EFFECTS OF HIGH INTENSITY INTERVAL TRAINING AND POSTNATAL GROWTH RESTRICTION ON MICROBIAL AND HOST METABOLISM

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PUBLIC ABSTRACT

Early life growth-restriction is a significant global issue, with over 161 million children experiencing growth-restriction under the age of 5 years. Undernutrition induced growth restriction during postnatal life (PNGR) is leads to permanent growth stunting. increased risk of cardiometabolic disease and reduced exercise capacity in adulthood. Exercise aids in preventing and treating cardiometabolic disease, however, the metabolic effects of exercise in the PNGR population is limited. Human metabolism is impacted by both host and gut microbial biochemical pathways, and thus studies should include impacts from gut microbes, human tissues, and the interplay between both entities. Recent literature in mice suggests the gut microbiome plays a role in exercise adaptations, both of which are altered in PNGR. Consistent evidence has shown the health benefits high-intensity interval training (HIIT) outweigh that of traditional moderate-intensity continuous training (MICT), and in a shorter timeframe in both healthy and chronic disease populations. Until now, HIIT has yet to be tested in the PNGR population, as well as its effects on the microbiome and metabolome.

The three objectives of this dissertation were to 1) determine the effect of a HIIT intervention on maximal exercise capacity (W_{max}) in adult PNGR mice compared to controls (CON), 2) to determine the effects of HIIT on the microbial compositions and 3) on the gut and serum metabolome in PNGR and CON. For the first objective, W_{max} was assessed via maximal treadmill tests performed at baseline, after one week of training, and post-intervention. W_{max} data analyzed via repeated measures ANOVA between baseline and post-intervention revealed improvements in all treatment groups. HIIT resulted in overall higher W_{max} in CON compared to PNGR, and in males compared females. HIIT successfully improved W_{max} in PNGR, which has yet to be shown in previous studies utilizing this mouse model. Future work is encouraged to

further examine physiological aspects allowing adaptation to HIIT in areas such as skeletal muscle or cardiovascular differentiation with training in PNGR compared to CON.

To accomplish the second objective, fecal samples were used to profile the gut microbiome. It was hypothesized that HIIT in CON and PNGR would increase microbial diversity as well as enrichment of unique taxa compared to their SED counterparts. Majority of the microbial differences, aside from a few genre and species differences, were not significant between treatment groups. Two microbes Christenellacae *g.s.* and Clostridiacae *g.s*. were altered in response to HIIT, although their specific roles in exercise capacity or adaptation are currently not clear.

To accomplish the third objective, fecal and serum samples collected post-intervention were processed for untargeted metabolomics. The effects of HIIT on host metabolism was evidently differential in PNGR compared to CON, with alternate patterns of heme, glutathione and acylcarnitine abundances post-intervention. Despite exposure to 4-weeks of HIIT, PNGR retained downregulated essential amino acids, acylcarnitines, glutathione and heme, all of which are potentially important components in the process of exercise adaptations. To summarize, PNGR leads to reduced exercise capacity which improves with HIIT, although the metabolome remains significantly altered compared to CON. By accomplishing the objectives of this dissertation, a more comprehensive profile of the metabolic baseline as well as responses to HIIT were characterized. It is encouraged that future studies further examine and compare the cardiovascular, skeletal muscle and mitochondrial activities in response to HIIT to determine where the alterations in metabolome derive from in PNGR.

ABSTRACT

Early life growth restriction impacts over 161 million children globally under the age of 5. Undernutrition induced growth restriction during postnatal life (PNGR) leads to permanent growth stunting, reduced exercise capacity and cardiometabolic disease risk in adulthood. High-intensity interval training (HIIT) results in exponential health benefits, especially in individuals with chronic disease compared to moderate-intensity continuous exercise. Furthermore, the gut microbiome plays a vital role in health and nutrient digestion, with a potential role in exercise adaptation. Previous evidence has shown both exercise capacity and the gut microbiome are altered in PNGR, however the metabolic responses to exercise are currently limited. Thus, the objective of this dissertation was to understand the metabolic and microbial responses to HIIT in PNGR and their potential interplay with exercise adaptations.

For the first objective, exercise capacity (W_{max}) was assessed via maximal treadmill tests performed at baseline and post-intervention. W_{max} data analyzed via repeated measures ANOVA between baseline and post-intervention revealed improvements in all treatment groups ($p<0.001$). Three-way ANOVA analyses showed improvements stronger improvements in W_{max} in males vs. females and in CON compared to PNGR ($p<0.0001$). HIIT improved W_{max} in PNGR-F-TRD compared to PNGR-F-SED ($p=0.0102$) and in PNGR-M-TRD compared to PNGR-M-SED ($p=0.0005$). HIIT was successful in both PNGR-M and PNGR-F which has yet to be shown in other studies utilizing this mouse model.

To accomplish the second objective, fecal samples were used to examine the gut microbiome profile. Microbial alpha diversity was not significantly altered in response to HIIT. However, level 7 taxonomic analysis revelated two microbes Christenellacae *g.s.* and Clostridiacae *g.s*. that were altered post-HIIT. Clostridiacae *g.s*. significantly increased in

PNGR-TRD compared to PNGR-SED ($p=0.007$), CON-TRD ($p=0.0124$) and CON-SED ($p=0.0237$). Christensenellaceae *g.s.* decreased the most in PNGR-TRD compared to CON-TRD ($p=0.01$), with trending decreases in CON-TRD vs. CON-SED and PNGR-TRD vs. PNGR-SED ($p<0.05$). While these two microbes may be beneficial to overall health or intestinal function, their roles in relation to HIIT or exercise capacity are currently unclear.

To accomplish the third objective, fecal and serum samples collected post-intervention were processed for untargeted metabolomics. The serum metabolites found showed distinct downregulation in energy regulation and mitochondrial oxidative stress metabolites in PNGR compared to CON, with downregulation of glutathione and xanthosine. PNGR and CON mice exhibited opposite effects of HIIT on heme metabolism, with downregulation in PNGR, and upregulation in CON. PNGR also showed signatures of altered fat oxidation with increased excretion and decreased circulating acylcarnitines. Other areas of concern in PNGR are the depleted essential amino acids: phenylalanine (PNGR-SED vs. CON-SED ($p=0.0001$) and CON-TRD ($p<0.0001$), L-tyrosine (PNGR-SED vs. CON-SED ($p=0.0001$), methionine (higher in CON-SED compared to PNGR-SED ($p<0.0001$) and PNGR-TRD ($p=0.0005$).

To summarize, HIIT improves exercise capacity, especially in PNGR. However, specific areas of host metabolism remain altered compared to CON, particularly in amino acids, energy regulation, mitochondrial fat oxidation and antioxidant capacity. By accomplishing the objectives of this dissertation, a more comprehensive metabolic profile in both sedentary and HIIT were characterized. Future studies are encouraged to further examine and compare the cardiovascular, skeletal muscle and mitochondrial activities in response to HIIT to determine where the alterations in metabolome derive from in PNGR.

This dissertation is dedicated to my loving and supportive husband, Rob, the notorious RPD (Roofus the Puppy Dog), and to my family who live near and far. Thank you for always believing in me and being there for me throughout this process. Without your never-ending support and love, this would not be possible.

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CHAPTER 1: INTRODUCTION

Early life growth restriction (GR) affects approximately 161 million children globally[1]. Undernutrition induced GR during postnatal life (PNGR) leads to permanent growth stunting, as shown by ~18% reduction in body mass, which is unresolved with proper nutrient intake and persists into adulthood[2–5]. The PNGR phenotype involves a multitude of cardiometabolic impairments[2,3,5–7] which increase the risk of cardiovascular disease (CVD) in adulthood by 47%[2]. Additionally, PNGR can have long-term effects on metabolic physiology, specifically reducing glucose tolerance and insulin sensitivity, which can increase the risk of developing diabetes later in life[8–11].

Exercise is highly effective in reducing chronic disease risk, especially CVD[12–15]. The American College of Sports Medicine (ACSM) defines moderate-intensity exercise as ranging between 40-60% of maximal oxygen capacity (VO_{2max}) while high-intensity exercise is generally considered to be performed above 65% VO_{2max}[16]. Both intensities have been shown to improve metabolic and cardiovascular health, and prevent disease onset[17,18].

Recent findings using the Neonatal Nutrition and Exercise Research Lab (NNERL) nutritional model for PNGR suggests that maximal exercise capacity is impaired in PNGR, especially in females[2,6,7] furthermore when PNGR mice engage in moderate intensity continuous exercise training there is adverse CV and skeletal muscle responses, resulting in signatures of cardiac fibrosis[2,19], pathological cardiac hypertrophy[2], altered Ca^{2+} handling[19], and an inability to induce skeletal muscle hypertrophy[4,6], which reflect an inability to adapt appropriately to the training stimuli.

Human variability in CV response to exercise has been widely studied since the 1980's and helped to expose the prevalence of 'non-responders' and 'adverse responders' to aerobic exercise. Exercise non-responders are typically identified as having little to no improvement in

one or more CRF related biomarkers (i.e., VO_{2max}, blood pressure, resting heart rate and cholesterol profile[20–22]) following exercise training[22]. Adverse responders are defined as having a change in response to exercise that worsens a risk factor beyond expected biological daily variation or measurement error[20].

Initial Heritage Family cohort studies investigated non-responders utilizing a progressive aerobic continuous training protocol (increase in exercise over the duration of the study) in healthy trained or untrained subjects, with a standardized total volume of exercise performed per week. Although majority (~69%) of individuals responded positively in at least one CRF outcome, some individuals either had no significant improvements or had declined in at least one variable[20,22–27]. While it is not uncommon to have some biological or technical variability in exercise response[22,28,29], the mechanisms underlying adverse responses in healthy individuals is not well explained. Studies investigating non-responders suggest there are multiple factors may that contribute to its occurrence including the training protocols used[30–38], genetic or epigenetic differences[39–46] and/or pre-existing cardiometabolic impairments which may alter their exercise response[47–52].

Since the emergence of non-responders more comparisons are being made between training variables (i.e., intensity, volume, etc.) to observe differences in outcomes. Many studies have highlighted that higher exercise intensities lead to exponential CRF outcomes compared to lower intensity exercise[53–60]. However, many have shown that increasing intensity alone may still lead to non- or adverse exercise responses[53,61,62]. The use of interval training in combination with higher exercise intensities has gained more attention within the last decade. HIIT protocols involve repeated bouts of high-intensity for a short period of time, followed by either a short passive (rest) or active recovery (low-moderate intensity) interval. HIIT also allows for altering

multiple variables simultaneously (e.g., duration and/or intensity of the work-rest intervals, number of repeating cycles) which allows for a more personalized approach to fit an individual's health restrictions or baseline exercise capacity[63].

HIIT in comparison to moderate-intensity continuous training (MICT) results in greater improvements in cardiometabolic biomarkers related to CRF in healthy individuals[57,64–68]. HIIT also outperforms MICT in many clinical trials involving patients with pre-existing cardiometabolic disease such as hypertension, coronary artery disease, heart failure with preserved ejection fraction, among many others[66,69–71]. HIIT in healthy cohorts also elicits greater improvements in vascular function[57] and left ventricular morphology[69], Ca^{2+} handling and reuptake[67], stroke volume (SV)[67], and cardiomyocyte remodeling[67]. Evidence has shown that the interval component of high-intensity interval training HIIT may be key in introducing a potent stimulus to induce physiological adaptations[55]. Utilizing active recovery intervals has shown to be more beneficial than passive, ultimately preventing fatigue through lactate buffering mechanisms and improving exercise response[72,73]. Due to repeated evidence of CV impairments found in PNGR mice[2,7,19,74] as well as their adverse response to MICT[2,19,75], HIIT could be an alternative therapeutic countermeasure for PNGR.

As previously shown in our lab, PNGR also leads to an altered gut microbiome and metabolome in adulthood. Despite feeding for several months on a normal protein diet post-weaning, PNGR led to altered fecal microbial β-diversity, driven primarily by increased abundance of Erysipelotrichales and Bacteroidales which in very high abundance is associated with gut inflammation and may signify disrupted lipid or glucose metabolism within the host[76–78]. PNGR also increased excretion of fecal peptides, which may be caused by impairments in gut permeability, nutrient absorption or normal signaling processes. Many studies aimed to

understand the link between exercise performance and capacity and the gut microbiome [79–86]. Recent evidence has shown that exercise results in increased diversity microbes that can produce beneficial metabolites (e.g., short-chain fatty acids, SCFAs) and may support host metabolism[79–86]. Alterations in microbial composition through exercise has shown to improve lipid and glucose control and improve nutrient absorption. Additionally, it has been shown that with all factors accounted for, including diet, that exercise capacity can account for more than 20% of variation in microbial composition[87], highlighting the importance of better understanding their underlying role.

Metabolomics research offers invaluable insights into the fluctuations of metabolic intermediates in response to different physiological stimuli, such as dietary modifications, diseases, or exercise. The field continues to advance through technological enhancements and database machine learning methods, rendering it an increasingly dependable technique for investigating alterations in our metabolism. Previous studies have shown that early life GR leads to alterations in metabolites in multiple organ systems, of which some are seemingly permanently altered from adolescence through adulthood. Additionally, human and animal models have shown alterations in metabolites in response to exercise training of various intensities and types. This evidence allows for further investigation of metabolic responses to specific exercise stimuli, to make association of these responses to exercise capacity, to detect or prevent biomarkers of cardiometabolic disease in response to nutritional insult, and to elucidate molecules that may support and interact with our gut microbiome.

Thus, it is hypothesized that HIIT could improve metabolic function and exercise tolerance by allowing for positive exercise adaptations to occur, which will subsequently enhance gut microbiome composition and metabolomic profile in the PNGR population.

The overarching goal of this dissertation is to characterize the effects of HIIT on exercise capacity and associated microbiome and metabolomic responses in the PNGR and CON mice in adulthood. To accomplish the goal of the dissertation the following aims were developed:

Specific Aim 1: To determine the effect of a HIIT intervention on maximal exercise capacity (Wmax) in PNGR mice as compared to controls (CON).

Hypothesis 1.1: HIIT will improve Wmax in both PNGR and CON mice post-intervention.

Hypothesis 1.2: Baseline Wmax will be higher in CON vs. PNGR mice.

Hypothesis 1.3: PNGR and CON trained females will have lower Wmax compared to PNGR and CON trained males.

Hypothesis 1.4: PNGR and CON SED females will have lower Wmax compared to PNGR and CON SED males.

Specific Aim 2: To determine the effects of HIIT on the microbial compositions in PNGR and CON mice.

Hypothesis 2.1: HIIT in CON and PNGR will increase abundance and enrichment of unique taxa compared to their SED counterparts.

Hypothesis 2.2: HIIT in CON and PNGR will increase microbial diversity compared to SED counterparts.

Hypothesis 2.3: HIIT will result in higher Bacteroidetes to Firmicutes ratio in the PNGR and CON mice compared to their SED counterparts.

Specific Aim 3: To determine the effects of HIIT on the gut and serum metabolome in PNGR and CON mice.

Hypothesis 3.1: SED PNGR mice will have serum and gut metabolomic profiles reflecting altered fatty acids and carnitines compared to the SED CON mice.

Hypothesis 3.2: HIIT trained PNGR mice will have serum and gut metabolomic profiles reflecting altered fatty acids and carnitines compared to the SED PNGR mice.

Hypothesis 3.3: HIIT in PNGR will lead to serum and gut metabolites that are more similar to those found in SED CON mice.

CHAPTER 2: LITERATURE REVIEW

This literature review will focus on characterizing the effect of undernutrition induced GR during postnatal life (PNGR) on reduced exercise capacity in adulthood, metabolic perturbations that have been previously established including alterations in the gut microbiome and metabolome, as well as additional mechanisms that may be involved. After the review is a justification of the methodology to be used in this dissertation. This literature review will provide the rationale for the aims and hypotheses proposed in this dissertation.

2.1 Defining Growth Restriction

In 2022, the world health organization (WHO) reported early life GR affected about 22.3% or 161 million children globally under the age of 5[88,89]. GR has repeatedly shown to increase prevalence of chronic non-communicable diseases in adulthood, such as cardiovascular disease (CVD)[3,5,90–92] and diabetes[93–95]. GR is an all-encompassing term of which is defined as 'the failure to meet growth potential' and is usually marked by weight or height z-scores >2 standard deviations (SD) below the growth standards median for children under the age of 5[96–98]. The two primary categories of GR tracked globally are wasting (low z-scores for weight-to-height) and growth stunting (low z-scores for height-for-age).

Undernutrition, which indicates an imbalance or deficiency in nutrient intake, is a primary contributor to both wasting and stunting forms of GR[81,83]. Early life GR is commonly associated with poor maternal nutritional status during gestation, deficiency in nutrients received during breastfeeding, or frequent illnesses or infections[89,99–102]. Wasting and stunting often result in long-term developmental delays, and if left untreated, or if the degree is too severe can result in permanent reductions in physical and cognitive potential.

GR is often isolated to two specific periods of growth: intrauterine (IUGR) occurs during gestation, and postnatal life (PNGR). PNGR begins after birth through the either late childhood

or early adolescent developmental periods[3,5,98,103,104]. IUGR is also sometimes referred to as GUN in the literature, while PNGR is often referred to as PUN.

Other terms used in the literature may include but are not limited to fetal GR (FGR), extrauterine GR (EUGR), small-for-gestational age (SGA), and 'failure to thrive'[105]. FRG is similar to IUGR, in that it affects the fetus during gestation. FGR and SGA are defined as the failure of the fetus to meet its growth potential due to a pathological factor. Placental dysfunction is the most common cause of FGR[106]. EUGR or 'failure to thrive' refer to those born prematurely and despite nutritional support, fail to reach their growth potential[107].

For the scope and purpose of this dissertation, PNGR will be used as a comprehensive overarching term for the permanent growth limitations as a result of undernutrition isolated to the postnatal window of development, as used in a nutritive mouse model to induce GR, of which will be detailed later in this review. The term IUGR will be used to refer to GR isolated to the gestational period. Additionally, early life GR ('perinatal') includes both gestational and postnatal periods.

2.2 Early Life Origins of Health and Disease

Early epidemiological studies, led by Dr. David Barker, revealed initially that non-communicable disease could be programmed by way of intrauterine or environmental stressors (e.g., nutritional insult or sedentary lifestyle), which became known as the 'fetal origins hypothesis' or the 'Barker Hypothesis'[101,108,109]. Later studies suggested programming effects may also occur postnatally, evident by slow growth patterns in infants and children born normal weight and height for their gestational age. This area of research soon evolved into what is now known as the 'developmental origins of health and disease' (DOHaD) hypothesis, which encompasses programming effects that may occur during gestational or postnatal life[100,110,111].

The ‘developmental programming’ that occurs is hypothesized to be the result of the body’s inability to maintain homeostasis when exposed to environmental stressors, leading to abnormal molecular and physiological activity (e.g., altered DNA methylation or histone acetylation resulting in hypertension or altered insulin secretion)[112–114]. The result of this programming effect is increased susceptibility and prevalence of chronic cardiometabolic disease later in life[111].

Primary epidemiology studies supporting the DOHaD hypothesis were derived from the Dutch Famine and Helsinki Birth Cohorts, which demonstrated that gestational or postnatal exposure to undernutrition (due to famine), increased risk of hypertension, atherosclerosis, coronary heart disease, stroke, and type 2 diabetes in adulthood[115–119].

2.3 Growth Restriction Animal Models

Animal models have become a widely used method to mimic and further investigate the molecular and physiological effects of early life GR[120,121]. Development of animal models has allowed for longitudinal and repeatable studies that would otherwise be unethical and arduous to perform in humans[120–122]. GR models have been used in various species of animals, including but not limited to, rodents, sheep, and pigs[3,8,123–125]. GR has also been observed globally in human studies and in some human interventions, however, most studies utilize animal models[95,126–128]. Studies examining the effects of IUGR or FGR typically use larger animals such as sheep or pigs[120]. IUGR is usually performed either by 1) nutrient restriction during gestation to mimic a chronic physiological effect or via 2) bilateral ligation of the uterine artery to mimic acute effects (e.g., pre-eclampsia) and results in GR isolated to the gestational development period[98,120,122,129]. FGR is typically performed by altering litter sizes or the maternal diet itself, which leads to GR in the offspring[120]. Recent studies have compared human FGR and IUGR research to these

animal models and have found consistent outcomes with reductions in organ and body weights at birth as well as altered metabolic physiology in result of maternal nutrient restriction[130,131]. However, these models yield low sample sizes, can induce unnecessary stress, tend to be less cost-effective, and are not commonly used to examine effects isolated to the postnatal period[120].

Rodent models, which include rats, guinea pigs and mice are mostly used to examine skeletal muscle development and structure, protein signaling pathways, and gene expression targets in response to either gestational or postnatally induced GR[103,121,130–132]. Positive aspects of using rodent models are their short lifespans and cost-effectiveness, which allows ease of completing a longitudinal experiment, of which in humans would otherwise be much more difficult to accomplish[103]. Mice are more commonly used for studying GR, especially during postnatal life, due to their genetic similarities to humans, as well as their physiological mechanisms of which closely mimic those seen in humans[121,133,134].

Majority of manuscripts published on early life GR have been focused on the gestational growth period, with a main concentration on IUGR, FGR, or GUN with very few studies examining the effects of PNGR in adulthood, especially with a focus on exercise capacity. Thus, the primary focus of this dissertation will be on characterizing and highlighting the programming effect on health outcomes and exercise capacity in adulthood specific to the postnatal development period.

2.4 Exercise Training and Responses – Comparison of Humans and Mice

In both mice and humans, heart rate, blood pressure, lactate, respiration, as well as other common biological variables measured during exercise all increase with increasing exercise intensity[135,136]. Like in humans, maximal aerobic capacity (VO_{2max}) can be measured in mice using a metabolic mouse treadmill, or it can alternatively be measured indirectly as maximal

work capacity (W_{max}) by using subjective performance criteria (e.g., mouse touching the shock grid 5 times in 30 seconds)[137–139]. Since a W_{max} test is highly correlated VO_{2max}[6,140–142], most studies (~78%) use this in place of the traditional (VO_{2max})[137].

HIIT leads to similar physiological responses in humans and in mice, including but not limited to improvements in cardiac output, stroke volume, cardiomyocyte remodeling, mitochondrial biogenesis, insulin sensitivity, skeletal muscle fiber type alterations, and increased exercise capacity[136–139,143,144]. Unlike in humans, there is no one suggested type or protocol of exercise suggested for rodents, and there is a wide variety used among studies with subsequent varying training responses. However, Massett et al. developed a validated HIIT protocol that works across a variety of strains of mice (especially the one we use – FVB/N) which has repeatedly shown to elicit positive exercise adaptations that are also seen in humans, and therefore is appropriate for use in this dissertation[39,138,139,143,145].

HIIT induces superior cardiovascular and exercise capacity adaptations compared to MICT in both humans and mice such as exacerbated increases in heart weight, cardiomyocyte size and length, improvements in stroke volume and ejection fraction, increases in maximal work capacity and VO_{2max}[60,67,68,146,147]. HIIT increases skeletal muscle citrate synthase (mitochondrial biogenesis) and succinate dehydrogenase in both humans and mice, primarily in mixed and glycolytic or type II fibers, unlike MICT which increases this activity primarily in type I or slow-twitch fiber types[137,148–152]. HIIT also results in an alteration in skeletal muscle fiber type composition, however the evidence is mixed in the literature based on the muscle group or protocols being tested. Generally, HIIT has shown to increase proportion of type II fibers, as well as increasing the cross-sectional area (CSA) of type I, type II fibers in both humans and mice[153–156]. In response to HIIT, mice like humans also exhibit increased translocation and expression of

GLUT4 in skeletal muscle (shown to improve insulin sensitivity) and increased cross-sectional area and muscle weight in the fast-twitch fibers[136,150,153,157,158].

2.5 Exercise Capacity and Evidence of Non-Responders in PNGR

Exercise decreases the incidence of CVD, type II diabetes, hypertension, and obesity and increases CRF. In a Helsinki Birth Cohort sub-study, the correlation of IUGR or PNGR to CRF in adulthood was further investigated. This sub-study included 606 men and women who participated in a UKK 2-km walk test, which at the time was used as a surrogate for a maximal exercise stress test to measure CRF in population studies. Data on body size at birth and growth during infancy and childhood were obtained from clinical records. Body size at birth was not associated with adult CRF. However, early postnatal growth was found to be a positive predictor for adult CRF, indicating postnatal GR has a stronger relationship to CRF in adulthood. Increases in height and lean body mass from ages 2-11 years was strongly associated with CRF ($p<0.001$)[159]. The result of this study highlights that early growth, particularly during postnatal life may plays a role in predicting adult CRF, however, underlying mechanisms warrant further investigation[159].

In a foundational study by Ferguson et al. 2019, adult female and male mice were subjected to either postnatal undernutrition [PNGR (denoted as PUN in the article) males (PNGR-M) and PNGR females (PNGR-F); 8% protein] or an isocaloric control diet [CON males (CON-M) and CON females (CON-F); 20% protein] from birth to PN21. At PN70, basal oxygen consumption (VO_2), maximal oxygen consumption (VO_{2max}), total work (Joules) and maximal work capacity (W_{max}; Joules/min) were measured by a treadmill stress test. Baseline VO_2, taken before the treadmill test and was confirmed to have a significant correlation to VO_{2max} ($r=0.85$, $P < 0.001$), and was thus used as a covariate in the VO_{2max} analysis. Myocardial performance and heart rate

was also measured as indicators of cardiovascular function with and without β-adrenergic stimulation, which acted as a simulation of exercise-related stimulus and resting basal conditions. Body composition was also measured in all treatment groups. At PN70, PNGR-F and PNGR-M had lower total body weight, lower lean mass and lower fat percentage compared to their CON counterparts. PNGR-M had higher lean mass and body weight compared to PNGR-F[7]. PNGR-F had an approximately 20% decrease in exercise capacity compared to CON-F, evident by lower W_{max} ($p<0.001$), VO_{2max} ($p<0.001$) and total work ($p<0.001$)[7]. PNGR-M did not have significantly lower total work, W_{max} or VO_{2max} compared to CON-M. PNGR-F had distinctly unfavorable MPI, decreased stroke volume (SV) and higher resting heart rate compared to CON-F, which are factors likely contributing to the impaired exercise capacity observed in the PNGR-F. Although this study did not measure these outcomes in response to a specific exercise training intervention, the foundational evidence provided allows for inferences of what could potential occur in response to training in the PNGR mice[7].

In a similar study, by Pendergrast et al. 2019, the effects of early life GR on maximal exercise capacity in adulthood was evaluated in the PNGR mice compared to CON. At PN67, mice performed a maximal treadmill test. Echocardiography and Doppler blood flow analysis was performed at PN72, following which skeletal muscle cross-sectional area (CSA) and fiber types were evaluated. Maximal running capacity was reduced ($p = 0.0002$) in the PNGR mice compared to CON[6]. CON-F mice sustained maximal speed for a significantly ($p = 0.012$) longer time than PNGR-M, PNGR-F and CON-M. Work performed at maximal speed was significantly ($p = 0.0002$) greater in CON compared to PNGR mice[6]. Results of total work performed throughout the test was not significantly different between groups. Similar to previous findings as Ferguson 2019, the PNGR mice had reduced stroke volume (SV) ($p = 0.04$), which likely

contributed to their reduction in maximal running capacity. PNGR mice had greater CSA of soleus fibers, reduced % of type-IIX fibers in the extensor digitorum longus (EDL; $p = 0.03$) and a greater % of type-IIB fibers in the EDL ($p=0.008$)[6]. The results of this study highlight the inability of PNGR mice to sustain work for long periods of time at maximal speeds compared to CON mice, which may indicate that interval training may be more ideal to cater to this nutritionally programmed phenotype. However, this study did not complete a training intervention. Thus, since the results are summarizing acute impaired responses, as opposed to chronic responses to exercise, a training intervention would be ideal to investigate the reduced exercise capacity further

In a follow-up study by Ferguson et al. in 2021, exercise capacity was tested in PNGR and CON mice after 3 weeks of access to wheels starting from PN45 and ending on PN67[2]. The CON mice had 45.6% higher maximal work ($p = 0.0390$) compared to the PNGR mice, despite equality in total accumulated wheel revolutions during the 3-week training period. These results strongly suggest PNGR mice have reduced exercise capacity with the same amount of training completed as CON. Also, there was no sex effect found for maximal work performed during the treadmill test. Interestingly, the PNGR-F mice exhibited a glycolytic shift in the soleus muscle, which is typically a highly oxidative fiber in this strain. It is possible that early postnatal life may have influenced the metabolic programming of the skeletal muscles, leading to a predisposition towards glycolytic metabolism, highly influencing the glycolytic shift observed in response to the intervention[2]. PNGR-F also developed signs of cardiac fibrosis development, evident by lower left ventricular volume ($p = 0.0540$) and lower left ventricle area ($p = 0.0585$). It was inferred that the limitations in exercise capacity in PNGR mice were most likely due to the

previously mentioned adverse cardiac and skeletal muscle adaptations that were unlike the CON mice[2].

A recent study by Wellette-Hunsucker et al. 2023, completed an eccentric downhill treadmill (DHR; -16%) training protocol using PNGR and CON mice to mimic resistance training responses in humans, to determine the programming effects of PNGR on muscle growth[75]. Mice were either DHR trained at a moderate-continuous pace[160] of 30 minutes at 18m/min, 3 days per week for 12 weeks or kept in a sedentary control group (SED). No maximal capacity test was performed in this study. The DHR protocol successfully elicited an increase in markers related to muscle protein synthesis (MPS) in CON evident by increased type IIa myofibril CSA. The PNGR DHR-trained mice did not see changes in CSA of type IIa skeletal muscle fibers. Additionally, type IIb fibers were much larger in CON DHR-trained mice compared to PNGR DHR mice[75]. The results of this study indicate early GR leads to a blunted exercise response in mice exhibited by inability for their skeletal muscle to adapt like their CON counterparts.

It is difficult to discern a true correlation or comparison of PNGR mice pattern of exercise response due to the Ferguson lab only having two previous papers showing non-response. Based off of evidence from the study by Ferguson et al. 2021, which utilized a 3-week wheel running intervention, there was very low within-group variability for the PNGR mice (<10%) which suggests that most responded very similarly (little to no response), with the few that did respond positively having <20% improvement which was not statistically significant[2]. Massett et al. 2009[161] provided vital information regarding how the FVB mouse strain typically responds to training and their variability. After 4 weeks of training the typical work values (work calculated as kg*m) as 4.66 +/- 0.23 SE with a mean change of 1.78+/- 0.17 SE from baseline work.

To summarize, the evidence from the previously mentioned studies further emphasizes the importance in further investigating the mechanisms underlying the atypical exercise responses observed in the PNGR mice to establish an optimal method to induce positive exercise adaptations.

2.6 Exercise Non-responders in Human Studies

CRF and physical activity (PA) level are strongly and inversely associated with risk of developing metabolic and cardiovascular related diseases[162]. Aerobic exercise is a well-known modality for improving CRF[13,162,163]. Current American College of Sports Medicine (ACSM) recommendations involve healthy adults (aged 18-65 years) engaging in a minimum of 150 minutes of moderate PA or 75 minutes of vigorous PA per week, with higher amounts of PA recommended to obtain more health benefits (300 min/week moderate PA or 150 min/week vigorous PA).

Individual responses in CRF to exercise have been tested as far back as the mid 1980's. Studies within the HERITAGE family cohort laid the foundation for investigating correlations between human variability to exercise response and inherited traits supporting CRF. One of the initial studies involved 742 healthy sedentary subjects from 200 families who underwent a standardized 20-week aerobic training program[164]. Evidence of atypical exercise response in this study was depicted as deviations from the means in CRF biomarkers measured from the participants. The study resulted in mean improvements of 400 ml VO_{2max}, but there was still a large range of interindividual variability with some improving to over 1000 ml O_2 per min and others seeing reductions from baseline VO_{2max} post-intervention[164].

According to the literature, the reported percentage of individuals that exhibit minimal or no improvements in CRF ranges from 7-45%[165–167]. However, the percentage of individuals who

are non-responders to aerobic exercise can vary depending on the specific parameters and outcomes measured in the study. In a review analyzing six different human studies, multiple CRF outcome variables were compared in response to training: VO_{2max}, high-density lipoprotein cholesterol (HDL-C), fasting insulin (FI), systolic blood pressure (SBP), and serum triglycerides (Tg)[168,169]. Out of a total of 1,687 subjects, 31% had at least one outcome measurement respond adversely, while 6% had two, and 0.8% had 3-4 adverse responses in healthy individuals[169].

The impact of genetics on exercise adaptations has been studied for the past 15 years, with recent findings elucidating a link between specific genetic variants or single nucleotide polymorphisms (SNPs) in exercise non-responders[41,43,44,170–172]. Recent studies have highlighted SNPs associated with mitochondrial oxidative phosphorylation (protein coding genes, NADH dehydrogenase 1-5 [mtND1, mtDS3, mtND4, mtND5], and cytochrome B [mtCYTB], as well as 7 hypervariable [HV] regions) which may help to explain inherited exercise trainability[44,45,171]. However, approximately only 20-50% of the variation in exercise trainability can be attributed to genetic factors[173–175], leaving the remaining 50% to be explained by either environmental (i.e., nutritional programming) and/or epigenetic factors which regulate gene expression and activity without altering the genetic code[175–177].

Although many biological factors can influence CRF, the most important is the CV pumping capacity, which can be improved with aerobic exercise. The most common biomarker used for CRF is maximal exercise capacity, usually measured as W_{max} or (VO_{2max}[22,38,47,178]. Other variations of CRF outcomes used to assess exercise adaptations and prescribe exercise intensity are peak oxygen consumption (VO2peak), lactate threshold (LT), and % maximum heart rate ($\%HR_{max}$).

In a retrospective review by Astorino et al. 2014, evidence of non-responders to two types of interval training were analyzed and compared, which derived from two separate studies in humans[27,68,69]. Study 1 involved twenty active men and women who performed 6 days of sprint interval training (4-6 Wingate tests per day), while Study 2 involved 20 sedentary women who performed 12 weeks of high-volume HIIT at workloads ranging from 60–90% Wmax. Individual changes in heart rate (HR), fat oxidation and VO_{2max} were also examined. It was found among the 40 total individuals in the study that only one was a 'non-responder' all variables when exposed to both interventions[27]. The frequency of non-responders was overall estimated to be 5-45% depending on the outcome variable being measured and the interval protocol used. More individuals responded positively in VO_{2max} (95% vs. 65%) and HR response (85% vs. 55%) to the HIIT protocol compared to the shorter, more intense sprint interval training (SIT) protocol. Most individuals responded similarly in terms of fat oxidation (60-65%)[27]. The results of this study suggest that simply increasing intensity itself is not as optimal in eliminating exercise non-responders and HIIT may be more ideal to improve CRF. Although, both protocols led to a very low prevalence of non-responders across all variables (1/40 participants) indicating a minimum intensity may be necessary to elicit responses in certain individuals.

A study found that the incidence of "responders" versus "non-responders" was higher in response to HIIT compared to MICT[53]. Prior to the intervention, an incremental max test was performed using a cycle ergometer to determine baseline VO_{2max}. The participants (n = 21 per group) were then assigned to either HIIT or MICT, of which they would complete 3x/week for 6 weeks. The HIIT group performed four cycles of 4-min intervals at 90% HR_{max}, followed by a 4-min active recovery at 30 W (1:1 work/rest ratio). The HIIT bout was preceded with a 10 min warm-up and followed by a 5-min cool-down at 30 W, for a total of 43 min of exercise. The

MICT group performed 60 min of continuous cycling at an output of ~90% of their LT. The VO_{2max} test was also performed at the end of the intervention. Criteria for determining the degree of response in this study was based on ΔVO_{2max} (ΔVO_{2max} = Post interventionVO_{2max} - Pre-intervention VO_{2max}). 'Responders' were then defined as individuals with positive improvements in ΔVO_{2max}. Alternatively, individuals with a negative ΔVO_{2max} response were considered 'non-responders'. Interestingly, the study found that the incidence of responders was much higher in the HIIT group (20 out of 21; 95.2%), compared to the MICT group (11 out of 21; 52.4%), which suggests that the HIIT enhances exercise response to a greater degree compared to MICT[53].

Montero et al. 2017, utilized an exercise protocol including two consecutive 6-week programs with 1-5 sessions per week. The average exercise intensity over the duration of each session was 65% W_{max}[62]. The groups that were given more sessions per week (4 and 5) after 6 weeks resulted in 0% non-responders while 1-3 sessions still contained non-responders, signifying a minimum physical activity volume or frequency requirement for this population[62]. After the second 6-week session there were 0% non-responders in all groups. The main caveat in this study is it only tested on healthy individuals and not those with pre-existing cardiometabolic conditions.

2.7 Moderate-Intensity Continuous vs. High-Intensity Interval Training and CRF

The exponential health benefits from high versus-moderate-intensity exercise in increasing VO_{2max}, has been demonstrated in both human clinical trials[58,179,180] and in rodents[65,67]. Initial evidence stems back to the late 1970s, with studies testing the effects of high- or moderate- intensity exercise in individuals with recent cardiac events[181] as well as functional

capacity in healthy adults. Many studies have shown exponential improvements in CRF with increases in exercise intensity[58,64,179].

Helgerud et al. 2007[58] compared the effects of four different aerobic protocols to compare the effects of intensity when matched for total work and frequency. Forty healthy moderately trained male participants were randomly assigned to one of four groups: MICT (70% HR_{max}), high-intensity continuous running at/or near LT (~85% HR_{max}), HIIT 15 x 15 (15 sec at 90-95% HR_{max}, 15 sec of active resting at 70% HR_{max}) and HIIT 4 x 4 min (4 min at 90-95% HR_{max}, 3 min of active resting at 70% HR_{max}). The exercises were performed 3 days per week for 8 weeks. Primary outcomes examined were VO_{2max}, stroke volume of the heart (SV), blood volume, lactate threshold (LT), and running economy (CR). Both HIIT protocols elicited greater improvements in VO_{2max}, SV, and CR, highlighting the importance of the work-matched interval component in conjunction with higher-intensity exercise to elicit advanced CRF outcomes.

A previous study using rodents aimed to assess the effect of increasing exercise intensity on functional cardiovascular outcomes post-10 weeks of high-intensity versus moderate-intensity interval training [67]. The protocol consisted of 5 intervals of 8 min at HIGH: 85%-90% VO_{2max} or MOD: 65%-70% VO_{2max} followed by 2 min active rest at 50%–60% VO_{2max}. The total training duration was 1 hour per day, 5 days per week. Weekly VO_{2max} testing was performed to adjust exercise intensity. HIGH training increased VO_{2max} by 71%, while MOD increased by 28%. Cardiomyocyte hypertrophy increased in an intensity-dependent fashion, with 14% increased with HIGH and only 5% MOD. Cardiomyocyte function improved exponentially with HIGH compared to MOD, exhibited by increased fractional shortening (45% vs 23%), and decreased contraction rate (43% vs. 39%) and relaxation rate (20% vs 10%; in HIGH and MOD, respectively). Ca^{2+} sensitivity increased more with HIGH (40%) compared to MOD (30%).

Endothelial function in the carotid artery improved similarly with both protocols. The current study demonstrates that CV adaptations to training are greater with increased intensity during interval. training. Higher intensity interval training outweighed improvements in VO_{2max}, cardiomyocyte contractility and relaxations, with equivalent increases in endothelial function versus training at moderate intensities.

The effects of HIIT vs. MICT has also been evaluated in individuals with pre-existing cardiometabolic disease. In a recent meta-analysis evaluating 10 studies with 273 total patients included, the aim was to compare the effects of HIIT vs. MICT in individuals with pre-existing cardiometabolic disease (e.g., heart failure, coronary artery disease, hypertension, obesity, and metabolic syndrome)[182]. HIIT led to significantly higher peak oxygen capacity (VO_{2peak}) compared to MICT (mean difference of 3.03 mL/kg/min, ~9.1%). Improvements in cardiovascular function in individuals with cardiometabolic disease should theoretically increase CRF, thereby decreasing all-cause mortality rates in this population. This study concluded that HIIT increases CRF by nearly double in comparison to MICT in individuals with pre-existing chronic disease[182].

2.8 Potential Benefits of HIIT for PNGR

There is evidence to suggest HIIT may help to overcome the physiological impairments in PNGR that are contributing to adverse exercise capacity. Primary detriments seen in PNGR that are proposed to contribute to decreased exercise capacity are reductions in SV, end-diastolic volume (EDV), and altered Ca^{2+} kinetics[2,6,7,19]. HIIT has shown to improve these biological factors healthy rodents. When subjected to a six-week interval treadmill program (6 cycles of 8 min @ 85-90% VO_{2max}: 2 min active recovery @ 50-60% $VO_{2max;}$ 5 days/week), HIIT increased exercise capacity by 35%, cardiomyocyte length by 20% ($p < 0.01$) and cardiomyocyte width by

30% ($p < 0.01$)[183]. Ca^{2+} handling also improved post-HIIT, with a 25% increase in Serca2. Serca2, an important protein that regulates Ca^{2+} reuptake, is downregulated in PNGR female sedentary mice, which only decreases further in response to moderate-intensity training[19]. Other studies have shown similar findings of improved Ca^{2+} reuptake by almost 50-60% in response to HIIT compared to MICT, which may mitigate fatigue and enhance adaptation to the training stimulus.

A previous study using a similar HIIT protocol in rodents led to improved exercise capacity and increased skeletal muscle mass of the primary locomotor muscles in mice, the soleus and extensor digitorum longus (EDL), by 12-18% [136]. PNGR typically have smaller skeletal muscles, with higher composition of glycolytic fibers and reduced ribosomal biogenesis[75] compared to their CON counterparts[6], which may contribute to their reduced exercise capacity. Fortunately, HIIT relies primarily on the glycolytic pathway during the high-intensity intervals[72], which may serve as a more appropriate metabolic fit to support exercise adaptations in PNGR. Although a previous study has showed non-response to skeletal muscle hypertrophy in PNGR[75], it was performed using moderate-intensity and may not be the potent stimulus required to support appropriate adaptations to improve exercise capacity.

2.9 Microbiome in PNGR & Exercise Capacity

The microbiome plays a significant role in regulating health and preventing disease development[184–188]. Research has shown that early life nutrition may have a strong influence on gut microbiome composition[103,185,188–190]. A distorted gut microbiome composition will lead to altered metabolism in the gut and systemically, because recent literature has shown that gut microbes significantly impact circulating blood metabolites and host metabolism as a whole[184].

Early life GR is associated with an altered gut microbiome. With most studies reporting altered diversity and abundance of specific microbiomes of which are divergent between normal and those affected by GR[103,191–194]. An altered gut microbiome as a result of early life GR has shown to not only affect nutrient absorption and utilization, but also plays a role in programming metabolic and immune functions[191,195–197]. Many studies highlight the importance of microbiome development during the first few years of life[196,198–200]. Quinn et al. in 2022, aimed to investigate the longitudinal gut microbiome and metabolomic profile in early life GR. Longitudinal fecal samples were collected for gut microbiome and metabolome analysis every week starting at PN21 until PN80 for gut microbiome and metabolome analyses. The results of this study showed that PNGR mice exhibited alterations in gut microbiome and gut metabolome compared with CON mice, evident by altered microbial β-diversity ($p = 0.001$) and exhibited higher proportions of Bifidobacteriales ($p = 7.1x10^{-6}$), Clostridiales ($p = 1.459 \times 10^{-5}$), Erysipelotrichales ($p = 0.0003$), and lower Bacteroidales ($p = 4.1x10^{-5}$)]. PNGR fecal metabolome had a reduced total bile acid pool ($p < 0.01$), as well as higher excreted fecal peptides ($p = 0.0064$) compared with CON[103]. Overall, these findings highlight the importance of investigating the relationship between PNGR and the gut microbiome to better understand the underlying mechanisms and potential health outcomes.

Studies examining the effects of aerobic exercise in human and animal cohorts have resulted in increased gut microbiome composition changes in abundance of specific microbial genre and species that may have beneficial functions in improving metabolic health and exercise capacity[201]. A study that investigated the effects of voluntary wheel running led to increases in the Bacteroidetes-to-Firmicutes ratio in healthy non-obese rats on a standard diet[202]. Treadmill running in mice has also been shown to significantly increase composition of other bacteria

genre such as *Tenericutes* and *Proteobacteria,* among many other types compared to the sedentary group[203]. In a recent study, Anhe et al. 2022 investigated the effects of aerobic exercise training (AET) on the gut microbiome composition in rodents selectively bred for low- or high capacity running but kept sedentary (LCR vs. HCR). The fecal microbiota of the sedentary mice had a higher relative abundance of Lachnospiraceae, Ruminococcaceae, Turicibacteriaceae, and *Allobaculum*, but lower *Bacteroidales*, *Alistipes*, *Akkermansia*, and *Anaeroplasma*. AET mice had a higher running capacity than SED mice. AET mice had lower fecal levels of Lachnospiraceae, Turicibacteriaceae, and *Allobaculum* and higher *Anaeroplasma* than SED mice. Recent findings show a specific microbe, *Eubacterium*, in humans, has been correlated with lower aerobic capacity[204].

In a recent study, participants with low VO_{2max} had lower abundance of *Bacteroides*, but higher *Eubacterium rectale-Clostridium coccoides* than the high VO_{2max} group[204]. Interestingly, VO_{2max} was found to be inversely associated with *EreC* but not with other bacteria. Unfortunately, the particular role of these microbes in terms of exercise capacity are still unclear. Most studies have characterized the microbiome from changes in specific taxa, which is challenging to translate to functional effects on the host. Some better characterized effects on host metabolism from the microbiome outside of exercise focused studies include their ability [177177]to ferment polysaccharides and other nutrients from diet into byproducts that improve metabolic health[187,205–207]. Exercise performance may also be enhanced by increasing gut microbial clearance and/or conversion of these byproducts from host circulation[208], such as those observed in *Veillonella,* which ferments lactate into propionate[209]. It is becoming evident from the microbiome literature that one of the major modalities that gut bacteria impacts human health is through their production of small molecules and metabolites. Perhaps most

significantly, a recent paper in *Nature* by Donhalova et al. showed that metabolites from the microbiome can impact the host's exercise performance[210]. Clearly, there is evidence for changes in the microbiome during exercise interventions and these changes can modify host metabolism, but how and via which metabolites and metabolic pathways remains mostly unknown.

2.10 Metabolic Perturbations in Early life GR

Nutritional insult during early life development has profound downstream effects on global protein synthesis and the growth and development of organs and tissues, such as in skeletal muscle and cardiomyocytes[5,126,211–216]. Dams undernourished during lactation impairs offspring pancreatic β-cell function which leads to increased risk of developing metabolic syndrome or diabetes[10,217]. Impairment in pancreatic β-cell function is likely due to a metabolic imprinting effect of undernutrition during postnatal development on the central nervous system (CNS) in offspring, leading to alterations in muscarinic acetylcholine receptors that are subsequently present in adulthood[10,217].

Other studies have further investigated subsequent effects of undernutrition during lactation on pituitary gland hormone regulation and found elevated leptin receptor expression with normal leptin concentration, low thyroid stimulating hormone (TSH) and high triiodothyronine (T3) and thyroxine (T4) levels in adulthood compared to CON[218,219]. There is some speculation that the metabolic perturbations are an attempt to compensate for impaired CNS signaling to improve energy homeostasis regulation[218]. The results from these studies strongly suggest that the undernutrition during lactation, of which is a vital period of CNS development in offspring, permanently programs the nervous system's control of insulin signaling, leading to impairments in energy regulation and metabolism into adulthood.

In addition to impairments shown in glucose regulation, alterations have also been found in amino acid as well as fatty acid profiles in offspring undernourished during lactation[11]. Early life undernutrition led to increased fatty acid (FA) β-oxidation and lower visceral, as well as total body fat, that persisted into adulthood[11]. In a previous study by the same lab, the undernourished dams resulted in offspring with divergent fatty acid profiles and altered amino acid levels compared to offspring from the control fed dams[220]. While it has been well established that amino acids play a vital part in early life growth and development[221–225], the role of fatty acids has not been elucidated well. These findings highlight the importance of understanding the role of early life nutritional insult on metabolic activity in adulthood.

2.11 Measuring Metabolic Response to Exercise and PNGR via Metabolomics

Metabolomics is a method to broadly identify, characterize and quantify metabolites from complex biological samples. Recent advancements in mass spectrometry-based metabolomics have allowed researchers to measure thousands of metabolites in a single sample. Further advances in the informatics of metabolomics data analysis have increased the ability to detect products of human metabolism in response to environmental stimuli, which can help identify metabolic biomarkers for predicting onset of disease and assess exercise performance and capacity[226–229]. However, a significant challenge in metabolomics science is that the vast majority of metabolites identified remain unknown and structurally uncharacterized, especially those associated with the diverse gut microbiome[230]. Despite these challenges untargeted metabolomics remains a powerful tool because it is a holistic approach that detects host molecules, xenobiotics and metabolites produced by the microbiome. Though the field is only in its infancy, recent advances in mass spectrometry-based metabolomics have enabled better

characterization of the metabolome and identification of metabolites associated with human health[230].

Exercise produces beneficial metabolic changes, which can be comprehensively measured via metabolomics. In 2012, a study by Chorell et al. aimed to characterize the human serum metabolome and establish correlations between metabolites and individual fitness level (as determined by VO_{2max})[231]. Untargeted metabolomics was used to characterize 460 plasma samples from 27 individuals taken pre- and post-exercise. The authors were able to see distinct differences in metabolites related to oxidative stress, energy metabolism and muscle leakage. A higher VO_{2max} was associated with decreased gamma-tocopherol (vitamin E isomer, GT), whereas a low VO_{2max} was with higher alpha-tocopherol (AT)[231]. Both metabolites are associated with oxidative stress and inflammation, with superior antioxidant support with GT in combating reactive nitrogen species and heat shock proteins. Increased association of GT with High VO_{2max} is likely due to enhanced adaptation to normal stress responses seen in exercise training, which is superior compared to individuals with low VO_{2max}. High VO_{2max} was also associated with elevated omega-3 (DHA) and decreased omega-6 polyunsaturated fatty acids (PUFAs), decreased saturated, monounsaturated, and trans- fatty acids compared to the low VO_{2max} group[231]. The lipid profile in total was decreased in the high VO_{2max} versus low VO_{2max} group, indicative of increased fatty acid utilization with increases in fitness level following exercise training. The authors also suggested that higher DHA associated with individuals with higher CRF (higher VO_{2max}) may reflect a cardioprotective effect due to other studies reflecting its anti-inflammatory and low-density lipoprotein (LDL) scavenging capabilities[231,232]. Overall, metabolites associated with higher fitness level reflected improved oxidative capacity, altered

fatty acid metabolism and anti-inflammatory metabolite activity compared to low fit individuals[231].

A recent systematic review highlighted the benefits of using high throughput metabolomic methods to characterize acute metabolic exercise responses in individuals. As expected, the metabolomics data overall supports what has been previously observed in the literature - alterations in intermediates associated with a specific pathway or area of metabolic activity (i.e., glycolysis, TCA cycle, etc.) and remains to be a reliable and reproducible approach[227].

[227227227]Metabolomics is also beneficial for comparing metabolic responses to specific exercise protocols, such as MICT versus HIIT. In a randomized cross-over design by Peake et al. 2014, blood samples were collected pre- and post-exercise to compare the metabolic effects of long duration (60 min) MICT (~67% VO_{2max}) and HIIT (~82% VO_{2max}) in 10 male cyclists[233]. Using a targeted metabolomics approach only 49 metabolites chemically identified, with 29 being altered in response to exercise. Immediately following HIIT, TCA cycle intermediates and monosaturated fatty acids were much higher compared to MICT. Interestingly, total serum free fatty acid concentration was not different between HIIT and MICT[233]. Thus, as expected, the evidence indicates HIIT relies more on glycolytic and TCA cycle activity compared to MICT, with differential utilization in the types of fatty acids between intensities. Although this study provides some framework into the metabolic demands between exercise protocols, an untargeted approach would allow for more comprehensive insight.

Metabolomics is generally categorized into either untargeted or targeted approaches. Untargeted metabolomics is a more comprehensive analysis of all the metabolites within a sample, including chemical unknowns[234]. Targeted metabolomics is a more restrictive technique, analyzing metabolites derived from more specific molecular pathways (i.e., hormones or

steroids). There are many benefits to using an untargeted approach, such as the ability to generate thousands of metabolites measured using a single sample[234]. Generating higher throughput datasets is an efficient way to facilitate foundational knowledge of the underlying metabolic activities in a study. This is especially helpful in novel areas of research which may have had less prior supporting evidence for directing study design or analysis (e.g., PNGR response to HIIT). Untargeted metabolomics also typically utilizes more generalized and flexible extraction methods, of which are less restrictive in comparison to targeted approaches. Targeted metabolomics requires highly specific and technical extraction methods, as well as molecular internal standards, in order to identify specific proteins of interest. While the targeted approach is beneficial in measuring proteins of interest, the number of molecules generated in one assessment is much smaller, which leaves thousands of remaining molecules in that sample left unstudied. However, targeted metabolomics is beneficial in reducing artifacts and chemical unknowns in the dataset and is an overall clearer outcome, assuming the investigator has molecules of interest that are already known[234]. Untargeted, while high throughput, produces many chemical unknowns which are generally unannotated and must be confirmed for their metabolite name via assessment of molecular weight and biochemical makeup, as well as their retention time[235].

2.12 Summary

It is well established that undernutrition induced GR in early life largely influences cardiometabolic health outcomes in adulthood. Exercise is a primary method known to reduce disease onset, however, individuals exposed to early life GR exhibit reduced exercise capacity and in turn respond poorly to previously tested exercise interventions. In humans, variability in exercise response is commonly seen [38,140,178,236–238], evident by atypical outcomes in CRF

biomarkers (e.g., VO_{2max}) post-intervention. Although various genetic and phenotypic hypotheses exist, the true underlying mechanisms behind adverse responses to exercise have not yet been established[168,239,240]. More focus has been placed investigating the effects of HIIT in chronic disease and healthy populations, with recent studies showing promising outcomes. Additionally, growing evidence supports evaluating metabolic associations with training, especially in the PNGR population, via characterizing the gut microbiome and metabolome and serum metabolic profile. Therefore, the purpose of this dissertation will be to evaluate the response to HIIT in the PNGR population in adulthood as well as determine the microbiome and metabolomic profile outcomes to prevent and ameliorate chronic disease risk.

2.13 Methodology and Rationale

To accomplish the previously described aims, specific methodologies to examine and improve exercise response will be utilized. The following section describes the supporting literature and mechanisms behind the methods utilized throughout this dissertation.

2.13.1 Mouse Strain & Nutritive Model

The FVB strain of mouse is the best fit for this experimental design due to the ease of cross-fostering, generation of large litters, as well as being genetically homogenous[161,241]. FVB mice have been reported to exhibit significant improvements in performance with exercise training, and these improvements were associated with expected cardiovascular and metabolic adaptations to training.

Avila & Massett et al. aimed to investigate exercise capacity and training responses in 24 genetically diverse inbred mouse strains[139]. The FVB strain of mouse were among the 24 investigated in this study. The FVB mice showed a significant increase in exercise capacity after 4 weeks of exercise training, with the highest change in work performed compared to the other

strains[139]. The change in work performed in the FVB mice was +2.30 kg/m (~22.6 J)[139], which indicated a substantial improvement was made in their endurance exercise capacity in just 4 weeks, similar to the CON mice described in the above studies.

Our lab utilizes a well-established, cross-fostering nutritive model to induce GR. Nutrient restriction is initiated immediately following cross-fostering of pups to their new dams, where they nurse from moms fed a low protein diet from postnatal days (PN) 1-21. This model allows for isolating GR to the postnatal period of development[2–7,19,74,103]. Two weeks prior to breeding animals were randomly assigned to one of two experimental diets: 1) Normal protein diet (NP, Research Diets) consisting of 20% protein (control diet), or 2) Low protein diet (LP, Research Diets), consisting of an isocaloric, 8% protein diet. The LP diet reduces the volume of milk produced in lactating dams, which restricts calories to suckling pups, thus growth-restricting them [242,243]. After the two-week diet acclimation, the males were introduced for a 24-hour period, so all pups born to be the same age. At postnatal day (PN) 1, the pups from dams fed the NP diet will be pooled, weighed, and cross-fostered to another NP fed dam, or CON. The other half of NP fed pups were cross-fostered to form the two experimental groups: 1) pups from the NP-fed dams cross-fostered to another NP-fed dam, or CON or 2) pups from a NP-fed dam cross-fostered to an LP-fed dam, or PNGR. Each litter was standardized to equivalent male/female ratio (4 males, 4 females) and number (8 pups per litter) to maintain equivalent milk production[243]. Pups were also given identifying tattoos and weighed biweekly from days PN1-21 (weighed weekly after PN21). At PN21 pups were weaned to the NP diet to isolate the window of GR to postnatal early life. This model has been adopted and repeatedly validated by the Ferguson lab at MSU as well as other labs across the globe[2,3,5,244,245].

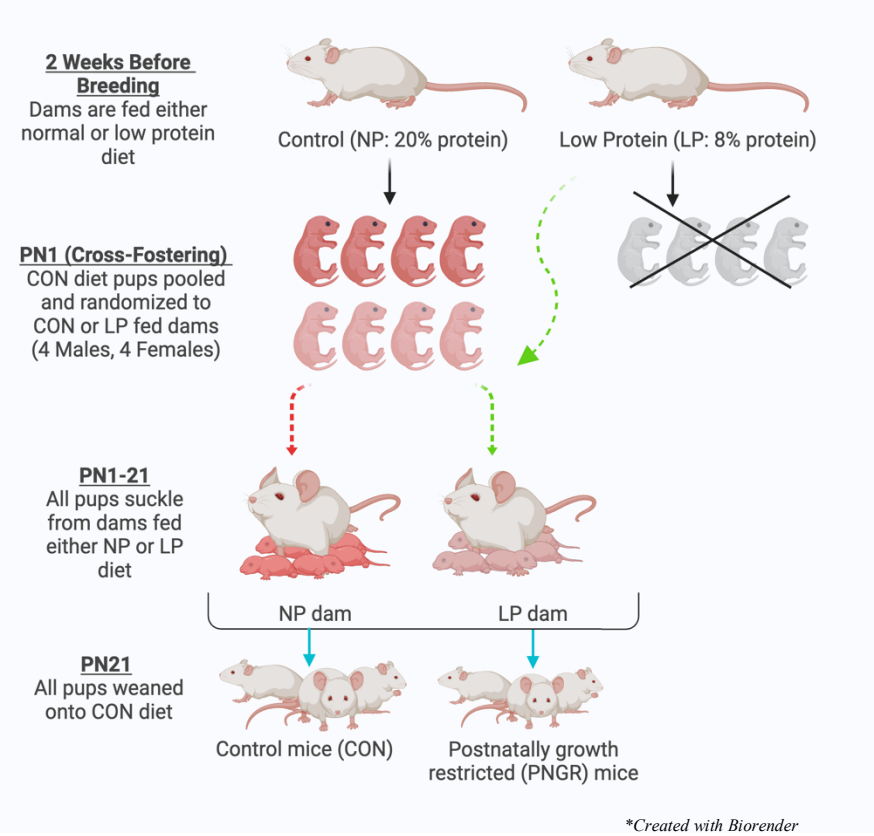

Figure 2.11 ***Cross-fostering Nutritive Model & HIIT Experimental Design.***
(Adapted from studies by Kim, Avila & Massett [39,138,139] *and Kemi et al.*[183]*)*

2.13.3 Treadmill Acclimation

Mice were acclimated to the treadmill for 2 consecutive days. Acclimation was performed 3 days prior to maximal exercise (W_{max}) testing to avoid any training effects. The acclimation was performed using an adapted version of a previously published treadmill acclimation protocol in FVB mice[39,241]. Based on previously validated methods[39,241], the initial days of acclimation involve very little movement of the treadmill to allow for full exploration, while the following days implemented more movement and exposure to the shock grid. While short, but efficient, these methods allow for the mice to be adjusted the least amount of stimulus necessary to properly adjust prior perform the exercise capacity testing to avoid undue stress responses[39,139,241].

2.13.4 Initial Exercise Capacity (W_{max}) Testing

The methodology utilized for W_{max} testing as well as HIIT were adapted from previous studies by Kim, Avila & Massett [39,138,139]. Exercise capacity was measured in terms of time and work performed during the graded exercise test, thus providing quantitative data on the performance levels of the mice at baseline, as well as at the end of the study.[34,225] Body mass was measured prior to W_{max} testing to avoid effects of the training bout on initial mass values. At the beginning of testing the mice were provided a 9-minute warm up at 9 m/min at 0° grade. After 9 min, the grade was increased to 5° and the speed increased to 10m/min for 3 minutes. Every 3 minutes thereafter, the speed was increased by 2.5 m/min (Fig. 2.12) and the grade was increased 5° every 9 min up to a final grade of 15° (or until exhaustion). Exhaustion was defined as contacting the shock grid at the back of the treadmill for a total of 5x in 30 seconds[6]. Once exhaustion is reached, the shock grid is turned off to allow the mouse to rest and the data is recorded for later use. All data was recorded upon exhaustion. W_{max} is calculated from the data as follows: [W_{max} (J/min) = (mass in kg x 9.81) x (final incline in radians) x (final speed in m/min) x (final distance in meters)].

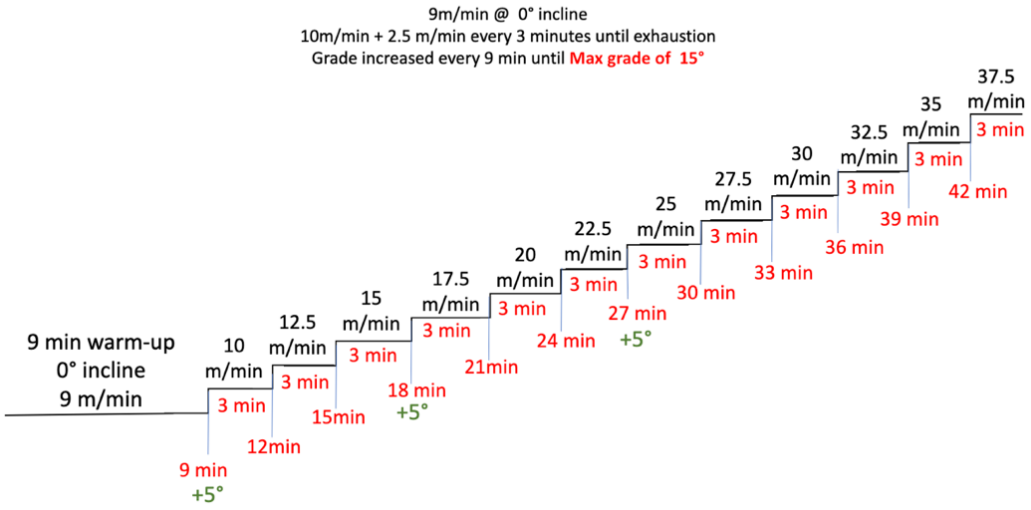

Figure 2.12 ***Exercise Capacity Testing Protocol.***
Protocol Adapted from Kim, Avila, & Massett [39,138,139]

2.13.5 High-Intensity Interval Training

The methodology utilized for HIIT was adapted from previous studies[135,138,246]. Training speeds were prescribed to each treatment group assigned to HIIT and are calculated via distance, final speeds, final time, body mass collected from the W_{max} tests. Speeds for each HIIT interval was calculated via W_{max} values of 85% W_{max} for the 8-min high-intensity intervals and 50% W_{max} for the 2-min active recovery. Due to there being six lanes on the mouse treadmill, if mice are given the same calculated running speed, then those mice would be trained at the same time.

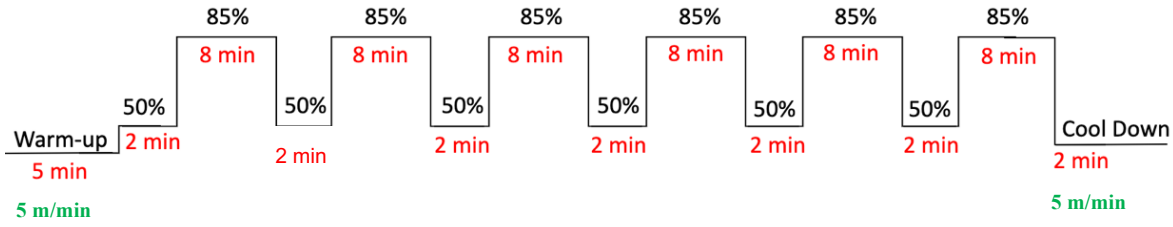

Figure 2.13 ***Detailed HIIT Protocol.***
Protocol adapted from Kim et al. 2019[138] & Wisloff et al. 2002[247]

The work:rest interval durations and intensity percentages were adapted from multiple studies that have repeatedly validated a very similar protocol setup which resulted in superior exercise capacity (40-70% VO_{2max}) improvements in rodents[248]. The authors of which originally developed the interval setup tested the intensity and duration repeatedly in rodents and found it was the highest attainable intensity that could be maintained over several weeks of training[147]. Similar adapted versions of this protocol have highlighted substantial cardiovascular benefits with a four-fold increase in acetylcholine sensitivity which improved endothelial-dependent vasodilation[249], decrease risk of arrhythmias via improved calcium spark control[250], increased cardiomyocyte length, left and right ventricular mass (~15-20%), and 40-50% increase in fractional shortening[135]. Cardiovascular contraction as well as relaxation rates also have shown to improve by 20-40% with use of this HIIT protocol[55,67,183,251].

The use of active recovery intervals compared to passive in HIIT are more optimal in increasing lactate clearance as opposed to passive recovery (full resting state), which leads to improved recovery and fatigue offset. Menzies et al. 2010, showed in humans using various interval intensities ranging from passive (0% lactate threshold; LT) to 100% active LT that active recovery allowed for faster lactate clearance and recovery compared to passive ($p<0.01$)[72]. Additionally, active recovery intensities between 60-100% of LT were more efficient for lactate clearance than lower intensity active recovery ($p<0.05$), although lower intensities were still superior to passive recovery ($p<0.05$)[72]. Measuring lactate threshold, however, can be very challenging to accomplish in rodents without introducing confounding stress or anxiety[135].

Additionally, a recent study in humans comparing rest durations in HIIT showed that rest time >80 sec allowed for increased work performed in the following work interval [252]. Shorter recovery (2 minutes, as compared to 3- or 4- minute recovery durations) activates glycolytic metabolism, especially in skeletal muscles to a greater extent[252], which may cater to the glycolytic muscle phenotypes observed in PNGR[2,6]. These findings in combination with the substantial positive evidence regarding the forementioned adapted protocol using 8 minutes of high-intensity (85%) followed by 2 minutes of low-intensity active recovery (50%), supported its use in the current study [55,67,135,136,183,246].

2.13.6 Follow-up Exercise Capacity (W_{max}) Testing

A W_{max} test was performed after one week of training in the pilot study of this dissertation for quality control purposes to ensure speed prescriptions derived from W_{max} values collected were optimum enough to produce positive training adaptations. Since the initial cohort was exposed to this additional test, to keep things consistent it was decided to keep all remaining cohorts on the same protocols and continue to include a test post one week of training. A final

W_{max} test was also performed at the end of the intervention (PN71) to compare to baseline W_{max} values to measure changes in exercise capacity in response to the training protocol.

2.13.7 Microbiome & Metabolome

The gut microbiome plays a critical role in host metabolism and can be influenced by factors such as host genotype, diet, and exercise training[253,254]. Studies have shown that exercise training can lead to enhanced exercise performance of which is mediated by the gut microbiota[80,82,253,255]. Additionally, exercise training has been shown to increase metabolites made by the host as well as by the microbes themselves, both of which potentially influence metabolic function and exercise adaptations[82,253,256–259]. Therefore, studying the microbiome in relation to exercise capacity can provide insights into the mechanisms underlying exercise-induced improvements in performance and metabolic profile. Fecal samples allow for collection of metabolic intermediates in a method that is non-invasive and prevents unnecessary euthanasia common with other methodology. Additionally, fecal samples allow for simultaneous characterization of host and microbial metabolites in addition to microbial composition and diversity metric analysis. Metabolomics analysis via blood collection by either plasma or serum are well-established methods in evaluating circulating metabolites and provides an overall insight into host metabolism as opposed to isolation of a specific tissue. Due to the high-throughput of these data collection types, and their ability to create a 'snapshot' in time of what is occurring endogenously and metabolically, they are viewed as preferred methods[85,260–262]

2.13.8 Statistics Rationale

Microbiome and metabolome research, especially within the niche research topic exploring the effects of HIIT in PNGR is still fairly novel. The primary aim was to perform an exploratory study. However, exploratory studies can come with confounding variables such as

breeding round (batch effects) as well as cage effects[263,264]. Aiming for very large sample sizes could lead to the cage and breeding effects masking any physiologically relevant variables of interest[263,264]. Additionally, due to the complexity and non-parametric nature of metabolomic and microbiome data, using power analyses to estimate sample size would lead to incorrect estimates due normality assumptions, of which this type of data is not normally distributed. Thus, instead this study implemented other common and previously validated methodology such false discovery rate (FDR) p-value correction[265,266]. Therefore, the aim is to generate enough mice and samples to see an effect while limiting the number of breeding rounds required. Based the previous study (Quinn et al. 2022), a sample size of n=5-6 per group was used (without inclusion of sex or training groups, total of 17 mice, total of 85 samples), thus this sample size for the microbiome and fecal metabolome data was extended to also include both sex and training groups (n=5-6; total of 46 mice; total of 230 samples; [CON-SED n=10, CON-TRD n=11, PNGR-SED n=13, PNGR-TRD n=12]. The serum metabolomic data sample sizes were as follows: [PNGR-SED n=14, PNGR-TRD n=14, CON-SED n=15, CON-TRD n=15].

For the W_{max} data, a power analysis (via GPower software) was utilized to estimate sample size for a 3-way interaction (diet x sex x treatment) with an alpha set at 0.1 and beta set at 0.8 and effect size at 0.4 (large) for 8 total groups of mice, which resulted in an estimated sample size of 96 mice or n=12/treatment group [PNGR-SED n=12, PNGR-TRD n=12, CON-SED n=12, CON-TRD n=12]. Effect size was calculated via a Cohen's f test based off of the sum of squared mean differences and standard deviations on data from Ferguson et al. 2019[7]. To analyze the repeated measures appropriately within and between groups, it is recommended to maintain equal sample sizes per group[267], thus the n remained at 12 for the W_{max} datasets. A Shapiro-Wilk

test for normality was used, which showed normal distribution of the HIIT data at each timepoint among treatment groups, thus no additional data transformations were utilized.

CHAPTER 3:

HIGH INTENSITY INTERVAL TRAINING IMPROVES EXERCISE CAPACITY IN POSTNATALLY GROWTH RESTRICED MICE

3.1 Abstract

Introduction: Early life growth restriction (GR) affects over 161 million children globally under the age of 5 per year. GR occurring during postnatal life (PNGR) increases the risk of cardiometabolic disease by 47%. Exercise is well-known to prevent and/or treat cardiometabolic disease, however previous studies have shown an exercise 'non-response' in PNGR mice. High-intensity interval training (HIIT) leads to more pronounced health benefits compared to traditional endurance training. The physiological effects of HIIT have yet to be established in PNGR. Thus, we aimed to establish metabolic evidence underlying exercise response to HIIT in PNGR.

Methods: To induce GR mice nursed from dams fed a low protein diet (LP; 8%) or control diet (CON; 20%) until weaning at postnatal age of 21 days (PN21). At PN21, all mice were fed the CON diet until adulthood (PN70). Exercise capacity (W_{max}) was tested a baseline (PN44) and at the end of the intervention (PN71). At PN45, mice were either HIIT trained (1 hour/day, 5 days/week @ 15º grade or kept sedentary. Interval speeds were calculated as high: 85% W_{max} and low: at 50% W_{max}. Serum samples were collected at the end of the intervention (PN73) following one day of rest. Fecal samples were collected for microbiome analysis at baseline through the end of the intervention.

Results: Repeated measures ANOVA of PN44 and PN71 showed that W_{max} improved in CON-M-TRD, CON-F-TRD, PNGR-F-TRD, and PNGR-M-TRD ($p<0.0001$) cohorts as a result of HIIT. All cohorts improved from baseline values, especially PNGR-F with a 43.9% increase in W_{max} between PN44 and PN71. Although exercise capacity improved, many serum metabolites remained blunted in response to training as compared to CON, including downregulated heme, acylcarnitines, glutathione, nicotinamide, and essential amino acids: phenylalanine and

methionine. Fecal palmitoylcarnitine excretion was the primary metabolite that did improve with HIIT in PNGR. Riboflavin was elevated in PNGR-SED and increased further with training. Microbial diversity metrics were not significantly altered with HIIT. However, HIIT resulted in increased composition of microbes Clostridiaceae *g.s.* (KW $p<0.001$) solely in PNGR-TRD, as well as in decreases of Christensenellaceae *g.s.* (KW $p=0.02$) in both CON-TRD and PNGR-TRD, although their physiological roles in this specific case are currently not clear.

Conclusion: Results from this study provide strong evidence that HIIT improves exercise capacity in PNGR. Some aspects of PNGR metabolism in response to HIIT are still blunted in comparison to CON, especially in areas of protein metabolism as well as mitochondrial oxidation and markers of oxidative stress regulation.

3.2 Introduction

Early life GR affects approximately 22% of children globally under the age of 5 per year[88]. Growth restriction during postnatal life (PNGR) significantly increases cardiometabolic disease risk in adulthood by 47%[2]. Exercise is a prominent method to either prevent or ameliorate cardiometabolic disease[15,268], however recent findings have shown PNGR exhibit a 'non-response' to moderate-intensity endurance exercise adaptations[2,6,7]. The gut microbiome is a vital component of host metabolism and may also improve exercise adaptations through interaction with nutrients taken in through diet as well as other metabolic intermediates. Although, the impact of exercise on the gut microbiome composition in PNGR has not yet been established. High-intensity interval training (HIIT) leads to exponential health benefits that outweigh those of traditional (moderate-intensity) endurance training, which has been shown in both healthy and diseased populations. HIIT has been shown to improve metabolic function via

increased insulin sensitivity, lipid oxidation as well as mitochondrial biogenesis. However, the physiological and metabolic effects of HIIT are currently not well characterized in PNGR.

The primary aim of this study is to determine the effect of a HIIT intervention on maximal exercise capacity (Wmax) in PNGR mice as compared to CON. Therefore, the overarching hypothesis is that HIIT could improve exercise adaptations via improved metabolic response which is reflected in enhanced gut microbiome composition and metabolomic profile in PNGR.

Additionally, we aim to determine the effects of HIIT on the microbial compositions in PNGR and CON mice. It is hypothesized that HIIT in CON and PNGR will result in increased abundance and enrichment of unique taxa as well as microbial diversity compared to their SED counterparts. Also, it is hypothesized that HIIT will result in higher Bacteroidetes to Firmicutes ratio (typically inversely associated with cardiometabolic disease) in the PNGR and CON mice compared to their SED counterparts.

Finally, we also aim to determine the effects of HIIT on the gut and serum metabolome in PNGR and CON mice. It is hypothesized that primary alterations in the serum and gut metabolome will reflect changes in fatty acid oxidation and carnitine activity between treatment groups. Completion of these aims will help to contribute valuable insights into the metabolic responses and potential benefits of HIIT in the context of PNGR individuals to prevent and treat cardiometabolic disease in adulthood.

3.3 Methods

3.3.1 Ethical Approval

This study has been approved by the MSU Institutional Animal Care and Use Committee (ID: PROTO202100230). All procedures were performed following the proper use and care of

laboratory animals. Experimental FVB mice were bred from an existing colony of breeders and kept in a vivarium maintained at 18-21°C on 12-hour day/light cycles (PROTO202100234). Food and water were provided *ad libitum*.

3.3.2 Nutritive Model

A previously validated cross-fostering nutritive model was used to induce GR. Two weeks prior to breeding mice were randomly assigned to one of two experimental diets: 1) Normal protein diet (NP, Research Diets) consisting of 20% protein, or 2) Low protein diet (LP, Research Diets), consisting of an isocaloric, 8% protein diet. Following the two-week diet acclimation, the males were introduced for a 24-hour period, so all pups born were the same age. At postnatal day (PN) 1, the pups from dams fed the NP diet were pooled, weighed, and cross-fostered to another NP fed dam (CON), while the other half of pooled NP fed pups were cross-fostered to a LP fed dam, which isolates GR to the period of postnatal development via lactation, thus creating the postnatally undernourished group (PNGR). All litters were standardized to have equal male/female ratio (4 males, 4 females) and total number (8 pups per litter) to maintain equivalent milk production[243]. At PN21 all pups were weaned onto the NP diet throughout adulthood.

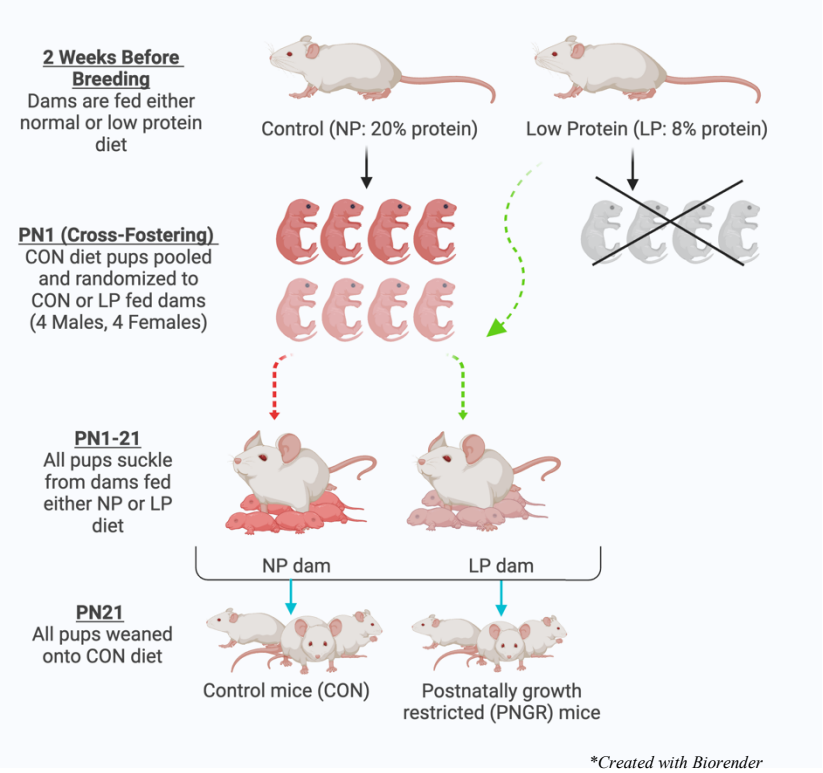

Figure 3.1 Cross-Fostering Nutritive Model

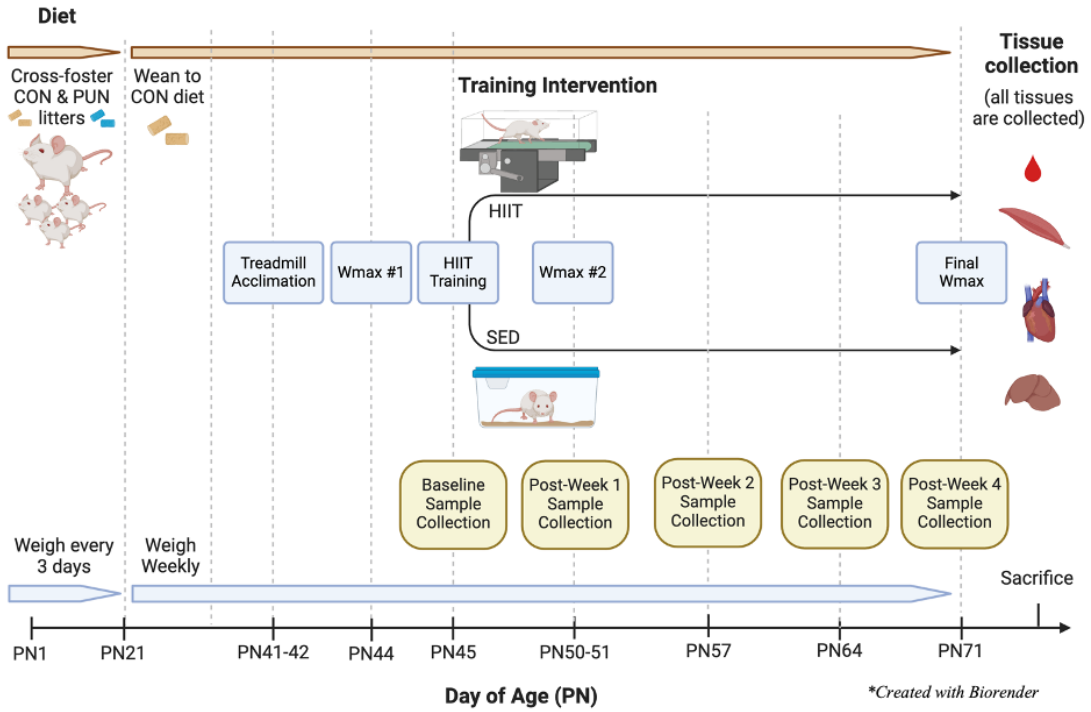

Figure 3.2 Comprehensive Study Timeline

3.3.3 Treadmill Acclimation

Mice were acclimated to the treadmill for 2 consecutive days, 3 days prior to maximal exercise (W_{max}) testing to avoid any training effects[39,241]. Acclimation involved initial exploration 5 minutes at 0 m/min, followed by a slow, gradual increase in speed to a maximum of 5 m/min for 5 minutes. The second day of acclimation began with 1-2 minutes for exploration similar to the previous day, followed by 2-3 minutes of walking pace at 5 m/min, and then a subsequent increase to 10 m/min for the remaining 5-6 minutes. The shock grid was utilized on both days to allow acclimation to low frequency (1/min) and low voltage (0.8 mHz) shock stimulus.

3.3.4 Exercise Capacity (W_{max}) Test

The methodology utilized for W_{max} testing as well as HIIT were adapted from previous studies by Kim, Avila & Massett [39,138,139]. Exercise capacity was measured in terms of time and work performed during the graded exercise test, thus providing quantitative data on the performance levels of the mice at baseline, as well as at the end of the study.[34,225] Body mass was be measured prior to W_{max} testing. The mice began the test with a 9-minute warm up at 9 m/min at 0° grade. After 9 min, the grade was increased to 5° and the speed increased to 10m/min for 3 minutes. Every 3 minutes thereafter, the speed was increased by 2.5 m/min and the grade was increased 5° every 9 min up to a final grade of 15° (or until exhaustion). Subjective criteria was used to define exhaustion as contacting shock grid at the back of the treadmill for a total of 5 times in 30 seconds[6]. Once exhaustion is reached, the shock grid is turned off to allow the mouse to rest and the data is recorded for later use until all mice in adjacent lanes have completed the test. A final W_{max} test was also performed post-intervention (PN71) to compare to baseline W_{max} values and measure changes in exercise capacity in response to HIIT protocol. W_{max} was

calculated as follows: [W_{max} (J/min) = (mass in kg x 9.81) x (final incline in radians) x (final speed in m/min) x (final distance in meters)].

3.3.5 High-Intensity Interval Training

The HIIT protocol was adapted from previous studies[135,138,246] and included 6 cycles of low intensity (50% W_{max}, 2 minutes), followed by high intensity (85% W_{max}, 8 minutes) intervals. The training was initiated with a warm-up of 5 m/min for 5 minutes and a cool down post-exercise of 2 min at 5 m/min for a total training duration of approximately 67 minutes.

3.3.6 Serum Metabolomic Sample Processing

The serum samples were collected post-training intervention following a minimum of 24 rest. To perform euthanasia, mice were first anesthetized with 1% isoflurane, followed by cervical dislocation. Sterile surgical scissors were then used to open the chest cavity and remove the heart. The pooled blood was then collected using a disposable pipette and placed in a centrifuge tube to be processed for serum isolation. The serum sat at room temperature and was disrupted periodically with a sterilized glass instrument to breakdown blood clots. The sample tubes were then centrifuged at room temperature at 2,500g for about 15 minutes until the hematocrit and serum have separated into two phases. The serum was then pipetted into a clean tube and placed into the -80C freezer for later use. To perform the extractions, the serum was combined with a chloroform-methanol mixture (1:2 v/v ratio) to form a monophasic solvent system to extract and dissolve the lipids and then subsequently vortexed. The resulting suspension consisted of chloroform/methanol/water mixture with volumetric ratios of 1:2:0.8 (v/v/v). A biphasic system was then produced in a purification step by the addition of water and more chloroform, which then shifted the mixture to volumetric ratios of 2:2:1.8 (v/v/v). The mixture was then filtered using medium flow filter paper, allowing for a separation of polar and

non-polar compounds into upper (polar) and lower (non-polar) phases. The samples were then separated based on polar (e.g., amino acids, salts, etc.) and non-polar compounds (e.g., lipids)[269].

3.3.8 Fecal Metabolomic Sample Processing

Mouse fecal samples were collected as previously described[103], starting from baseline at PN44 and once per week until the end of the intervention (PN71). Fecal homogenates were then processed using methods from our previous study[103]. All metabolomics data was generated during the run on the LC-MS/MS system as a single batch. The metabolomics raw data was converted to the .mzXML format, and the area-under-curve abundance of each feature was calculated using MZmine 2 software. Molecules found in blanks were removed from the data set prior to analysis. The mass spectrometry MZmine 2 output files were uploaded to the Global Natural Products Social Molecular Network (GNPS) database. Subsequent library searching and feature-based molecular networking were performed using parameters as previously described[103]. The mass spectrometry data will be deposited on a public repository once completed prior to publication.

3.3.9 Metabolomic Data Analysis

In order to generate a list of metabolites driving differences in the dataset by treatment group, a principal coordinate analysis (PCA) plot and PERMANOVA were used. Subsequent testing of the dataset as a whole involved generating volcano plots of the log-transformed fold-change differences between treatment groups (Log2FC) and the use of a false discovery rate (FDR<1.0) p-value adjustment (-log(FDR)). In order to further analyze differences between treatment groups, a subsequent Kruskal Wallis and post-hoc Dunnet's test were used.

3.3.10 Microbiome Data Analysis

Microbiome data was analyzed via Qiita[270]. The data was first rarefied to avoid potential false positive results and to standardize sampling distribution. The microbial differences in diversity within each sample population was analyzed via alpha Faith's test of alpha diversity. Beta diversity was analyzed via PERMANOVA to determine differences in distance between treatment groups. Phylogenetic differences were analyzed via non-parametric Kruskal Wallis (alpha<0.05) and post-hoc Wilcoxon Pairwise testing in Rstudio.

3.3.11 Statistics

Treadmill data was analyzed via Graphpad Prism v.10 and JMP (v.14.0). Initial analyses for differences between treatment groups at each individual timepoint involved a three-way factorial ANOVA with diet (CON vs. PNGR), sex (M vs. F), and treatment (SED vs. TRD) as the main independent categorial fixed variables and W_{max} (J/min) as the primary continuous outcome variable. A Shapiro-Wilk test for normality was used, which showed normal distribution of the HIIT data at each timepoint among treatment groups. Body mass was tested with all three main predictor variables to determine significance in use as a covariate. Due to insignificance with the interaction tests, the addition of body mass as a covariate was not used in this analysis. To measure differences in means between timepoints and treatment groups a repeated measures ANOVA was used (e.g., Baseline vs. Final W_{max} Test). An alpha level of 0.10 was set for testing 3-way interactions, and a 0.05 was set *a priori* for all other interactions. When appropriate, a Tukey's HSD post hoc test for multiple comparisons was performed. Percent improvements in W_{max} were calculated from the average improvement of each treatment group as follows: [percent improvement = (final-baseline)/(baseline)*100], with variance reported as standard deviation (SD). Litters were used as the statistical unit for the HIIT data which was generated previously via a power analysis (alpha=0.01; power=0.08; effect size=0.4) which

estimated an n = 98 total mice. The final sample size for the HIIT data was as follows: [PNGR-F-SED n=12, PNGR-F-TRD n=12, PNGR-M-SED n=12, PNGR-M-TRD n=12, CON-F-SED n=12, CON-F-TRD n=12, CON-M-SED n=12, CON-M-TRD n=12]. Due to the non-parametric nature of the microbiome and metabolome data, a power analysis was not utilized, but instead were based off of sufficient sample sizes used in previous studies. Limited breeding rounds were implemented to reduce occurrence of batch effects. The resulting sample sizes for the microbiome/metabolome were as follows: [Microbiome: n=5-6; total of 46 mice; total of 230 samples; [CON-SED n=10, CON-TRD n=11, PNGR-SED n=13, PNGR-TRD n=12]; [Metabolome: PNGR-SED n=14, PNGR-TRD n=14, CON-SED n=15, CON-TRD n=15].

3.4 Results

3.4.1 Effects of HIIT, Diet and Sex on Growth Rates and Body Mass

Longitudinal growth curves (Fig 4.1) were generated to confirm GR via the previously established and validated cross-fostering nutritive model achieved GR. Analysis of body mass over time confirmed significant divergence of mass by diet group (CON vs. PNGR) initiating at PN4 (Fig. 4.1) with PNGR having lower body mass compared to CON. At PN19-20 a secondary shift occurred, which separated males from their female counterparts, with males being heavier than females. After one week of training (PN49) there was a third separation of body mass by treatment group (SED vs. TRD), leading to lower body mass as a result of HIIT which lasts until the end of the study.

3.4.2 Effects of HIIT and Diet on Exercise Capacity

3.4.2.1 Exercise Capacity at Baseline

As expected, exercise capacity at baseline (PN44) was higher overall in CON vs. PNGR($p<0.0001$) and higher in males vs. females (M vs. F $p<0.0001$). Post-hoc tests revealed

that baseline W_{max} was reduced in PNGR-F vs. all other groups (PNGR-F vs. CON-F $p<0.0001$; PNGR-F vs. PNGR-M $p=0.0295$; PNGR-F vs. CON-M $p<0.0001$). CON-F also had lower baseline W_{max} compared to CON-M ($p=0.0066$). There was also no effect of treatment (TRD) at baseline – which was expected.

3.4.2.2 Exercise Capacity Post-Intervention

At PN71, W_{max} was higher in CON compared to PNGR ($p<0.0001$), higher in TRD compared to SED ($p<0.0001$), as well as higher in M vs. F ($p<0.0001$); (Fig. 4.2). Post-hoc multiple comparisons showed a higher W_{max} in CON-M-TRD vs. CON-F-TRD ($p=0.0003$) with CON-M-TRD also improving compared to CON-M-SED ($p=0.0349$). It was confirmed that W_{max} was higher in response to HIIT in both PNGR-F-TRD vs. PNGR-F-SED ($p=0.012$), and in PNGR-M-TRD vs. PNGR-M-SED ($p=0.0005$). However, PNGR-F-TRD had lower W_{max} compared to PNGR-M-TRD ($p<0.0001$), which highlights a blunted response to training in PNGR-F versus their male counterparts (Fig. 4.2).

A RM ANOVA was also performed to examine differences in W_{max} within treatment groups between baseline and post-intervention with all TRD cohorts: PNGR-M, PNGR-F, CON-F and CON-M all improving from baseline ($p<0.0001$) with HIIT in comparison to their SED cohorts (Fig. 4.2). Percent improvements of W_{max} were calculated between baseline and post-intervention in both SED and HIIT groups. For the HIIT trained mice, PNGR-M-TRD had the largest percent improvement at 61.0 ± 40.2%, followed by 46.1 ± 13.1% in CON-M-TRD, 44.9 ± 17.2% in PNGR-F-TRD and 43.9 ± 36.7% in CON-F-TRD. Most of the SED mice had % improvements below 20%, aside from PNGR-M-SED which had a 28.8 ± 10.0% improvement [PNGR-F-SED: 12.5 ± 8.8%, CON-F-SED: 16.0 ± 7.7%, CON-M-SED: 14.6 ± 8.9%,].

3.4.3 Effects of Diet and HIIT on the Serum Polar Metabolome

Polar serum metabolites were analyzed for signatures of clustering or separation showing effects of effects of diet, sex and/or treatment on the molecules in the entire dataset (Fig. 4.3). The PERMANOVA by both diet and sex in the polar serum metabolites was not significant (R^2= 0.035; p=0.451). However, after further initial separation by diet groups, and analyzed for an effect of treatment, the PNGR group (R^2=0.122; p=0.036) had the strongest signature, while CON was not significant (R^2=0.067; p=0.422); (Fig.4.3). These results indicate a greater separation of serum metabolites in the PNGR group as a response to HIIT compared to CON.

3.4.3.1 Effects of Training on the PNGR Serum Metabolome

Differential analysis of serum metabolites in PNGR in response to 4 weeks of HIIT (Fig. 4.4) revealed distinct up- and downregulated intermediates primarily in areas of mitochondrial fatty acid oxidation, mitochondrial reactive oxygen species (ROS) and antioxidant regulation, protein metabolism and heme. There was an upregulation (FC >1.0; FDR>0.1) of Riboflavin, and a downregulation (FC <1.0; FDR>0.1) of heme, glutathione, and amino acids.

3.4.3.2 Effects of Training on the CON Serum Metabolome

Differential analysis of serum metabolites (Fig. 4.5) revealed primary areas of up- and downregulated metabolites in response to 4 weeks of HIIT were primarily in areas of energy regulation and fatty acid metabolism. There was an upregulation (FC >1.0; FDR>0.1) of Xanthosine, Heme, and a downregulation (FC <1.0; FDR>0.1) of Taurocholic acid.

3.4.4 Individual Metabolite Analysis

3.4.4.1 Fat Oxidation Intermediates - Acylcarnitines

Multiple acylcarnitines, which are vital intermediates in mitochondrial fat oxidation were altered in the PNGR group (Fig. 4.6). Butyrylcarnitine, propionylcarnitine, succinylcarnitine,

hexanoylcarnitine, and palmitoylcarnitine were all downregulated in PNGR-SED and did not improve with training in PNGR-TRD [Butyrylcarnitine: PNGR-SED vs. CON-SED ($p<0.0001$) and CON-TRD ($p<0.0001$); PNGR-TRD was lower than CON-TRD and CON-SED ($p<0.0001$; Propionylcarnitine (D) lower in PNGR-SED vs. CON-SED and CON-TRD ($p<0.0001$), lower in PNGR-TRD vs. CON-TRD and CON-SED ($p<0.0001$); Succinylcarnitine (E) was lower in PNGR-SED vs. CON-SED ($p=0.0441$) and PNGR-TRD was lower than both CON-TRD ($p=0.0441$) and CON-SED ($p=0.0005$). Hexanoylcarnitine (F) was lower in PNGR-SED vs. CON-SED and CON-TRD ($p<0.0001$), and lower in PNGR-TRD vs. CON-TRD and CON-SED ($p<0.0001$); Palmitoylcarnitine (G) was lower in PNGR-SED vs. CON-SED and CON-TRD ($p<0.0001$), and lower in PNGR-TRD vs. CON-TRD and CON-SED ($p<0.0001$);].. Myristoylcarnitine, a long-chain acylcarnitine was upregulated in PNGR-SED (Fig. 4.6) compared to CON-SED ($p<0.0001$), and higher in PNGR-TRD vs. CON-TRD ($p=0.0429$)]. Off-set in abundance of acylcarnitines in PNGR reflects altered mitochondrial fat oxidation activity of which does not improve with HIIT.

3.4.4.2 Mitochondrial Metabolites Related to Oxidative Stress, Energy Regulation and Heme Metabolism

In addition to finding downregulation of multiple metabolites related to mitochondrial fatty acid oxidation in PNGR (acylcarnitines), there are other serum metabolites that indicate errors in normal metabolic processes compared to their CON counterparts. Xanthosine, and adenosine intermediate was upregulated in CON-SED compared to PNGR-SED ($p<0.0001$) and compared to PNGR-TRD ($p=0.0002$); (Fig. 4.10). Xanthosine was also higher in CON-TRD compared to PNGR-SED ($p<0.0001$) and PNGR-TRD ($p<0.0001$). Inosine Monophosphate (IMP) was lower in CON-SED compared to PNGR-SED ($p<0.0001$) and PNGR-TRD

($p<0.0001$). IMP was also lower in CON-TRD compared to PNGR-SED ($p<0.0001$) and PNGR-TRD ($p<0.0001$). IMP was not different between SED and TRD within their respective diet groups. Inosine (C) was higher in CON-SED compared to PNGR-SED ($p=0.0005$) and PNGR-TRD ($p=0.0005$). Inosine was also higher in CON-TRD compared to PNGR-SED ($p=0.0005$) and PNGR-TRD ($p=0.0011$). Methylthioadenosine (D), a metabolite related to mitochondrial oxidative stress regulation, was higher in PNGR-SED compared to CON-SED ($p<0.0001$) and CON-TRD ($p<0.0001$), and higher in PNGR-TRD compared to CON-TRD ($p<0.0001$) and CON-SED ($p<0.0001$). HIIT was not successful in improving energy regulating metabolites found in the serum and reflect a programming effect of which may play a role in adaptation to exercise. A hemoglobin precursor metabolite, heme, was found to be in much lower abundance in PNGR-SED compared to their CON counterparts ($p=0.0001$), as well as lower in PNGR-TRD vs. CON-TRD ($p<0.0001$). Heme did not improve in abundance in response to HIIT (Fig. 4.7).

3.4.4.3 Mitochondrial Metabolites Reflecting Altered Amino Acid Metabolism

Essential amino acids methionine and phenylalanine were significantly depleted in PNGR as compared to CON and did not improve with HIIT (Fig. 4.9). Riboflavin was upregulated in PNGR-SED compared to CON-SED ($p=0.0094$) and in PNGR-TRD compared to CON-TRD ($p<0.0001$) and PNGR-SED ($p=0.0450$).

Methionine was significantly higher in CON-SED compared to PNGR-SED ($p<0.0001$) and PNGR-TRD ($p=0.0005$), and higher in CON-TRD compared to PNGR-SED ($p=0.0159$). L-Tyrosine was decreased in PNGR-SED vs. CON-TRD ($p=0.0003$) and CON-SED ($p=0.0001$) and decreased in PNGR-TRD vs. CON-TRD ($p=0.0249$) and CON-SED ($p=0.0123$). As per the initial volcano plot analysis (Fig. 4.4), L-Tyrosine was upregulated in response to HIIT in

PNGR-TRD (2.6 Log_2FC), however, follow-up analysis via Kruskal Wallis testing led this comparison to not be significant (p=0.14).

DL-Phenylalanine was decreased in PNGR-SED vs. CON-SED (p=0.0001) and CON-TRD (p<0.0001), as well as in PNGR-TRD vs. CON-TRD (p<0.0001) and PNGR-TRD vs. CON-SED (p=0.0002). The remaining amino acids remained blunted in their response to HIIT in PNGR, which highlights a key area for future investigation.

3.4.4.4 Effect of HIIT on the Fecal Metabolome in PNGR at PN71

PCA Plot and PERMANOVA analysis revealed very few signatures of clustering by diet and treatment group or by treatment group alone (Fig. 4.12). Majority of metabolites differentially affected via HIIT were downregulation of palmitoylcarnitine, upregulation of peptide excretion and downregulation of heme metabolites such as Protoporphyrin IX.

Fecal metabolites were analyzed post-intervention at PN71 to assess differences between diet and treatment groups, which revealed higher excretion of palmitoylcarnitine in the PNGR-SED vs. PNGR-TRD p=0.0029 and trending higher excretion in PNGR-SED vs. CON-SED p=0.0968.

3.4.4.5 Effects of HIIT on the Fecal Metabolome in CON at PN71.

Very minimal effects of HIIT were found in the fecal metabolome of CON post-intervention (Fig. 4.13). The primary downregulated metabolites found were various forms of Lyso-phosphocholines (Lyso PC) and medium-chain dicarboxylic acid Undecenedioic acid. The main two metabolites found to be upregulated were Taurousodeoxycholic acid and 9-OxoODE. Follow-up analysis of each specific metabolite via Kruskal Wallis and FDR correction resulted in no significant differences as a result of HIIT within CON.

3.4.4.6 Effects of HIIT on Microbial Diversity and Composition in CON and PNGR

Analysis of the microbiome dataset via PERMANOVA revealed no strong signatures of clustering or separation due to an effect from HIIT among treatment groups ($R^2 = 0.064$; p=0.576). Further separation of microbes between diet groups also showed very minimal effects (CON: $R^2 = 0.039$; p=0.678; PNGR $R^2 = 0.025$; p=0.698;). (Fig. 4.15)

Alpha Diversity was analyzed at PN71 and was not found to be significant. Thus, longitudinal analysis was not performed since there were no significant differences in diversity at the final timepoint. However, further analysis did involve testing diet and training effects on the microbial composition post-intervention within the different taxonomic levels. Level 2 analysis of Bacteroidetes: Firmicutes ratio resulted in no significant differences as a result of HIIT (data not shown). Downstream analysis of Level 7 (species level) revealed increases in Clostridiaceae *g.s.* (KW p<0.001) with post-hoc comparisons showing a strong increase in PNGR-TRD compared to PNGR-SED (p=0.007), CON-TRD (p=0.0124) and CON-SED (p=0.0237); Fig. 4.16. Another microbe within the same level, Christensenellaceae *g.s.* was also significantly altered (KW p=0.02) and decreased in response to HIIT in both CON and PNGR at PN71; Fig. 4.16. Christensenellaceae *g.s.* decreased the most in PNGR-TRD compared to CON-TRD (p=0.01), with trending decreases in CON-TRD vs. CON-SED and PNGR-TRD vs. PNGR-SED (p<0.05).

3.5 Discussion

3.5.1 Effects of Diet, Sex and Training on Growth Rate and Body Mass

PNGR-SED and CON-SED growth rates (Fig. 4.1) were as expected based on previous studies in the Ferguson lab, which exemplify that the nutritive cross-fostering postnatal GR model was successful[3,4,6,103], especially the initial separation due to diet effects shown as early as PN4. A significant sex effect on body mass was observed around PN21, with males having

higher body mass compared to their female counterparts. This divergence between sexes has been previously shown[6], although the mechanism behind this occurrence is currently unknown. It is possible that there is an increase in sex hormone production around this timepoint that may increase the ability for males to gain mass at a faster rate than females, however those factors were not measured nor confirmed in the current investigation.

Interestingly, as a result of HIIT, body mass in all HIIT groups were lower than their SED counterparts, which initiated after about one week of training (PN49) and continued until the end of the intervention. Since mice were allowed to feed *ad libitum* and the specialized feed was not weighed to avoid additional outside factors that may impact the gut microbiome portion of the current study, it is unknown whether there were any potential alterations in feeding behavior or amount over the duration of the study. However, this finding was previously seen in another study in the Ferguson lab by Wellette-Hunsucker et al. with the training groups having smaller body mass compared to the sedentary groups over time[75], despite increased food intake in the trained mice. Thus, the impact of increased food intake was likely not a significant contributor to body mass alterations and was decidedly not tracked in the current study.

[269]PNGR-SED mice have previously exhibited lower lean mass and fat mass compared to their CON counterparts[3], which is clearly a programming effect. A further relative decrease in fat and/or lean mass due to HIIT may contribute to more metabolic challenges that outweigh the benefits of HIIT in PNGR. Future investigations should consider the potential impact of body composition alterations due to HIIT on the PNGR population.

3.5.2 Effects of HIIT and Diet on Exercise Capacity

As hypothesized exercise capacity was initially lower in PNGR compared to CON and lower in females as compared to males (Fig. 4.2). Baseline exercise capacity was also the lowest

in PNGR-F compared to all other groups (roughly 17-32%), which closely reflects similar previously published findings using this mouse model [2,6,7]. Based on other literature, what is considered a good improvement varies widely, with average ranges between 10-18%[58,137,271], with 1.7-fold improvement in final distance and 2.4-fold improvement in W_{max} (calculated as kg*m; FC=post/pre) shown in mice. However, these studies utilized distance as a metric in the W_{max} calculation in place of final speed.

PNGR-F-TRD successfully improved W_{max} from baseline ($p<0.0001$); and was not significantly lower in W_{max} compared to their CON trained counterpart (Fig. 4.2). Furthermore, the degree of improvement in PNGR-F-TRD was also similar to that of CON-F-TRD with about a 1% difference in percent improvement (43.9% vs. 44.9%, respectively)[2,6,7]. PNGR-M-TRD also showed significant improvements in exercise capacity and had the greatest improvement relative to their baseline W_{max} (61%) compared to the other cohorts, and to a greater degree than in CON-M-TRD. An atypical response was also found within the PNGR-M-SED group, which showed a 28% improvement in W_{max} from baseline, which was not otherwise shown in the other groups. A potential explanation for this could be due to body mass (a major component of the W_{max} calculation) in PNGR-M-SED, which had the greatest average change between baseline and post-intervention compared to the other cohorts (PNGR-M-SED: 15.3%; all other cohorts: <11%). This has been shown before in the study by Avila et al. 2017 where some continued growth and changes in body mass (in their study average age of PN56), may have confounded the changes in exercise capacity from baseline and post-intervention[139].

HIIT elicited positive improvements in maximal work capacity in PNGR mice, that have not otherwise been observed utilizing other intensities or types of training regimens. The mechanisms underlying the increases in maximal work capacity in PNGR despite continued

metabolic challenges is not clear. It is possible that although the circulating metabolic intermediates reflect deficits in major areas of metabolism, there are other areas that have not yet been well investigated (e.g., glycolytic) of which may allow for appropriate adaptations to occur. As previously mentioned, PNGR typically respond to exercise with a glycolytic skeletal muscle fiber shift[2,4,6], which leads to alteration in fiber type distribution and size in their main locomotor muscles. Theoretically, if glycolytic adaptations still occur, then this would assist in muscular adaptations despite depletion of the forementioned metabolites. Structural and functional cardiovascular impairments in PNGR could inherently explain the consistent limitations in W_{max} that are seen in PNGR, however, even small improvements in this area in combination with glycolytic adaptations to HIIT could aid in their ability to overcome these detriments. Additionally, HIIT leads to increased mitochondrial biogenesis in glycolytic fiber types which is atypical in moderate-intensity continuous training[154,272], therefore allowing for further adaptations in PNGR. Future directions of this project are aimed to further elucidate skeletal muscle alterations (i.e., fiber type distribution), characterize cardiovascular structure and function as well as potential oxidative adaptations to HIIT. In a more broad perspective, it could be speculated that HIIT leads to improvements in PNGR due to a combination of both the intense stimulus, which caters to the glycolytic skeletal muscle phenotype previously observed[137,148,149,273], as well as the interval component, which serves to improve lactate oxidation and allow for periods of active recovery[72,73]. Further molecular investigation is encouraged to pinpoint the underlying mechanisms behind the improved work capacity achieved in PNGR with HIIT.

3.5.3 Alterations in Metabolites Associated with Exercise Adaptations at PN71

There were various energy regulating metabolites found to be altered as a result of diet in PNGR compared to CON. Energy regulation aids in improving exercise capacity as well as cellular and skeletal muscle exercise adaptations during recovery. Xanthosine, a metabolite involved in adenosine triphosphate (ATP) synthesis and degradation. Xanthosine was downregulated in PNGR-SED compared to CON-SED. Xanthosine trended higher in CON-TRD vs. CON-SED ($p>0.05$), however PNGR did not see an increase in Xanthosine with training (Fig. 4.10). In human studies, post-training intervention Xanthosine levels typically increase. Some speculate this may reflect processes involved in tissue (skeletal muscle) regeneration or reestablishing the purine nucleotide pool[274].

Inosine Monophosphate (IMP), another nucleotide degradation metabolite was elevated in PNGR-SED and did not decrease back to the levels of CON with training (Fig. 4.10). IMP is derived from deamination of adenosine monophosphate (AMP), of which is an irreversible reaction. Since the serum samples were collected at rest (after a minimum of 24-hr), elevated IMP above that of CON, suggests that IMP formation is not reflective of a training response but rather a programming effect of regulating energy production in PNGR. It is possible elevated IMP is reflective of the glycolytic muscle phenotype seen in PNGR. It has been previously shown that manual stimulation of fast-twitch muscles leads to a substantial increase in IMP as compared to slow-twitch muscle fibers[275], which do not experience an increase in IMP. Alternatively, elevated circulating IMP is also associated with cardiovascular and skeletal muscle impairments (e.g., heart failure and/or mitochondrial defects)[276], which is plausible considering the extensive cardiovascular and mitochondrial impairments observed in PNGR[3,5,74]. While more evidence is necessary to establish a mechanistic role for these metabolites, definitive alterations

in abundance between CON and PNGR, with blunted improvements with training, may be a key area for future investigation.

Nicotinamide was also found to be downregulated in PNGR-F-SED and PNGR-M-SED and remained depleted in PNGR-TRD (both sexes) compared to their respective CON groups (Fig. 4.7). Nicotinamide is a key molecule for regulating energy production, nucleotide synthesis, lipid metabolism and cell proliferation[277]. Nicotinamide is also a precursor of Nicotinamide Adenine Dinucleotide (NAD+) and Nicotinamide Mononucleotide (NMN) which are both known to contribute as cofactors in a variety of cellular pathways, including those in energy regulation of many forms[277]. Methionine, which was similarly downregulated (Fig. 4.9), is a protein that acts in parallel pathways of Nicotinamide metabolism and aids in its production. Methionine restriction or depletion has previously been linked with disrupted nicotinamide metabolism.[278]. It is also possible that most nicotinamide is being scavenged and recycled to maintain energy balance and production in PNGR.

Riboflavin (RF) in its cofactor forms FMN and FAD also play a role in energy homeostasis and act as folate pathway co-factors. RF was found to be in much higher concentration in PNGR compared to CON and also significantly increased with training in PNGR but not in CON. Generally, it is recommended to have sufficient B vitamins through proper dietary intake since they are considered essential and not made endogenously. However, overabundance of RF, specifically in the blood, is associated with cardiovascular disease[279]. Cardiovascular impairments have been repeatedly shown in PNGR[2,3,5,7,74], however the mechanistic role of RF in these detriments are currently not clear. RF also works with oxidized glutathione to reduce oxidative stress (ROS), of which was decreased in PNGR and could potentially be acting to combat ROS at rest and in response to training.276,[278]

3.5.4 PNGR Altered Mitochondrial Fat Oxidation

There are signatures in the serum metabolomic data that suggest that PNGR have altered fatty acid metabolism.[279,280] PNGR-SED had a low abundance of short-chain acylcarnitines (SC-ACs) compared to their CON counterparts (Fig. 4.6), which also did not improve with HIIT. The primary SC-ACs downregulated in PNGR were butyrylcarnitine, succinylcarnitine and propionylcarnitine. SC-ACs are suggested to be the most abundant type of acylcarnitine, with some individuals having circulating levels close to 80% compared to other types[280,281]. The most common SC-AC is Acetylcarnitine which aids in energy production and acts as an acetyl group donor increase synthesis of molecules (e.g., acetylcholine). Altered abundance of SC-ACs are associated with various genetic diseases, especially inborn errors of metabolism, as well as cardiometabolic diseases[280–282]. Butylrylcarnitine, succinylcarnitine and propionylcarnitine are all Beta-oxidation intermediates. A possible mechanism underlying downregulation of these SC-ACs is likely due to either depleted methionine, which aids in carnitine production, or an impairment in the Carnitine acetyltransferase (CrAT) enzyme, which is responsible for the synthesis of all short-chain and short branched-chain acylcarnitines[281,283]. The specific roles of each of these individual acylcarnitines is not clearly defined. Succinylcarnitine is strongly correlated with insulin sensitivity[284]. Propionylcarnitine in low abundance is associated with systolic heart failure, malonyl-coA decarboxylase deficiency, methylmalonic acidemia, and type II diabetes[281]. In addition to acylcarnitines providing a role in Beta-oxidation, they also are acyl-CoA donors and contribute to the production of other molecules such as acetylcholine, which is an important molecule in exercise adaptations and other processes associated with cholinergic neurotransmission[281].

PNGR-SED also had very low abundance of medium-chain acylcarnitine (MC-AC), Hexanoylcarnitine. MC-ACs are much less common than SC-ACs but are still considered a common circulating metabolite. There are much fewer types of SC-ACs as compared to MC-ACs seen in the literature (~50 SC vs. ~400 MC), with the latter being formed via L-carnitine esterification processes as well as via branched-chain fatty acid peroxisomal metabolism[280,281]. The primary outcome of MC-ACs is to be shuttled into the mitochondria and metabolized. Metabolic diseases associated with altered MC-ACs are usually those resulting in altered fatty acid metabolism, as well as type 2 diabetes, cancer, and cardiovascular disease (CVD)[281]. Interestingly, PNGR-SED had significantly high long-chain acylcarnitines (LC-ACs) compared to CON-SED. LC-ACs are a product of L-carnitine esterification with long-chain fatty acids, which aid in transport of the lipid into the mitochondria for oxidation and require a transporter to enter the inner mitochondrial membrane. The long-chain fatty acids are usually either obtained from the diet or generated from *de novo* lipogenesis[280,281]. In comparison to the SC-ACs and MC-ACs, LC-ACs have the highest amount (~500) which vary widely depending on how they are formed. The most common LC-AC in serum is palmitoylcarnitine. PNGR-SED and PNGR-TRD both had very low levels of palmitoylcarnitine (C16:0) compared to CON-SED and CON-TRD. In contrast, myristoylcarnitine (C14:0), which is also a LC-AC was significantly higher in PNGR-SED compared to CON-SED and CON-TRD (Fig. 4.6). There is evidence to support alterations in both directions – elevated or depleted and their association with metabolic disorders. Decreased levels of palmitoylcarnitine is associated with carnitine palmitoyltransferase 1A deficiency. Increased myristoylcarnitine is associated with carnitine/acylcarnitine translocase deficiency, carnitine palmitoyl transferase 2 deficiency, glutaric acidemia type 2, type 2 diabetes, and CVD[280,281].

Very minimal effects were observed throughout this study in CON-SED and CON-TRD, especially within the fecal metabolome. The largest signatures or patterns observed consistently were in PNGR which highlights a strong programming effect that persists despite treatment with HIIT. In the fecal metabolome, a positive response to training was seen in PNGR-TRD which decreased palmitoylcarnitine excretion to levels similar to that of CON (Fig. 4.14). However, in the serum palmitoylcarnitine was still downregulated with no effect of HIIT on relative abundance. HIIT unfortunately did not significantly alter LC-ACs that were abnormally high in abundance or to increase serum SC-ACs. HIIT did, however decrease abnormal fecal excretion of palmitoylcarnitine, which may still benefit health and disease prevention in PNGR.

3.5.5 PNGR Induced Programming of Circulating Essential Amino Acids

There were many deficits in essential amino acids (EAAs) such as phenylalanine, methionine, and tyrosine in PNGR compared to CON (Fig. 4.9), of which did not change in abundance with HIIT. It is not surprising that there is a dysregulation in amino acid metabolism in PNGR, exemplary of an ongoing programming effect of GR and has also been shown to continue into adulthood in stunted individuals[221].

The mechanisms behind decreased EAA abundance in PNGR is unclear but could potentially be related to an error in metabolic regulation within the pancreas, liver, and/or skeletal muscle[285,286]. Decreased circulating methionine can also occur through decreased methionine synthase activity, which requires vitamin B_{12} also called cobalamin, as a cofactor[287]. Cobalamin was not found within the current dataset. However, it is recommended that follow-up studies using this model aim to perform more direct assays or targeted protein analysis of the B vitamin profile in PNGR due to their vital role in many methylation and growth-related pathways.

Methionine is also an important precursor for cysteine production. Alterations in concentrations of both methionine and cysteine have been previously shown in protein undernourished children[288–290]. Plausible underlying mechanisms of low circulating methionine in this case could be due to alterations in nitrogen balance and slower rates of protein degradation[288,290]. Downstream implications of low methionine and cysteine also contribute to depleted glutathione[288], as also shown in the current study.

By the time the serum samples were collected from this cohort, the mice had been weaned onto the specialized CON diet for at least 50 days, which is sufficient with amino acids (especially methionine), however the continued depletion despite this reintroduction of nutrients signifies an inability to either convert between homocysteine and methionine within the tissues or a lack of enzymes required for those pathways to be successful[290]. It has been previously shown that supplementing with additional methionine or EAAs might alleviate some of the epigenetic effects previously seen in GR later in life[291,292]. It would be interesting to determine if supplementing with additional methionine, and ultimately providing more cellular methylation potential, in PNGR would prevent many of the metabolic signatures shown in the current investigation.

Interestingly, a methionine salvage pathway metabolite, methylthioadenosine (MeSAdo), (Fig. 4.10) was found to be upregulated in PNGR-SED and PNGR-TRD compared to CON and may be a key indicator of increased methionine scavenging activity. Methionine scavenging typically occurs in order to maintain cellular abundance of methionine or its associated intermediates (e.g., homocysteine)[293]. Other major players within the pathways associated with methionine production were unfortunately not found in the current dataset, such as cysteine,

homocysteine, or lysine. However, future investigations are encouraged to measure these specific proteins to determine the source of methionine depletion in PNGR.

3.5.6 Altered Biomarkers of Mitochondrial Oxidative Stress & Oxygen-Carrying Capacity in PNGR

The oxidized form of glutathione was downregulated in PNGR compared to CON (Fig. 4.7). Downregulated glutathione is associated with many metabolic diseases, especially CVD. Glutathione is generally located exclusively in the cytoplasm and must be actively transported into the mitochondria via various enzymes.

Heme, a precursor to the molecule hemoglobin, was found to be downregulated in relative abundance in PNGR compared to CON. Metabolism of heme is essential for maintaining and improving exercise capacity as well as other roles such as skeletal muscle and tissue cellular health. Heme is synthesized via heme oxygenase-1 (HO-1)[294]. In a study investigating a heme oxygenase-1 knockout (HO-1$^{-/-}$KO) mouse model, not only did it result in disrupted heme production, but it also decreased mitochondrial content and function as well as decreased exercise capacity[294]. Interestingly, the HO-1$^{-/-}$KO mice also had increased glycolytic muscle fiber types compared to the wild-type CON mice, which is a common phenotype seen repeatedly in the PNGR mouse studies within the Ferguson lab[2,4,6]. Unfortunately, heme production did not improve with HIIT in PNGR, however it did maintain much higher abundance in CON-SED and CON-HIIT groups. Heme did not increase in response to HIIT as it did with CON which remained lower in concentration. Additionally, these samples were collected in resting conditions, thus the downregulation is not a response of training but rather a snapshot of regulation at rest in the PNGR. Signs of development of porphyria, a heme disorder, would be first indicated via lower levels of Coproporphyrin III[295], however this metabolite was not

downregulated in PNGR and had levels similar to both CON-SED and CON-TRD (figure not shown), indicating this mechanism likely is not the cause.

3.5.7 Microbial Abundance and HIIT in PNGR and CON

Overall, effects of HIIT on the gut microbiome of both PNGR and CON mice were minimal. There was little difference in alpha diversity in response to HIIT, which has been shown previously in a few studies[296–298]. A recent systematic review highlighted that most studies exhibiting strong alterations in alpha diversity had much longer training intervention durations (>12 weeks), while shorter durations demonstrated differences in specific microbial abundances[297]. HIIT did alter the abundance of specific microbes among treatment groups. The two primary microbes that were altered in composition in response to HIIT were from the Clostridiaceae and Christensenellaceae families. Both microbes come from the firmicutes phylum. Clostridiaceae increased primarily in PNGR-TRD compared to all other groups. Previous observational studies have demonstrated positive correlations between higher exercise performance and increased abundance of microbes within the Clostridiaceae family[299]. Christensenellaceae decreased abundance in both CON-TRD and PNGR-TRD. Many studies have shown opposite effects of exercise on Christensenellaceae abundance, however, many of those were of moderate-intensity exercise or in studies with very long intervention durations[300–302].

3.6 Conclusion

In conclusion, the PNGR nutritive model was successful, with an additional effect of decreased body mass in all groups in response to training across the duration of the intervention relative to their respective sedentary cohorts. Due to the loss of body mass occurring much later (PN49) post-refeeding at PN21, we can conclude the loss of mass is solely an effect of HIIT and

likely not a disadvantage to PNGR, especially since the same effects were also mirrored in CON. Exercise capacity testing was successful in all groups post-intervention from baseline. HIIT was successful in improving exercise response as compared to results from previous studies utilizing other exercise modalities and intensities, indicating this form of exercise may be a better fit for this cohort due to their phenotypic profiles as a result of GR in early life. Metabolic profiling of PNGR and CON and their responses to HIIT were evidently differential in nature and highlight key areas for future investigation, such as in mitochondrial fat oxidation regulation and oxidative stress regulation, heme metabolism, and the etiology of consistent amino acid depletion in adulthood despite refeeding fully nutritive diet for ~50 days. Additionally, it is clear that the microbial signatures in PNGR are not direct indicators of exercise capacity, but may play other roles (e.g., inflammation or cellular signaling) not investigated further in this dissertation.

3.7 Limitations and Future Directions

A primary limitation in this study were the very small effects seen in the microbiome data. A cross-fostering animal model is key for inducing growth restriction during specific timepoints (i.e., gestationally, or postnatally). However, it is very difficult to control for biological alterations in the microbiome during this process due to immediate sharing of bacteria between the mother and pups[263,303]. Although the pups were ultimately pooled and reinstated into their new dams upon birth, the initial birth itself provides a set of microbes which is highly likely to be different from the other dams. However, it is nearly impossible to control for microbial composition differences between dams[263].

Another limitation in the current study is the lack of significant effects seen in response to HIIT in the serum metabolome. One potential explanation is the overpowering effects of programming via PNGR that greatly shadow that of HIIT[303]. It would be interesting to see if the

acute effects can also be seen in PNGR immediately following a single bout of HIIT, which may eliminate some confounding longitudinal aspects of this study (e.g., changes in age or body mass).

Additionally, while this cross-fostering animal model provides novel insight into the underlying metabolic activity in PNGR, it is difficult to determine the true generalizability of this specific HIIT protocol for its use in human PNGR populations. A primary concern is the decrease in body mass seen over time in response to HIIT in a cohort that is already growth restricted at baseline. Future investigations are encouraged to determine the best dose-response that is translatable from mice to humans with PNGR to ensure it is prescribed safely.

While previous studies have shown high correlations between VO_{2max} and W_{max} in humans and mice, there are some limitations with using W_{max} in the current study. To measure VO_{2max}, a metabolic cart or metabolic treadmill is required to collect and calculate inhaled and exhaled breath gases to determine where the true maximal oxygen consumption plateau is occurring[137]. In the current study, this equipment for mice was not available, thus use of observational criteria was implemented (e.g., changes in gait; repeatedly touching a shock grid in a set amount of time)[137]. Using observational techniques in mice to determine the point of fatigue leaves room for technical error and variability compared to using a more accurate quantitative measurement such as VO_{2max}[135,137]. The benefit of using the forementioned protocols is that they have been previously validated and correlated to improve exercise capacity and to induce or enhance physiological improvements in both cardiac and skeletal muscle[137,139,143,145]. However, it is recommended to implement VO_{2max} testing if possible in future investigations to pinpoint the true maximal exercise capacity more precisely in mice, or in conjunction with the forementioned protocols for quality control[304]. Additionally, if the use of a metabolic treadmill is not available,

it is also recommended to implementing the use of technical error in W_{max} calculations (standard TE previously reported as 5% for W_{max}[304,305]), which may help to correct for some of the variability observed in this study[304,305]. In summary, by acknowledging the forementioned limitations, future research in this area are better equipped to further enhance our understanding of the metabolic response to HIIT in PNGR.

4.0. Figures

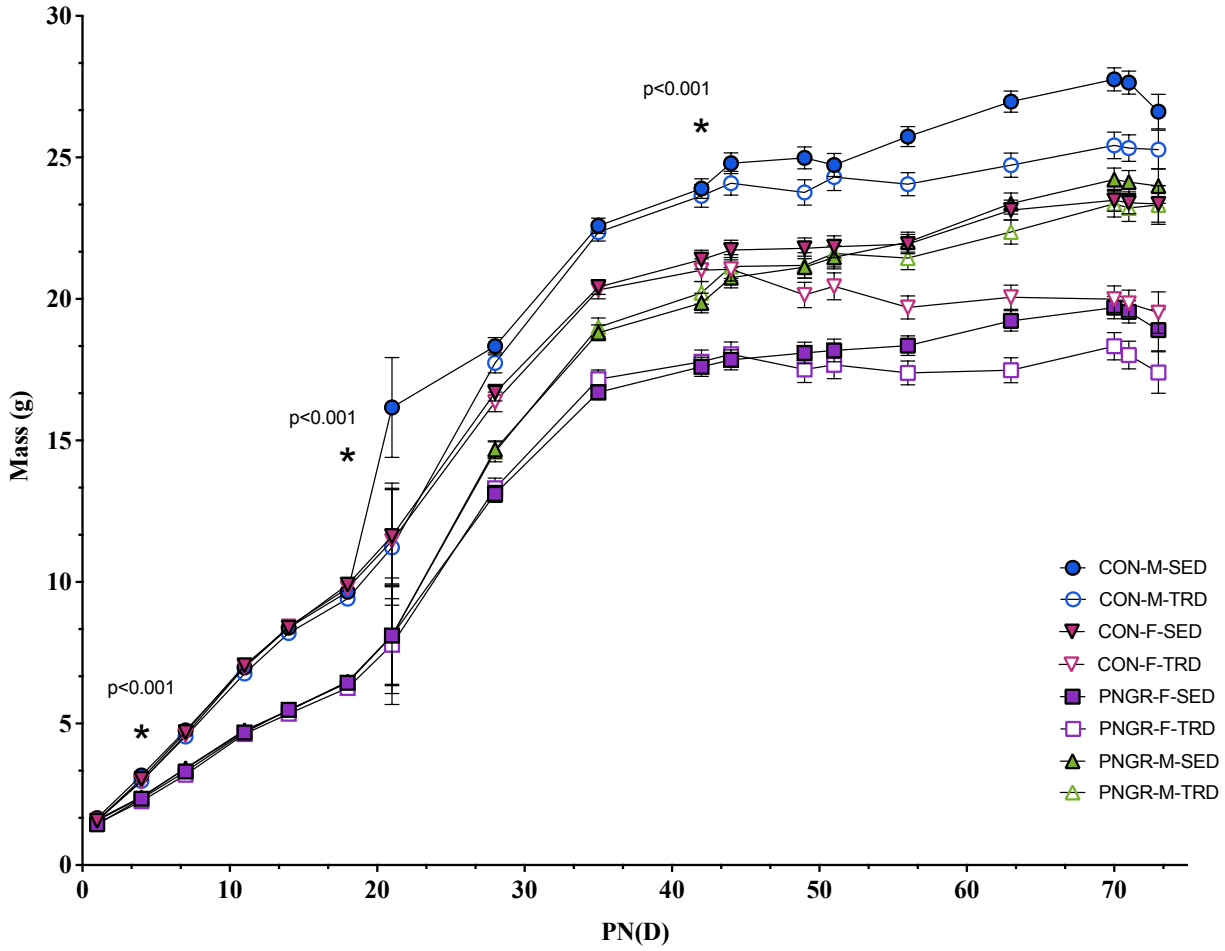

Figure 4.1 Longitudinal Growth Curve by Diet, Sex and Treatment Group.
Analysis of mouse body mass between treatment groups confirmed significant separation of body mass by diet group (CON vs. PNGR; $p<0.001$) at PN4 (indicated as *). At PN19-20 a secondary shift separates males from their female counterparts ($p<0.001$). After one week of training (~PN49) there is a third separation of body mass by treatment group (SED vs. TRD; $p<0.001$)), which lasts until the end of the intervention. Mass on the y-axis is in grams (g) and Age in postnatal days PN(D) is on the x-axis. Individual symbols represent mean and error bars represent standard deviation.

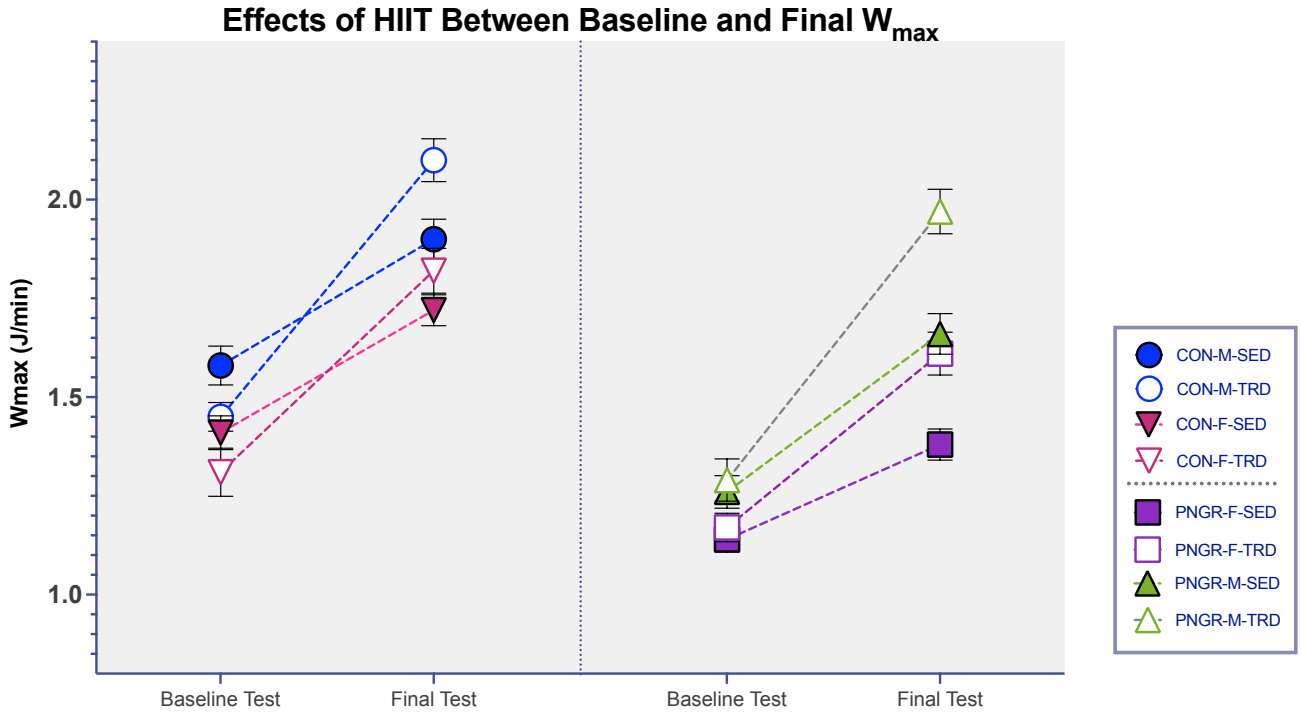

Figure 4.2 Effects of HIIT on Exercise Capacity at Baseline and Post-Intervention. Exercise capacity measured at baseline and post-HIIT intervention at PN71 was analyzed via repeated measures ANOVA in trained mice to examine within group differences. The results showed significant training effects in each respective cohort ($p<0.0001$). Error bars are representative of mean and SD. Individual points represent individual mice within each treatment group. Sample sizes were as follows [PNGR-F-SED n=12, PNGR-F-TRD n=12, PNGR-M-SED n=12, PNGR-M-TRD n=12, CON-F-SED n=12, CON-F-TRD n=12, CON-M-SED n=12, CON-M-TRD n=12].

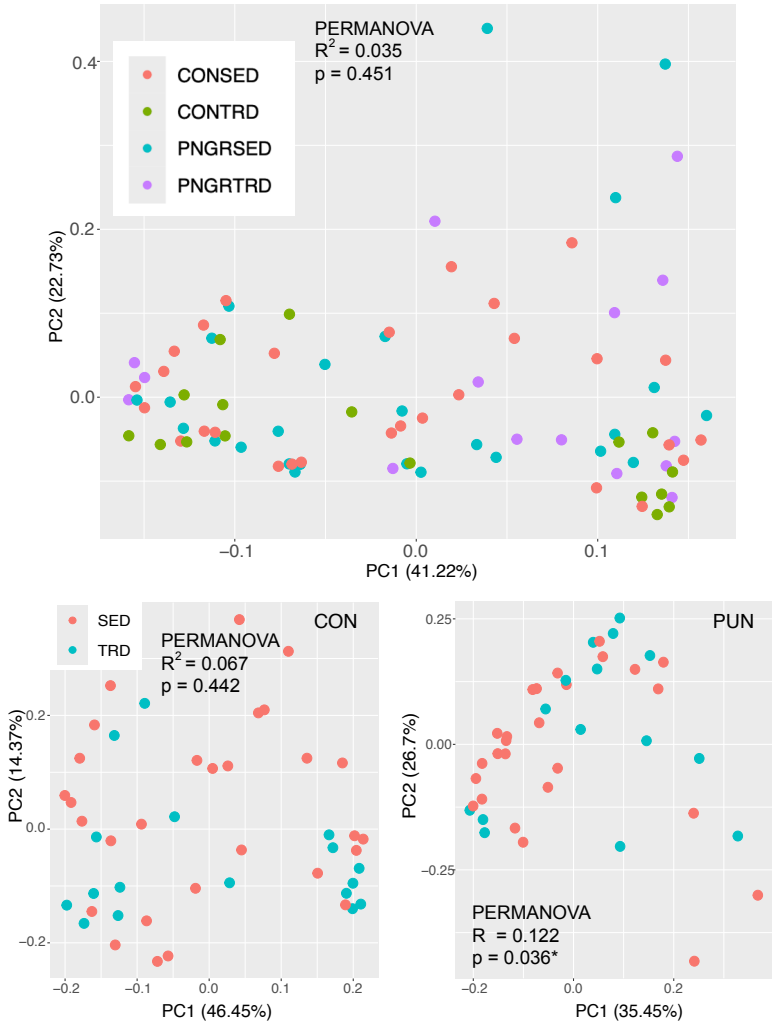

Figure 4.3 Effects of HIIT and Diet on Polar Serum Metabolites at PN71.
Polar serum metabolites were analyzed for signatures of differences among the molecules due to effects of diet, sex and/or treatment. The PERMANOVA by both diet and sex in the polar serum metabolites was not significant (R^2= 0.035; p=0.451). However, after further separating out the metabolites into their respective diet groups, an effect of treatment was found primarily in the PNGR group (R^2=0.122; p=0.036), while CON was not significant (R^2=0.067; p=0.422); [PNGR-SED n=14, PNGR-TRD n=14, CON-SED n=15, CON-TRD n=15].

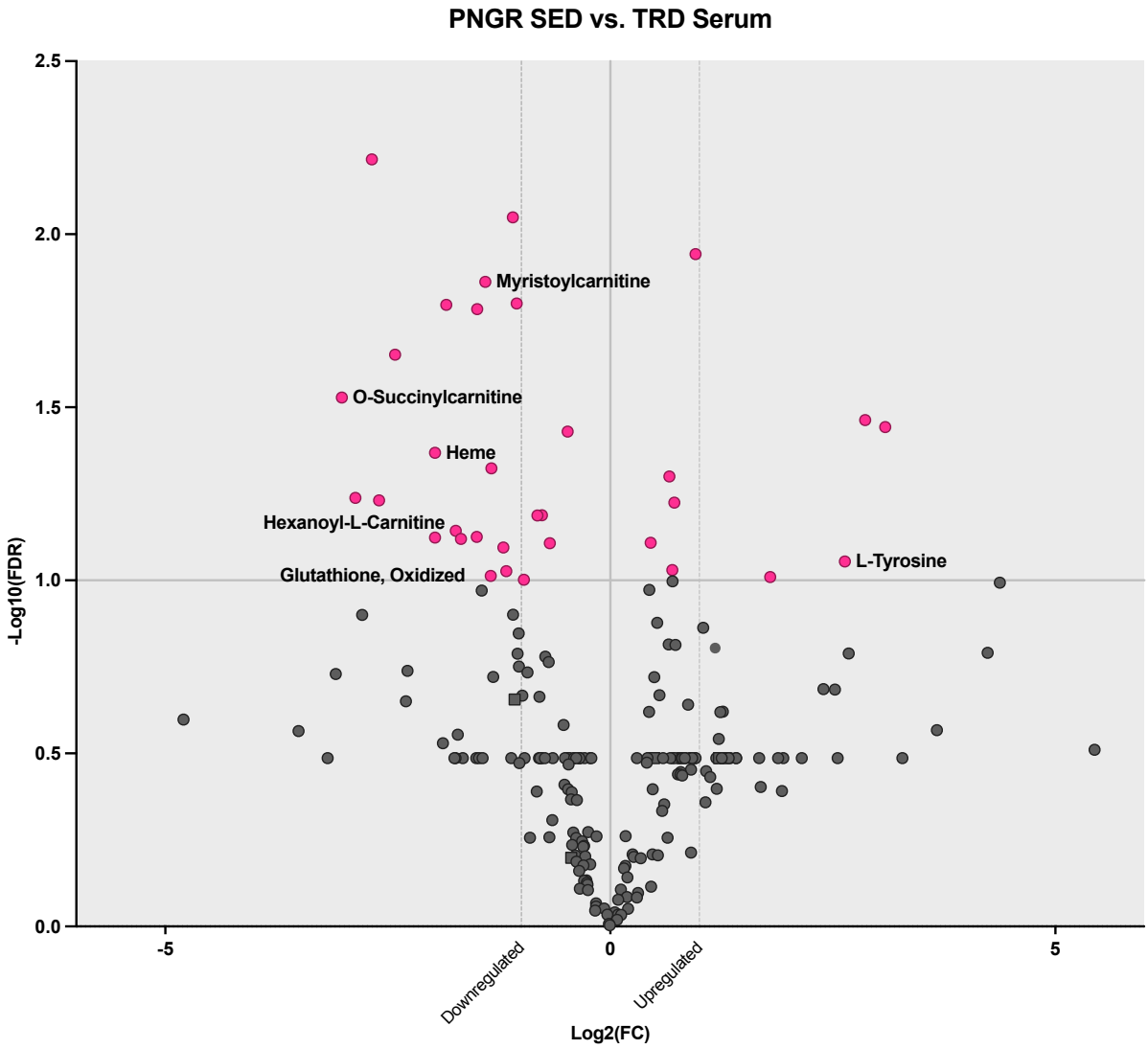

Figure 4.4 Volcano Plot – Investigating the Effects of Training in the PNGR Group.
Majority of metabolites differentially affected via HIIT were downregulation of acylcarnitines, glutathione and its intermediates, essential amino acids, and heme. Tyrosine was also upregulated in response to HIIT in PNGR. FC: Fold-change was calculated as differences between treatment and sedentary assignment among the diet group. FDR: False discovery rate is set at 0.10. All metabolites above the threshold are labeled in pink and all non-significant findings are in black. Upregulated and downregulated metabolites are based on the Log2(FC) which is set at >1.0 for Up, and <1.0 for down and is set on the x-axis; [PNGR-SED n=14, PNGR-TRD n=14].

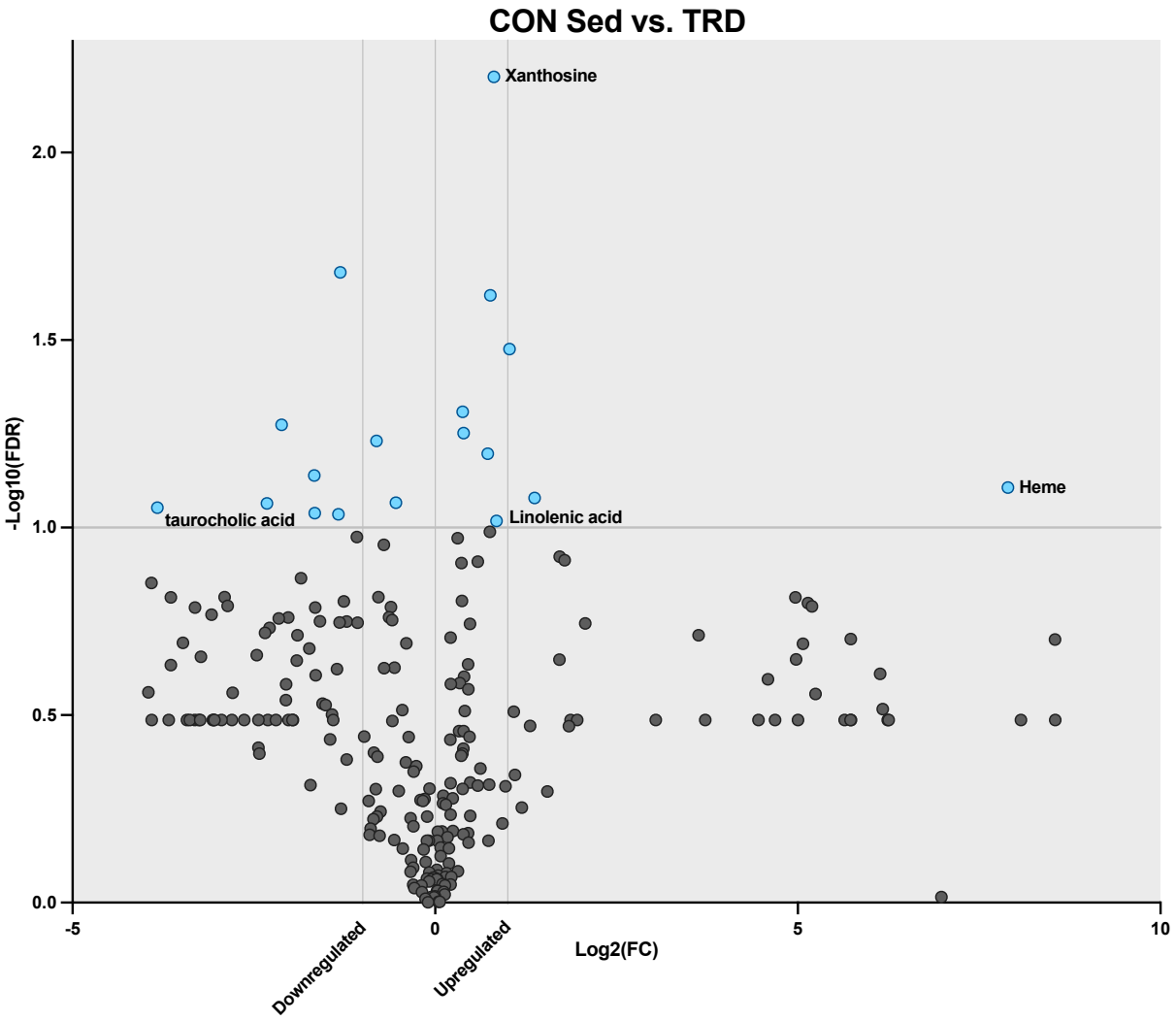

Figure 4.5 Volcano Plot – Investigating the Effects of Training on CON.
FC: Metabolites that were altered significantly in response to HIIT in CON were upregulation of xanthosine and heme and downregulation of taurocholic acid. Fold-change calculated as differences between treatment and sedentary assignment among the diet group. FDR: False discovery rate is set at 0.10. All metabolites above the threshold are labeled in blue and all non-significant findings are in black. Upregulated and downregulated metabolites are based on the Log2(FC) which is set at >1.0 for Up, and <1.0 for down and is set on the x-axis; [CON-SED n=15, CON-TRD n=15].

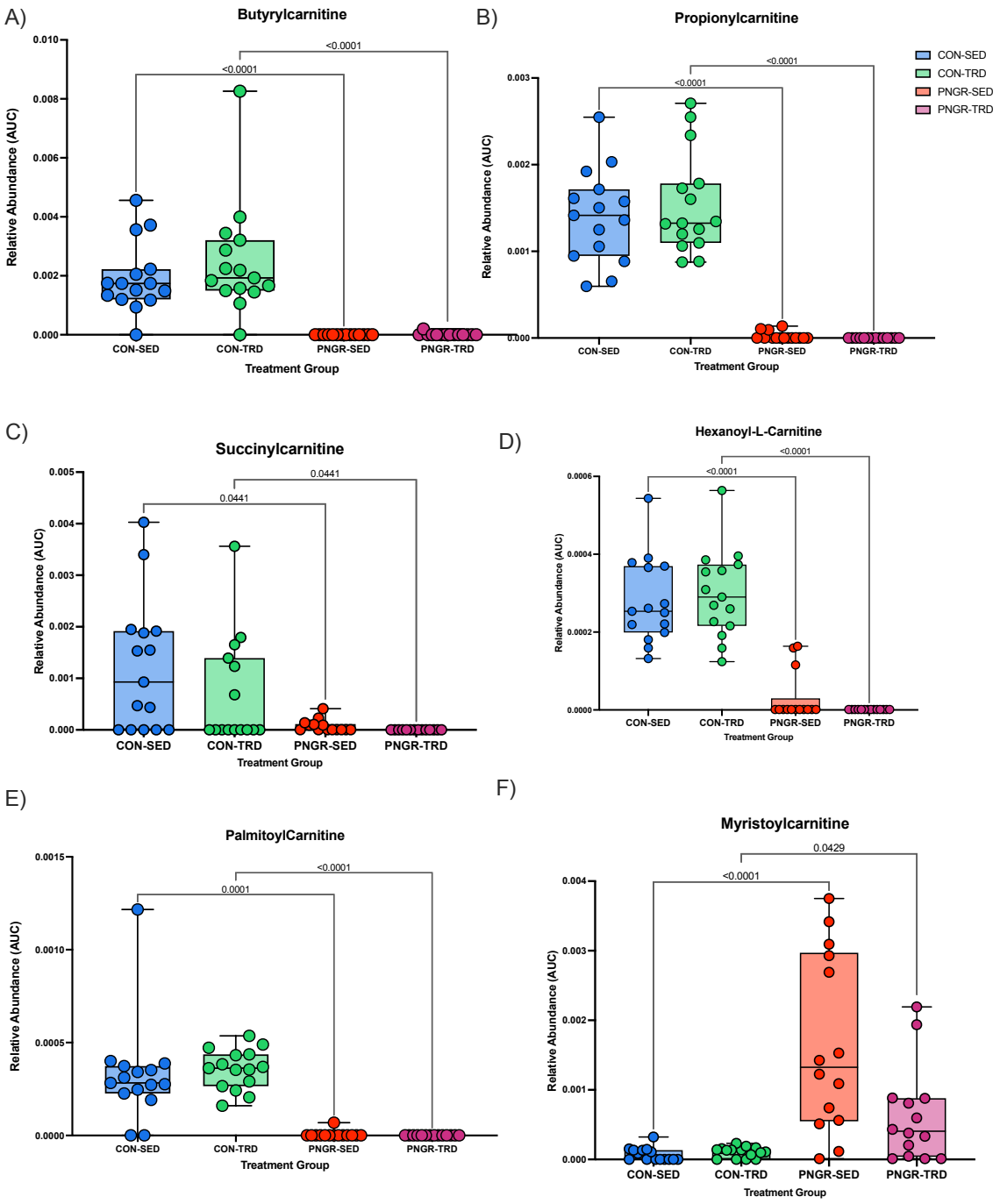

Figure 4.6 Effects of HIIT on Short, Medium and Long-Chain Acylcarnitines.
Serum Butyrylcarnitine (A), Propionylcarnitine (B), Succinylcarnitine (C) Hexanoylcarnitine (D) and PalmitoylCarnitine (E) were all downregulated in PNGR-SED and did not increase in response to HIIT. Alternatively, long chain acylcarnitine Myristoylcarnitine (F) was significantly higher in PNGR-SED compared to CON-SED and did not significantly decrease with HIIT. Error bars shown are mean and SD. Individual points are representative of individual mice; [PNGR-SED n=14, PNGR-TRD n=14, CON-SED n=15, CON-TRD n=15].

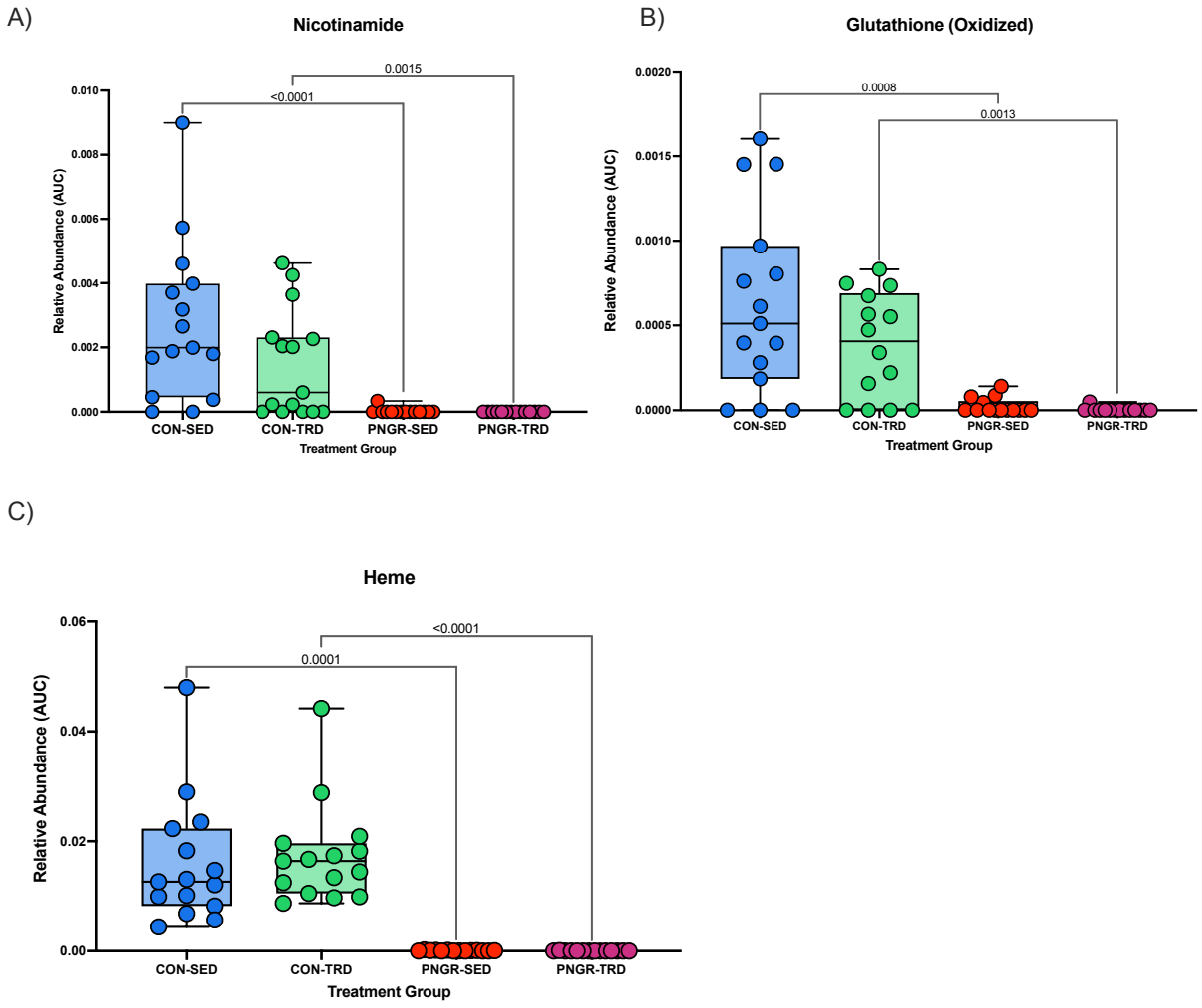

Figure 4.7 PNGR Alters Metabolites Related to Mitochondrial Oxidative Stress and Heme Metabolism.

Multiple mitochondria related metabolites were significantly affected by PNGR. Nicotinamide (A) was downregulated in PNGR-SED compared to all other groups and did not improve with training. Oxidized Glutathione (B) was lower in abundance in PNGR and did not increase to CON levels with HIIT. Heme (C) abundance was lower in PNGR-SED vs. their CON counterparts, which also did not increase in response to HIIT. Error bars shown are mean and SD. Individual points are representative of individual mice; [PNGR-SED n=14, PNGR-TRD n=14, CON-SED n=15, CON-TRD n=15].

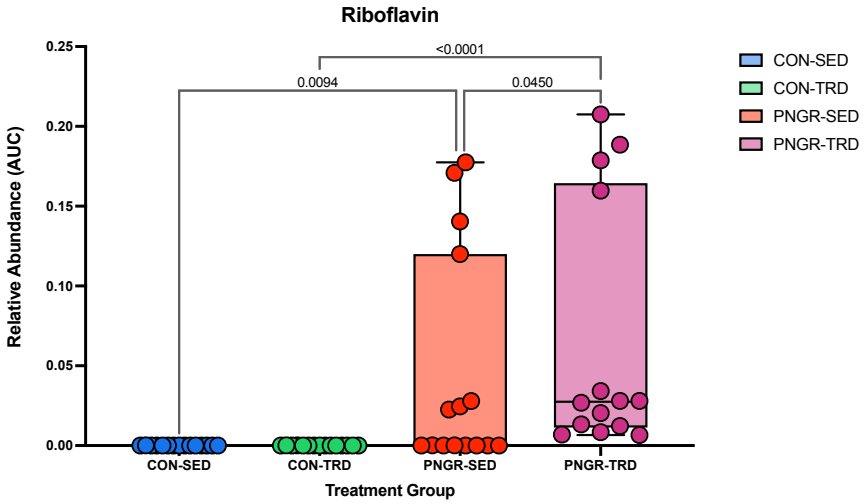

Figure 4.8 Effects of Diet and HIIT on Serum B Vitamins at PN71.
Riboflavin (A) was upregulated in PNGR-SED compared to CON-SED ($p=0.0094$) and in PNGR-TRD compared to CON-TRD ($p<0.0001$) and PNGR-SED ($p=0.0450$). Error bars shown are mean and SD. Individual points are representative of individual mice. Sample sizes were as follows: [PNGR-SED n=14, PNGR-TRD n=14, CON-SED n=15, CON-TRD n=15].

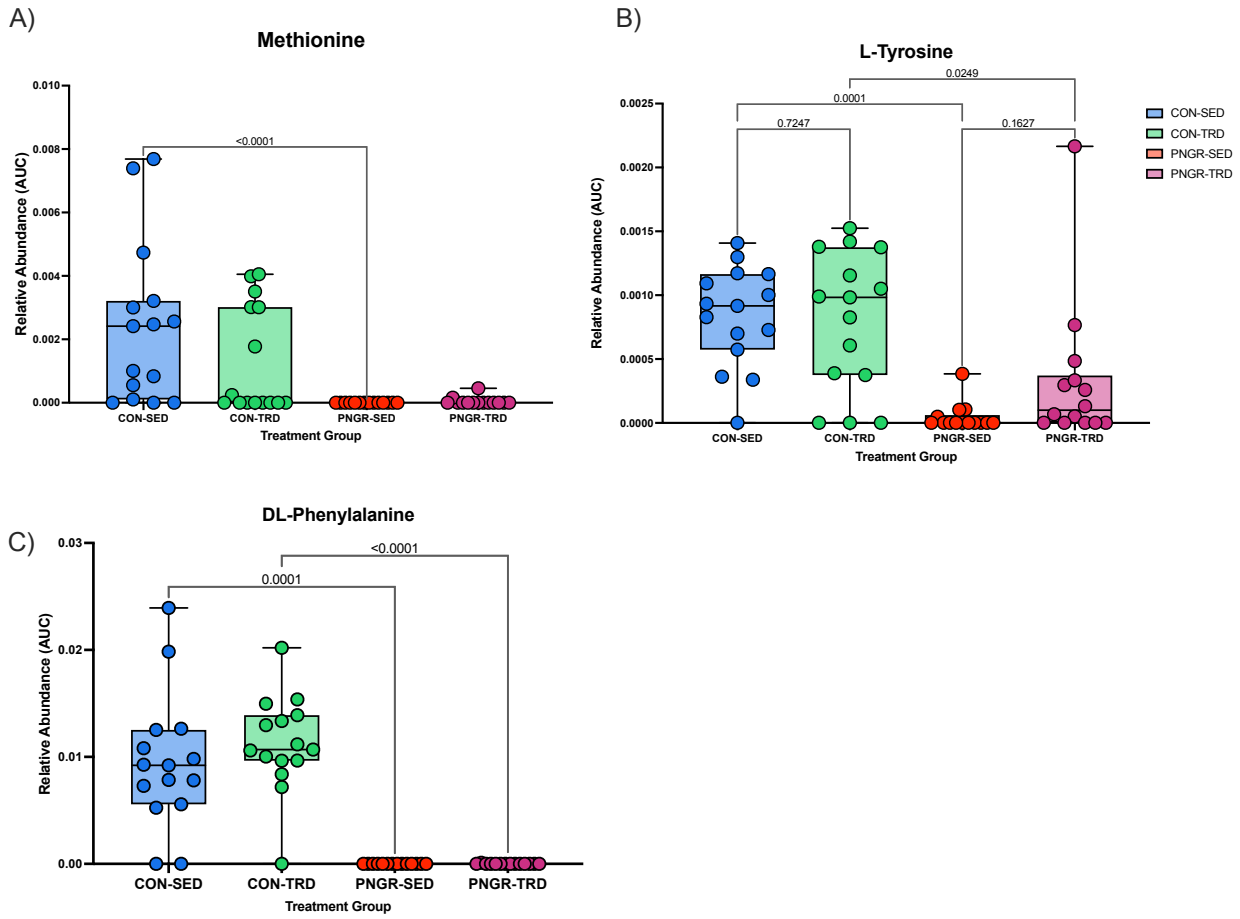

Figure 4.9 Effects of HIIT and Diet on Serum Amino Acids at PN71.
Methionine (A) was significantly higher in CON-SED compared to PNGR-SED ($p<0.0001$) and PNGR-TRD ($p=0.0005$), and higher in CON-TRD compared to PNGR-SED ($p=0.0159$). L-Tyrosine (B) was decreased in PNGR-SED vs. CON-TRD ($p=0.0003$) and CON-SED ($p=0.0001$) and decreased in PNGR-TRD vs. CON-TRD ($p=0.0249$) and CON-SED ($p=0.0123$). DL-Phenylalanine (C) was decreased in PNGR-SED vs. CON-SED ($p=0.0001$) and CON-TRD ($p<0.0001$), as well as in PNGR-TRD vs. CON-TRD ($p<0.0001$) and PNGR-TRD vs. CON-SED ($p=0.0002$). Error bars shown are mean and SD. Individual points are representative of individual mice. Sample sizes were as follows: [PNGR-SED n=14, PNGR-TRD n=14, CON-SED n=15, CON-TRD n=15].

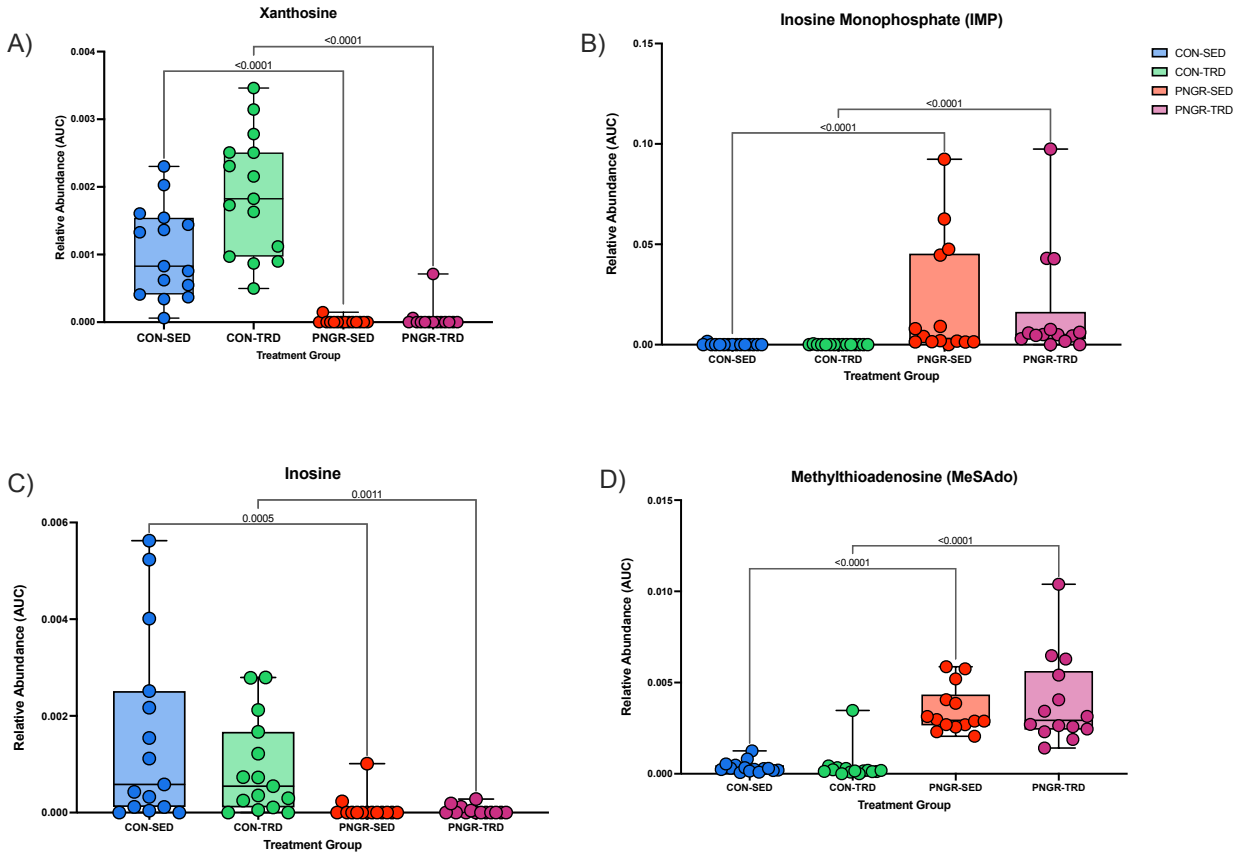

Figure 4.10 Effects of Diet and HIIT on Serum Metabolites Associated with Energy Regulation.

Xanthosine (A) was upregulated in CON-SED compared to PNGR-SED ($p<0.0001$) and compared to PNGR-TRD ($p=0.0002$). Xanthosine was also higher in CON-TRD compared to PNGR-SED ($p<0.0001$) and PNGR-TRD ($p<0.0001$). Inosine Monophosphate (IMP) was lower in CON-SED compared to PNGR-SED ($p<0.0001$) and PNGR-TRD ($p<0.0001$). IMP was also lower in CON-TRD compared to PNGR-SED ($p<0.0001$) and PNGR-TRD ($p<0.0001$). IMP was not different between SED and TRD within their respective diet groups. Inosine (C) was higher in CON-SED compared to PNGR-SED ($p=0.0005$) and PNGR-TRD ($p=0.0005$). Inosine was also higher in CON-TRD compared to PNGR-SED ($p=0.0005$) and PNGR-TRD ($p=0.0011$). Methylthioadenosine (D) was higher in PNGR-SED compared to CON-SED ($p<0.0001$) and CON-TRD ($p<0.0001$), and higher in PNGR-TRD compared to CON-TRD ($p<0.0001$) and CON-SED ($p<0.0001$). Error bars shown are mean and SD. Individual points are representative of individual mice. Sample sizes were as follows: [PNGR-SED n=14, PNGR-TRD n=14, CON-SED n=15, CON-TRD n=15].

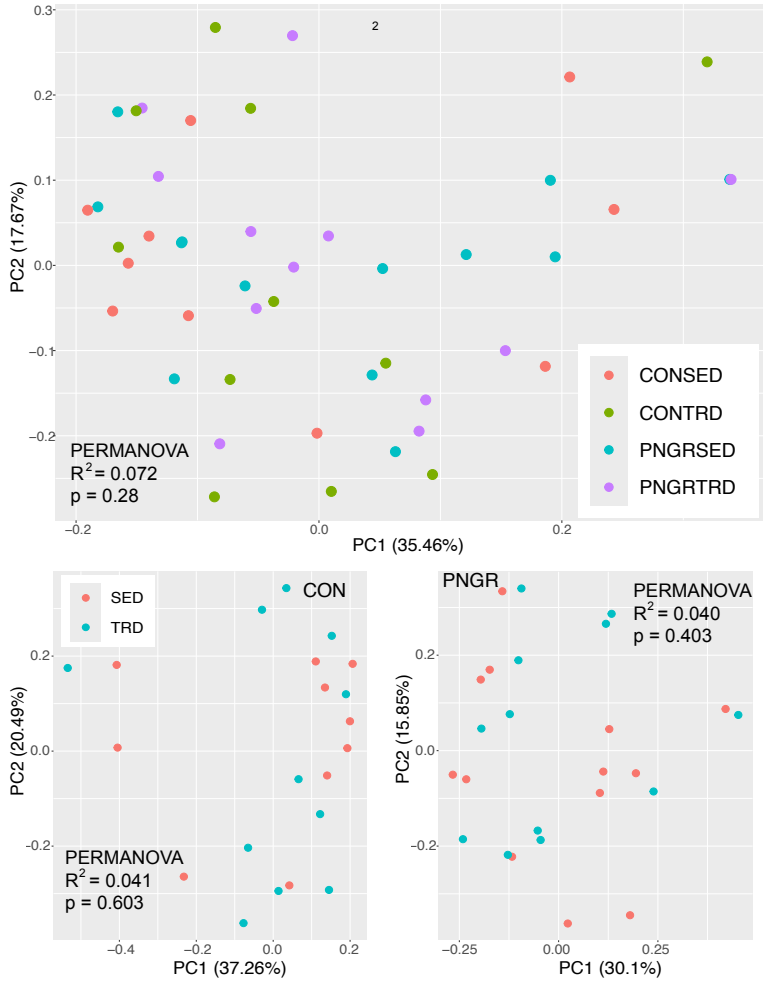

Figure 4.11 Testing Distances of Fecal Metabolites as an Effect of Diet and Training. Principle component analysis (PCA) Plot and PERMANOVA analysis revealed very few signatures of clustering or separation of metabolites by among treatment groups within the dataset as a whole (top) indicating a weak individual association of treatment or diet on the fecal metabolome (R^2=0.072; p=0.28). After further separation by diet and testing the effects of HIIT, there were still minimal effects found in the fecal metabolome (bottom left, CON: R^2=0.041; p=0.603; bottom right, PNGR: R^2=0.040; p=0.403). Individual data points are colored by their respective treatment group; [CON-SED n=10, CON-TRD n=11, PNGR-SED n=13, PNGR-TRD n=12].

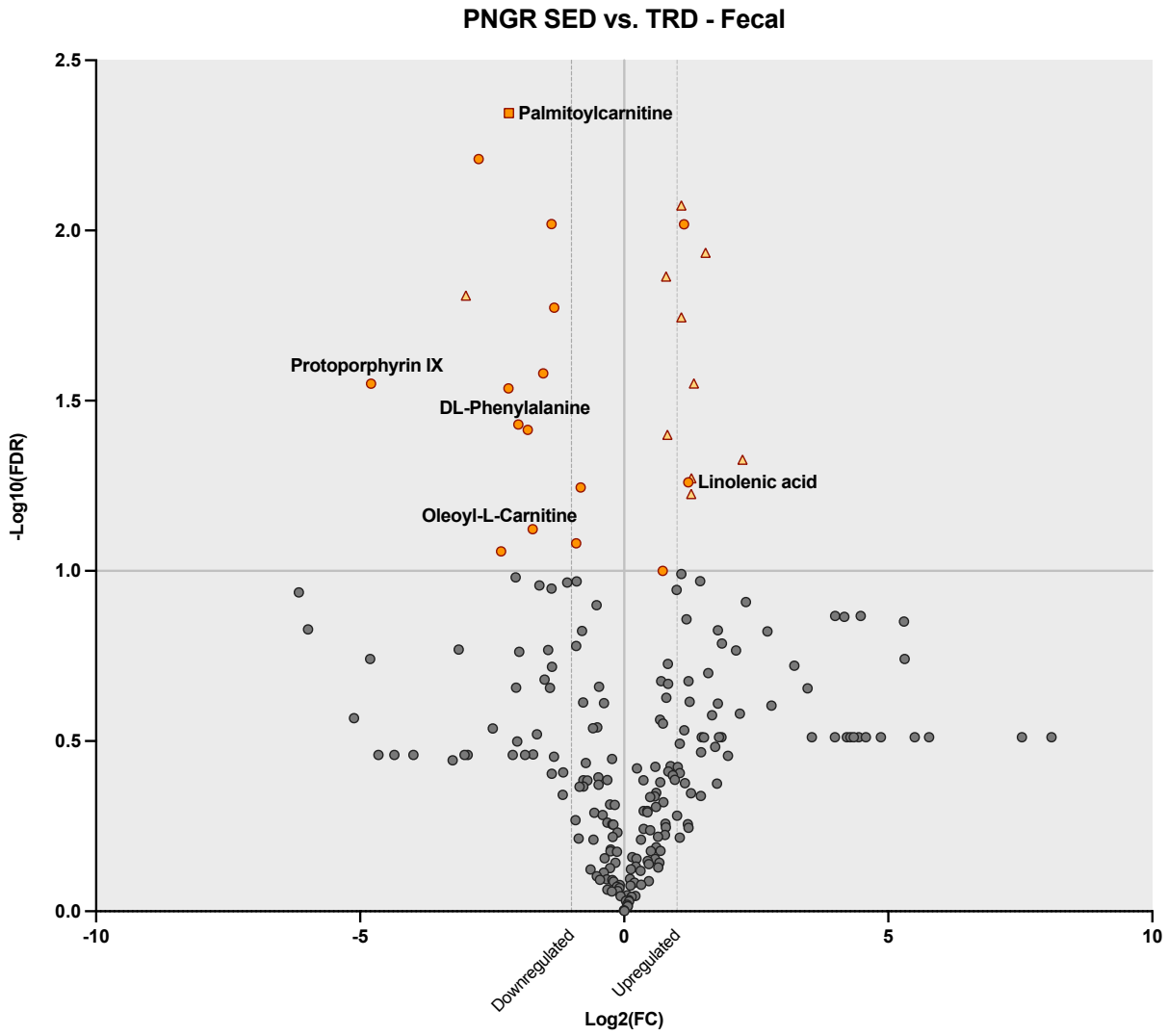

Figure 4.12 Investigating the Effects of HIIT on Fecal Metabolome in PNGR.
Majority of metabolites differentially affected via HIIT were downregulated palmitoylcarnitine, oleoyl-l-carnitine, phenylalanine and protoporphyin IX, and upregulation of excreted peptides and linolenic acid. FC: Fold-change was calculated as differences between treatment and sedentary assignment among the diet group. FDR: False discovery rate is set at 0.10. All metabolites above the threshold are labeled in orange and all non-significant findings are in black. Upregulated and downregulated metabolites are based on the Log2(FC) which is set at >1.0 for upregulated, and <1.0 for downregulated and is set on the x-axis. Triangles represent peptides and squares represent acylcarnitines; [PNGR-SED n=13, PNGR-TRD n=12].

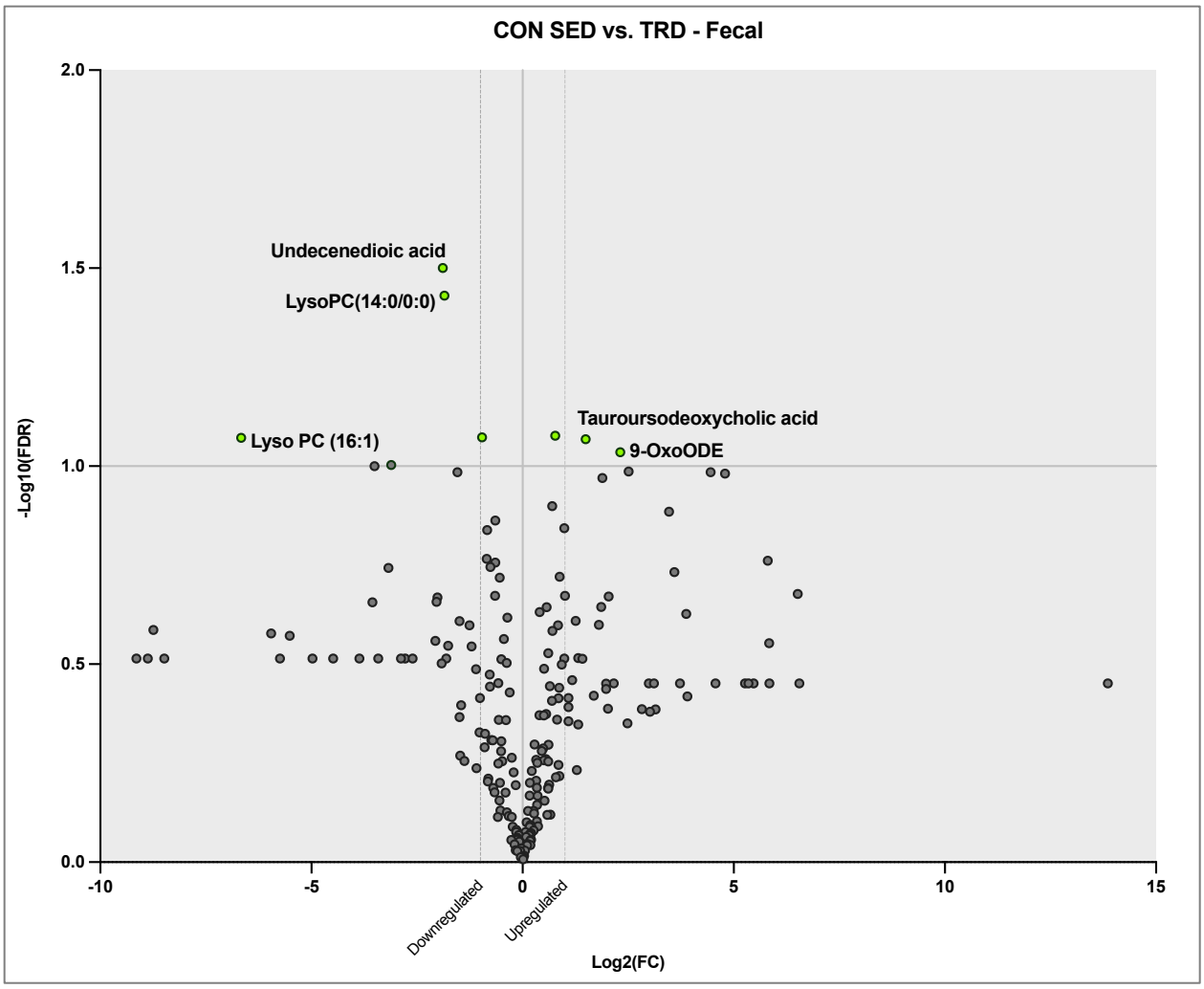

Figure 4.13 Minimal Effects of HIIT on Fecal Metabolome in CON at PN71.
Very minimal effects of HIIT were found in the fecal metabolome of CON post-intervention. The primary downregulated metabolites found were various forms of Lyso-Phosphocholines (Lyso PC) and medium-chain dicarboxylic acid Undecenedioic Acid. The main two metabolites found to be upregulated was a bile acid, Taurousodeoxycholic acid and a lipid mediator, 9-OxoODE. FC: Fold-change was calculated as differences between treatment and sedentary assignment among the diet group. FDR: False discovery rate is set at 0.10. All metabolites above the threshold are labeled in green and all non-significant findings are in gray. Upregulated and downregulated metabolites are set on the x-axis based on the Log2(FC) which is set at >1.0 for upregulated, and <1.0 for downregulated. Triangles represent peptides and squares represent acylcarnitines; [CON-SED n=10, CON-TRD n=11].

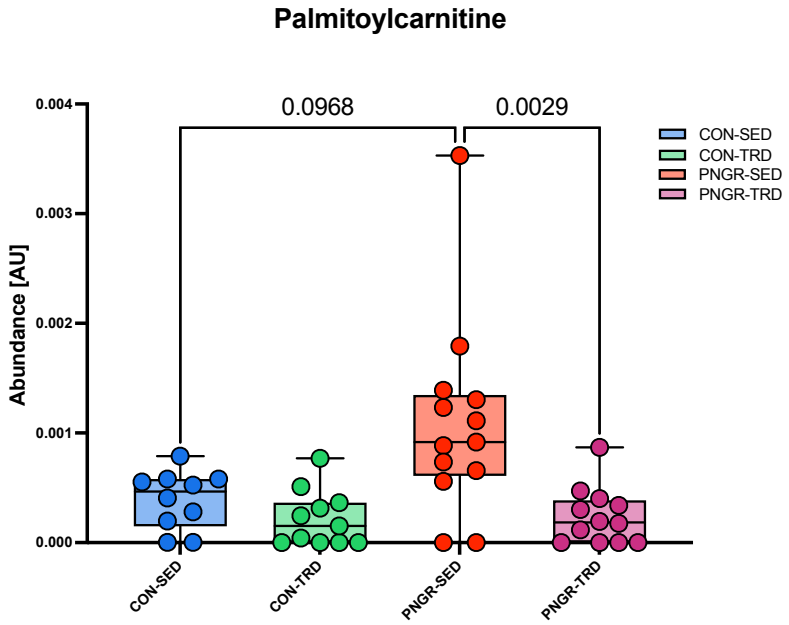

Figure 4.14 Effects of HIIT on Fecal Acylcarnitines at PN71.
Palmitoylcarnitine excretion was found to be significantly higher in PNGR-SED vs. PNGR-TRD (p=0.0029), which indicates a decrease in excretion in response to HIIT, leading to levels that appear more similar to CON. PNGR-SED also trended higher than CON-SED (p=0.0968). Error bars shown are mean and SD. Individual points represent individual mice within each treatment group; [CON-SED n=10, CON-TRD n=11, PNGR-SED n=13, PNGR-TRD n=12]

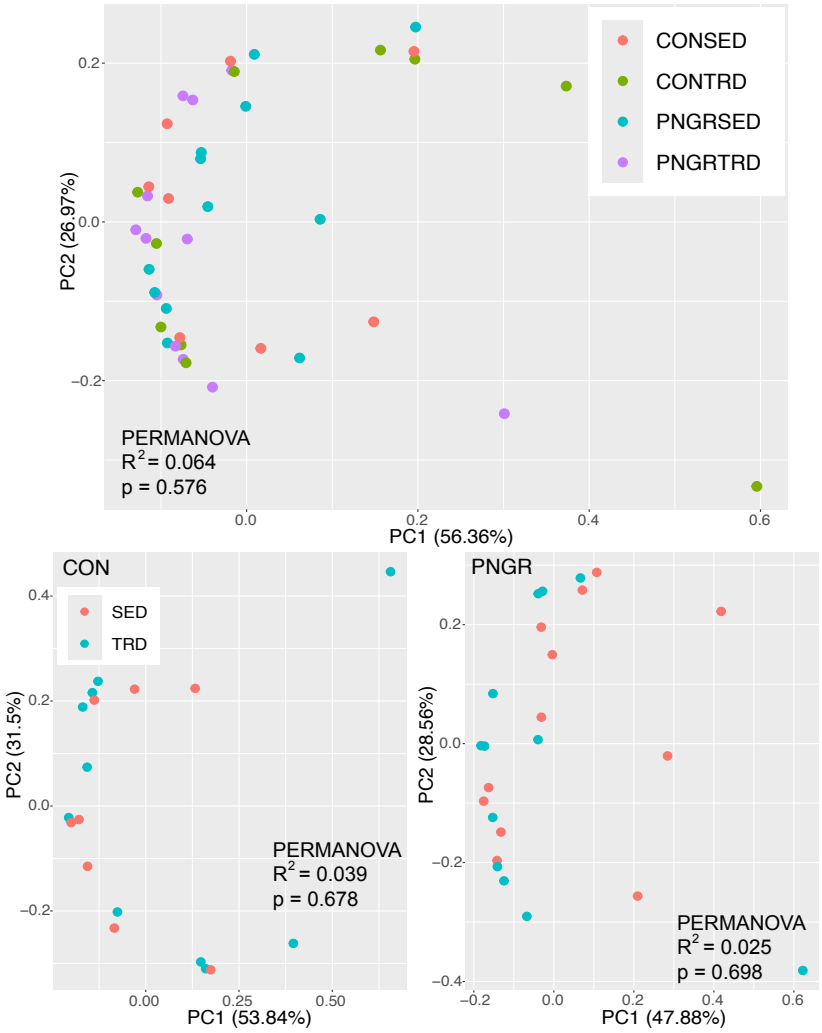

Figure 4.15 PERMANOVA Analysis of Microbes Post-HIIT.
Analysis of the microbiome dataset as a whole (top) revealed HIIT did not lead to strong separation or clustering of microbes between treatment groups (R^2 = 0.064; p=0.576). Further separation of microbes between diet groups also showed very minimal effects (bottom left: CON R^2 = 0.039; p=0.678; bottom right: PNGR R^2 = 0.025; p=0.698;). PC: Principal component analysis; [CON-SED n=10, CON-TRD n=11, PNGR-SED n=13, PNGR-TRD n=12].

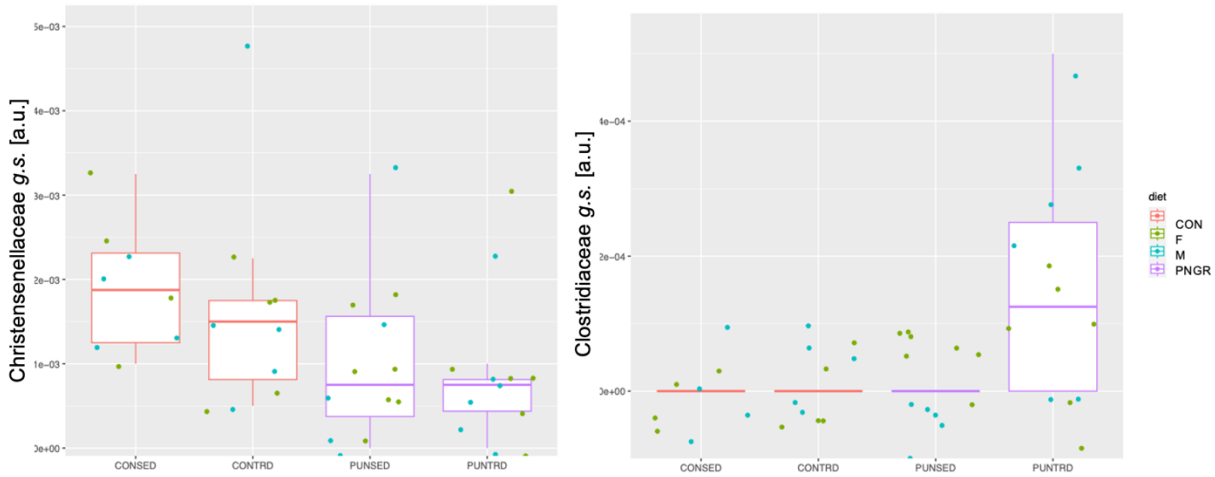

Figure 4.16 Level 7 Taxonomic Analysis of Microbes Post-HIIT.
Level 7 analysis revealed HIIT significantly altered Clostridiaceae *g.s.* (left; KW $p<0.001$) with post-hoc comparisons showing an increase in PNGR-TRD compared to PNGR-SED ($p=0.007$), CON-TRD ($p=0.0124$) and CON-SED ($p=0.0237$). Another microbe within the same level, Christensenellaceae *g.s*. (right) was also significantly altered (KW $p=0.02$) and decreased in response to HIIT in both CON and PNGR at PN71. Christensenellaceae *g.s.* decreased the most in PNGR-TRD compared to CON-TRD ($p=0.01$), with trending decreases in CON-TRD vs. CON-SED and PNGR-TRD vs. PNGR-SED ($p<0.05$); [CON-SED n=10, CON-TRD n=11, PNGR-SED n=13, PNGR-TRD n=12].

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APPENDIX A: SERUM COLLECTION PROTOCOL

Supplies:

- Label & autoclave tubes (x2)
    - 1 set of 2mL tubes
    - 1 set of 1mL tubes
- 70% ethanol to sanitize stir rod between samples
- Sharp tweezers and dissection scissors
- Small disposable pipettes

Steps:

1. Anesthetize and sacrifice the mouse according to the protocol
2. First carefully open the chest cavity and if possible, keeping the diaphragm intact
3. Then collect the heart and snip the main arteries to help blood pool in the chest cavity
4. Use a small disposable pipette to collect blood – work quickly to avoid clotting
    a. Aim for at least 200ul of whole blood per mouse
5. Once blood is in tube, let it sit in tube rack for 30-45 minutes to allow to clot at room temp
6. Then use a glass rod to disrupt clots from tube wall
7. Centrifuge tubes at room temperature for 15 minutes at 2000-2500xg
8. Aliquot serum from tube into new clean 1mL autoclaved labeled tubes
9. Serum will have a pinkish tint, with separation of phases between hematocrit and serum
10. Store all samples at –80C for future use

APPENDIX B: SERUM METABOLOMIC EXTRACTION PROTOCOL

- Monophasic solvent system (initial extraction step):
    - First, combine the serum with a chloroform-methanol mixture (1:2 v/v ratio) to form to extract and dissolve the lipids
    - Once dissolved, then vortex the samples
    - The resulting suspension will consist of a 1:2:0.8 (v/v/v) chloroform/methanol/water mixture
- Biphasic system (purification step):
    - add water and more chloroform
    - this now shifts the mixture to volumetric ratios of 2:2:1.8 (v/v/v)
- Filtration step:
    - Using medium flow filter paper, allowing for a separation of polar and non-polar compounds into upper (polar) and lower (non-polar) phases
    - Once the phases are separated, pipette the phases into separate sample tubes labelled as ‘polar’ and ‘non-polar’ which are then plated and subjected to LC-MS metabolomics processing

APPENDIX C: EXERCISE CAPACITY TESTING PROTOCOL

Note: The HIIT methodology utilized for this dissertation was adapted from previous studies[135,138,246].

1. Warm-up: Start the treadmill at 9 m/min at 0° grade for 9 min (machine will be flat/level).

2. After 9 min, increase the grade to 5° (first notch on the machine) and the speed to 10m/min for 3 minutes.

3. Every 3 minutes thereafter, the speed will be increased by 2.5 m/min (increments shown below) and the grade is increased 5° every 9 min up to a final grade of 15° (or until exhaustion).
    a. **Exhaustion:** contacting shock grid at the back of the treadmill for a total of 5x in 30 seconds.
        b. To track: set the stopwatch located next to the treadmill or use iPhone/watch. Once you see the mouse touch the shock grid the 1st time and then count the subsequent times the mouse touches the shock grid (up to 5 times) within 30 seconds.

4. At time of exhaustion:
    a. Turn off shock grid and leave mouse in the lane to rest (until all mice have finished) and record: the final time, final speed, final incline, and final distance for that mouse.

5. When all mice have finished the test, turn the treadmill off and make sure to clean the treadmill belt with the Virkon cleaner and paper towels.

6. To calculate W_{max}: [W_{max} (J/min) = (mass in kg x 9.81) x (final incline in radians) x (final speed in m/min) x (final distance in meters)]

APPENDIX D: HIIT PROTOCOL

- Note: The HIIT methodology utilized for this dissertation was adapted from previous studies[135,138,246].

- Prescribed training speeds are calculated via distance, final speeds final time, final incline and body mass collected from the W_{max} test (See APPENDIX C for formula).

1. To Begin, place the mouse in a lane on the treadmill and use dry erase marker to write the mouse ID for that lane. Make sure to turn the shock grids 'on' by flipping the switch for each lane, 'on' is indicated by a red flashing light.

2. Follow the prescribed speeds for each mouse and for each interval as previously calculated: (high intervals are 85% W_{max}, low intervals are 50% W_{max}).

3. Using either a stopwatch or interval timer App, begin with a 5-minute warm-up at 5 m/min. After 5 minutes, increase the speed to the first low interval (50%) for 2 minutes. Then, increase the speed to the high interval (85%) for 8 minutes. Repeat for a total of 6 cycles, followed by a 2-minute cool down at 5 m/min.

4. After the cool down has ended, flip the switch on the treadmill to 'stop' to freeze the distance on the treadmill. Record the final distance for each mouse and on each training day.

5. When you are ready to train the next mouse, keep the machine switched to 'stop' until you are ready to begin. Then, press the 'odometer reset' button to reset the distance to "0 meters". Then flip the switch on the machine to the 'run' position to begin the training session.

6. When all mice have finished the training session and have been returned to their cages, turn the treadmill off and make sure to clean the treadmill belt with Virkon cleaner and paper towels.

APPENDIX E: MICROBIOME/METABOLOME COLLECTION & PROCESSING

1. Grab a rolling cart from the hallway of the mouse facility into the mouse room

2. Collect a stack of 'white take out boxes' from the gray plastic hallway closet and a box of gloves from the wall (you will need to change gloves in between sample collection).

3. Make sure the cart/table used is clean (no visible debris or liquid) and if possible, use a Clorox or ethanol wipe to sanitize the area – do not use Vikron.

4. Take each mouse, one at a time, out of their cage and place them into a white take out box by themselves. Make sure to label the mouse ID on the white box to keep track of which mouse is in each container. Start with a round of about 2-3 mice at a time. Close the container to keep the mouse from jumping out.

5. Once all mice are in a labeled box, set a timer for 1-2 minutes, then check each box carefully to see if the mouse has left a sample in the container.

6. If there is no sample yet, continue the timer for another 1-2 minutes and check again. If the mouse has left a sample, carefully return the mouse to their respective cage – being very mindful not to turn the white take out box over. (It's best to collect the mouse by the base of their tail with the box upright and gently return them to their cage).
    a. *It is strongly recommended to include a helper so that one person can collect the mice, while the other collects the samples.*

7. To transfer the samples, first pre-label the tube for the respective mouse ID and date of collection. Put on a new clean pair of gloves.

8. Submerge the tweezers in the ethanol to sanitize them - wait a minimum of 10-15 seconds. Remove the tweezers and take a clean Kim wipe and dry off the tweezers to remove the excess ethanol

9. Use the now sanitized tweezers to collect 1-2 samples from the box making sure the mouse ID on the tube matches the mouse ID on the box. Seal the tube and place it in the box of dry ice

10. Place the tweezers back into the tube of 70% ethanol, throw away mouse take out box and change gloves. Make sure to change your gloves after each sample transfer.

11. Repeat the same process until all samples are collected and are on dry ice.

12. Store samples for future processing in the -80 freezer.