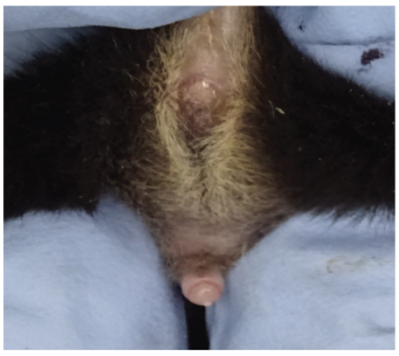


Figure 13. Impact of DSS on timing of pubertal onset. **A)** Heat map showing the progression of puberty onset in DSS treated females and age-matched controls (n=5/condition). The results show that DSS administration does not affect the timing of puberty onset in a statistically significant manner (t=-0.201, p=0.841). **B)** Heat map showing the progression of puberty onset in DSS treated males and age-matched controls (n=5/condition). The results show that DSS administration does not affect the timing of puberty onset in a statistically significant manner (Mann-Whitney U=20.0, p=0.114). **C)** Photograph showing pre-pubertal female mouse. The absence of a visible vaginal opening indicates immaturity and the pre-pubertal state in female mice. This would have been coded as 1 (no vaginal opening**). D)** Photograph showing post-pubertal female mouse. This image shows the open vaginal orifice below the anus. This would have been coded as 3 (vagina open and puberty complete). **E)** Photograph showing pre-pubertal male mice where the foreskin remains intact and preputial separation has not yet occurred. This mouse would have been coded as 1 (no preputial extrusion). **F)** Photograph of extruded penis after prepuce has completely separated marking the completion of the puberty milestone. This mouse would have been coded as 3 (prepuce completely extrudes).

4.1.3 DSS administration does not appear to alter timing of pubertal onset

Previous studies have reported the role of DSS in delaying pubertal onset. I sought to build on this work by investigating whether there are critical windows during which mice are particularly susceptible to DSS in terms of its effect on puberty. In order to do this we timed our experiments to coincide with known developmental and endocrine milestones. Male and female mice were treated with DSS before and during puberty (**Figure 6A** and **7A**). **Figure 13A** shows a heatmap showing the progression of puberty onset in DSS treated females and age matched controls. The results show that DSS did not affect the timing of puberty onset in a statistically significant manner. In fact, contrary to findings in the literature that show that DSS delays puberty, DSS treated male mice commenced puberty earlier than age-matched controls albeit in a non-significant manner.

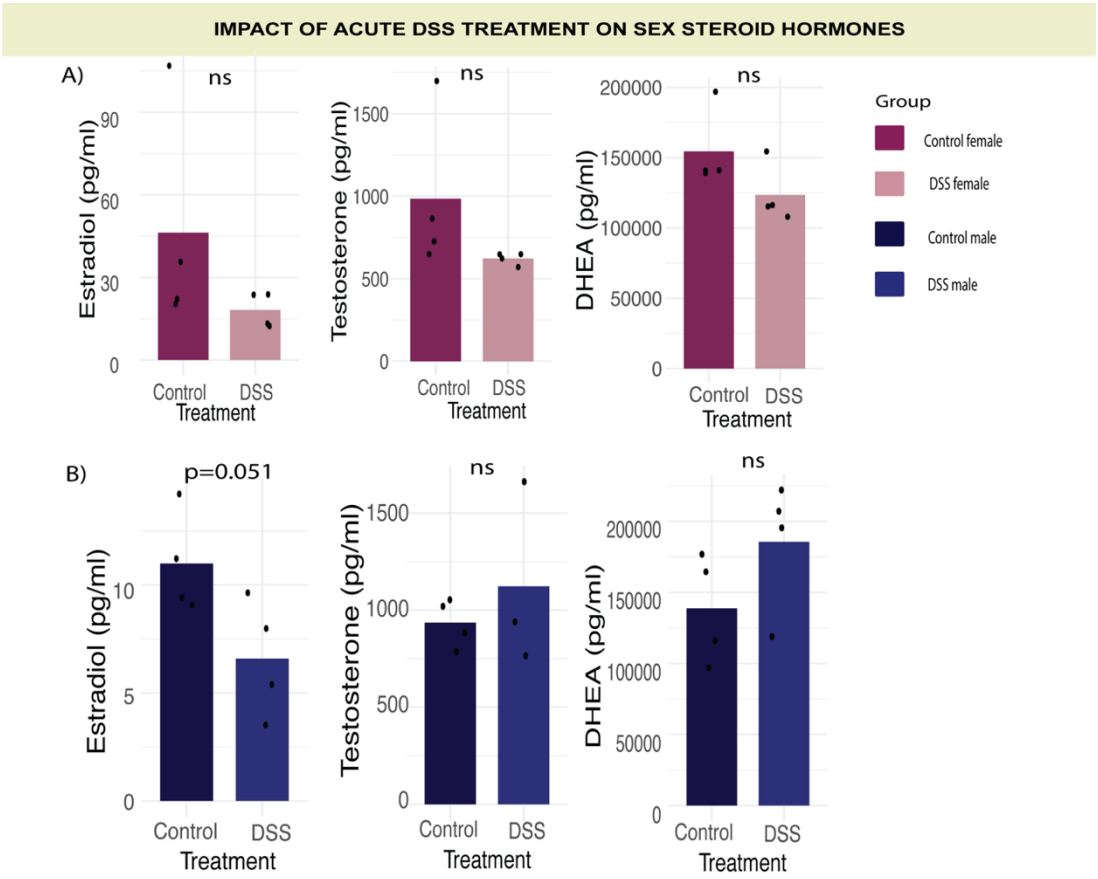


Figure 14. Impact of acute DSS treatment on sex steroid hormones. Acute time point specific DSS administration does not appear to be sufficient to significantly alter serum sex steroid levels. **A)** Bar graphs show the difference in the serum sex steroid concentration between female mice that received acute DSS treatment (at the post-puberty time point post natal day 43-49) and age matched controls (4/condition). Shapiro-Wilk tests and Levene's tests were used to test for normality and equality of variance. Independent t-tests were conducted to reveal no significant difference in the estradiol (t=-1.349, p=0.226), testosterone (t=-1.49, p=0.187), or DHEA (t=-1.76, p=0.128). **B)** Bar graphs show the difference in serum sex steroids between male mice that received acute DSS treatment (at the post-puberty time point post natal day 43-49) and age matched controls (4/condition). Shapiro-Wilk tests were used to test for normality. Independent t-tests were conducted to reveal a close to significant difference in estradiol (t=2.44, p=0.051), no significant difference in testosterone (t=0.77, p=0.48) or DHEA (t=1.567, p=0.168).

4.1.4 Acute DSS administration does not alter systemic levels of androgenic steroids but may alter estrogen levels in males

Figure 13 shows that acute administration of DSS at the post puberty time point (post-natal day 43-49) did not significantly alter systemic levels of testosterone or DHEA. Interestingly, **Figure 13B shows that** all of the DSS treated male mice exhibited a decrease in estradiol levels that approached significance (p=0.05). The female mice also showed a decline in estradiol levels however it was not significant. These results match similar findings in the literature showing that DSS seems to have an effect on reproductive organs, sex steroid hormones can remain unaffected [11]. Despite this, we wanted to test if chronic DSS administration would induce changes in sex steroids.

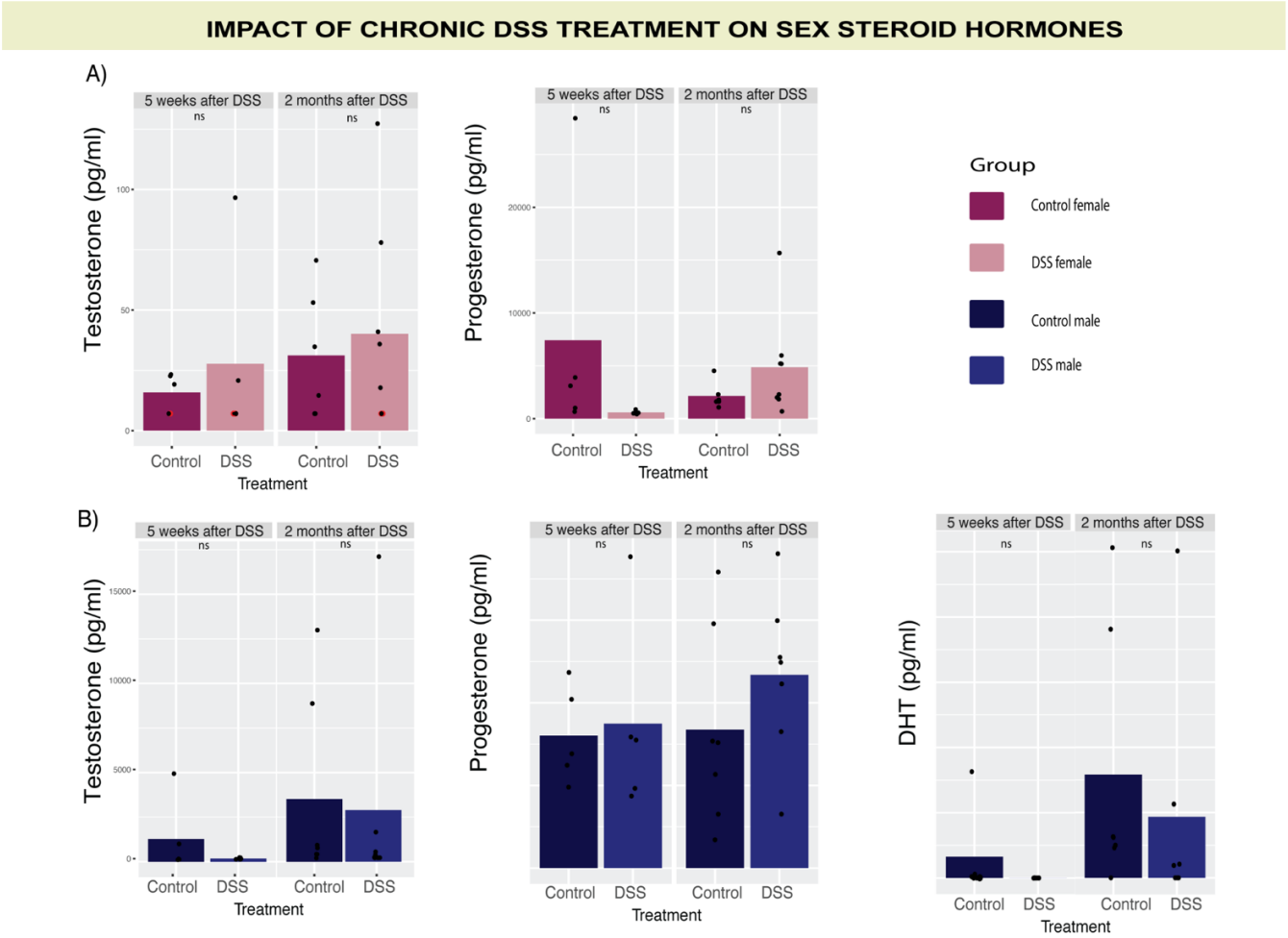


Figure 15. Impact of chronic DSS treatment on sex steroid hormones. Chronic DSS administration does not induce changes in systemic sex steroids. **A)** Bar graphs show serum testosterone and progesterone levels in females treated with 3 rounds of DSS and healthy age matched controls at two time points. The first time point was 5 weeks after the 3rd and final round of DSS administration (DSS n=5, control n=5), and the second time point was two months after the 3rd and final administration of DSS (DSS n=8, control n=7). Shapiro-Wilk tests and independent t-tests were carried out to test for normality and significance respectively, however none of the results returned a significant p-value. **B)** Bar graphs depict serum testosterone, progesterone and DHEA levels in male mice that received 3 rounds of DSS and healthy age-matched controls. Samples were collected 5 weeks after the final DSS administration (DSS n=8, control n=7). Shapiro-Wilk tests and independent t-tests were carried out to test for normality and significance respectively, however none of the results returned a significant p-value.

4.1.5 Chronic DSS treatment does not induce lasting changes in systemic sex steroids. After examining the effects of acute DSS administration of serum sex steroids I wanted to investigate whether chronic DSS administration would yield a different result. Unfortunately, due to sample size and practical limitations, we were unable to test the sex steroids immediately after DSS treatment. However, in our collaboration with the Ciernia lab, Dr. Jordan Hamden did test sex steroids 5 weeks and 2 months after DSS administration and the results are shown in **Figure 14.** There were no significant differences in sex steroids in males or females but there was a trend in the testosterone levels in the females as the DSS treated females had increased testosterone relative to controls at both the 5 week and 2 month time point. To further probe this trend, I analyzed the steroid data by the estrus stage of the females (**Figure 16)**.

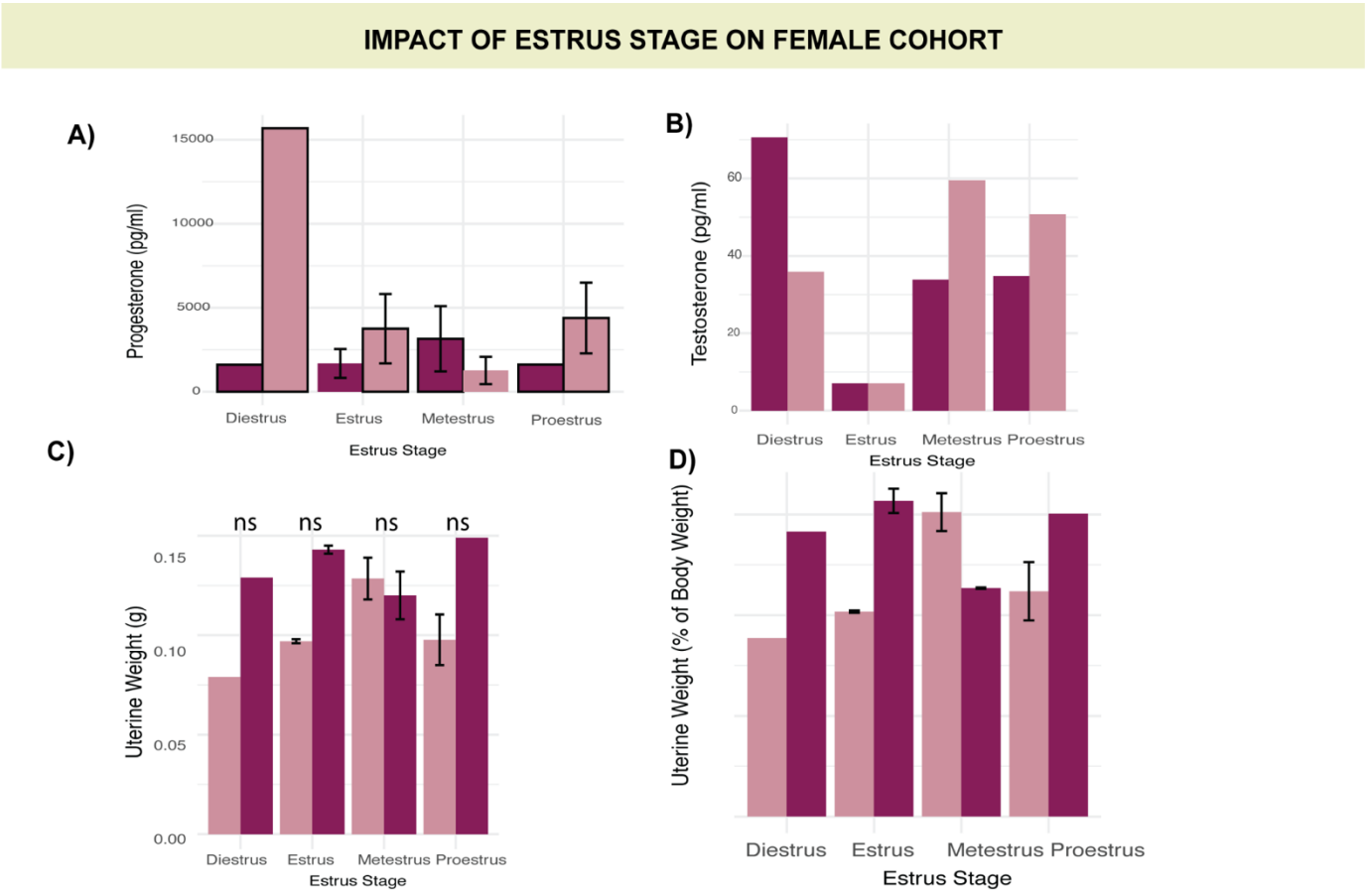


Figure 16. Influence of murine female estrus stage on sex steroids and reproductive organ weight. Murine hormones fluctuate throughout the course of the estrus cycle. **A)** Bar graph depicts the changes in progesterone in DSS treated mice and age matched controls. In healthy control there is a peak during diestrus and lower levels during estrus, metestrus and proestrus. In DSS treated mice there is a peak in diestrus and a lower peak in proestrus. **B)** Bar graph showing testosterone levels in mice chronically treated with DSS and age matched controls. **C)** Bar graph depicts uterine weights for chronically treated DSS mice and age matched controls sorted by estrus stage. **D)** Bar graph depicts uterine weights as percentage of body weight sorted by estrus stage.

4.1.6 Murine estrus stage may influence endocrine susceptibility to DSS disruptions but does not seem to drive changes in reproductive organ weight

Figure 12A shows that progesterone levels varied significantly by estrus stage between chronically treated DSS mice and healthy age matched controls. The progesterone levels for controls represent expected progesterone concentrations for each estrus stage in a healthy mouse. Progesterone levels are elevated in estrus and metestrus and peak during metestrus. The progesterone levels for the DSS treated mice. **Figure 16 C** and **D** show that estrus stage does not have a significant effect in driving the differences between uterine weights in DSS treated females and healthy age matched controls. This contradicts the results we anticipated from the literature that suggest the uterus is highly sensitive to cyclical changes [173–175].

4.2 Discussion:

The results in this chapter both add evidence to recent observations of the impact of DSS on sexual development, and also contradict some recent findings. While the sample sizes are low, the trends represented suggest further work is needed to understand the complex interplay between microbial, immune, dermatological and endocrine systems at work in the progression of DSS colitis.

Impact of DSS on reproductive development

The quantification of the observation that DSS treatment in juvenile mice can lead to reproductive underdevelopment of the seminal vesicles in mice is significant. Further, the fact that this finding persists far beyond the time of DSS exposure points towards lasting extraintestinal consequences in diseases such as IBD. The lack of histopathological changes in our data is not dissimilar to other results published in DSS experiments. Chen et al., 2022 found no difference in testicular weight or morphology between DSS mice and control mice had no obvious difference [102]. Intriguing, this echoes a sound often heard by patients with chronic disease such as IBD as their lived experience is not always easily verified by clear pathological markers. Findings like this provide unbiased reason to aim to understand patterns even in the common absence of histopathological markers in chronic inflammatory diseases.

Impact of DSS on pubertal timing

Our investigation into the timing of pubertal onset following DSS treatment reveals that the impact of DSS on timing of pubertal onset may not be as straightforward as previously thought. The lack of significant delay in puberty onset in DSS-treated mice, and in some cases, an earlier

onset, contradicts some existing literature and suggests that the relationship between intestinal inflammation and reproductive development is complex and possibly influenced by other unidentified factors. One factor that we have not seen discussed in the IBD or DSS literature is the role of early life stress, a factor that is certainly at play in juvenile DSS treatment. Other models of early life stress have shown that chronic childhood stress can disrupt timing of pubertal onset. For example Cowan & Richardson (2019) showed that when female rats experienced early life stress they experienced earlier puberty onset, however when male rats were exposed to early life stress they experienced late onset puberty [176]. Therefore, it could be possible that the stressful nature of the DSS colitis is a factor in influencing pubertal timing. Interestingly, the microbiota can have a role in mediating this. Cowan & Richardson were able to show that probiotic treatment was able to counteract the effect of early life stress and was capable of restoring the healthy timing of puberty onset in both males and females [176]). As common in microbiota studies, it is challenging to disentangle how much the gut microbiota influences the variable and how much the variable influences the microbiota. However, it would be interesting to decipher the mechanism by which probiotics are able to reverse early life stress puberty changes and investigate if they are at play in the absence of our delayed puberty results.

Hormonal Changes in DSS colitis

The investigation of the impact of DSS treatment on systemic sex steroids yielded interesting results. Although not statistically significant, the progesterone in DSS treated mice increased out of proportion to expected peaks throughout the estrous cycle. Intriguingly, progesterone is increasingly being studied for its anti-inflammatory effects and has previously been studied for its role in attenuating colitis in other mouse models of IBD [177].

Estradiol has been widely investigated for its role in modulating risk and severity of IBD [53,144,178]. Unfortunately, due to practical limitations we were not able to test for estradiol levels in the chronically treated mice. Interestingly however, the trends in susceptibility to DSS in our experiments correspond with fluctuating levels of estrogen during development. Firstly, the experimental group least affected by DSS-colitis was the pre-puberty female group which corresponds with the age when mice undergo a period of “mini-puberty” that is characterized by a surge in estradiol [179,180]. Secondly, the time period that the female mice responded with the most susceptibility to DSS (during the chronic puberty administration) period in development when estrogen levels have dropped and have not yet re-risen to their biologically significant

concentrations. Although it is not possible to further validate this observation, it is interesting and it does correspond with the ‘estrogen protective effect’ experienced by women in regard to IBD [181]. Another interesting observation is that women with IBD often report worsening IBD symptoms during these menses [182–184], which is the phase during the reproductive cycle when estrogen levels are at the lowest [185].

Conclusion

In summary, this chapter both confirms and challenges existing literature about the effects of DSS-induced colitis on endocrine and reproductive development. The quantification of the observation of seminal vesicle underdevelopment in juvenile mice confirms reports of disrupted sexual development in DSS treated juveniles, however the lack of reproducibility of pubertal onset results points to the need to decipher if there are aspects of the DSS colitis experiments that could contribute to developmental delays. The nuanced and at times contradictory trends reported in this chapter highlight the need for more research into the complex interactions between the microbiota, immune system, and the environment.

Chapter 5: Conclusion

Inflammatory bowel disease is increasing worldwide at an alarming pace, and up to 50% of IBD patients experience at least one extraintestinal manifestation of the disease [186]. Of the many multi-system extraintestinal manifestations that exist, endocrine and reproductive abnormalities are known to significantly impact quality of life for patients [94]. Despite the high prevalence of these endocrine and reproductive abnormalities, very little is known about the IBD mechanisms that drive them. The work presented in this thesis attempts to explore the endocrine and reproductive abnormalities that have been described in the widely used DSS mouse model. Chapter 1 provided the background to link the often distinct areas of research - microbiology, immunology, gastroenterology, endocrinology and reproductive health. Chapter 2 described the methods used in our experiments, primarily focusing on the procedures involved in our DSS mouse model. Chapter 3 provided results and a discussion from the perspective of the gastrointestinal side of the project. Chapter 4 provided the results and discussion from the endocrine and reproductive side of the project.

Limitations

Despite the valuable insights gained from this work, there were a number of limitations. Firstly, the small sample sizes made testing for statistical significance challenging. A number of the effects we were interested in are small and therefore could be present but undetectable due to the sample sizes. Addressing this limitation in future could allow for more subtle patterns in the data to emerge and strengthen the validity of the existing observations.

Although checking for onset of vaginal opening and preputial separation is a standard measure of assessing the timing of pubertal onset, it is prone to bias, it is qualitative, and can often be hard to distinguish. Although labor intensive, other methods such as sperm analysis and ovarian follicle count would provide more robust data.

Practical constraints prevented a number of other assays that would have greatly contributed to the study. For example, further tests on the gut such as lipocalin assays, colonic myeloperoxidase assays, and tests for inflammatory markers could provide more specific information on the extent of the DSS damage. On the endocrine side, assessment of estrogens in the serum could significantly assist in understanding some of the trends we observed.

Future directions

This work indicated that age and sex influence the severity of DSS-induced colitis in mice. The trends observed in the sex steroids, although statistically insignificant, indicate that it would be valuable to repeat these experiments with larger sample sizes, and more precise measures for detecting changes in the sex steroids. Moreover, adopting more quantitative methods for assessing the timing of pubertal onset would allow for the possibility of understanding the contradiction between our findings and that which is in the literature. Finally, extending the investigations beyond the DSS mouse model would be an exciting first step at understanding if there is the possibility for generalizability to our understanding of humans with IBD.

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