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# Exercise and the Gut Microbiome in Mice Selectively Bred for High Voluntary Wheel-Running Behavior

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

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by

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ABSTRACT OF THE DISSERTATION

Exercise and the Gut Microbiome in Mice Selectively Bred for High Voluntary Wheel-Running Behavior

by

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Doctor of Philosophy, Graduate Program in Evolution, Ecology, and Organismal Biology
University of California, Riverside, June 2022
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The gut microbiome is essential for normal host function. My dissertation examined the gut microbiome in juvenile and adult mice, including effects of exercise, diet, and antibiotics. As a model, I studied mice from 4 replicate High Runner (HR) lines that are bred for voluntary wheel-running behavior and their 4 non-selected Control (C) lines. Chapter 1 examined the juvenile gut microbiome in females from generation 81. Fecal samples were taken 24 hours after weaning. HR mice had higher relative abundance of the family *Clostridiaceae* compared to C mice. Based on beta diversity metrics, the 4 replicate HR lines differed from one another as did the 4 C lines. As part of a larger study, Chapter 2 examined effects of exercise training on adult body composition, organ masses, and food and water consumption. Four weeks of wheel access increased heart mass and decreased body fat for both HR and C mice. Chapter 3 tested for long-lasting effects of early-life Western diet and/or exercise. Males from generation 76 were given 3 weeks of Western or standard diet and/or wheel

access or no wheels starting at 3 weeks of age, then placed on a standard diet without wheels for 8 weeks, followed by fecal sampling. Western diet, exercise, and linetype (HR versus C lines) had an interactive effect on alpha diversity; in addition, Western diet decreased alpha diversity in all groups. Early-life Western diet also decreased the relative abundance of *Muribaculum intestinale*. Based on beta diversity metrics, HR and C mice had differing adult gut microbiome communities. Chapter 4 investigated effects of antibiotic treatment on wheel-running behavior in adult females from generation 89. Mice were given wheels for 2 weeks, then antibiotics in drinking water for 10 days, then returned to tap water for 12 days. Antibiotics reduced aerobic colony forming units from fecal samples to non-detectible levels and reduced wheel-running behavior in the HR, but not C lines. Antibiotics did not affect food consumption, nor did they appear to cause sickness behavior. These results suggest the HR microbiome is an important component of their high wheel-running phenotype.

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# 1. Introduction

## 1.1 A brief history of microbial research

Interest in the microorganisms inhabiting the body has skyrocketed over the past three decades, but our familiarity with bacteria started hundreds of years ago. In 1683, Antonie van Leeuwenhoek used a microscope to examine microbial life in plaque sampled from his teeth. Interest in pathogenic bacteria causing diseases (Koch's Postulates: Tabrah 2011) continued to grow, and the study of microbial ecosystems developed (Winogradsky column: Zavarzin 2006). In the 1970s, microbiome research made an important leap forward when Carl Woese discovered the potential of the 16s rRNA gene as a way of identifying microorganisms without culturing them in the lab (Woese and Fox 1977). Since then, other technological advances and widespread research efforts, such as the Human Microbiome Project, have greatly expanded our knowledge of the microbiome (The integrative human microbiome project 2019).

## 1.2 The gut microbiome

The gut microbiome encompasses the trillions of microorganisms inhabiting the host gastrointestinal tract. We now understand that the microbiome is an essential contributor to the host's physiology, and can also have important effects on behavior (McFall-Ngai et al. 2013; Cryan and Dinan 2014; Dominguez-Bello et al. 2019). The focus of microbiome research has primarily been on bacterial

species, though research on the virome (Liang and Bushman 2021) and mycobiome (Forbes et al. 2019) are emerging.

The gut microbiome is shaped by both host genetics and environmental factors (Benson et al. 2010; Spor et al. 2011; Gacesa et al. 2022; Ryu and Davenport 2022). In placental mammals, it is initially inoculated with bacteria from the environment, including those passed on from the mother, e.g., during nursing (Funkhouser and Bordenstein 2013; Milani et al. 2017; Sprockett et al. 2018; Ge et al. 2021). Subsequently, both intrinsic (e.g., species and strain competition in the gut) and extrinsic (e.g., diet and antibiotics) factors affect the community composition of the gut microbiome, through maturation and continuing into adulthood (Rawls et al. 2006; Cox et al. 2014; McLoughlin et al. 2016; Snijders et al. 2016). In C57BL/6 mice, fecal sampling for days 0-25, 45, 65, 125, and 364 after weaning demonstrated that the gut microbiome reached a stable, adult-like state 15 days after weaning and remained stable up to and including 364 days post-weaning (Schloss et al. 2012). In humans, the juvenile gut microbiome begins to resemble an adult gut microbiome at ~3 years of age (Yatsunenko et al. 2012).

### 1.3 Microbiota-gut-brain axis

The microbiota-gut-brain axis describes bidirectional communication between the host and the gastrointestinal tract through the central, autonomic, and enteric nervous systems, the endocrine system (including the hypothalamic-pituitary-

adrenal axis), the immune system, and bacterial metabolites (Grenham et al. 2011; Cryan and Dinan 2014; Toribio-Mateas 2018; Fülling et al. 2019). Bidirectional interactions between gut microbes and hosts is maintained through a mucus layer, epithelial cells, and immune cells that separate hosts from microbes found in the lumen of the gastrointestinal tract (Nehra et al. 2016). Microbial metabolites that can potentially act as host metabolic modulators and signals include B vitamins, K vitamins, glycine betaine, tryptophan, shorty-chain fatty acids, secondary bile acids, neurotransmitters, and urolithins (Cryan and Dinan 2014; Schroeder and Bäckhed 2016; Ticinesi et al. 2017). In addition, a small fraction of short-chain fatty acids not metabolized by epithelial cells of the colon can pass through the wall of the colon into the host's circulation, and in some cases, be used as energy sources (den Besten et al. 2013; Silva et al. 2020). Short-chain fatty acids are transported across the epithelial cells of the colon via passive diffusion or active transport (see review: den Besten et al. 2013)

Microorganisms of the gastrointestinal tract are in constant contact with immune cells. The epithelial cells lining the gastrointestinal tract provide a physical and chemical barrier separating host from bacteria in the lumen. One of the ways bacteria interact with the host immune system is through recognition of microbial-associated molecular patterns (MAMPs) that allow the immune system to identify "good" versus "bad" bacteria (Cerdá et al. 2016; Sprockett et al. 2018; Eshleman and Alenghat 2021). Additionally, microbial metabolites released by

bacteria in the gut can affect gut epithelial cells and stimulate an immune response via various host receptors for microbial metabolites (Kim 2018). Thus, the gut microbiome can function as an important contributor to host immune homeostasis.

Research on the microbiota-gut-brain axis indicates that species found in the gut microbiome are linked to such behaviors as sociability and anxiety in rodents (Bercik et al. 2011; Dinan et al. 2015). For example, germ-free mice inoculated with fecal bacteria from mothers fed a standard diet expressed more social behavior compared to germ-free mice lacking a microbiome (Buffington et al. 2016). In addition, behavior can be transplanted via microbiomes. In a study on BALB/c and NIH Swiss Webster mice, the BALB/c mice had lower levels of exploratory behavior in a step-down test compared to the NIH Swiss Webster (Bercik et al. 2011). Transplantation of the BALB/c microbiome into germ-free NIH Swiss mice decreased exploratory behavior while transplantation of the NIH Swiss microbiome into germ-free BALB/c mice increased exploratory behavior.

Many years of research has demonstrated that probiotics can be used to confer a health benefit to the host by contributing to intestinal microbial balance (Fuller 1991). The potential benefit of probiotic foods, especially the consumption of bacteria-rich yogurt was first recognized by Ilya Metchinkoff in the early 1900s, who suggested yogurt consumption was associated with long life in Bulgarian peasants (Metchnikoff 2004). Recently, probiotics have been suggested to improve host health by potentially colonizing the digestive tract

rather than harmful or potentially pathogenic bacteria (Nagpal et al. 2012; Amara and Shibl 2015). In addition, probiotic supplementation can have positive effects on behavior. For example, adult male BALB/c mice given *Lactobacillus rhamnosus* had reduced stress-induced corticosterone levels, less anxiety-like behavior in an elevated plus maze, and less depressive behavior during forced swim tests (Bravo et al. 2011).

### 1.4 Exercise and the microbiome

The primary behavior of interest in my dissertation is voluntary wheel running, which is an established model for human voluntary exercise (Eikelboom 1999; Garland et al. 2011; Novak et al. 2012). Exercise behavior is of crucial importance for human health and well-being, as it can prevent, ameliorate, and even cure some chronic diseases (Booth et al. 2012; Silverman and Deuster 2014). Conversely, a lack of sufficient exercise can contribute to the development of such chronic diseases as cardiovascular disease, type 2 diabetes, cancer, and mental disorders (Booth et al. 2012; Lee et al. 2012).

The effects of exercise on mammalian physiology have been the subject of intensive study for decades (Hawley et al. 2014; Heinonen et al. 2014; Silverman and Deuster 2014), but understanding of the effects of exercise on the microbiome is just emerging (Mailing et al. 2019; Mohr et al. 2020; Clauss et al. 2021). In the absence of compensatory reductions in other aspects of physical activity, exercise leads to increased energy expenditure and hence

necessitates greater food consumption (Garland et al. 2011), which should directly impact the gut microbiome. Exercise also causes many acute changes in physiology, including increases in body temperature, changes in hormone levels, intestinal barrier function, and digestive transit time that could feedback into the gut environment (Campbell and Wisniewski 2017; Mach and Fuster-Botella 2017).

Differences in the gut microbiome associated with exercise can be a result of genetic background, phenotypic plasticity, and/or evolutionary change (see Table 0.1 for further descriptions and examples). Unique microbiomes may reflect both genetically based, evolved differences in genetic composition and/or the effects of training (physical conditioning) that have accrued across their ontogeny. For example, endurance runners and body builders (Jang et al. 2019) are elite athletes that likely have inherent genetic differences and therefore phenotypic differences that can lead to differences in their microbiome. In addition, changes in the gut microbiome could be caused by training to improve their performance abilities (phenotypic plasticity). In amateur half-marathon runners, the relative abundance of several bacterial taxa and also fecal metabolites were significantly different pre- and post-race (Zhao et al. 2018). In ultramarathoners (96 km in 38-44 hours), as compared with fecal samples taken before a race, the relative abundance of several bacterial taxa changed when feces was sampled immediately after and also 10 days after the race (Sato and Suzuki 2022).

Chronic exercise (e.g., persistent daily jogging or cycling) is also likely to lead to natural selection for particular bacterial taxa and potentially evolutionary changes in the microbiome composition. To our knowledge, no studies have yet addressed this possibility. Demonstrating coevolution of the microbiome and host would require documentation of reciprocal genetic change; evolution in one species would lead to adaptative change in the other species, and vice versa (Douglas 2018; O'Brien et al. 2019; Groussin et al. 2020; Medina et al. 2022). My dissertation sets the stage for future studies that might be able to demonstrate host-microbiome coevolution in the selectively bred High Runner lines of mice (see below).

### 1.4.1 Exercise affects the gut microbiome community

The first paper highlighting the relationship between exercise and the microbiome found that cecal samples from adult rats given wheel access for five weeks had an increased amount of n-butyrate, a short-chain fatty acid byproduct of bacterial fermentation (Matsumoto et al. 2008). Since this paper was published, it has been suggested that butyrate can be transported from the small intestine to muscles, where it can lead to activation of several regulatory pathways linked to ATP production as well as muscle integrity, thus potentially altering athletic ability/performance (Ticinesi et al. 2017). For example, 12 weeks of dietary supplementation of butyrate in dietary-obese C57BL/6J mice increased the ratio of type 1 fibers in the vastus lateralis, gastrocnemius, and soleus muscles and

increased spontaneous physical activity in the home-cage at night compared to controls (Gao et al. 2009).

Some general patterns regarding the effects of exercise on the microbiome -- or the effects of the microbiome on exercise propensity or ability -- are emerging (Table 0.2). For instance, some studies have reported that voluntary wheel running increases the relative abundance of Bacteroidetes taxa in rodents after various lengths of exercise treatment (Queipo-Ortuño et al. 2013; Evans et al. 2014; Mika et al. 2015; Brandt et al. 2018), although the functional significance of these increases in unclear. The functionality of species in the Bacteroidetes phyla has been linked to the production of propionate, a type of short chain fatty acid, and research has shown a that high-protein diet can increase the diversity of Bacteroidetes species in the gut (Aguirre et al. 2016; Wexler and Goodman 2017). However, other studies using similar methods found no statistically significant increase in Bacteroidetes (Matsumoto et al. 2008; Cook et al. 2013; Campbell et al. 2016; Welly et al. 2016; Lamoureux et al. 2017; Liu et al. 2017). Typically, obesity in humans and rodents has been associated with a decreased ratio of Bacteroidetes to Firmicutes (John and Mullin 2016; Aoun et al. 2020).

### 1.4.2 Type and intensity of exercise affect the gut microbiome community

The type and intensity of exercise can differentially alter the gut community. Voluntary wheel running in rodents compared to forced treadmill exercise results

in differential increases in the two most abundant phyla of the gut, Bacteroidetes and Firmicutes. Specifically, in both laboratory rats and mice, wheel access (6 days-12 weeks) increased the relative abundance of Bacteroidetes compared to Firmicutes (Queipo-Ortuño et al. 2013; Evans et al. 2014), whereas treadmill training (4-6 weeks) had the opposite effect (Lambert et al. 2014; Petriz et al. 2014). As noted in the previous section, the functional significance of these changes is unclear, although any change in microbiome composition could influence short-chain fatty acid production, which are a potential energy substrate.

### 1.4.3 Exercise affects gut microbial diversity

Aerobic exercise can affect gut bacterial diversity. Exercise in elite athletes and healthy young adults has been associated with increased bacterial diversity (Clarke et al. 2014; Estaki et al. 2016; Barton et al. 2018). In the first microbiome study of human athletes, elite Irish rugby players had higher alpha diversity of the gut microbiome compared to sedentary adults (Clarke et al. 2014). However, the rugby players also had higher dietary protein intake, so the cause of the microbiome differences is unclear. Both voluntary wheel running and treadmill training in rodents for 4 to 12 weeks has been associated with increased bacterial diversity in some studies (Evans et al. 2014; Petriz et al. 2014; Denou et al. 2016; Liu et al. 2017), but not in others (Queipo-Ortuño et al. 2013; Mika et al. 2015; Welly et al. 2016; Batacan et al. 2017; Lamoureux et al. 2017).

### 1.4.4 Effects of the microbiome on host exercise behavior and/or ability

In principle, the gut microbiome might influence exercise behavior and/or ability in various ways, and several empirical studies show this to be the case. For example, both specific pathogen-free mice and mice mono-colonized with *Bacteroides fragilis* (a common gut symbiont) had increased swimming endurance time compared to germ-free mice in an unweighted swim performance test (Hsu et al. 2015). In a separate study, mice gavaged with *Veillonella atypica*, a species isolated from human marathon runners, had longer maximum treadmill run times than mice gavaged with a common probiotic species, *Lactobacillus bulgaricus* (Scheiman et al. 2019). However, this study did not include any physiological verification of exhaustion, which is essential when measuring endurance capacity (see Meek et al. 2009; Booth et al. 2010). In addition, Carmody and Baggish (2019) highlight that *L. bulgaricus* synthesizes lactate, which could explain the decrease in maximum treadmill run time in the mice that received this bacterium.

Additionally, the gut microbiome can influence skeletal muscle adaptation to exercise, demonstrated by reduced hypertrophy of soleus Type 1 and 2A fibers in adult female C57BL/6J mice with 8 weeks of progressive weighted wheel running while treated with antibiotics (Valentino et al. 2021 Note antibiotics did not reduce wheel running [cf. Chapter 4]). In a separate study on C57BL/6J male mice, germ-free mice had reduced gastrocnemius and quadriceps skeletal

muscle mass and reduced gene expression of the myosin heavy chain genes in tibialis anterior muscle (Lahiri et al. 2019). Furthermore, transplantation of the pathogen-free microbiome into germ-free mice increased skeletal muscle mass compared to germ-free controls.

### 1.4.5 Possible mechanisms by which bacteria affect exercise

The gut microbiome has the potential to influence exercise ability through bacterial-derived metabolites (Bindels and Delzenne 2013; Ticinesi et al. 2017; Grosicki et al. 2018). Bacterial symbionts produce short-chain fatty acids (SCFAs) from the fermentation of carbohydrates in the gastrointestinal tract, which can affect skeletal muscle performance (Przewłócka et al. 2020). Short-chain fatty acids can enter circulation and reach muscles to be utilized as an energy source (Grosicki et al. 2018). Metabolite byproducts from gut bacteria can travel to mitochondria in the muscles, potentially influencing endurance performance ability. For example, butyrate, a short-chain fatty acid, can activate PGC-1α, a biomarker of mitochondrial function that has been linked to increased expression in skeletal muscle during exercise (Clark and Mach 2017). Butyrate, one of the three most abundant SCFAs, is associated with increased epithelial cell wall integrity and increased glucose uptake in skeletal muscle (Ticinesi et al. 2019). In addition, aging (16-month-old) female mice fed a butyrate- containing diet for 10 months lost significantly less hindlimb muscle mass and had elevated

markers of mitochondrial biogenesis in skeletal muscle compared to mice on a control diet (Walsh et al. 2015).

### 1.5 My dissertation

To further our knowledge on the relationship between exercise and the gut microbiome, I used experimental manipulations of exercise, diet, and antibiotics with mice from the High Runner mouse model. The 4 replicate High Runner (HR) lines are bred for high levels of voluntary wheel-running behavior and compared with 4 non-selected Control (C) lines (Swallow et al. 1998). For the HR lines, the highest running males and females from at least 10 families are chosen as breeders for the next generation, based on average wheel revolutions on days 5 and 6 of a 6-day period of wheel access as young adults. Breeders in the C lines are chosen without regard to how much they run. Sibling pairings are not allowed.

HR mice are highly athletic as compared with C mice, and they have several key physiological and behavioral differences, suggesting that their gut microbes are likely to be different under baseline conditions and also potentially respond differently to exercise. First, and most obviously, HR mice run ~3-fold more on a daily basis (Careau et al. 2013; Copes et al. 2015; McNamara et al. 2021, 2022). Second, when housed without wheels, HR mice have higher home-cage physical activity (Malisch et al. 2008, 2009; Copes et al. 2015). HR mice are smaller in overall body mass and length, and have lower body fat (Swallow et

al. 2001; but see Hiramatsu and Garland 2018), despite eating more even when housed without wheel access (Swallow et al. 2001; Copes et al. 2015). High Runner mice differ from C mice for circulating concentrations of some hormones, including lower leptin (even accounting for their lower body fat) and higher adiponectin and corticosterone (Garland et al. 2016). Some aspects of gastrointestinal tract morphology do not appear to differ between the linetypes Specifically, HR and C mice at generation 37 had no significant difference in either large or small intestine mass or length, suggesting that HR mice might have faster digestive throughput (Kelly et al. 2017).

Several of the evolved traits in the HR mice, such as physical activity, hormone levels, food consumption, and body temperature could have potentially led to selection on the gut microbiome. The HR microbiome might have coevolved after many generations of selective breeding for high voluntary wheel-running behavior, but differences in the microbiome could also be reflective of acute effects of exercise or other phenotypic characteristics of the HR mice. Possible coevolutionary changes in the microbiome might facilitate higher levels of endurance exercise, e.g., by enhancing availability of substrates for mouse energy metabolism (Clark and Mach 2017; Grosicki et al. 2018). Alternatively, signals from the microbiome to the central nervous system (Cryan and Dinan 2014; Carabotti et al. 2015) might affect motivation for running, as opposed to physical abilities for exercise. These are important areas for future research (see Chapter 4).

My dissertation constitutes the first studies of the microbiome in High Runner mice. In addition, in Chapter 2, I present results of a study on exercise-training effects, from the same group of mice used in Chapter 1. These two chapters are part of a larger effort to explore the HR microbiome, done in collaboration with Dr. Rachel Carmody: the longitudinal effects of exercise on the gut microbiome will be reported in a separate manuscript by Dr. Carmody's lab. In this dissertation, I report the effects of 4 weeks of wheel access on exercise-associated traits (Chapter 2). These results are import because if exercise for only 4 weeks affects the masses of such key organs as the heart, then it likely also affects the gut microbiome community. In previous studies of laboratory rodents, it is well established that 8 weeks of wheel access affects organ masses, but relatively few studies have used exercise treatments of lengths as short as 4 weeks.

### 1.6 Chapter 1: the juvenile microbiome

I took fecal samples 24 hours after weaning, and the V4 region of the 16s RNA gene was sequenced. I hypothesized the linetypes would have diverged in the gut microbiome community, resulting in unique microbiomes between the HR and C mice.

### 1.7 Chapter 2: training effects on exercise-associated traits

For Chapter 2, I studied the effects of 4 weeks of voluntary exercise on adult body composition, organ masses, and food and water consumption in adult female mice. I hypothesized the linetypes would “train” in response to 4 weeks of voluntary exercise, and that interactive effects between exercise and linetype would occur (i.e., the HR mice would have greater training effects).

### 1.8 Chapter 3: effects of early-life exercise and diet on the adult microbiome

I examined the effects of juvenile diet and exercise manipulation on the adult gut microbiome, taking advantage of mice from another study (Cadney et al. 2021). Starting at three weeks of age, males were provided with Western diet (or standard diet) and/or wheel access (or no wheel access) for three weeks, followed by an 8-week "washout" period on standard diet with no wheel access. Finally, fecal samples were taken at 14 weeks of age and the hypervariable bacterial rRNA Internal Transcribed Spacer region was sequenced. I hypothesized that, in addition to the HR and C mice having different microbiomes, exercise and/or Western diet during early life would have a long-lasting influence on the gut microbiome community.

### 1.9 Chapter 4: effects of antibiotics on wheel-running behavior

Here, I studied the effects of greatly reducing the gut microbiome community via antibiotics. Adult female mice were provided with two weeks of wheel access, followed by 10 days of antibiotic treatment, then mice were provided with tap water while still housed with wheel access for 12 days. I recorded wheel-running behavior, home-cage activity, body mass, and food and water consumption. I hypothesized that removal of the gut microbiome community would alter voluntary wheel-running behavior, especially in the HR lines.

**Table 0.1**. Known or expected types of association between hosts and their gut microbiomes, along with some examples. These categories are not always mutually exclusive. For example, elite athletes (body builders, distance runners: Jang et al. 2019) and Irish athletes competing in 16 different events at the 2016 Summer Olympics (O'Donovan et al. 2019) had differences in their gut microbiome communities. In these cases, training is almost certainly a cause of differences in the microbiome, but some evidence indicates that human athletic ability has a genetic component (Bouchard et al. 2011; Mattsson et al. 2016; Pitsiladis et al. 2016; Lin et al. 2017; Williams et al. 2017).

| Type of Association | Example |
|---|---|
| 1. Inherent genetic and therefore phenotypic differences among hosts that lead to differences in their microbiome | Genetically differentiated strains and lines of mice **(Chapter 1, Chapter 3)** (Carmody et al. 2015; Kohl et al. 2016; Org et al. 2016; Pekkala et al. 2017; McNamara et al. 2021) |
| 2. Changes in the microbiome caused by training over various time scales (hours, days, months, years) (phenotypic plasticity) | Comparing athletes pre-and post-race (Zhao et al. 2018; Scheiman et al. 2019; Sato and Suzuki 2022)<br><br>Randomized controlled trials of prescribed exercise in healthy and overweight populations (Cronin et al. 2018; Kern et al. 2020; Cheng et al. 2022)<br><br>Voluntary wheel running in rodents (Matsumoto et al. 2008; Queipo-Ortuño et al. 2013; Evans et al. 2014; Mika et al. 2015; Welly et al. 2016; Liu et al. 2017)<br><br>Forced treadmill training in rodents (Lambert et al. 2014; Petriz et al. 2014; Denou et al. 2016; Liu et al. 2017) |
| 3. Evolutionary changes in the microbiome caused by training over various time scales (days, months, years) | No studies have examined microbial evolution within the host in response to training<br><br>Experimental evolution of *E. coli* within a mouse model (Barroso-Batista et al. 2014) |
| 4. Coevolutionary changes in the microbiome and the host species across many generations | Pea aphids and bacterial symbiont *Buchnera* (Smith and Moran 2020) |

**Table 0.2.** Examples of studies that provide evidence of exercise affecting the microbiome or the microbiome affecting exercise behavior or performance ability.

| **Type of Evidence** | **Human Examples** | **Rodent Examples** |
|---|---|---|
| Association/ observational study | (Clarke et al. 2014). Rugby players had increased fecal microbial diversity<br><br>(Petersen et al. 2017). Competitive cyclists had increased *Prevotella* genus in fecal samples and the abundance correlated with hours exercising | (Pekkala et al. 2017). Selection experiment: high and low aerobic capacity rats differed in gut microbiome composition, based on fecal samples from adults<br><br>(Kohl et al. 2016). Selection experiment: herbivorous, predatory, and high and low $VO_2$max-selected lines of voles differed in diversity and community structure, based on cecal samples from adults<br><br>McNamara et al. (Chapter 1). Selection experiment: HR and C lines differed in community structure and composition, based on fecal samples taken from weanling mice |
| Exercise affects the gut microbiome | (Scheiman et al. 2019). Marathoners had increased *V. atypica* post-race<br><br>(Zhao et al. 2018). Half-marathoners had altered taxa abundance post-race | Many publications:<br><br>(Matsumoto et al. 2008). Voluntary wheel access for 5 weeks increased cecal butyrate in adult rats<br><br>(McNamara et al. 2021: Chapter 3). Early-life exercise, diet, and linetype interactively affect alpha diversity of fecal samples taken from adult mice |
| Microbiome affects exercise behavior or performance ability | **Probiotics.** Probiotics associated with decreased severity of G.I. symptoms<br><br>Potentially improve exercise performance? | (Nay et al. 2019). Antibiotics decreased endurance of isolated muscles in adult mice<br><br>(Okamoto et al. 2019). Antibiotics reduced treadmill endurance in adult mice<br><br>(Oyanagi et al. 2018). Transplanted “exercised” microbiome after antibiotic treatment increased wheel running in adult mice<br><br>(McNamara et al. 2022: Chapter 4). Antibiotics reduced voluntary wheel-running behavior in HR lines of mice but not in non-selected C lines |

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# CHAPTER 1

Juvenile microbiome composition of a mouse model selectively bred for high voluntary wheel-running behavior

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**Abstract**

We compared the gut microbial community composition and diversity of four replicate lines of mice selectively bred for high wheel-running activity over 81 generations (HR lines) and four non-selected control (C) lines. We performed 16S rRNA gene sequencing on fecal samples taken 24 hours after weaning, identifying a total of 2,074 bacterial Operational Taxonomic Units. HR and C mice did not significantly differ for measures of alpha diversity, but HR had a higher relative abundance of the family *Clostridiaceae*. These results differ from a study of rats, where a line bred for high forced-treadmill endurance and that also runs more on wheels had lower relative abundance of *Clostridiaceae*, as compared with a line bred for low endurance that runs less on wheels. Within the two selection treatments, replicate HR and C lines had unique microbiomes based on unweighted UniFrac beta diversity, suggesting the possibility of multiple adaptive responses to selection.

## 1. Introduction

The mammalian gut microbiome plays an essential role in various aspects of the host's biology, including immune system function, energy extraction, and protection from pathogens (Dominguez-Bello et al., 2019; Eshleman and Alenghat, 2021; Gilbert et al., 2018; Kohl and Carey, 2016). Within an individual, the gut microbiome is shaped by both host genetics and environmental factors (Benson et al., 2010; Carmody et al., 2015; Tamburini et al., 2016).

Both acute and chronic voluntary exercise have been shown to affect the gut microbiome in rodents and humans (Campbell and Wisniewski, 2017; Mailing et al., 2019; Mohr et al., 2020). For example, the first study to examine the effects of exercise on the gut microbiome, demonstrated that adult rats with 5 weeks of wheel access had more butyrate-producing bacteria in their ceca compared to sedentary controls, as well as an increased amount of cecal n-butyrate, an essential short-chain fatty acid for intestinal epithelial cell health (Matsumoto et al., 2008). In human marathon and half-marathon runners, analyses of fecal samples pre- and post-event demonstrated rapid changes to the athlete’s gut microbiome (Scheiman et al., 2019; Zhao et al., 2018).

Conversely, the gut microbiome can affect exercise behavior and ability. For example, among adult male C57BL/6N mice treated with antibiotics then gavaged with cecal microbial communities harvested from sedentary versus exercised mice, recipients of the exercised microbiome ran more revolutions per day compared with recipients of the sedentary microbiome (Oyanagi et al.,

2018). With respect to exercise ability, mice gavaged with a lactate-metabolizing strain of *Veillonella atypica* cultured from human athletes after a marathon race had significantly longer run times to "exhaustion" than those gavaged with *Lactobacillus bulgaricus*, a common bacterial symbiont that cannot metabolize lactate (Scheiman et al., 2019: measured five hours after gavage; method of treadmill motivation not stated).

In the present study, we compared the juvenile gut microbiota among lines of mice that differ in both exercise behavior and ability. Specifically, we studied four replicate High-Runner (HR) lines of mice that have been selectively bred for high voluntary wheel-running behavior for more than 81 generations, as well as 4 non-selected Control (C) lines (Swallow et al., 1998). The selection criterion is the amount of wheel running revolutions on days 5 and 6 of a 6-day wheel-access period when mice are ~6-8 weeks old. Mice from the HR and C lines differ in several ways that potentially correlate with unique gut microbial communities. First, as compared with C mice, HR mice run ~3-fold more revolutions per day (Careau et al., 2013; Copes et al., 2015) and also have higher activity levels when housed individually without wheels (Copes et al., 2015; Malisch et al., 2009). These high activity levels are accompanied by elevated food consumption relative to body size (Copes et al., 2015; Hiramatsu and Garland, 2018; Swallow et al., 2001), which could directly affect gut microbial community composition via changes in luminal resources (Alcock et al., 2014). The HR and C mice have not been found to differ in small or large intestine mass

or length, which would suggest a faster digestive throughput in the HR mice (Kelly et al., 2017). Mice from the HR lines also have higher body temperature when active (Rhodes et al., 2000), altered hormone levels (Garland et al., 2016; Malisch et al., 2009), and tend to be smaller at weaning, although the difference in body mass is not always statistically significant (Cadney et al., 2021; McNamara et al., 2022; Swallow et al., 1999).

Previously, we reported that adult male HR and C mice differ in their gut microbial community composition, regardless of diet or exercise manipulation during early life (McNamara et al., 2021). In addition, when the microbiome is reduced with oral antibiotics, mice from the Control lines do not change their wheel-running behavior, whereas HR mice run significantly fewer revolutions per day (McNamara et al., 2022), suggesting the gut microbiome is an important component to the high wheel-running phenotype. Unique gut microbial phenotypes in HR mice could be a direct result of acute effects of differences in the traits unique to HR mice and/or changes in the selective regime experienced by the microbiota. The two previous studies mentioned above cannot distinguish between these possibilities because adult mice will have experienced many weeks of differences in physical activity, food consumption, and other physiological or developmental factors that could have independently led to gut microbial differentiation between HR and C mice. Although physical activity of HR and C pups prior to weaning has not been quantified, related aspects of pup behavior prior to weaning do not significantly differ: first day for eye opening,

moving, and feeding on solid food; locomotor play behavior (see Fig. 2A-C in Hiramatsu et al., 2017, 10-20 days old; Whitehead et al., in preparation, 15 days old). Therefore, the purpose of the present study was to compare the gut microbiome in juvenile mice at weaning. At weaning, any inherent differences in activity levels would have had less time to induce changes in the gut microbiome, as compared with sampling adult mice.

## 2. Methods

All experiments and methods were approved by the Institutional Animal Use and Care Committee of the University of California, Riverside.

### 2.1 Experimental animals

Mice for the present study were sampled evenly from generation 81 of an ongoing selection experiment for high voluntary wheel-running behavior. Briefly, four replicate High Runner (HR) lines are bred for voluntary wheel-running behavior and compared with four non-selected Control (C) lines. The selection experiment began in 1993 with a population of 224 outbred Hsd:ICR mice (Swallow et al., 1998). Mice are weaned at 21 days of age and housed 4 per cage separated by line and sex. At ~6-8 weeks of age, mice are housed individually for six days in cages attached to a 1.12 m circumference wheel with a sensor to measure daily total revolutions (Swallow et al., 1998). Each generation, the highest-running male and female from within each of 10 families

are chosen as breeders, based on the average revolutions on days 5 and 6 of the 6-day period of wheel access. For the C lines, one male and one female are taken from each family without regard to wheel running. Mice are paired within their line, and no sibling matings are allowed. Immediately following weaning from the mother, mice are provided with Standard Laboratory Rodent Diet from Harlan Teklad (Envigo) (W-8604), which contains 32% kJ from protein, 14% kJ from fat, and 54% from carbohydrate. Pregnant dams are given Harlan Teklad (Envigo) Lab Mouse Breeder Diet [S-2335] 7004 through weaning. In the present experiment, 100 females (12 from each line and 6 additional mice from line 6) were weaned from the mother at 21 days of age and housed individually for 24 hours prior to fecal sampling. Each subject was from a different litter, with 6 exceptions.

### 2.2 Fecal sampling

All mice were fecal sampled 20-24 hours after weaning. Mice were scruffed by the neck until defecation into a sterile tube, which was immediately placed on dry ice and stored at -80°C. All fecal samples were shipped on dry ice to the Nutritional and Microbial Ecology Lab at Harvard University, where they were stored at -80°C until processing.

### 2.3 DNA extraction

We used an established 16S ribosomal RNA (rRNA) gene sequencing pipeline to assess gut microbial community composition in each sample (Carmody et al., 2015; Carmody et al., 2019). Briefly, we isolated DNA using the Qiagen PowerSoil DNA Isolation Kit (Catalog no. 12888). Next, we PCR amplified the V4 region of the 16S rRNA gene using custom barcoded 515F and 806R primers (Caporaso et al., 2011; Caporaso et al., 2012). PCR amplification was performed in triplicate for each sample using the following reaction recipe: 11 µl nuclease-free $H_2O$, 1 µl 25mM $MgCl_2$, 10 µl Quantabio 5Prime Hot MasterMix (Cat. No. 2200410), 2 µl primers (1 µl of forward primer and 1 µl of reverse primer), and 1 µl of template DNA. We included a negative control reaction per sample to ensure that primers and reagents were not contaminated. PCR was performed using BioRad T100 thermocyclers and the following protocol: 94°C for 3 minutes; 35 cycles of 94°C for 45 seconds, 50°C for 30 seconds, and 72°C for 90 seconds; and 10 minutes at 72°C. PCR amplicons were checked by running recombined triplicate reactions, negative controls, and a 100 bp DNA ladder on a 1.5% agarose gel in an electrophoresis chamber. Amplicons were purified using Agencourt AMPure XP solution and resuspended in 40 µl of 1X TE buffer. Cleaned amplicons were quantified using the Quant-iT PicoGreen dsDNA Assay Kit, with fluorescence measured with a Spectramax Gemini XS Plate Reader set to 480 nm excitation / 520 nm emission. Cleaned amplicons were pooled at variable volumes to obtain 80 ng DNA per sample. We purified 100 µl of the

pooled solution using the Qiaquick MinElute kit (Cat. No. 28004). The eluted DNA was then gel-purified by 1.5% agarose gel electrophoresis. Band size was compared against a 100 bp DNA ladder, and the targeted 381 bp band was cut from the gel with a sterile razor and resuspended using the Qiaquick PCR Purification kit (Cat. No. 28104). The pool was diluted to 10 nM and submitted for sequencing on one lane of an Illumina HiSeq rapid flow cell (1 x 150 bp) at the Harvard Bauer Core.

### 2.4 Analysis of 16S rRNA gene sequences

Raw sequences were processed using the Quantitative Insights Into Microbial Ecology (QIIME) software package version 1.8 (Caporaso et al., 2010). After quality filtering, we obtained a mean sequencing depth of 123,557 ± 50,871 (SEM) reads per sample. Operational taxonomic units (OTUs) were picked at 97% similarity (Caporaso et al., 2010). Bacterial relative abundances at taxonomic levels from phylum to genus were generated using the summarize_taxa.py script. Prior to alpha diversity analysis, we subsampled the dataset at 25,000 reads. Alpha diversity (Shannon diversity index, Chao1, unique OTUs, and Faith's phylogenetic diversity) was analyzed using the alpha_diversity.py script. Prior to beta diversity analysis, we subsampled the dataset at 33,600 reads and used the beta_diversity_through_plots.py script to generate Bray-Curtis, unweighted UniFrac, and weighted UniFrac distance matrices and associated principal coordinates.

The bacterial 16S rRNA sequences will be deposited in the National Center for Biotechnology Information (NCBI)'s Sequence Read Archive (SRA).

### 2.5 Statistical analyses

We used mixed model ANOVAs to analyze alpha diversity metrics and taxonomic relative abundances in SAS 9.4 Procedure Mixed (SAS Institute, Cary, NC, USA). Our models tested the effects of linetype (HR versus C lines) against the variance among replicate lines, nested as a random effect within linetype. We also tested the effect of mini-muscle status, a phenotype currently present in two of the four HR lines that is caused by a single base pair change in the *Myosin heavy polypeptide 4* gene (Kelly et al., 2013). As noted above, the mini-muscle phenotype is characterized by a ~50% reduction in hindlimb muscle mass, larger internal organs, and a variety of other differences as compared to normal-muscle mice (e.g., see Garland et al., 2002; Swallow et al., 2009; Wallace and Garland, 2016). In the present study, 26 of the 95 mice had the mini-muscle phenotype (all 12 in HR line 3 and 6 of 14 in HR line 6).

Bacterial relative abundances were log or arcsine square root transformed to improve normality of residuals (Brown et al., 2020; Kohl et al., 2016). Based on simulations by Aschard et al. (2019), we limited analyses to taxa found with at least 85% prevalence amongst samples (see also McNamara et al., 2021). We then used a targeted approach to test for differentially abundant bacteria between the HR and C linetypes. Specifically, we analyzed taxa previously

associated with exercise in rodents (Codella 2018, Hughes 2020, Mach and Fuster-Botella 2016): Proteobacteria (phylum), Bacteroidetes (phylum), Firmicutes (phylum), Tenericutes (phylum), Actinobacteria (phylum), *Rikenellaceae* (family), *Lactobacillaceae* (family), *Clostridiaceae* (family), *Clostridium* (genus: endemic in laboratory house mice colonies, and potentially pathogenic under some conditions e.g. see Krugner-Higby et al., 2006), *Lactobacillus* (genus), *Bifidobacterium* (genus), and *Oscillospira* (genus). We were also interested in the genera *Veillonella* and *Akkermansia* for their prior associations with endurance activity (Clarke et al., 2014; Munukka et al., 2018; Scheiman et al., 2019), but *Veillonella* was not present and *Akkermansia* was found in only 58% of the samples. Statistical significance was judged at the $P$=0.05 level. For completeness, analyses of additional taxa with at least 85% prevalence among samples are presented in Supplemental File 1.1. A measure of effect size (in this case, Pearson's r) was calculated for all main and interactive effects for bacterial relative abundance and alpha diversity (Sullivan and Feinn, 2012).

Beta diversity of the gut microbiome was assessed by calculating unweighted UniFrac, weighted UniFrac, and Bray-Curtis distance matrices and performing principal coordinate analyses (PCoA) to visualize the microbial community clustering based on distance. We used the adonis function within the vegan package in R to perform permutational analysis of variance (PERMANOVA) tests to determine significant clustering within the dataset

(Anderson, 2001; Anderson, 2017). For this analysis, we permutated the distance matrix over linetype and mini-muscle status 999 times. Replicate line was not treated as a nested random effect because this feature is not available in the vegan package in R; therefore, we also permuted the distance matrix over line for separate analyses of the 4 replicate HR and 4 replicate C lines 999 times. One clear visual outlier was removed from the unweighted UniFrac distance matrix.

## 3. Results and Discussion

Note: Supplemental File 1.1 (LG_82_Microbiome_Supplemental_Table_1.xlsx) is an excel file with a table of p values from analyses of alpha diversity and taxa relative abundance with at least 85% prevalence among samples for phylum through genus. Significant ($P \leq 0.05$) P values are highlighted in red.

### 3.1 Alpha diversity of the juvenile gut microbiome

Based on ANOVA, the average unique OTUs per mouse and other alpha diversity metrics did not statistically differ between juvenile HR and C mice or between mini- and normal-muscle mice (Fig. 1 and Supplemental File 1.1). Within the two linetypes, the 4 individual replicate C lines significantly differed in the number of unique OTUs ($F_{3,41} = 3.14$, $P$=0.0353) and Faith's phylogenetic diversity metric ($F_{3,41} = 3.06$, $P$=0.0386), but not Chao1 ($F_{3,41} = 2.53$, $P$=0.0707) or Shannon index ($F_{3,41} = 1.28$, $P$=0.2954). The 4 individual replicate HR lines

did not significantly differ in the number of unique OTUs ($F_{3,45}$ = 0.93, $P$=0.4356), Faith's phylogenetic diversity ($F_{3,45}$ = 0.49, $P$=0.694), Chao 1 ($F_{3,45}$ = 0.96, $P$=0.4220), or Shannon index ($F_{3,45}$ = 0.62, $P$=0.6081).

The gut microbiome community typically becomes more diverse with age in both humans and rodents (Koenig et al., 2011; Schloss et al., 2012; Yatsunenko et al., 2012). Consistent with this general pattern, our weanling mice had fewer average OTUs (N = 373) compared with our previous study of adult HR and C mice (N = 430), although it is important to note we are comparing numbers from different sequencing methods (the present study sequenced the 16S rRNA gene, whereas the previous study sequenced the Internal Transcribed Spacer region: see McNamara et al. 2021).

Two prior studies of rodents examined the gut microbiome in response to selective breeding for aspects of exercise capacity, although not in weanlings. Two lines of rats bred for either high (HCR) or low (LCR) endurance capacity during forced treadmill exercise did not differ in alpha diversity metrics at either 7 or 40 weeks of age (Pekkala et al., 2017: weaning occurs at 4 weeks of age in these rats). Four replicate lines of bank voles selectively bred for oxygen consumption during swimming exercise at a temperature below the thermal neutral zone also did not differ in alpha diversity from four control lines when measured as adults (mean age: 166 days) (Kohl et al., 2016).

### 3.2 Dominant phyla of the juvenile gut microbiome

A total of 2,074 OTUs were identified across the entire sample (N = 95 mice) of weanling mice, representing 11 phyla, 21 classes, 38 orders, 108 families, and 218 genera of microbes. As is typical for mice, community composition for the entire sample was dominated by the phyla Firmicutes (48.4 ± 14%; mean ± S.D.) and Bacteroidetes (37.5 ± 17.1%), with additional phyla being much less abundant (Fig. S1). Based on the mixed models in SAS, neither linetype (HR vs. C) nor mini-muscle status statistically affected the relative abundance of the phyla Bacteroidetes, Firmicutes, Proteobacteria, Tenericutes or Actinobacteria (Supplemental File 1.1).

Previously, we reported that the adult gut microbiota in these mice was dominated by Bacteroidetes (~68%), with Firmicutes (~28%) being the second most abundant (McNamara et al., 2021). In addition, the phylum Proteobacteria comprises a much larger portion of the juvenile (11.8 ± 8.7%) compared to the adult (~1%) gut microbiome. Thus, our results are consistent with previous studies in lab rodents showing that the juvenile gut microbiome is initially dominated by Firmicutes (Cox et al., 2014; Pantoja-Feliciano et al., 2013) followed by a shift towards Bacteroidetes as adults (Cox et al., 2014; Nagpal et al., 2018).

### 3.3 Lower-level taxonomic comparisons

The HR mice had significantly higher relative abundance of the family *Clostridiaceae* compared to C mice (LS Means for arcsine square root transformed values ± SE: HR = 0.0698 ± 0.0082; C = 0.0279 ± 0.0117) (ANOVA, $F_{1,6}$ = 10.54, $P$=0.0175, Fig. S2), with no statistical difference for the families *Rikenellaceae* or *Lactobacillaceae,* and genera *Clostridium, Bifidobacterium, Lactobacillus,* and *Oscillospira* (Supplemental file 1.1). Mini-muscle mice had a significantly higher relative abundance of the genus *Clostridium* (note: this genus is not in *Clostridiaceae*) compared to normal-muscle mice (LS Means of arcsine square root transformed values ± SE: Mini = 0.1844 ± 0.0146; Normal = 0.1373 ± 0.0064) (ANOVA, $F_{1,86}$ = 8.24, $P$=0.0052; Fig. S3). In our previous study of adults, HR mice also had a higher relative abundance of the family *Clostridiaceae* compared to controls, although the difference was not statistically significant (P=0.0750: Table S2 in McNamara et al., 2021).

The HCR and LCR lines of rats (see above) also differed in their gut microbiome community composition (Liu et al., 2015; Pekkala et al., 2017). HCR rats have higher maximal aerobic capacity ($VO_2$max) and higher voluntary wheel running compared to the LCR line (Karvinen et al., 2015; Park et al., 2016; Swallow et al., 2010), paralleling the elevated endurance capacity (Meek et al., 2009) and $VO_2$max (Rezende et al., 2005) of our HR mice. In addition, the HCR rats are smaller than the LCR rats (Pekkala et al., 2017; Wisløff et al., 2005) and HR mice are smaller than C mice (Dumke et al., 2001; Kelly et al., 2017). Thus,

we expected to see some similar patterns of differentiation in the gut microbiome communities. However, whereas adult rats from the HCR line had significantly lower relative abundance of *Clostridiaceae* compared to the LCR line (Liu et al., 2015), HR mice had a higher relative abundance of *Clostridiaceae* compared to the non-selected Control lines for both adults (McNamara et al., 2021) and weanlings (present study). One obvious explanation for this difference is that they are different species, and rats and mice are known to differ in various ways with respect to exercise physiology and responses to exercise training (e.g., see Dumke et al., 2001; Kowalski and Bruce, 2014). Future studies in rodents bred for exercise-related traits, including our own HR mice, should examine the gut microbiome community across various timepoints and generations to illuminate if the abundance of *Clostridiaceae* changes during development and/or drifts over generations.

### 3.4 Beta diversity of the juvenile gut microbiome

Principal coordinate analysis based on unweighted UniFrac distances did not indicate separation between HR and C mice along the first two principal coordinate axes (Fig. 2A: although together they account for only 14% of the total variance in the data). PERMANOVA of the unweighted UniFrac distance matrix also indicated no significant differentiation between HR and C mice ($R^2$=0.013, $P$=0.080). This lack of statistical differentiation contrasts with our previous results for adults, where HR and C mice clustered separately, regardless of diet

and/or exercise treatment during early life, based on unweighted UniFrac distances (PERMANOVA P=0.009: McNamara et al., 2021). Fig. S4 shows the corresponding plots for Bray-Curtis and weighted UniFrac distances and also the PERMANOVA results, which again indicated no significant separation based on linetype.

Mini- and normal-muscle mice separate somewhat on the third PCoA axis for unweighted UniFrac distances, and PERMANOVA indicates statistically significant separation for unweighted UniFrac (Fig. 2B*;* $R^2$=0.014, $P$=0.047). Thus, weanling mice have distinct bacterial communities based on mini-muscle status. However, no significant separation was detected when separation was measured by Bray-Curtis or weighted UniFrac distances, suggesting that the effects of mini-muscle status may be limited to differences in phylogenetic representation in the microbiome and not differences in relative abundance (Fig. S4). In our previous study, we did not test for effects of mini-muscle status on adult microbiome beta diversity (McNamara et al., 2021).

Finally, we considered potential separation among the four replicate HR lines and among the four replicate C lines (Fig. 3). Differentiation among replicate lines was statistically significant within both linetypes based on PERMANOVAs on unweighted UniFrac distances (HR lines with one outlier removed: $R^2$=0.028, $P$=0.038; C lines: $R^2$=0.037, $P$=0.006). Based on Bray-Curtis distances, the separation among C lines was statistically significant, but

not among the HR lines, and weighted UniFrac distances indicated no separation among the replicate lines for either linetype (Fig. S5).

When measured as adults (37 weeks of age after 11 weeks of individual housing with or without voluntary wheel access), the HCR and LCR lines of rats mentioned above clustered separately, based on unweighted and weighted UniFrac distances (Liu et al., 2015). In the bank vole selection experiment, a total of 16 lines were included: four bred for aerobic capacity, four for the ability to maintain body weight when fed a low-quality diet for four days, and four for predatory behavior towards crickets. However, when measured as adults (mean age: 166 days), none of the sets of selected lines had gut microbial profiles that differed from those of the control lines using weighted UniFrac distances (Kohl et al., 2016). Unweighted UniFrac distances indicated the herbivorous lines differed in gut microbial profiles compared to the other groups.

**Figure 1.1**

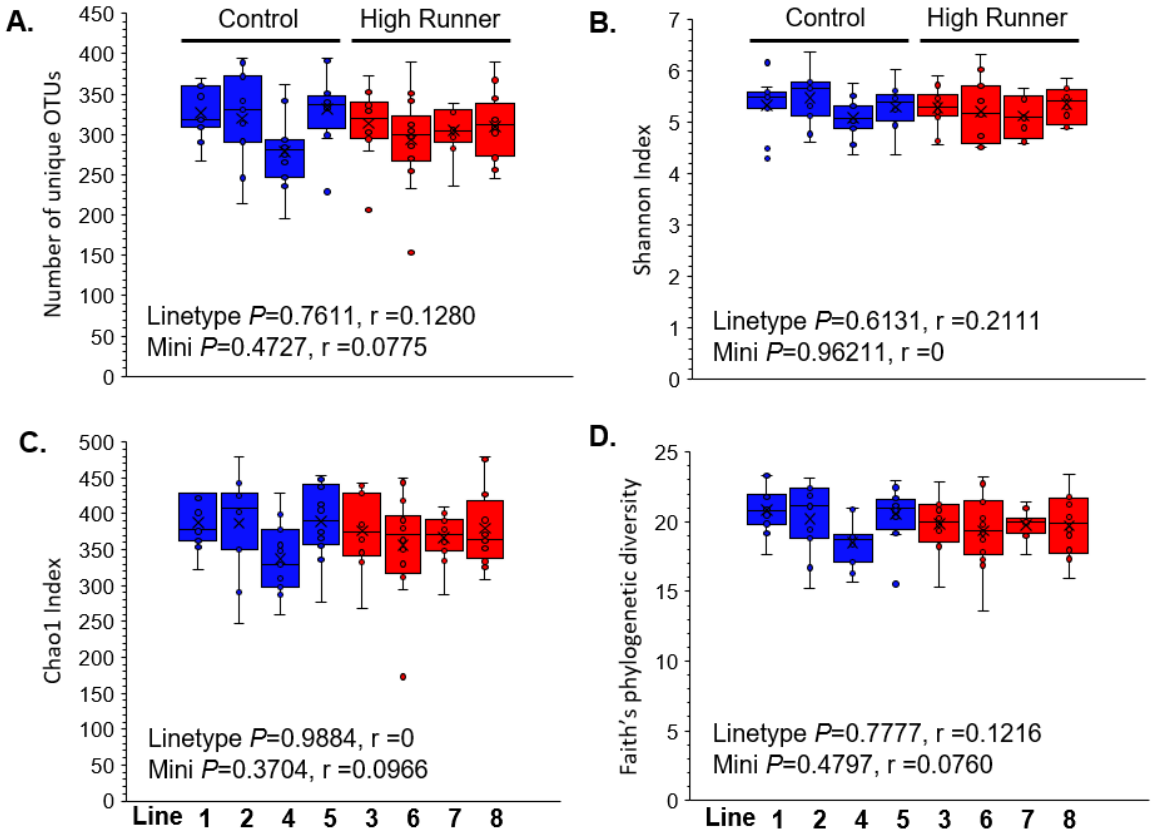
A.
Control
High Runner
Number of unique OTUs
Linetype P=0.7611, r =0.1280
Mini P=0.4727, r =0.0775
B.
Control
High Runner
Shannon Index
Linetype P=0.6131, r =0.2111
Mini P=0.96211, r =0
C.
Chao1 Index
Linetype P=0.9884, r =0
Mini P=0.3704, r =0.0966
Line 1 2 4 5 3 6 7 8
D.
Faith's phylogenetic diversity
Linetype P=0.7777, r =0.1216
Mini P=0.4797, r =0.0760
Line 1 2 4 5 3 6 7 8

Fig. 1.1. Box and whisker plots (raw data) for 4 measures of alpha diversity in the juvenile fecal microbiome, separated by line (raw data). Boxes encompass the interquartile range, horizontal lines within the boxes are the median, the x is the mean, and the ends of the whiskers are the highest and lowest non-outlier value. Based on mixed models comparing HR and C lines, as well as mini-muscle and normal-muscle mice (not indicated in the figures) (SAS Procedure Mixed), alpha diversity did not significantly differ between HR and C mice (Unique OTUs, $F_{1,6} = 0.01$, $P$=0.7611; Shannon Index, $F_{1,6} = 028$, $P$=0.6131; Chao1, $F_{1,6} = 0$, $P$=0.9884; Faith's phylogenetic diversity, $F_{1,6} = 0.09$, $P$=0.7777;) or between mini-muscle and normal-muscle and individuals (Unique OTUs, $F_{1,86} = 0.52$, $P$=0.4727; Shannon Index, $F_{1,86} = 0.0$, $P$=0.9621; Chao1, $F_{1,86} = 0.81$, $P$=0.3704; Faith's phylogenetic diversity, $F_{1,86} = 0.5$, $P$=0.4797). Pearson's r is a measure of effect size (see Methods).

**Figure 1.2**

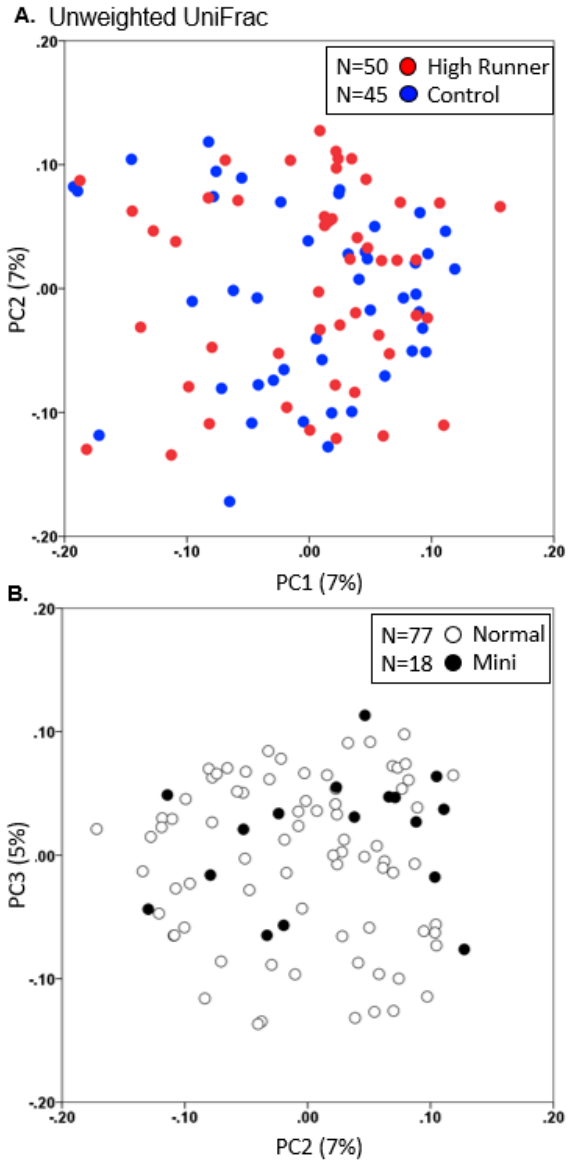


Fig. 1.2. Principal coordinate analysis of unweighted UniFrac distances between the 16S rRNA gene sequencing-based profiles of juvenile fecal microbiomes in relation to (A) linetype and (B) mini-muscle status. One outlier was removed. PERMANOVAs indicated significant separation based on mini-muscle status mice ($R^2$=0.014, $P$=0.047), but not between linetypes ($R^2$=0.013, $P$=0.080).

**Figure 1.3.**

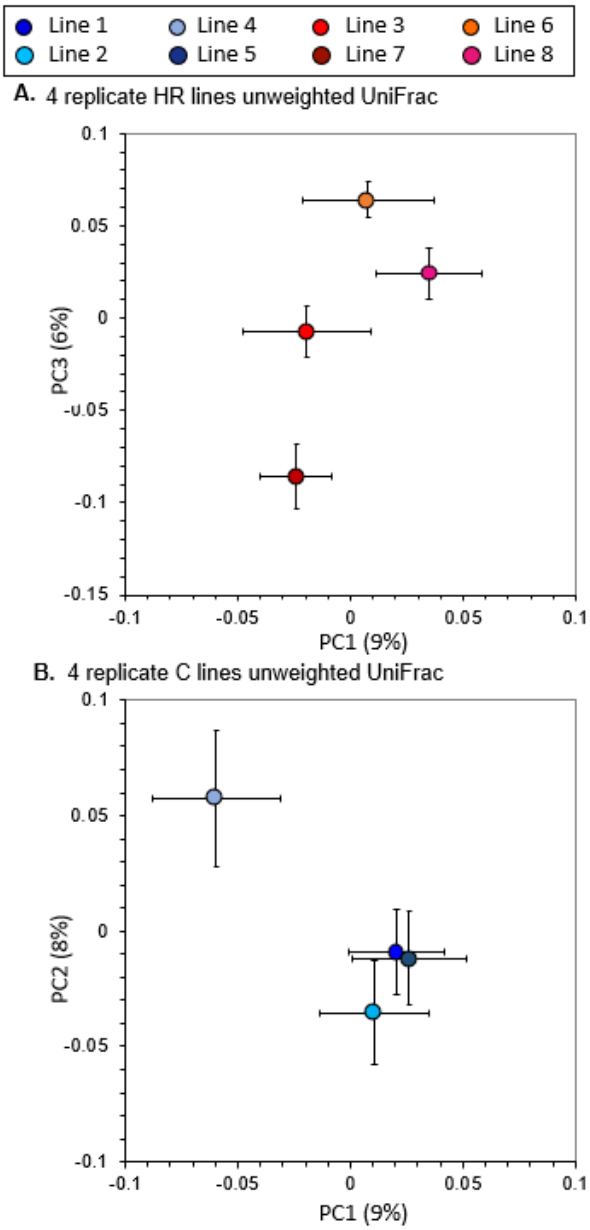
Line 1
Line 2
Line 4
Line 5
Line 3
Line 7
Line 6
Line 8
A. 4 replicate HR lines unweighted UniFrac
PC3 (6%)
PC1 (9%)
B. 4 replicate C lines unweighted UniFrac
PC2 (8%)
PC1 (9%)

Fig. 1.3. Principal coordinate analysis of unweighted UniFrac distances between the 16S rRNA gene sequencing-based profiles of the 4 HR lines (A) and the 4 C lines (B). Principal coordinate analysis was performed for each linetype separately. Values are means and standard errors for scores on PCoA axes. PERMANOVAs indicated significant separation among the 4 replicate HR lines and among the 4 non-selected C lines (HR lines: $R^2$=0.028, $P$=0.038; C lines: $R^2$=0.037, $P$=0.006).

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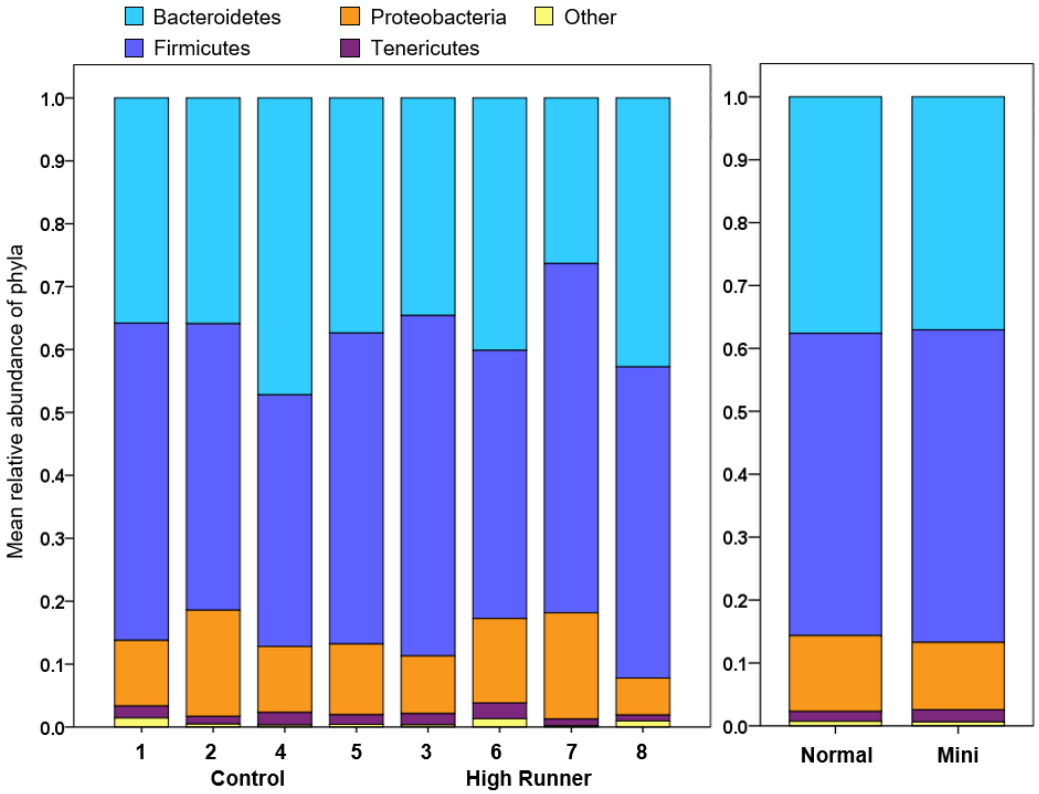
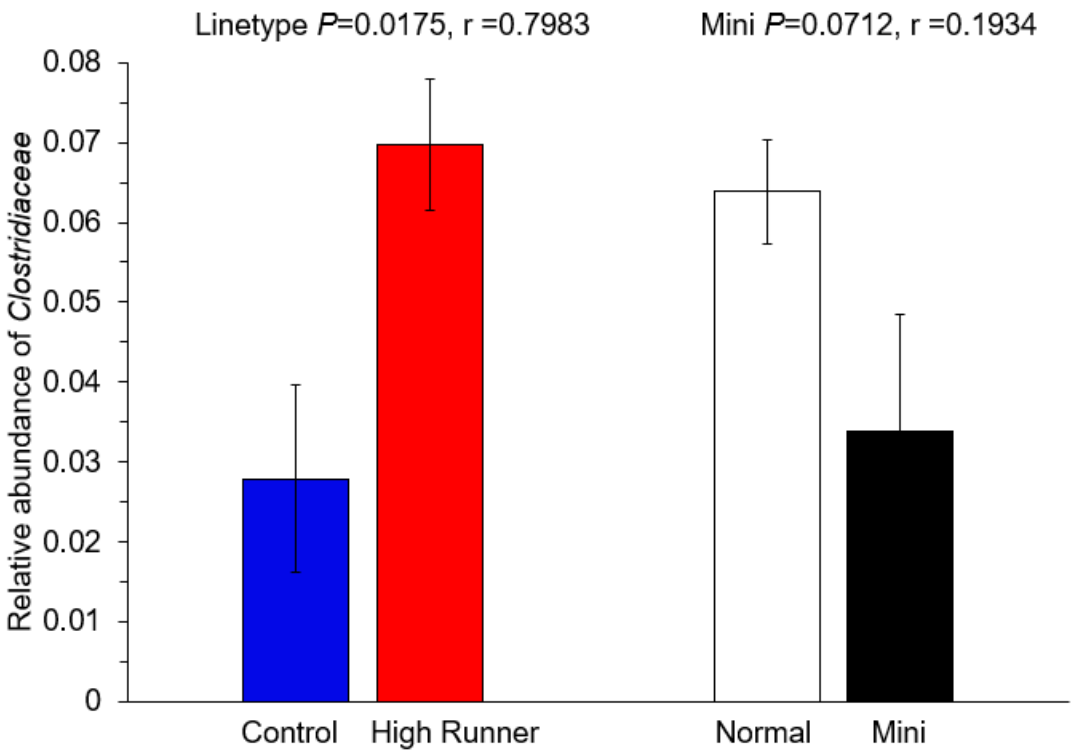
# Appendices

## Appendix 1.1



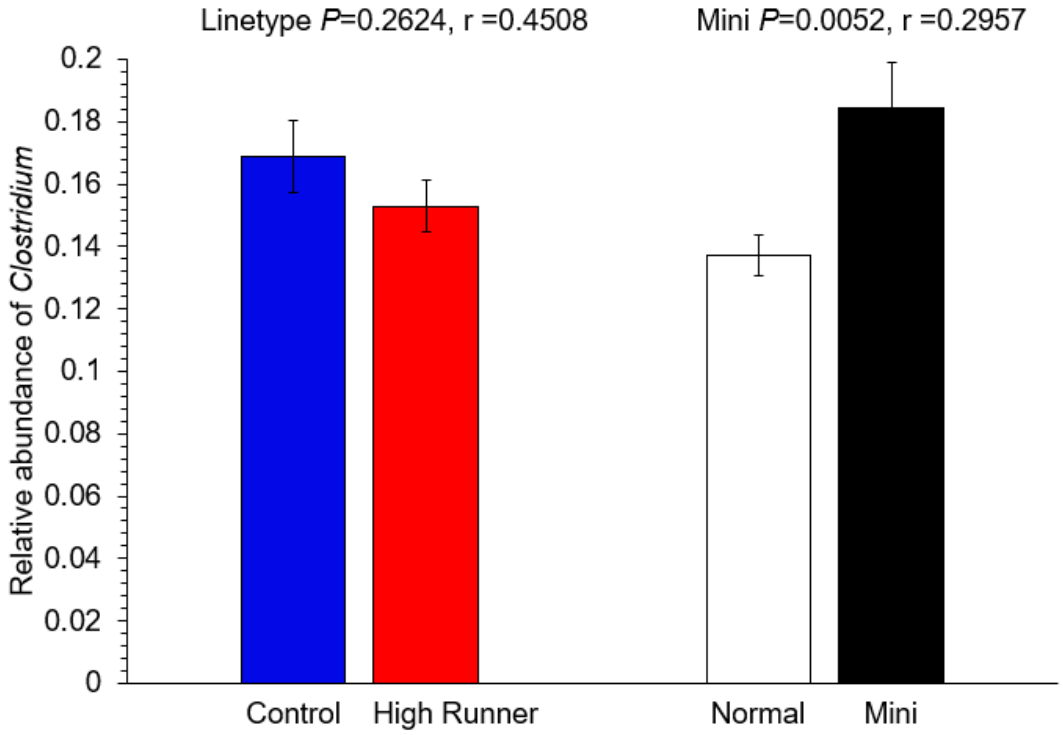

Appendix 1.1. Community composition of the juvenile gut microbiome for all experimental mice (N=95) was dominated by Firmicutes (48.4 ± 14%) (mean ± S.D.) and Bacteroidetes (37.5 ± 17.1%), with additional phyla being much less abundant: Proteobacteria (11.8 ± 8.7%), Tenericutes (1.6 ± 2%), Cyanobacteria (0.28 ± 0.53%), Verrucomicrobia (0.24 ± 1.1%), Actinobacteria (0.09 ± 0.16%), Deferribacteres (0.08 ± 0.24%), Fusobacteria (0.0002 ± 0006%), and TM7 (0.0001 ± 0.0004%).

**Appendix 1.2**



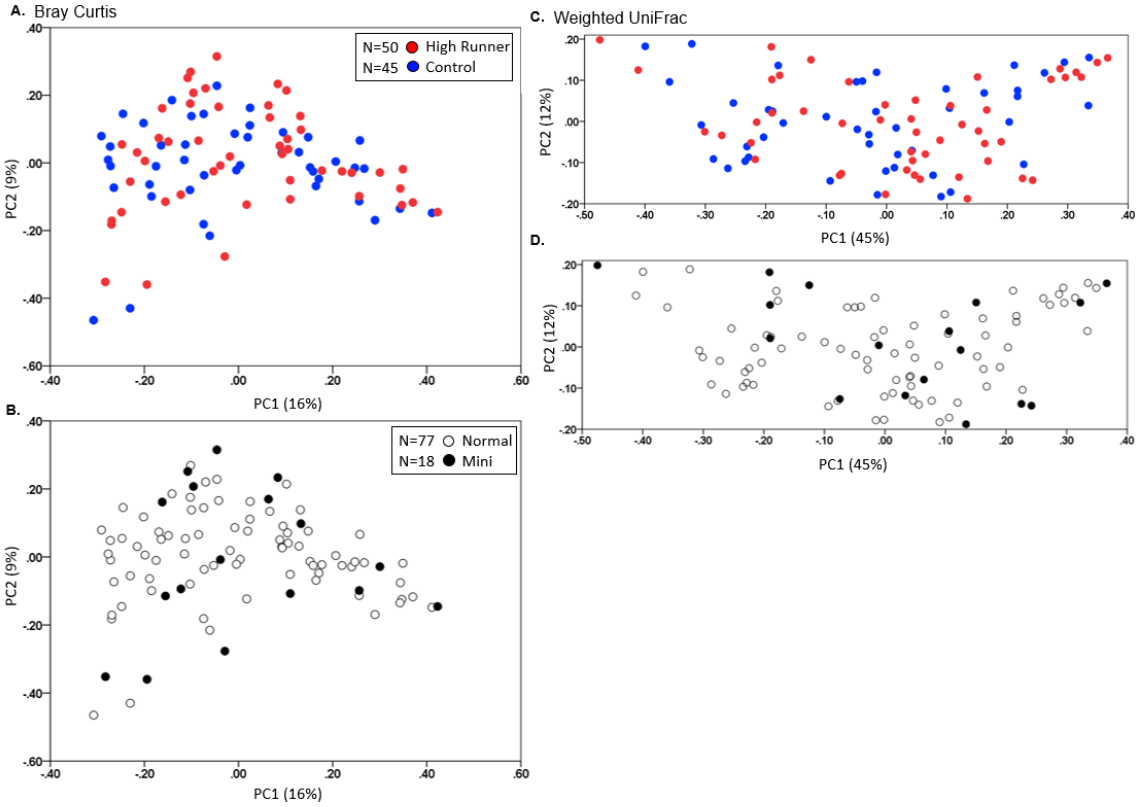

Appendix 1.2. Relative abundance of the family *Clostridiaceae.* Based on mixed models simultaneously comparing HR and C lines, as well as mini-muscled and normal-muscle mice (SAS Procedure Mixed), HR mice had significantly higher relative abundance of the family *Clostridiaceae* compared to Control mice (ANOVA, $F_{1,6} = 10.54$, $P = 0.0175$), with no effect of mini-muscle status. Shown are least squares means ± s.e.m for arcsine square root transformed values. Pearson's r is a measure of effect size (see Methods).

**Appendix 1.3**



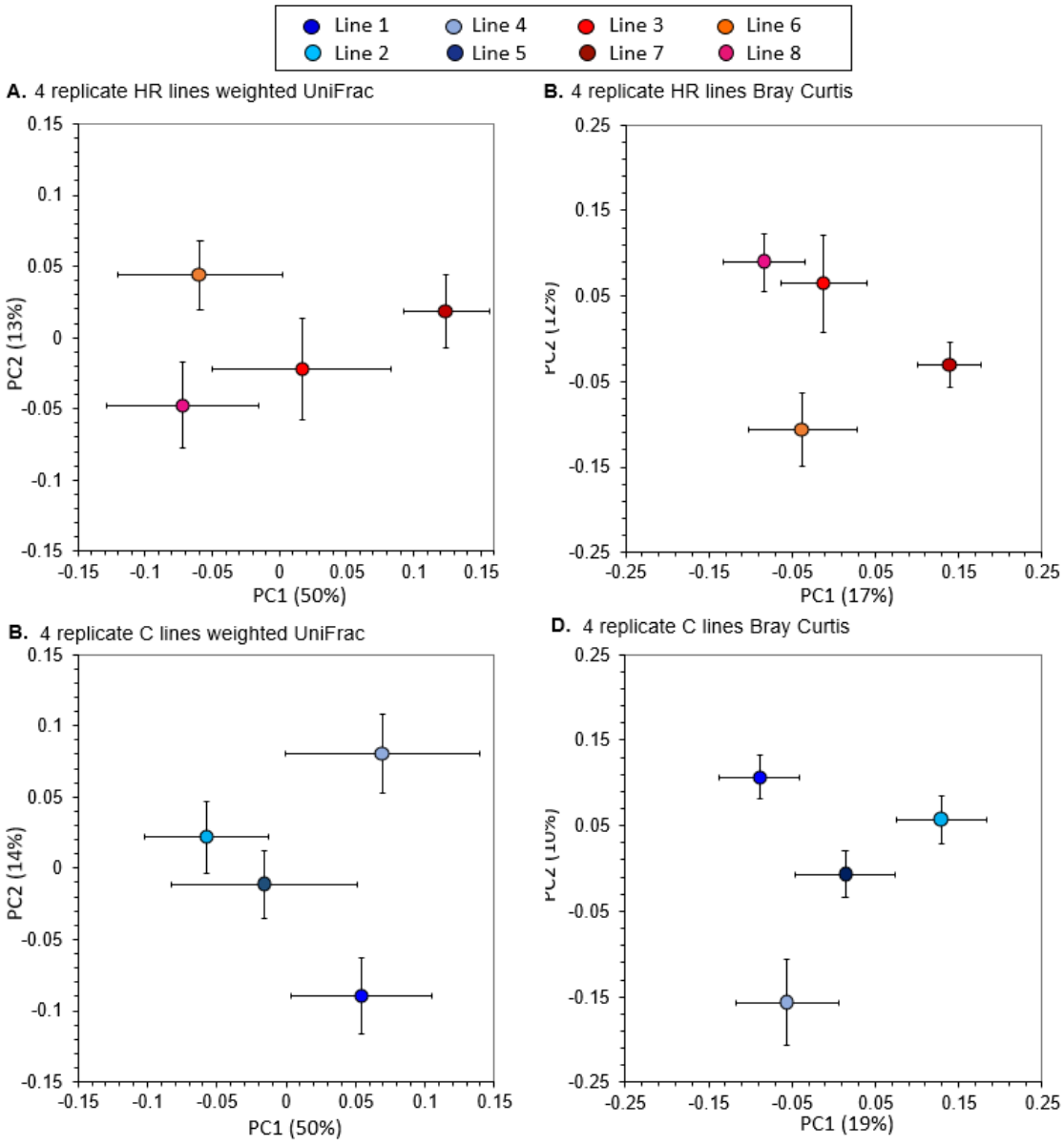

Appendix 1.3. Relative abundance of the genus *Clostridium.* Based on mixed models comparing HR and C lines, as well as mini-muscle and normal-muscle mice (SAS Procedure Mixed), the mini-muscle mice had significantly higher relative abundance of the genus *Clostridium* compared to normal-muscle mice (ANOVA, $F_{1,66} = 8.24$, $P$=0.0052), with no effect of linetype. Shown are least squares means ± s.e.m for arcsine square root transformed values. Pearson's r is a measure of effect size (see Methods).

## Appendix 1.4


A. Bray Curtis
N=50 High Runner
N=45 Control
PC2 (9%)
PC1 (16%)
B.
N=77 Normal
N=18 Mini
PC2 (9%)
PC1 (16%)
C. Weighted UniFrac
PC2 (12%)
PC1 (45%)
D.
PC2 (12%)
PC1 (45%)

Appendix 1.4. Beta diversity (among experimental groups) of the juvenile fecal microbiome based on 16S rRNA sequence data. A and B are PCoA plots for Bray-Curtis distance matrices (bacterial OTU sequence relative abundance considered). C and D are for weighted UniFrac distance matrices (bacterial OTU sequence relative abundance and phylogenetic distances considered). PERMANOVAs based on the Bray-Curtis or on the weighted UniFrac distance matrix indicated no statistically significant separation based on either linetype (Bray-Curtis: $R^2$=0.0147, $P$=0.108, weighted Uni-Frac: $R^2$=0.0116, $P$=0.307) or mini-muscle status (Bray-Curtis: $R^2$=0.009, $P$=0.638, weighted Uni-Frac: $R^2$=0.005, $P$=0.773).

## Appendix 1.5


Line 1
Line 2
Line 4
Line 5
Line 3
Line 7
Line 6
Line 8
A. 4 replicate HR lines weighted UniFrac
PC2 (13%)
PC1 (50%)
B. 4 replicate HR lines Bray Curtis
PC1 (17%)
B. 4 replicate C lines weighted UniFrac
PC2 (14%)
PC1 (50%)
D. 4 replicate C lines Bray Curtis
PC1 (19%)

Appendix 1.5. PCoA plots from separate weighted UniFrac analyses and Bray-Curtis of the 4 HR lines (A and C) and of the 4 C lines (B and D). Values are means and standard errors for scores on PCoA axes. Separately, we used PERMANOVAs to test for significant separation among the 4 replicate HR lines and among the 4 non-selected C lines. Differences among the 4 replicate C lines were statistically significant based on Bray-Curtis ($R^2$=0.051, $P$=0.005), but not among the 4 replicate HR lines ($R^2$=0.014, $P$=0.839). Weighted UniFrac indicated no significant separation (HR lines: $R^2$=0.005, $P$=0.957; C lines: $R^2$=0.030, $P$=0.228).

# CHAPTER 2

Four weeks of voluntary wheel running alters exercise-associated traits in mice

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**Abstract**

Phenotypic plasticity potentially affects all aspects of an organism's phenotype. As an example, many studies have demonstrated (adaptive) training effects in response to exercise. In lab rats and mice, 8 weeks of forced or voluntary exercise generally causes numerous such changes. However, for many traits, the amount of training is quantitatively related to the amount of exercise and/or depends on genetic background (e.g., not all individuals train). In the present study, we examined training effects in adult female mice (7 weeks of age) given 4 weeks of wheel access, using 4 replicate, selectively bred High Runner (HR) and 4 non-selected control (C) lines. Consistent with previous studies, HR mice ran ~3-fold more revolutions/day than C over the course of the study. Throughout the experimental period, HR mice tended to be smaller in body mass than C, but the difference never reached statistical significance. Mice with wheel access tended to be larger than those without, though the difference was significant ($P < 0.05$) only after two weeks of wheel access. In addition, mice with wheels had significantly more lean mass after weeks two and three. With body mass as a covariate, wheel access increased food and water consumption throughout the experiment. In addition, HR mice generally ate and drank more than C mice, although statistical significance was only attained for food consumption during the second week. After 4 weeks, wheel access significantly reduced total fat mass for both linetypes, and HR mice had less total fat mass than C. With body mass as a covariate, 4 weeks of wheel access also

reduced both reproductive and subdermal fat pad masses, but increased heart mass, with no statistical effects on hematocrit or the masses of triceps surae muscles, liver, spleen or brain. We detected no significant differences between HR and C mice for organ masses, and no interactive effects between linetype and wheel access for body mass, body composition, or organ masses. Mini-muscle mice, which occur in two of the four HR lines and have an approximately 50% reduction in hindlimb muscle mass, had reduced lean mass and increased fat mass compared to normal-muscle mice, and tended to be smaller in body mass. With body mass as a covariate, mini-muscle mice drank more and had larger reproductive fat pads, livers, and brains. Overall, results show that voluntary exercise for as little as 4 weeks can alter morphological phenotypes of adult female mice, even those that do not engage in high levels of exercise.

## 1. Introduction

Phenotypic plasticity refers to a pattern in which one genotype produces different phenotypes in different environments (Garland and Kelly, 2006; Kelly et al., 2012; Piersma and Gils, 2010). If the difference in the value of the phenotype between or among genotypes is not constant, then genotype-by-environment interaction exists. An example of phenotypic plasticity is an organism's response to physical activity. In humans, various types of exercise routinely lead to changes in physiological traits, including: increased maximal oxygen consumption (Ruegsegger and Booth, 2018), improved cardiovascular health (Nystoriak and Bhatnagar, 2018), and lower blood pressure (Pescatello et al., 2004). Additionally, in humans, the degree of training response varies among individuals, in part because of differences in their genetic background (Bouchard et al., 1999). Rodent models provide a useful tool to elucidate the effects of physical activity, genetic components, and their interaction on traits (e.g., see Feng et al., 2019; Harpur, 1980).

Training effects from forced treadmill exercise in laboratory house mice were reviewed by Massett et al. (2021), who report studies ranging in length from 2-16 weeks. Mouse training studies that used voluntary wheel running were reviewed by Manzanares et al. (2019), with study lengths ranging from 1 week to 12 months. Perhaps surprisingly, training effects have been shown to occur on short time scales. For example, the effects of exercise on organ masses can occur in as little as 6 days: male mice with 6 days of wheel access had

significantly increased ventricle mass (Kay et al., 2019). As another example, 7 days of wheel access significantly increased levels of hippocampal brain-derived neurotrophic factor in mice from lines selectively bred for high voluntary wheel-running behavior, but not in mice from non-selected control lines (Johnson et al., 2003).

In the present study, we used mice from a long-term experiment in which 4 replicate lines of High Runner (HR) mice have been selectively bred for voluntary wheel-running behavior, while 4 replicate non-selected lines are maintained as controls (C) for random genetic drift (Swallow et al., 1998). Since reaching selection limits, the HR mice have consistently run ~2.5-3 times as many revolutions per day as C mice (Careau et al., 2013; McNamara et al., 2022), primarily by increased speed rather than minutes ran per day. In addition, the HR mice have higher endurance (Meek et al., 2009) and maximal oxygen consumption ($VO_2max$) during forced treadmill exercise (e.g., see Cadney et al., 2021a; Dlugosz et al., 2013; Hiramatsu et al., 2017; Kolb et al., 2010), and are more active in home cages when housed without wheels (Copes et al., 2018; Malisch et al., 2009; Thompson et al., 2018). HR and C mice also differ for various suborganismal traits, including circulating hormone levels, hematocrit or blood hemoglobin concentration, and relative masses of brain, heart ventricle, liver, and kidney (Cadney et al., 2021a; Copes et al., 2015; Dumke et al., 2001; Garland et al., 2011; Garland et al., 2016; Kelly et al., 2017; Kolb et al., 2013; Swallow et al., 2005; Thompson, 2017; Wallace and Garland, 2016).

Previous experiments have also demonstrated that the two linetypes differ in plasticity (training effects) for some morphological and physiological traits. For example, in a study of both sexes at generation 14, of the 14 traits reported from separate analyses of males and females (24 total analyses), 7 had a statistically significant ($P \leq 0.05$) interaction between exercise treatment and linetype, all for females: body-mass adjusted spleen, liver, and gonad mass, as well as blood hematocrit and hemoglobin content (Swallow et al., 2005). In a separate study of female mice from generation 16 housed with or without wheels for 20 months, there was a statistically significant interaction between exercise treatment and linetype for the cross-sectional area of the femoral mid-shaft: wheel access increased the cross-sectional area in HR lines but decreased it in C lines (Middleton et al., 2008a). On the other hand, in a study of females given wheel access for 13-14 weeks, none of the ~25 traits studied had a wheel-access-by-linetype when judged at $P \geq 0.05$ (Kelly et al., 2017). For some traits, the greater training responses of HR mice as compared with C mice can be explained (statistically) by their greater amount of running on wheels, but in other cases they have altered plasticity in the strict sense (i.e., a greater amount of change for a given amount of wheel running) (Garland and Kelly, 2006; Gomes et al., 2009; Kelly et al., 2017; Middleton et al., 2008b).

The purpose of the present study was to determine if traits related to the capacity for sustained aerobic exercise would respond to 4 weeks of wheel access. We measured wheel-running behavior, spontaneous physical activity in

home cages, body mass, lean and fat mass, organ masses, hematocrit, and food and water consumption. Voluntary wheel running is more strenuous for HR than C mice because the former run at higher average and maximum speeds (e.g., Claghorn et al., 2017b) and closer to their maximum aerobic capacity ($VO_2$max) (see Rezende et al., 2005; Rezende et al., 2006). Therefore, we expected the higher levels of exercise to result in greater training effects in the HR mice. We studied females because they run on wheels more than males (e.g., see Careau et al., 2013; Claghorn et al., 2017b) and because Swallow et al. (2005) found significant wheel access-by-linetype interactions only for females.

## 2. Methods

All experiments and methods were approved by the Institutional Animal Care and Use Committee of the University of California, Riverside.

### 2.1 Experimental animals and design

Female mice from generation 81 of an ongoing selection experiment were evenly sampled from four replicate High Runner (HR) lines selectively bred for high voluntary wheel running behavior and four non-selected Control (C) lines of mice. The selection experiment began with a population of 224 outbred genetically variable Hsd:ICR strain house mice (Swallow et al., 1998). After two generations of random breeding, mice were randomly assigned into 8 lines, 4 selectively bred for high voluntary wheel running and 4 non selected control. Each generation,

mice are weaned at 21 days of age and housed in same-sex groups of four. At ~6-8 weeks of age, mice are placed into individual home cages attached to a 1.12 m circumference wheel (Lafayette Instruments, Lafayette, IN, USA). Sensors attached to the wheel record the number of revolutions per minute for six consecutive days. Each generation, the highest-running male and female (based on the number of average revolutions on days 5 and 6) from each HR family are selected as breeders for the next generation (within-family selection). In the Control lines, breeders are chosen without regard to their wheel running, again sampling one male and one female from each family. Pairing of breeders within lines does not allow sibling pairs. Mice are maintained in rooms at approximately 22°C on a 12 h:12 h L:D photoperiod, with ad lib food and water.

Here we used 12 female mice from each of the 8 lines (mainly from different families), with 6 extra mice from HR line 6, which is polymorphic for the mini-muscle phenotype (see 2.7 Statistics section). Figure 1 provides the experimental timeline. Mice were weighed at weaning, then co-housed in groups of four in standard cages without regard to line or linetype. Each week thereafter, mice were again randomized and co-housing continued. At seven weeks of age, half of the mice were placed into home cages with attached wheels (as described above for the routine selective breeding protocol). The other half of the mice were placed into home cages without access to wheels but with a passive infrared sensor (see below). Wheel access or no wheel access

treatment continued for four weeks. Body mass, body composition, food consumption, and water consumption were measured weekly (see below).

### 2.2 Wheel running

Wheel running was measured during experimental weeks 8-11. Sensors attached to the wheels record the number of revolutions in 1-minute intervals over a 23-hour period (Swallow et al., 1998). On days when body composition was measured, the wheel-running measurement period was reduced to 21 hours. We calculated the total distance run (revolutions/day), duration of activity (number of 1-min intervals with at least one revolution), average speed (revolutions/minute), and maximum revolutions observed for any 1-minute interval. We analyzed mean values for wheel running for each week of the experiment, and a measure of wheel freeness was included as a covariate.

### 2.3 Spontaneous physical activity (SPA)

To measure spontaneous physical activity (SPA: Garland et al., 2011) in the home cages, half of the mice without wheel access were placed into home cages with a corner-placed infrared sensor (Talon TL-Xpress-A; Crow Electronics, Fort Lee, New Jersey), as previously described (Copes et al., 2015). The sensor measures whether movement or no movement is detected 3 times per second, and then calculates the mean value (between 0 and 1) for every minute during the measurement period of 23 hours. We also calculated the total daily activity

levels (the sum of all daily activity), duration of activity (the number of 1-minute intervals with any movement), mean intensity (daily activity divided by the number of 1-minute intervals with any activity), and maximum intensity (the single minute with the highest amount of activity). We analyzed SPA for each week from experimental week 8 to 11, with a measure of sensor sensitivity used as a covariate (Copes et al., 2015).

### 2.4 Body composition

Weekly lean and fat mass were measured using a non-invasive, quantitative magnetic resonance EchoMRI machine (EchoMRI-100, Echo Medical Systems, Houston, TX), as previously described (e.g. see Cadney et al., 2021a; Cadney et al., 2021b). Briefly, mice were restrained in a tube while their body composition was analyzed. The EchoMRI machine reports fat mass and lean mass in grams. Body composition measurements occurred weekly, starting when mice were placed into treatment groups, and at the end of the experiment (Figure 1). Due to time constraints, 27 of the 100 mice did not have body composition measurements prior to the start of the 4-week wheel or no wheel treatments.

### 2.5 Food and water consumption

Apparent food and water consumption were measured during experimental weeks 8 to 11. Daily water consumption was calculated by subtracting the water bottle mass at the end of a 6-day measurement period from when the water

bottle was placed on the cage. Food consumption was measured over a 7-day period. We weighed the food hopper before and after the 7-day measurement period. We accounted for food wastage by weighing orts found in the bedding (Koteja et al., 2003). We did not measure water consumption during the first week of wheel access, as we were taking daily fecal samples for a separate study.

### 2.6 Dissections

After four weeks of wheel or no wheel access, mice were killed by decapitation with decapicones to dissect organs. The day prior to dissections, a subset of mice were removed from their cages (with or without wheels) and housed individually overnight without wheel access, but with continued food and water. Blood samples were taken from the trunk in heparinized micro-hematocrit tubes and centrifuged for 12 minutes at 4°C to determine hematocrit. Organs were dissected, weighed, and flash frozen in liquid nitrogen. As in Singleton and Garland (2019), we dissected reproductive fat pads and subdermal (inguinal, gluteal, and lumboidal) fat pads (Cinti, 2005). We report liver, heart ventricle, spleen, retroperitoneal fat pad, subdermal fat pad, total fat pad (retroperitoneal plus subdermal), hematocrit, and average triceps surae mass (calf muscle).

## 2.7 Statistical analyses

Following numerous previous publications on the HR and C lines of mice, we used linear mixed models in SAS 9.4 Procedure Mixed (SAS Institute, Cary, NC, USA) to test the effect of linetype against the variance among the replicate lines nested within linetype (1 and 6 d.f.). Effects of exercise and of the linetype x exercise interaction were also tested with 1 and 6 d.f. Some traits were transformed to improve normality of residuals data. Depending on the trait, we used body mass, time of day, and z-transformed time squared (orthogonal polynomial) as covariates.

In addition, the presence/absence of a mini-muscle phenotype was used in the model with 1 and the residual d.f. The mini-muscle phenotype is identified as a greatly reduced (~50%) hindlimb muscle mass, and has a variety of pleiotropic effects, such as enlarged internal organs (Garland et al., 2002; Kelly et al., 2017; Swallow et al., 2005). This phenotype is caused by a Mendelian recessive allele that is a single nucleotide substitution in an intron of the myosin heavy polypeptide 4 gene (Kelly et al., 2013). The mini-muscle allele is currently fixed in HR line 3 (100% of individuals express the phenotype) and polymorphic in HR line 6. We determined mini-muscle phenotype status from muscle mass data and included the status in our analyses.

Excluding nuisance variables (time of day and time squared, wheel freeness, sensor sensitivity), the statistical analyses resulted in 276 p values, with 89 of them having $p < 0.05$. To address the likelihood of inflated

experiment-wise Type I error rates when making multiple comparisons on related data, we used the positive False Discovery Rate Q-value procedure, as implemented in SAS Procedure Multtest. This indicated that only the lowest 75 would have a corrected $p \leq 0.05$, with the cutoff being $p < 0.025$.

## 3. Results

Note: Supplemental File 2.1 (SAS_Tables_11.xlsx) is an excel file with least squares means and P values from SAS Proc Mixed.

### 3.1 Wheel running and spontaneous physical activity

Figure 1 presents the experimental timeline. The HR mice ran significantly more than C mice during all four weeks of wheel treatment (all *P* values <0.001; Fig. 2A). The HR mice also had significantly higher duration of activity, mean speed, and maximum speed (all *P* values <0.0001 except for duration during week 9: Appendix 2.1). HR mice housed without wheels tended to be more active in their home cages than C mice, though the difference never reached statistical significance (Fig. 2B). Mini-muscle mice had a significantly lower maximum SPA during week 9 (*P* = 0.0116: Appendix 2.2).

### 3.2 Body mass

Wheel access tended to increase body mass, but the effect was only significant at the end of week 9. Mice from the HR lines tended to be smaller than the C

lines, but the difference was never statistically significant (Table 1). Mini-muscle individuals tended to weigh less than those with normal muscles across weeks 8-11, although the significance level only fell below $P = 0.05$ at the start of week 8.

### 3.3 Body composition

Wheel access generally increased lean mass, but decreased fat mass when analyzed without or with lean mass as a covariate (Table 2). Mice from the HR lines had less fat mass than those from C lines at the end of week 11. Mini-muscle individuals had reduced lean mass as compared with normal-muscled individuals, but more body fat when analyzed either without or with lean mass as a covariate.

### 3.4 Food and water consumption

With body mass as a covariate, mice with wheel access ate significantly more during all weeks, with a linetype x wheel access interaction during week 9 ($P = 0.0470$), indicating that the effect was somewhat greater in HR mice (Table 3). Also, with body mass as a covariate, wheel access significantly increased water consumption during weeks 9, 10, and 11 (no data available for week 8). In addition, mini-muscle mice drank significantly more than normal-muscled individuals.

Considering only mice housed without wheels, HR and C lines did not differ statistically in mass-adjusted food or water consumption, although mini-

muscle mice had higher water consumption (Table 3). For mice housed with wheels, HR had higher food consumption than C in weeks 9 ($P$ = 0.0161) and 11 ($P$ = 0.0696), but the linetypes did not differ in water consumption. With weekly wheel revolutions as a covariate, HR and C lines did not significantly differ in food or water consumption (Table 3). The same was true when weekly home-cage activity was included as a covariate.

### 3.5 Organ masses

Four weeks of voluntary wheel-running activity significantly increased heart ventricle mass ($P$ = 0.0012; Table 4), decreased subdermal fat pad mass ($P$ = 0.0192), and decreased reproductive fat pad mass ($P$ = 0.0118). Similar to previously reported results, mini-muscle mice had a 47% reduction in average triceps surae mass ($P$ <0.0001). The mini-muscle mice also had significantly larger livers ($P$ = 0.0001), brains ($P$ = 0.0129), and reproductive fat pads ($P$ = 0.0495; Table 4).

## 4. Discussion

### 4.1 HR mice sometimes train more than C mice

Several experiments using mice from the HR selection experiment have tested for differences in phenotypic plasticity between the selected HR and non-selected C lines by comparing mice housed with versus without wheel access for varying periods of time. Previously, we found that longer periods of wheel

access (8 to 13 weeks) sometimes result in differential training effects between the HR and C mice (e.g., see Garland and Kelly, 2006; Houle-Leroy et al., 2000; Middleton et al., 2008b; Swallow et al., 2005). In other words, the effects of exercise training are contingent on genetic selection history. Table 5 provides a summary for several studies that measured one or more of the traits measured in the present study. In the present study, the only trait for which we observed a linetype-by-wheel access was food consumption during week 9. Taken together, existing training studies in the HR mice suggest that durations of voluntary wheel access longer than 4 weeks are required to observe greater training effects in the HR mice.

### 4.2 Wheel running and home-cage activity

Consistent with many previous publications, HR mice ran ~3 times more revolutions/day than did the C mice (e.g. see Cadney et al., 2021b; Copes et al., 2015; Copes et al., 2018; McNamara et al., 2021; McNamara et al., 2022). In addition, the HR mice had a higher average speed, maximum speed, and duration of wheel running (Appendix 2.1).

For the individuals housed without wheels, the HR mice tended to be more active in their home cages (Supplemental File 2.1), although the difference never reached statistical significance (Appendix 2.2). In previous studies, the HR mice sometimes have significantly higher SPA than C mice when housed without wheel access (e.g., Malisch et al., 2008; Malisch et al., 2009; Thompson et al.,

2018). For example, female HR mice had higher SPA than C mice across the course of a 12-week experiment beginning at weaning (Copes et al., 2015; Copes et al., 2018).

### 4.3 Body mass and composition

In both rats and mice, previous studies have often found that females better "protect" themselves from body mass loss when given wheel access (Cortright et al., 1997; Swallow et al., 1999). Consistent with these general findings, several studies from the selection experiment have demonstrated that the effects of wheel access on body mass depend on sex (Castro and Garland, 2018; Claghorn et al., 2017b; Swallow et al., 1999; Swallow et al., 2005). In the present study of females, 4 weeks of exercise tended to increase body mass in both HR and C lines for the duration of the experimental treatment (Table 1). In contrast, female HR and C mice with wheel access for 5 to 80 weeks had decreased body mass compared to sedentary controls (Kelly et al., 2017; Middleton et al., 2008a; S. A. Kelly and T. Garland unpublished).

In the present study, two weeks of wheel access, significantly increased lean mass measured by EchoMRI, although the effect was no longer significant by week 11 (Table 2). Previously in mice from the selection experiment, 6 days of wheel access resulted in a tendency for increased lean mass, with no significant effect of sex, linetype, or interaction (Hiramatsu and Garland, 2018).

Four weeks of wheel access significantly decreased total body fat mass measured by EchoMRI for both linetypes for the duration of the present experiment (except week 11; Table 2). In a separate study, female HR and C mice had significantly reduced fat mass after 6 days of wheel access compared to before wheel access began (Hiramatsu and Garland, 2018). Finally, at the end of the 4 weeks of experimental treatment, mice with wheel access had decreased reproductive fat pad mass and subdermal fat pad mass (Figure 3, Table 4).

### 4.4 Food consumption

To support higher levels of daily physical activity, increased energy is needed in the form of food consumption (Garland et al., 2011; Swallow et al., 2001). As expected, both HR and C mice with wheel access ate significantly more than those without, with body mass as a covariate (Table 3), a result that is consistent with previous studies (Copes et al., 2015; Swallow et al., 2001).

For mice housed without wheels, linetype never had a significant effect on mass-adjusted food consumption. For mice housed with wheels, HR tended to consume more food than C, although the difference only reached significance during week 9 of the experiment (Table 3). This results is consistent with previous studies in these mice (Copes et al., 2015; Copes et al., 2018), and sometimes even when accounting for the high amount of wheel running (Hiramatsu and Garland, 2018).

### 4.5 Water consumption

Mice with wheel access had significantly higher water consumption compared to those housed without wheels (Table 3). In addition, the HR mice tended to consume more water, but the difference never approached statistical significance. Previous studies have also reported a trend for greater water consumption by HR mice, but a statistically significant difference has never been found (Claghorn et al., 2017a; McNamara et al., 2022; Singleton and Garland, 2019).

### 4.6 Changes in organ masses

As noted previously, cardiac hypertrophy is a robust indicator of exercise-training effects in laboratory mice (Feng et al., 2019; Manzanares et al., 2019; Massett et al., 2021). In the present study, four weeks of voluntary exercise increased ventricle mass (adjusted for body mass), with no significant effect of linetype or interaction (Table 4, Fig 3). Previous research has demonstrated that 8 to 13 weeks of voluntary wheel access in both sexes resulted in significant ventricle hypertrophy in mice from the selection experiment (Copes et al., 2015; Kelly et al., 2017; Swallow et al., 2005; S. A. Kelly and T. Garland unpublished). Moreover, 6 days of voluntary wheel access is sufficient to illicit ventricular hypertrophy in both HR and C mice (Kay et al., 2019).

In the present study, we found no significant effect of linetype, wheel access, or their interaction on liver mass, spleen mass, brain mass, triceps surae mass, or hematocrit (Table 4). Thus, longer periods of time are required for exercise-training effects via voluntary wheel running in these lines of mice (Copes et al., 2015; Kelly et al., 2017; Swallow et al., 2005; S. A. Kelly and T. Garland unpublished). In future studies, it would be of interest to conduct training studies by use of forced treadmill exercise so that the amount and intensity of exercise could be equilibrated between the HR and C mice.

Previous studies (see above and Introduction) have sometimes reported differences in hematocrit, ventricle, liver, spleen, brain, or triceps surae mass (aside from effects in mini-muscle mice), but in the present study we found no statistical differences (with body mass as a covariate, all $P > 0.1$).

### 4.7 Differences associated with the mini-muscle phenotype

In the present study, mini-muscle mice differed from normal-muscle individuals in several ways. However, they did not differ in either wheel running (Appendix 2.1) or spontaneous physical activity in the home cage (Appendix 2.2).

Mini-muscle mice tended to be smaller in body size than normal-muscle mice for the duration of wheel access (only significant at the beginning of week 8, Table 1). Mini-muscle mice had significantly less lean mass for the duration of the experimental treatment (except at the end of week 11, Table 2), and tended

to have more fat (statistically significant during week 9 and at the end of the experiment) compared to normal-muscle mice.

With body mass as a covariate, mini-muscle mice consumed significantly more water compared to normal-muscled mice (Table 3). In a previous study, HR male mice with the mini-muscle phenotype housed without wheels drank significantly more water than normal-muscled HR mice (Singleton and Garland, 2019). Moreover, previous research has demonstrated that the mini-muscle mice tend to have larger kidneys (Garland et al., 2002 [males and females]; Kelly et al., 2017 [females]; Swallow et al., 2005 [males only]).

Consistent with previous publications, the mini-muscle mice had larger livers (Table 4) (Copes et al., 2015; Kelly et al., 2017; Swallow et al., 2005 [females only]). Mini-muscle mice tended to have larger ventricle mass, but the difference was not statistically significant. Previous studies have generally reported significantly larger heart ventricles in mini-muscle mice (e.g., Kay et al., 2019; Kelly et al., 2017; Swallow et al., 2005).

Finally, mini-muscle mice had significantly larger brain mass (Table 4). Previous studies have noted that mini-muscle mice tend to have larger brains (Hiramatsu et al., 2017; Kolb et al., 2013; Thompson, 2017), but a statistically significant difference has only been reported once previously (Cadney et al., 2021a).

**Table 2.1.** Significance levels for analyses of body mass (SAS Proc Mixed). Bold values indicate $P$ <0.05). + and – symbols indicate direction of the effect: $P_{linetype}$ + indicates HR>C; $P_{wheel\ access}$ + indicates mice with wheels > than mice without wheels; $P_{mini}$ + indicates mini-muscle mice > than normal mice.

| Trait | N | $P_{linetype}$ | $P_{wheel\ access}$ | $P_{interaction}$ | $P_{mini}$ |
|---|---|---|---|---|---|
| Mass at weaning | 100 | 0.2084 - | | | 0.1025 + |
| Mass when placed into treatment groups at the beginning of week 8 | 100 | 0.4109 - | | | **0.0356 -** |
| Mass end of week 8 | 97 | 0.2119 - | 0.8992 + | 0.7983 | 0.0940 - |
| Mass end of week 9 | 98 | 0.2308 - | **0.0387 +** | 0.4832 | 0.0933 - |
| Mass end of week 10 | 91 | 0.1269 - | 0.1022 + | 0.2572 | 0.1150 - |
| Mass end of week 11 | 93 | 0.1395 - | 0.1747 + | 0.9958 | 0.0861 - |

**Table 2.2.** Significance levels for lean and fat mass (SAS Proc Mixed). Bold values indicate $P$ <0.05). + and – symbols indicate direction of the effect: $P_{linetype}$ + indicates HR>C; $P_{wheel\ access}$ + indicates mice with wheels > than mice without wheels; $P_{mini}$ + indicates mini-muscle mice > than normal mice. Fat mass results presented with and without lean mass as a covariate.

| Trait | N | $P_{linetype}$ | $P_{wheel\ access}$ | $P_{interaction}$ | $P_{mini}$ | $P_{lean\ mass}$ |
|---|---|---|---|---|---|---|
| log lean mass start of wheels/no wheels | 72 | 0.5755 – | | | **0.0185 -** | |
| log fat mass start of wheels/no wheels | 72 | 0.3987 - | | | 0.0627 + | |
| log fat mass start of wheels/no wheels | 72 | 0.5242 - | | | **0.0151 +** | 0.1099 |
| log lean mass start of week 9 | 97 | 0.2643 - | 0.1088 + | 0.1765 | **0.0279 -** | |
| log fat mass start of week 9 | 96 | 0.1722 - | **0.0003 -** | 0.0911 | **0.0121 +** | |
| log fat mass start of week 9 | 96 | 0.1787 - | **0.0003 -** | 0.0973 | **0.0149 +** | 0.9536 |
| log lean mass start of week 10 | 95 | 0.3012 - | **0.0060 +** | 0.2554 | **0.0146 -** | |
| log fat mass start of week 10 | 95 | 0.1633 - | **0.0036 -** | 0.0786 | 0.0671 + | |
| log fat mass start of week 10 | 96 | 0.1752 - | **0.0082 -** | 0.0683 | 0.0853 + | 0.7671 |
| Lean mass start of week 11 squared | 91 | 0.2098 - | **0.0145 +** | 0.1299 | **0.0181 -** | |
| log fat mass start of week 11 | 92 | 0.1139 - | 0.1437 - | 0.6159 | 0.1064 + | |
| log fat mass start of week 11 | 94 | 0.1746 - | 0.1665 - | 0.2500 | 0.0781 + | 0.1214 |
| log lean mass at the end of week 11 | 93 | 0.1404 - | 0.1114 + | 0.5321 | 0.1138 - | |
| log fat mass at the end of week 11 | 93 | **0.0235 -** | **0.0292 -** | 0.8565 | **0.0370 +** | |
| log fat mass at the end of week 11 | 93 | **0.0371 -** | **0.0307 -** | 0.8407 | **0.0419 +** | 0.8072 |

**Table 2.3.** Significance levels for analyses of food and water consumption (SAS Proc Mixed). Bold values indicate $P$ <0.05). + and – symbols indicate direction of the effect: $P_{linetype}$ + indicates HR>C; $P_{wheel\ access}$ + indicates mice with wheels > than mice without wheels; $P_{mini}$ + indicates mini-muscle mice > than normal mice. Food and Water Consumption presented for all of the mice, mice without wheels and HCA as a covariate, and mice with wheels and weekly wheel revolutions as a covariate. Water consumption was not measured during week 8.

| Trait | N | $P_{linetype}$ | $P_{wheel\ access}$ | $P_{interaction}$ | $P_{mini}$ | $P_{mass}$ | $P_{Total\ HCA}$ | $P_{Wheel\ revs}$ |
|---|---|---|---|---|---|---|---|---|
| Average daily food consumed week 8 | 96 | 0.5879 + | **0.0096 +** | 0.3813 | 0.5221 + | **<0.0001** | | |
| Mice WITHOUT wheels | 48 | 0.9360 + | | | 0.9763 - | **0.0025** | | |
| Mice WITHOUT wheels with weekly HCA as a covariate | 46 | 0.9672 - | | | 0.8748 - | **0.0075** | 0.4176 | |
| Mice WITH wheels | 48 | 0.3424 + | | | 0.2925 + | **0.0022** | | |
| Mice WITH wheels with weekly revs as a covariate | 44 | 0.2872 - | | | 0.2358 + | **0.0157** | | 0.0557 |
| Average daily food consumption week 9 | 97 | **0.0401 +** | **0.0001 +** | **0.0470** | 0.8336 - | **<0.0001** | | |
| Mice WITHOUT wheels | 48 | 0.9955 - | | | 0.6782 - | **0.0158** | | |
| Mice WITHOUT wheels with weekly HCA as a covariate | 46 | 0.6920 - | | | 0.8825 + | **0.0066** | 0.0992 | |
| Mice WITH wheels | 49 | **0.0161 +** | | | 0.9288 + | **<0.0001** | | |
| Mice WITH wheels with weekly revs as a covariate | 45 | 0.5714 + | | | 0.5455 + | **0.0002** | | 0.0748 |
| Average daily food consumption week 10 | 92 | 0.2105 + | **<0.0001+** | 0.4423 | 0.9878 - | **<0.0001** | | |
| Mice WITHOUT wheels | 48 | 0.8070 + | | | **0.0453 +** | **<0.0001** | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Mice WITHOUT wheels with weekly HCA as a covariate | 46 | 0.8792 - | | | 0.0682 + | **<0.0001** | **0.0078** | |
| Mice WITH wheels | 44 | 0.1835 + | | | 0.5108 - | **0.0033** | | |
| Mice WITH wheels with weekly revs as a covariate | 45 | 0.8921 + | | | 0.3186 - | **0.0028** | | 0.3396 |
| Average daily food consumption week 11 | 93 | 0.0796 + | **0.0001 +** | 0.6582 | 0.5607 + | **<0.0001** | | |
| Mice WITHOUT wheels | 44 | 0.4274 + | | | 0.4227 + | **0.0035** | | |
| Mice WITHOUT wheels with weekly HCA as a covariate | 45 | 0.6310 + | | | 0.8024 - | **0.0051** | 0.0934 | |
| Mice WITH wheels | 46 | 0.0696 + | | | 0.8961 - | **<0.0001** | | |
| Mice WITH wheels with weekly revs as a covariate | 46 | 0.9359 - | | | 0.9893 + | **<0.0001** | | **0.0246** |
| No water consumption data were gathered for week 8 | | | | | | | | |
| Average daily water consumed week 9 | 84 | 0.7199 + | **0.0104 +** | 0.1160 | 0.0562 + | **0.0170** | | |
| Mice WITHOUT wheels | 37 | 0.5342 + | | | 0.2393 - | **0.8343** | | |
| Mice WITHOUT wheels with weekly HCA as a covariate | 35 | 0.4322 + | | | 0.2182 - | 0.7207 | 0.6303 | |
| Mice WITH wheels | 50 | 0.6421 - | | | 0.0679 + | **0.0085** | | |
| Mice WITH wheels with weekly revs as a covariate | 45 | 0.7361 + | | | **0.0246 +** | **0.0093** | | 0.2305 |
| Average daily water consumption week 10 | 90 | 0.8145 + | **0.0164 +** | 0.9064 | **0.0055 +** | **0.0015** | | |
| Mice WITHOUT wheels | 45 | 0.7405 - | | | **0.0138 +** | 0.4622 | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Mice WITHOUT wheels with weekly HCA as a covariate | 43 | 0.6296 - | | | **0.0256 +** | 0.5875 | 0.1389 | |
| Mice WITH wheels | 45 | 0.6430 + | | | 0.1470 + | **0.0015** | | |
| Mice WITH wheels with weekly revs as a covariate | 45 | 0.4677 + | | | 0.1761 + | **0.0021** | | 0.5031 |
| Average daily water consumption week 11 | 91 | 0.8063 + | **0.0015 +** | 0.9824 | **0.0036 +** | **0.0013** | | |
| Mice WITHOUT wheels | 46 | 0.8735 + | | | **0.0215 +** | **0.0390** | | |
| Mice WITHOUT wheels with weekly HCA as a covariate | 44 | 0.8323 + | | | **0.0163 +** | **0.0314** | 0.4195 | |
| Mice WITH wheels | 45 | 0.7808 + | | | **0.0236 +** | **0.0032** | | |
| Mice WITH wheels with weekly revs as a covariate | 45 | 0.3357 + | | | **0.0174 +** | **0.0028** | | 0.0889 |

**Table 2.4.** Significance levels for analyses of organ masses and hematocrit (SAS Proc Mixed). Bold values indicate $P$ <0.05). + and – symbols indicate direction of the effect: $P_{linetype}$ + indicates HR>C; $P_{wheel\ access}$ + indicates mice with wheels > than mice without wheels; $P_{mini}$ + indicates mini-muscle mice > than normal mice. We included body mass (mass), time of day (time), and the z-transformed squared term for time of euthanasia (Time2) as covariates. All organ masses and body mass were log transformed.

| Trait | N | $P_{linetype}$ | $P_{wheel\ access}$ | $P_{interaction}$ | $P_{mini}$ | $P_{mass}$ | $P_{time}$ | $P_{time^2}$ |
|---|---|---|---|---|---|---|---|---|
| log ventricle mass | 92 | 0.1032 + | **0.0012 +** | 0.9718 | 0.2065 + | **<0.0001** | 0.6687 | 0.9731 |
| log liver mass | 91 | 0.4107 + | 0.4230 - | 0.3466 | **0.0001 +** | **<0.0001** | **<0.0001** | 0.7064 |
| log spleen mass | 92 | 0.8060 + | 0.1770 - | 0.8954 | 0.7102 + | **0.0029** | 0.4362 | 0.8122 |
| Hematocrit | 92 | 0.8141 - | 0.3517 + | 0.2409 | 0.8278 + | | 0.1646 | 0.2511 |
| log brain mass | 91 | 0.1458 + | 0.1342 + | 0.1387 | **0.0129 +** | **0.0002** | 0.3283 | 0.0637 |
| log XTS (R and L tricep average) | 91 | 0.6724 + | 0.7651 - | 0.2010 | **<0.0001 -** | **<0.0001** | 0.9654 | 0.7595 |
| log subdermal fat pad mass | 92 | 0.4794 - | **0.0192 -** | 0.0896 | 0.1295 + | **0.0426** | 0.8863 | **0.0217** |
| log reproductive fat pad mass | 92 | 0.2702 - | **0.0188 -** | 0.2803 | **0.0495 +** | **<0.0001** | 0.0953 | 0.5171 |
| log fat total (sum of subdermal + reproductive) | 92 | 0.3461 + | **0.0076 -** | 0.0935 | 0.0745 + | **0.0008** | 0.2910 | 0.1233 |

**Table 2.5.** Examples of interactive effects (P values) between linetype and wheel access in mice from the High Runner selection experiment.

| | **Generation 14 (Swallow et al. 2005)** | | **Generation 37 (Kelly et al. 2017)** | | **Generation 49 (Kelly et al. unpublished)** | | **Generation 57 (Copes et al. 2015)** | | **Generation 81 (Present study)** | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Start Age** | 22 days | | 53 days | | 21 days | | 24 days | | 49 days | |
| **Duration of wheel access** | 8 weeks | | 13 weeks | | 8 weeks | | 13 weeks | | 4 weeks | |
| **Trait[&]** | Males | Females | Males | Females | Males | Females | Males | Females | Males | Females |
| Body mass | 0.4665 | 0.1333 | | 0.2571 | | 0.4776 | | 0.5230 | | 0.9958 |
| Ventricle mass | 0.4508 | 0.4539 | | 0.0648 | | **0.0339** | | 0.4808 | | 0.9718 |
| Liver mass | 0.7484 | **0.0383** | | 0.1136 | | | | 0.2177 | | 0.3466 |
| Spleen mass | 0.8329 | **0.0304** | | 0.5417 | | | | | | 0.8954 |
| Hematocrit | 0.3450 | **0.0465** | | 0.2003 | | | | | | 0.2409 |
| Brain mass | | | | | | 0.3756 | | | | 0.1387 |
| Triceps Surae mass | 0.7881 | 0.9832 | | | | | | | | 0.2010 |
| Food consumption | | | | | | | | 0.0743 | | **0.0470**[%] |

[&]For all traits except body mass, body mass was used as a covariate in statistical analyses.
[%]During week 9.
Statistically significant ($P \leq 0.05$) interactive effects are in **bold**

.

**Figure 2.1**

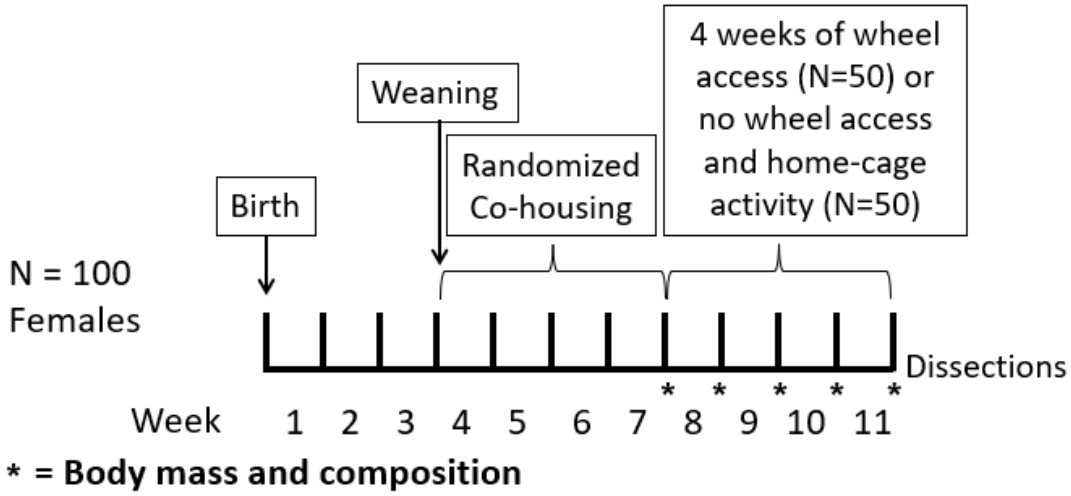


**Figure 2.1.** Experimental design. 12 females from each of the 4 replicate High Runner and 4 replicate Control lines were used, with 6 additional Line 6 mice. N =73 for Week 8 body composition measurements.

**Figure 2.2**

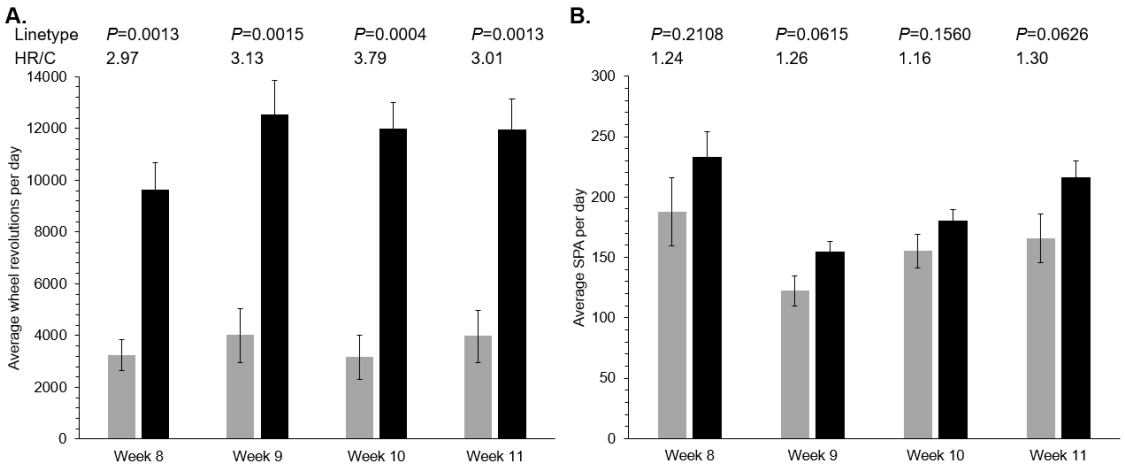


Fig. 2.2. A. Average wheel revolutions per day during experimental weeks 8 to 11 for the Control (grey) and HR (black) mice. B. Average spontaneous physical activity (SPA) in home cages per day during experimental weeks 8 to 11 for the mice that did not have wheel access. *P* values are from nested ANCOVAs in SAS Proc Mixed unadjusted for multiple comparisons. The HR/C ratio of least squares means is reported below the *P* values. Additional analyses of wheel-running duration, average speed, and maximum speed can be found in Appendix 2.1; analyses of duration, intensity, and maximum intensity of SPA are in Appendix 2.2.

**Figure 2.3**

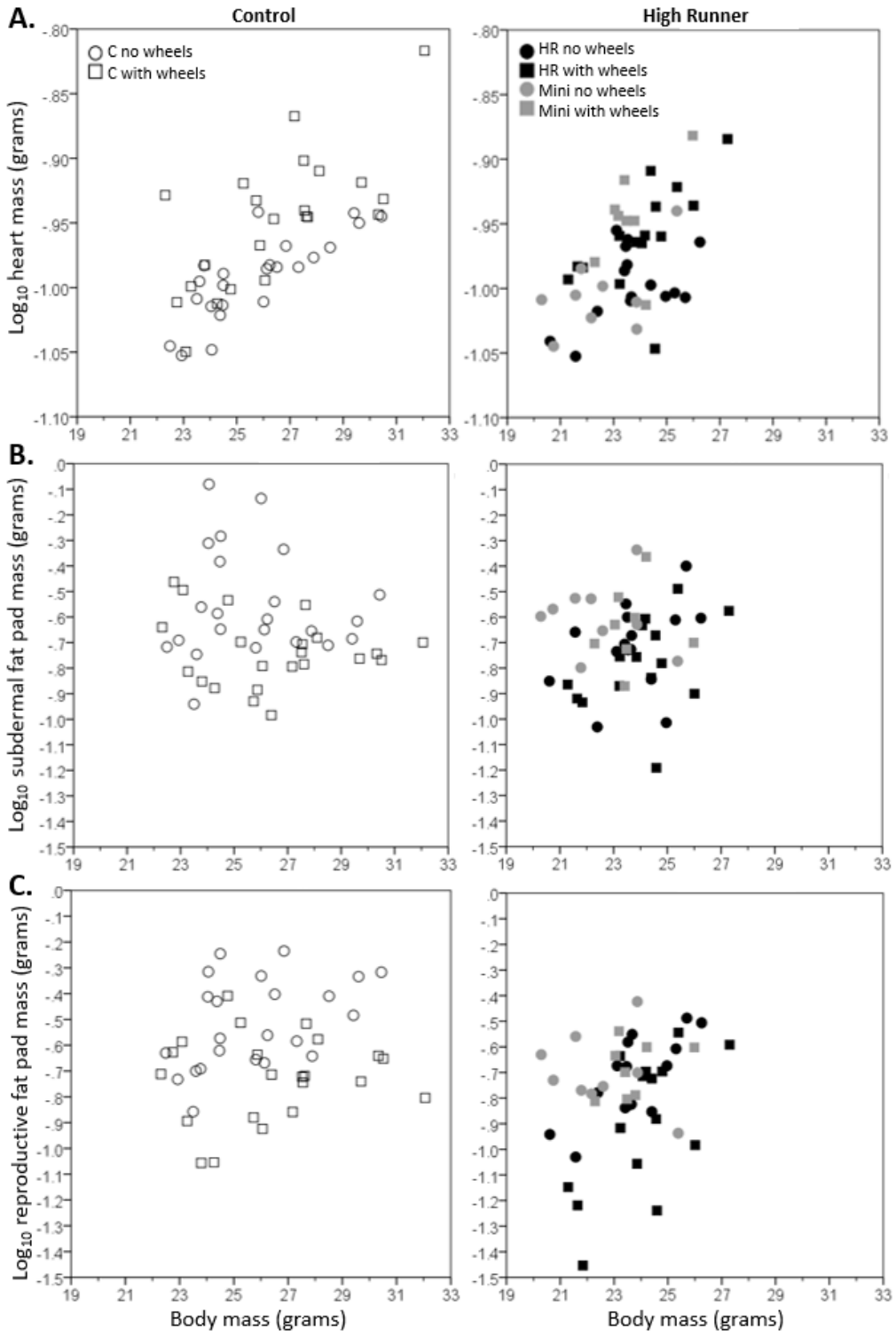
A.
Control
High Runner
C no wheels
C with wheels
HR no wheels
HR with wheels
Mini no wheels
Mini with wheels
Log10 heart mass (grams)
B.
Log10 subdermal fat pad mass (grams)
C.
Log10 reproductive fat pad mass (grams)
Body mass (grams)
Body mass (grams)

Fig. 2.3. A. Heart ventricle mass logged in relation to body mass, separated by linetype. B. Subdermal fat pad mass logged in relation to body mass, separated by linetype. C. Reproductive fat pad mass logged in relation to body mass, separated by linetype. See Table 4 for statistical analyses.

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## Appendices

**Appendix 2.1.** Additional analyses of wheel-running behavior (SAS Proc Mixed). Bold values indicate p<0.05). + and – symbols indicate direction of the effect: $P_{selection}$ + indicates HR>C; $P_{mini}$ + indicates mini-muscle mice > than normal mice.

| Trait | N | $P_{linetype}$ | $P_{mini}$ |
|---|---|---|---|
| Average wheel running revs week 8 | 46 | **0.0013 +** | 0.7400 + |
| Average duration week 8 | 46 | **0.0191 +** | 0.5985 - |
| Average speed week 8 | 46 | **0.0015 +** | 0.3106 + |
| Average maximum speed week 8 | 46 | **0.0007 +** | 0.2176 + |
| Average wheel running revs week 9 | 46 | **0.0015 +** | 0.9239 + |
| Average duration week 9 | 45 | 0.0891 + | 0.8318 - |
| Average speed week 9 | 45 | **0.0028 +** | 0.8175 + |
| Average maximum speed week 9 | 46 | **0.0008 +** | 0.9341 - |
| Average wheel running revs week 10 | 46 | **0.0004 +** | 0.2906 + |
| Average duration week 10 | 45 | **0.0191 +** | 0.2402 - |
| Average speed week 10 | 46 | **0.0008 +** | 0.4615 - |
| Average maximum speed week 10 | 46 | **0.0027 +** | 0.7159 - |
| Average wheel running revs week 11 | 46 | **0.0013 +** | 0.8160 + |
| Average duration week 11 | 46 | **0.0077 +** | 0.4024 - |
| Average speed week 11 | 46 | **0.0016 +** | 0.8650 + |
| Average maximum speed week 11 | 46 | **0.0027 +** | 0.7159 - |

**Appendix 2.2.** (SAS Proc Mixed). Bold values indicate $P<0.05$). + and – symbols indicate direction of the effect: $P_{selection}$ + indicates HR>C; $P_{mini}$ + indicates mini-muscle mice > than normal mice.

| Trait | N | $P_{linetype}$ | $P_{mini}$ |
|---|---|---|---|
| Average total SPA week 8 | 48 | 0.2108 + | 0.4741 + |
| Average SPA duration week 8 | 48 | 0.2308 + | 0.7822 - |
| Average SPA intensity week 8 | 48 | 0.1568 + | 0.1867 + |
| Average SPA maximum week 8 | 48 | 0.1785 + | 0.8842 + |
| Average total SPA week 9 | 45 | 0.0615 + | 0.3235 - |
| Average SPA duration week 9 | 45 | 0.1348 + | 0.4215 - |
| Average SPA intensity week 9 | 45 | 0.1030 + | 0.4073 - |
| Average SPA maximum week 9 | 46 | 0.1652 + | **0.0116 -** |
| Average total SPA week 10 | 46 | 0.1560 + | 0.9815 + |
| Average SPA duration week 10 | 46 | 0.5035 + | 0.6510 + |
| Average SPA intensity week 10 | 46 | 0.5480 + | 0.8236 - |
| Average SPA maximum week 10 | 45 | 0.2399 - | 0.7867 - |
| Average total SPA week 11 | 44 | 0.0626 + | 0.8780 - |
| Average SPA duration week 11 | 44 | 0.2521 + | 0.6780 + |
| Average SPA intensity week 11 | 44 | 0.0570 + | 0.4380 - |
| Average SPA maximum week 11 | 45 | 0.1401 + | 0.5405 - |

# CHAPTER 3

Early-life effects of juvenile Western diet and exercise on adult gut microbiome composition in mice

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**Abstract**

Alterations to the gut microbiome caused by changes in diet, consumption of antibiotics, etc., can affect host function. Moreover, perturbation of the microbiome during critical developmental periods potentially has long-lasting impacts on hosts. Using four selectively bred High Runner and four non-selected Control lines of mice, we examined the effects of early-life diet and exercise manipulations on the adult microbiome by sequencing the hypervariable Internal Transcribed Spacer region of the bacterial gut community. Mice from High Runner lines run ~3-fold more on wheels than do Controls, and have several other phenotypic differences (e.g., higher food consumption and body temperature) that could alter the microbiome, either acutely or in terms of coevolution. Males from generation 76 were given wheels and/or a Western diet from weaning until sexual maturity at 6 weeks of age, then housed individually without wheels on standard diet until 14 weeks of age, when fecal samples were taken. Juvenile Western diet reduced bacterial richness and diversity after the 8-week washout period (equivalent to ~6 human years). We also found interactive effects of genetic linetype, juvenile diet, and/or juvenile exercise on microbiome composition and diversity. Microbial community structure clustered significantly in relation to both linetype and diet. Western diet also reduced the relative abundance of *Muribaculum intestinale*. These results constitute one of the first reports of juvenile diet having long-lasting effects on the adult microbiome after a substantial washout period. Moreover, we found interactive effects of diet with

early-life exercise exposure, and a dependence of these effects on genetic background.

## 1. Introduction

Animals have evolved in a bacterial world. Coevolution between hosts and symbionts has resulted in complex relationships, wherein the diverse community of species inhabiting the gastrointestinal tract in mammals is essential for breaking down nutrients from ingested food, normal metabolic function, and protection through enhanced immunity (Dominguez-Bello et al., 2019; Gilbert et al., 2018; Kohl and Carey, 2016). Many factors have been shown to influence the gut microbial community and diversity, including diet, exercise, antibiotics, and age (Bokulich et al., 2016; Clark and Mach, 2016; Lozupone et al., 2012; Yatsunenko et al., 2012). Alterations to the community can result in potentially irreversible (Dethlefsen and Relman, 2011; Langdon et al., 2016) changes in the microbiome. Compositional changes in the gut microbiome can, in turn, affect many aspects of host biology, including physiology and behavior.

Diet can rapidly alter the gut microbiome community in as short as 24 hours (David et al., 2014). For example, many laboratory studies of adult rodents have shown that a typical Western diet (high in fat and sugar) alters the gut microbiome community and reduces diversity of bacterial species (Becker et al., 2020; Beilharz et al., 2018; Leamy et al., 2014; Pindjakova et al., 2017; Turnbaugh et al., 2008). In multiple strains of inbred, outbred, and transgenic mice, a shift in diet can have lasting effects on the community, as repetitive switching from a high-fat, high-sugar diet to a low-fat diet results in altered community membership and composition that does not revert to the original state

(Carmody et al., 2015). Rodent studies also indicate that diet can alter microbial function. For example, adult mice fed a high-fat diet for 12 weeks had unique gut microbiome communities, increased body mass, and altered gut bacterial function as measured by metaproteome and metabolome analyses (Daniel et al., 2014). In that study, high-fat diet led to an increase in amino acid metabolism and enzymes involved in the oxidative stress response, possibly in response to the shift in nutrient availability within the gut.

Acute and chronic exercise can also affect the microbiome (Clark and Mach, 2016; Codella et al., 2018; Mach and Fuster-Botella, 2017; Mailing et al., 2019; O'Sullivan et al., 2015; Scheiman et al., 2019). The first paper highlighting the relationship between exercise and the microbiome found that adult rats with wheel access for five weeks had an increased amount of cecal n-butyrate, a short-chain fatty acid byproduct of bacterial fermentation (Matsumoto et al., 2008). Butyrate can be transported from the small intestine to muscles, where it can lead to activation of several regulatory pathways linked to ATP production as well as muscle integrity, thus potentially altering athletic ability and/or performance (Ticinesi et al., 2017; Walsh et al., 2015). Approaches for measuring the effect of exercise on the gut microbiome vary widely in the literature, but consistent trends in results are emerging. For example, both rodent and human studies have reported increased butyrate-producing bacteria (Barton et al., 2018; Matsumoto et al., 2008), and also increases in taxa such as *Lactobacillus* (Batacan et al., 2017; Lambert et al., 2014; Petriz et al., 2014;

Queipo-Ortuño et al., 2013), *Bifidobacterium* (Bressa et al., 2017; Lambert et al., 2014; Queipo-Ortuño et al., 2013), and *Akkermansia* (Barton et al., 2018; Bressa et al., 2017; Clarke et al., 2014; Liu et al., 2015). In amateur half-marathon runners the relative abundances of several bacterial taxa and also fecal metabolites were significantly different pre- and post-race (Zhao et al., 2018).

Diet and exercise have also been shown to interactively influence the gut microbiome community and diversity in rodents (Batacan et al., 2017; Denou et al., 2016; Evans et al., 2014). Mice placed on a high-fat diet for 6 weeks followed by 6 weeks of high-intensity interval training had greater bacterial diversity in the feces compared with sedentary mice on standard chow (Denou et al., 2016). Exercise-trained mice on a high-fat diet had significant changes in the relative abundance of the phylum *Bacteroidetes* in the small intestine, cecum, and colon compared with mice on a high-fat diet without exercise training. In another study on the interactions between exercise and diet, mice given 12 weeks of voluntary wheel access on a standard or high-fat diet had higher diversity than sedentary controls as well as significant main effects of diet, exercise, and their interactions on taxa relative abundance (Evans et al., 2014). More specifically, that study found an increase in the relative abundance of butyrate-producing taxa in the *Clostridiales* order compared with sedentary mice. In rats, high-intensity and light-intensity interval training regimens resulted in unique microbiome communities regardless of whether they were on a high-fat, high-fructose diet or

a standard diet (Batacan et al., 2017). The scarcity of studies examining diet-exercise interactions highlights the need for more research in this growing field.

In mammals, the period of development from weaning to sexual maturity is a crucial time during which environmental conditions can have a lasting impact on many traits (Garland et al., 2017), including normal development of the microbiome (Kerr et al., 2015). Immediately after birth, initial colonizers of the gut microbiome in placental mammals are dominated by microbes from the mother, followed by further acquisitions from the early-life environment (Funkhouser and Bordenstein, 2013; Milani et al., 2017). A clear example of developmental effects on the gut microbiome is early-life diet: babies that are breastfed have a unique microbiome compared with those fed formula (Sprockett et al., 2018), and have higher bacterial diversity during the first 12-24 months of age (Bokulich et al., 2016). In mice, early-life antibiotic treatment followed by placement on a high-fat, high-sugar diet as adults results in increased adult adiposity and an increase in the ratio of *Firmicutes* to *Bacteroidetes* as compared with mice on a normal diet (Schulfer et al., 2019). In a recent study, juvenile mice given 3 weeks of high-fat diet or cafeteria diet starting at 4 weeks of age followed by an approximately 7-week long washout period had altered adult gut microbiome communities (Fülling et al., 2020). More specifically, mice with a juvenile high-fat diet had reduced diversity of the adult gut microbiome at approximately 14 weeks of age. However, only one study has tested whether early-life effects of exercise on the microbiome can persist after a substantial

washout period. Mika et al. (2015) found that after a 25-day washout period, rats with 6 weeks of juvenile wheel access tended to have decreased *Firmicutes* abundance as adults.

The first goal of the present study was to test for long-lasting effects of early-life Western diet and exercise on the adult microbiome. To do so, we used a unique animal model: four lines of High Runner (HR) mice that have been selectively bred for high voluntary wheel-running behavior and their four non-selected Control (C) lines (Swallow et al., 1998). The HR mice differ from C mice in several ways that might affect the microbiome through alterations in the gut environment. HR mice have higher activity levels and food consumption even when housed without wheels, and increased body temperature when active (Copes et al., 2015; Malisch et al., 2009; Swallow et al., 2009; Wallace and Garland, 2016), all of which might affect the gut environment. In the absence of compensatory reductions in other aspects of physical activity, exercise leads to increased energy expenditure and hence necessitates greater food consumption (Garland et al., 2011), which should directly impact the gut microbiome. Exercise also causes many acute changes in physiology, including increases in body temperature, and changes in hormone levels, intestinal barrier function, and digestive transit time that could feedback into the gut environment (Campbell and Wisniewski, 2017; Mach and Fuster-Botella, 2017). HR and C mice also differ in circulating concentrations of hormones (Garland et al., 2016). When housed without wheels, HR and C mice do not differ in small or large intestine mass or

length, suggesting that the former might have faster digestive throughput (Kelly et al., 2017). Therefore, our second goal was to test for microbiome differences between the HR and C lines, which could result from acute effects of the noted phenotypic differences. Another possibility is coevolution of the gut microbiome across many tens of generations of selective breeding, but we cannot differentiate that from acute/chronic effects of exercise with the present experimental design. Our analyses also considered the possibility of interactive effects, e.g., that genetic background (Benson et al., 2010; Carmody et al., 2015; Leamy et al., 2014) might influence whether and how early-life Western diet or exercise opportunity affects the adult microbiome.

## 2. Methods

All experiments and methods were approved by the Institutional Animal Use and Care Committee of the University of California, Riverside.

### 2.1 Experimental animals

Mice were sampled from generation 76 of an ongoing selection experiment selecting for high voluntary wheel-running behavior. Four replicate HR lines were bred for high levels of voluntary wheel running and were compared with four non-selected C lines. The base population was 224 outbred Hsd:ICR laboratory house mice (Swallow et al., 1998). Mice were weaned at 21 days of age and housed 4 per cage separated by line and sex until ~6-8 weeks of age.

Mice were then placed into individual cages attached to a 1.12 m circumference wheel (Lafayette Instruments, Lafayette, IN, USA) with a sensor to record the total number of revolutions per day (e.g. see Swallow et al., 1998). For HR mice, the highest running male and female from each family based on the average revolutions on days 5 and 6 of a 6-day period of wheel access were chosen as breeders for the next generation. Breeders in the C lines were chosen without regard to how much they run. Each generation had ~10 breeding pairs per line, and sibling pairings were not allowed.

**2.2 Early-life diet and exercise treatment**

A total of 165 male mice, sampled approximately equally from the 4 replicate HR and 4 non-selected C lines, were weaned at 21 days of age and placed into one of 4 treatment groups for 3 weeks: (1) standard diet, no wheels; (2) Western diet, no wheels; (3) standard diet, wheels; and (4) Western Diet, wheels (see Fig. 1). Mice were provided with *ad libitum* food and water for the duration of the experiment. Standard Laboratory Rodent Diet (SD) from Harlan Teklad (W-8604) contained 4% kJ from fat and the Western diet (WD) from Harland Teklad (TD.88137) contained 42% kJ from fat. After the 3 weeks of juvenile exposure, which allowed them to reach sexual maturity, all mice were housed individually without wheel access on standard diet for an 8-week washout period (equivalent to approximately 6 human years: Dutta and Sengupta, 2016). Mice were maintained in rooms with lights on at 0700 Pacific Standard Time for a 12h:12h

L:D photo period, and at approximately 22°C.

### 2.3 Juvenile wheel running

Juvenile wheel running was measured during weeks 3-6 of the early-life diet and/or exercise manipulation. Mice were housed individually in home cages with attached wheels, as used during the routine selective breeding protocol (Swallow et al., 1998). Sensors attached to the wheel record the number of revolutions in each 1-minute interval during a 23 hr measurement period. We measured wheel freeness by recording the number of revolutions per wheel until it reached a stop after accelerating each wheel to a constant speed (Copes et al., 2015).

### 2.4 Juvenile food consumption

Juvenile food consumption was measured during weeks 3-6 of the early-life diet and/or exercise manipulation. Food hoppers were weighed at the start and end of each week to measure apparent food consumption after accounting for food wasting (Koteja et al., 2003). Food consumption was converted to caloric intake as the diets differed in energy content (Meek et al., 2010).

### 2.5 Fecal sampling

At 14 weeks of age, individual mice were placed into a clean, empty cage and watched until defecation. We obtained fecal samples from 149 individuals. The samples were placed into a sterile tube and held on dry ice prior to storage at -

80°C, where they remained until DNA extraction.

### 2.6 Bacterial rRNA ITS analysis

Illumina bacterial rRNA internal transcribed spacer (ITS) libraries were constructed as follows. PCRs were performed using a DNA Engine thermal cycler (Bio-Rad Inc., Hercules, CA, USA) as 25-µl reactions containing: 50 mM Tris (pH 8.3), bovine serum albumin (BSA) at 500 µg/ml, 2.5 mM $MgCl_2$, 250 µM of each deoxynucleotide triphosphate (dNTP), 400 nM of the forward PCR primer, 200 nM of each reverse PCR primer, 2.5-µl of DNA template, and 0.625 units JumpStart Taq DNA polymerase (Sigma-Aldrich, St. Louis, MO, USA). PCR primers targeted a portion of the small-subunit (ITS-1507F, GGTGAAGTCGTAACAAGGTA) and large-subunit (ITS-23SR, GGGTTBCCCCATTCRG) rRNA genes and the hypervariable ITS (Ruegger et al., 2014), with the reverse primers including a 12-bp barcode and both primers including the sequences needed for Illumina cluster formation; primer binding sites are the reverse and complement of the commonly used small-subunit rRNA gene primer 1492R (Frank et al., 2008) and the large-subunit rRNA gene primer 129F (Hunt et al., 2006). PCR primers were only frozen and thawed once. Thermal cycling parameters were as follows: 94°C for 5 min; 35 cycles of 94°C for 20 s, 56°C for 20 s, and 72°C for 40 s; followed by 72°C for 10 min. PCR products were purified using a Qiagen QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. DNA

sequencing (single-end 250 base) was performed using an Illumina MiSeq (Illumina, Inc., San Diego, CA, USA). Clusters were created using template concentrations 2.5 pmol $1^{-1}$ and phi X at 107,000 $mm^{-2}$.

Data processing was performed with USEARCH v10.0 (Edgar, 2010). We used the UPARSE pipeline for de-multiplexing, length trimming, quality filtering and operational taxonomic unit (OTU) picking using default parameters or recommended guidelines that were initially described in (Edgar, 2013) and which have been updated at https://www.drive5.com/usearch/manual10/uparse_pipeline.html. Briefly, after demultiplexing and using the recommended 1.0 expected error threshold, sequences were trimmed to a uniform length of 248 bp and then dereplicated. Dereplicated sequences were subjected to error-correction (denoised) and chimera filtering to generate zero-radius operational taxonomic units (ZOTUs) using UNOISE3 (Edgar, 2016b). An OTU table was then generated using the otutab command. ZOTUs with non-bacterial DNA were identified and enumerated by performing a local BLAST search (Altschul et al., 1990) of their seed sequences against the nucleotide database. ZOTUs were removed if any of their highest scoring BLAST hits contained taxonomic IDs within the rodent family, Fungi, Viridiplantae, or phi X. Taxonomic assignments to bacterial ZOTUs were made with the SINTAX taxonomy prediction algorithm (Edgar, 2016a) on an updated SSU-ITS database (Ruegger et al., 2014). This resulted in 2,730 OTUs with an average of 47,851 sequences per sample. Data were

normalized within each sample by dividing the number of reads in each OTU by the total number of reads in that sample.

The bacterial rRNA ITS sequences were deposited in the National Center for Biotechnology Information (NCBI)'s Sequence Read Archive (SRA) under SRA BioProject Accession is PRJNA624662.

## 2.7 Statistical analyses

### 2.7.1 Juvenile wheel running and food consumption

As used in numerous previous studies of these lines of mice, we used linear mixed models in SAS 9.4 Proc Mixed (SAS Institute, Cary, NC, USA). The effect of linetype is tested against the variance among replicate lines, which are a nested random effect within linetype. Wheel Access×Line(Linetype), Diet×Line(Linetype), and Wheel Access×Diet×Line(Linetype) were also nested random effects. In these full models, the effects of Wheel Access, Diet, Linetype, and their interactions were tested with 1 and 6 degrees of freedom. If the covariance parameter estimate for higher-order random effects was zero, we removed them in a stepwise fashion. In other words, if the covariance parameter estimate for the 3-way interaction was 0, we removed the Wheel Access×Diet×Line(linetype) random effect. Then, if one of the two-way random interaction effects was also zero, we removed it. However, we always retained the line(linetype) random effect, given the nature of the experimental design (e.g. see Castro and Garland, 2018; Castro et al., 2020; Swallow et al., 1998). For

juvenile wheel running, we included wheel freeness as a covariate in the model. For caloric intake, we included body mass as a covariate.

In these statistical models, we also tested for effects of the mini-muscle phenotype (present in 2 of the HR lines) on juvenile wheel running, juvenile caloric intake, adult gut microbiome richness and relative abundance. The mini-muscle phenotype is caused by an autosomal recessive allele, a single base pair change in a myosin heavy chain gene (Kelly et al., 2013). Homozygotes for this naturally occurring mutation are characterized by a 50% reduction in hindlimb muscle mass, larger internal organs, and various other differences as compared with unaffected individuals (Garland et al., 2002; Swallow et al., 2009; Wallace and Garland, 2016). In the present study the number of mini-muscle individuals varied among analysis. For example, of the 88 mice for which we obtained wheel-running data during week 1 of juvenile exposures, 12 had the mini-muscle phenotype (all 9 in line 3 and 3 of the 11 in line 6). Of the 165 mice for which we obtained week 1 food consumption data, 43 had the mini-muscle phenotype (all 21 in line 3 and 5 of 22 in line 6). Of the 149 mice for which we obtained microbiome data, 25 had the mini-muscle phenotype (all 20 in line 3 and 5 of 20 in line 6).

### 2.7.2 Beta diversity of the adult gut microbiome

Gut microbiome membership and community structure were compared by calculating unweighted UniFrac and Hellinger distance matrices in QIIME version

1.9.1. Unweighted UniFrac distance utilizes the presence and absence of bacterial species while accounting for the phylogenetic relationship between bacterial species. For statistical and graphical representation, we used an OTU table rarified to an even sequencing depth of 14,000 reads per sample. We used a Principal Coordinates Analysis (PCoA) to visualize the communities in a 3D space. For beta diversity, we used a PERMANOVA test in QIIME to determine statistical significance (Anderson, 2001). For these tests we did not treat replicate line as a nested random effect because the software to do this is not currently available.

**2.7.3 Alpha diversity of the adult gut microbiome.**

To determine the effects of diet, exercise, linetype, and their interactions on alpha diversity of the adult gut microbiome, we used the Chao1 Index and Shannon Index calculated in QIIME Version 1.9.1 from an OTU table rarified to the lowest common sequencing depth of 14,000 reads. We also totaled the number of non-zero OTUs identified in each mouse using the rarified OTU table. We used the statistical procedures described above in *Juvenile wheel running and food consumption*. Because ANOVAs have relatively low power to detect interactions (Wahlsten, 1990), and following our laboratory's previous analyses of these mice (e.g., Belter et al., 2004; Houle-Leroy et al., 2000), we considered interactions significant if $P<0.10$.

### 2.7.4 Lower-level taxa summary comparisons.

We compared the relative abundance data of identified phylum, class, order, family, genus, and species groups produced by the summarize_taxa.py script in QIIME. Based on the simulations reported by Aschard et al. (2019), we only analyzed taxa found in >85% of the mice [phylum (*N*=6), class (*N*=9), order (*N*=8), family (*N*=16), genus (*N*=17), species (*N*=26), and OTUs (*N*=140, of the total 2,730 identified OTUs)], which totaled 221 tests and 1,761 *P* values. We used the statistical procedures described above in *Juvenile wheel running and food consumption*. Bacterial relative abundance data were log or arcsine square root transformed to normalize residuals (Brown et al., 2020; Kohl et al., 2016). *P* values were corrected for multiple comparisons using the false discovery rate (FDR; Benjamini and Hochberg, 1995). For these analyses, we accepted statistical significance at $P<0.05$ after adjustment for FDR.

## 3. Results

Note: Supplemental File 3.1 (Significant_Taxa_Differences_Table_10.xlsx) is an excel file with a table of p values (448) for phylum through genus. P values before FDR ≤ 0.1 are highlighted in red.

### 3.1 Linetype, diet, and exercise affect juvenile wheel running and food consumption

Diet had an interactive effect on wheel running across the three weeks of early-life exposure (full statistical results are in Appendix 3.3). During the first week, Western diet increased wheel running, but the effect was greater in HR mice (interaction $F_{1,76} = 7.62$, $P$=0.0072, Fig. 2A), and mini-muscle mice ran more than normal-muscle mice ($F_{1,76} = 6.12$, $P$ =0.0156). During the second week, mice with a Western diet continued to run significantly more than those with standard diet, and HR mice ran 2.6-fold more revolutions per day than C mice, with no interaction between diet and linetype (interaction $F_{1,76} = 0.51$, $P$=0.4765, Fig. 2A). By the third week of juvenile wheel access, HR mice ran 3.4-fold more than C mice and diet no longer significantly affected wheel running.

During the first week of early-life exposure, diet and wheel access had an interactive effect on caloric intake (interaction $F_{1,143} = 26.62$, $P$<0.0001, Fig. 2B). Western diet increased caloric intake in all groups, by ~21% on average ($F_{1,143} = 313.25$, $P$<0.0001, Fig. 2B). However, wheel access increased intake in mice on a standard diet but decreased it in those on a Western diet. During the second week, mice on the Western diet had increased caloric intake ($F_{1,6} = 37.71$, $P$=0.0009, Fig. 2B) and those with wheels consumed more than mice without wheels ($F_{1,6} = 25.18$, $P$=0.0024, Fig. 2B). In the third week, mice with wheels again consumed more calories than those without wheels ($F_{1,6} = 84.23$, $P$<0.0001, Fig. 2B), but the effect of diet was no longer significant. Mini-muscle

mice consumed significantly more food than normal-muscle mice during both weeks 2 ($F_{1,137}$ = 5.55, $P$=0.0199) and 3 ($F_{1,136}$ = 4.97,$P$=0.0274).

### 3.2 Dominant phyla of the adult gut microbiome

The 2,730 identified OTUs were classified into 7 phyla, 22 classes, 36 orders,58 families, 79 genera, and 112 species. Community composition for the entire set of experimental mice ($N$=149) was dominated by the phylum *Bacteroidetes* (68.1 ± 17.4%) (mean ± S.D.) and *Firmicutes* (27.9 ± 16.7%), with additional phyla being much less abundant: *Proteobacteria* (1.2 ± 2.1%), *Candidatus Melanobacteria* (0.3 ± 0.6%), *Tenericutes* (0.2 ± 0.3%), and *Actinobacteria* (0.05 ± 0.04%) (Fig. 3).

### 3.3 Juvenile diet and linetype affect adult community membership (Beta diversity)

Community membership measured by unweighted UniFrac distance and by Hellinger distance plotted in a PCoA plot (Fig. 4 and 5, respectively; corresponding statistical results in Tables 1 and 2, respectively) showed clustering of mice by linetype and by juvenile diet exposure. HR and C mice significantly clustered independent of one another (PERMANOVA, $F_{1,147}$ = 1.56, $P$=0.009, Fig. 4A; PERMANOVA, $F_{1,147}$ = 2.31, $P$=0.001, Fig. 5A). Mice fed a juvenile Western diet resulted in significant clustering of samples compared with mice fed a juvenile standard diet (PERMANOVA, $F_{1,147}$ = 2.72, $P$=0.001, Fig. 4B;

PERMANOVA, $F_{1,147}$ = 2.85, $P$=0.001, Fig. 5B). Within both HR and C linetypes, mice clustered together by diet (C, $F_{1,75}$ = 1.64, $P$=0.007; HR $F_{1, 70}$ = 0.001, $P$=0.001: Appendix 3.1). Wheel Access did not result in significant clustering within linetypes (PERMANOVA, $F_{1, 70}$ = 1.30, $P$=0.072, Appendix 3.1). HR mice also clustered independently by diet (PERMANOVA, $F_{1, 70}$ = 3.783, $P$=0.001, Appendix 3.2).

### 3.4 Early-life exposures, linetype, and their interactions affect adult gut microbiome richness (Alpha diversity)

For the total number of OTUs, early-life diet and exercise exposures altered the adult gut microbial richness in a linetype-dependent manner: the three-way interaction of juvenile diet, wheel access, and linetype was significant (interaction $F_{1,128}$ = 2.83, $P$=0.095; Fig. 6A). Early-life Western diet tended to have a lasting impact on gut microbiome diversity by reducing the total OTUs (ANOVA, $F_{1,6}$ = 5.67, $P$=0.055; Fig. 6A).

The three-way interaction of juvenile diet, exercise, and linetype was significant for the Chao1 Index, a corrected index of gut microbial richness that accounts for rarer taxa (interaction $F_{1,128}$ = 2.83, $P$=0.013; Fig. 6B). Early-life exposure to Western diet tended to have a lasting impact on the gut microbiome by reducing adult gut community richness (ANOVA, $F_{1,6}$ = 5.68, $P$=0.054; Fig. 6B). The Shannon Index, another measure of gut microbial richness that

accounts for the abundance of taxa in a sample, was not statistically different among groups (Fig. 6C).

### 3.5 Juvenile Western diet affects adult gut microbiome community

Of the 1,760 *P* values tested, only 2 remained significant at $P<0.05$ after correcting for multiple comparisons using a Benjamini and Hochberg FDR (See Supplemental File 3.1 for phylum through genus *P* values before FDR). Western diet significantly reduced the relative abundance of the family *Muribaculaceae*, which is commonly found in the mouse gut microbiome (ANOVA, $F_{1,128} = 19.2$; $P=0.021$). This decrease is explained by the gut bacterial species *Muribaculum intestinale*, which was found in all mice from our study (ANOVA, $F_{1,128} = 19.2$; $P=0.021$; Fig. 7). *Muribaculum intestinale* made up 0.38% of the identified OTUs. Mini-muscle mice did not significantly differ in the relative abundance of any of the tested taxa.

## 4. Discussion

Our results constitute one of the first reports of juvenile diet having long-lasting effects on the adult microbiome after a substantial washout period (equivalent to ~6 human years). Moreover, we found interactive effects of diet with early-life exercise exposure, and a dependence of these effects on genetic background. The overall bacterial community composition that we found (Fig. 3) is similar to that reported in many other studies of adult laboratory house mice (e.g. Benson

et al., 2010; Lamoureux et al., 2017). However, beta diversity metrics indicated that community membership was unequal between the two genetic linetypes we studied (replicate, selectively bred HR and C lines of mice), and was also affected by early-life Western diet (Fig. 4, 5). Bacterial richness and alpha diversity were also affected by an interaction of juvenile diet, exercise, and linetype (Fig. 6). Finally, juvenile Western diet significantly decreased the relative abundance of the *Muribaculaceae* family driven by the species *Muribaculum intestinale* (Fig. 7).

Selective breeding for high voluntary wheel running resulted in unique clustering of gut microbiomes by linetype (Fig. 4, 5). These results are consistent with the fact that selection for wheel-running behavior has caused many exercise-associated biological changes that could influence the gut environment, including higher food consumption even when housed without wheels, higher body temperatures when active, and differences in circulating concentrations of multiple hormones, including corticosterone, a classic "stress hormone" (Copes et al., 2015; Garland et al., 2016; Malisch et al., 2009; Swallow et al., 2009; Wallace and Garland, 2016). Our results and those of other recent studies also demonstrate the utility of selectively bred rodent models for understanding possible coevolutionary changes in the microbiome (e.g., see Kohl et al., 2016; Liu et al., 2015; van der Eijk et al., 2020; Zhang et al., 2020).

A Western diet can negatively impact the host's normal gut barrier function by increasing intestinal permeability (Martinez-Medina et al., 2014) and by

increasing inflammation of the gut environment (Agus et al., 2016). Several studies have demonstrated effects of a Western diet on the gut microbiome in adult rodents. For example, Western diet results in unique clustering of microbiome communities (Carmody et al., 2015; Pindjakova et al., 2017). We also found significant clustering of microbiome communities by diet (Figs 4, 5). Previous studies of adult mice have reported that a high-fat or high-sugar diet can decrease bacterial diversity (Pindjakova et al., 2017; Sonnenburg et al., 2016; Turnbaugh et al., 2008). Adult rats on standard chow supplemented with 10% sucrose solution and a selection of cakes, biscuits, and high-protein foods continuously for 25 days had a significantly reduced alpha diversity, evidenced by a reduction in the total number of OTUs compared with control rats (Beilharz et al., 2018). In our study, Western diet during the juvenile period increased wheel-running behavior and food consumption in both selectively bred HR mice and non-selected C mice (Fig. 2). Both altered diet and increased food consumption can affect the gut environment and thus alter the bacterial community. In principle, early-life Western diet could have altered the gut microbiome in a way that persists into adulthood, an effect that we did indeed find (Figs 4-7).

Only one other publication has examined the long-lasting effects of juvenile diet on the adult gut microbiome after a significant washout period in mice. Mice with 3 weeks of juvenile high-fat diet followed by a 7-week washout period had decreased alpha diversity as measured by the Shannon Index as

adults (Fülling et al., 2020). In our study, perturbation of the juvenile gut microbiome with Western diet also had long-lasting effects on species community indicators of adult gut microbial richness by reducing the total number of OTUs and the Chao1 index, though no differences in Shannon diversity were found (Fig. 6). Similarly to Carmody et al. (2015), who demonstrated that a high-fat, high-sugar diet in multiple inbred, outbred, and transgenic strains of mice resulted in clustering of mice by both diet and genotype within diet treatment, we found significant clustering of genetic lines within diet treatment (Appendix 3.1), showing the response to diet can be genotype-dependent.

After correction for multiple comparisons of 1,760 *p* values comparing taxa at the level of phylum, class, order, family, genus, species, and OTU, we found one species (and its family) whose relative abundance was significantly decreased by juvenile Western diet, *Muribaculum intestinale* (Fig. 7, Supplemental File 3.1). The *Muribaculaceae* family is commonly found in mouse (but not human) gut microbiomes (previously referred to as S24-7; Lagkouvardos et al., 2016; Seedorf et al., 2014). *Muribaculaceae* has been linked with propionate production, a short-chain fatty acid, in a mouse longevity study (Smith et al., 2019). This family was also seen to increase in abundance in mice given voluntary wheel access while on a high-fat or standard diet, and decrease in relative abundance in mice on a high-fat diet with or without exercise (Evans et al., 2014). This finding is similar to our study in which the relative abundance of *Muribaculum intestinale*, a species of the *Muribaculaceae* family, was unaffected

by exercise but decreased in abundance with juvenile Western diet (Fig. 7). *Muribaculaceae* belongs to the phylum *Bacteroidetes*, one of the two most abundant phyla in the gut microbiome. A Western diet has been shown to usually decrease the relative abundance of *Bacteroidetes*, a primarily acetate and propionate producing phylum while increasing the relative abundance of *Firmicutes*, a primarily butyrate producing phylum (Carmody et al., 2015; den Besten et al., 2013; Ley et al., 2006). If species in the *Muribaculaceae* family could potentially influence the energy substrate availability to the host, this could lead to a differential effect of diet and exercise treatments on normal host function. As *M. intestinale* is a newly cultured species, it remains to be seen what other functions it might have (Lagkouvardos et al., 2019). In a small sample of adult wild-type and AC5KO mice (known for their exercise-associated traits of longevity and increased mitochondrial metabolism in skeletal muscle (Ho et al., 2015)), a taxon with high sequence similarity to *M. intestinale* was enriched in adult AC5KO mice after 5 weeks of treadmill training, suggesting that *M. intestinale* is a potentially exercise-associated species (Dowden et al., 2020).

To our knowledge, only one previous study of rodents has tested for long-lasting effects of juvenile exercise on the adult microbiome. Mika et al. (2015) found that juvenile rats given 6 weeks of wheel access, followed by a 25-day washout period, tended (not statistically significant) to have a decreased abundance of the *Firmicutes* phylum compared with sedentary juveniles. We found that early-life exercise significantly interacted with diet and linetype to

influence gut microbial diversity (Fig. 6). Given that we have shown long-lasting effects of relatively mild and natural early-life changes (diet, exercise), more severe treatments, such as antibiotics, might have even stronger, long-lasting effects (Ma et al., 2020).

### 4.1 Limitations and future directions

When examining the gut microbiome, variation in sequencing methods can lead to different results under similar experimental conditions. Much of the literature consists of 16S rRNA analysis. Instead, we sequenced the ITS rRNA gene for finer resolution of the gut microbial community (Ruegger et al., 2014). This poses a challenge when comparing ITS data with 16S data. Nevertheless, by examining broad patterns in diversity and community structure (Figs 4-6) we were able find similar patterns between our data and the literature (see above). For example, a Western diet tends to decrease gut microbiome diversity (Fig. 6) and alters the gut microbiome community measured by beta diversity (Figs 4, 5).

We were only able to sample feces and obtain microbial sequence data for one time point. Logistical constraints precluded our obtaining fecal samples at the beginning of the study. In future studies, repeating this experiment with a baseline sample at weaning and immediately after the juvenile exposure to diet and/or exercise would increase the power to detect longitudinal changes. As we had only the microbiome data after the washout period, we cannot know when the effects of the experimental treatments first appeared. They might have

appeared during the 3-week treatment period, which seems likely, or they might have appeared later, at any time prior to when we took fecal samples. Regardless of when the effects first appeared, they were detectable when we analyzed the adult fecal samples. This is an important result, even in the absence of information regarding the longitudinal trajectory of the effects. Future studies should examine the time course of early-life effects. In addition, study of the cecum would allow a more in situ view of the microbiome.

We did not separate or sterilize cages, bedding, food, or water, thus giving the mice constant exposure to environmental bacteria. This exposure should have tended to homogenize the gut microbiome, thus possibly erasing any early-life effects of diet or exercise. Nevertheless, we were able to detect such effects after a substantial washout period, supporting the idea that the early-life developmental period of the microbiome is sensitive and responsive to change, and can be impacted in ways that resist subsequent environmental perturbations.

Future experiments involving antibiotic reduction and transplantation of the microbiome will be required to determine whether the unique microbial community of HR mice (Figs 4, 5), which has potentially co-evolved during the selection experiment, contributes to their high motivation and/or ability for sustained, aerobically supported exercise (Hsu et al., 2015; Nay et al., 2019; Okamoto et al., 2019; Scheiman et al., 2019). More specifically, one could administer antibiotics to eliminate the existing gut microbiome, monitor changes in wheel running, and then transplant the HR microbiome into C mice and vice

versa. Additional groups would receive their own line-type-specific microbiome in the reseeding phase of the experiment (i.e., HR to HR and C to C). If a unique microbiome is partly responsible for the HR phenotype, then we would predict that (1) antibiotics would reduce their wheel running and (2) reseeding with HR (but not C) microbiome would recover the normal wheel-running behavior for HR mice. It is also possible that transplanting the HR microbiome to C mice would increase their wheel running, at least if some other inherent factor does not limit their running motivation or ability.

Overall, we found that an early-life Western diet had more long-lasting effects on the microbiome than did early-life exercise. Future studies will be required to determine whether this is a general result. In particular, we need dose-response studies of how much exercise, and what type of exercise, is needed to elicit a permanent, potentially beneficial, change in the gut microbiome. The field also needs more studies of how voluntary exercise can acutely change the gut microbiome (e.g., by short-term or alternate-day wheel access), combined with longitudinal sampling. Finally, milder diet alterations should be examined, in addition to effects of probiotics (Sanders et al., 2019).

**Table 3.1.** Community membership of the adult gut microbiome assessed by PERMANOVA statistical tests using unweighted UniFrac distances. Statistical analyses corresponding to Fig. 4.

| | SS | d.f. | *F* | $R^2$ | *P* | Figure |
|---|---|---|---|---|---|---|
| Line type | 0.213 | 1, 147 | 1.560 | 0.010 | 0.009 | 4A |
| Diet | 0.369 | 1, 147 | 2.719 | 0.018 | 0.001 | 4B |
| Wheel access | 0.170 | 1, 147 | 1.243 | 0.008 | 0.096 | 4C |
| C:Diet | 0.225 | 1, 75 | 1.644 | 0.021 | 0.007 | 4B, S1F |
| HR:Diet | 0.328 | 1, 70 | 2.462 | 0.034 | 0.001 | 4B, S1F |
| C:Wheel access | 0.116 | 1, 75 | 0.838 | 0.011 | 0.832 | 4C, S1G |
| HR:Wheel access | 0.176 | 1, 70 | 1.304 | 0.018 | 0.072 | 4C, S1G |

HR, high runner; C, control. Statistical analyses corresponding to Fig. 4.

**Table 3.2.** Community membership of the adult gut microbiome assessed by PERMANOVA statistical tests using a Hellinger distance matrix. Statistical analyses corresponding to Fig. 5.

| | SS | d.f. | *F* | $R^2$ | *P* | Figure |
|---|---|---|---|---|---|---|
| Line type | 1.150 | 1, 147 | 2.310 | 0.015 | 0.001 | 5A |
| Diet | 1.414 | 1, 147 | 2.851 | 0.019 | 0.001 | 5B |
| Wheel access | 0.497 | 1, 147 | 0.989 | 0.007 | 0.483 | 5C |
| C:Diet | 0.534 | 1, 75 | 1.043 | 0.014 | 0.384 | 5B, S2F |
| HR:Diet | 1.753 | 1, 70 | 3.783 | 0.051 | 0.001 | 5B, S2F |
| C:Wheel access | 0.385 | 1, 75 | 0.749 | 0.010 | 0.843 | 5C, S2G |
| HR:Wheel access | 0.458 | 1, 70 | 0.951 | 0.013 | 0.518 | 5C, S2G |

Statistical analyses corresponding to Fig. 5.

**Figure 3.1**

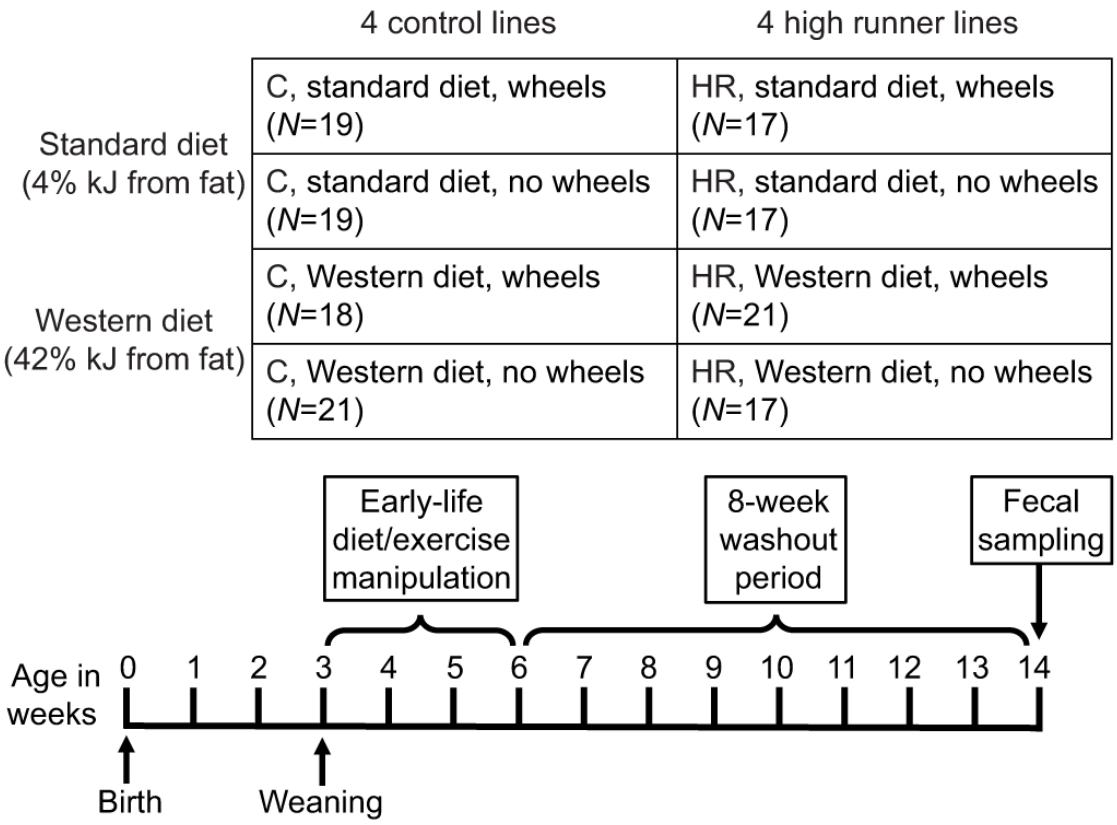


Fig. 3.1. Early-life experimental design and treatment groups (*N*=149 mice). Fecal sampling occurred as adults (14 weeks of age) after the eight-week washout period on standard diet with no wheel access.

**Figure 3.2**

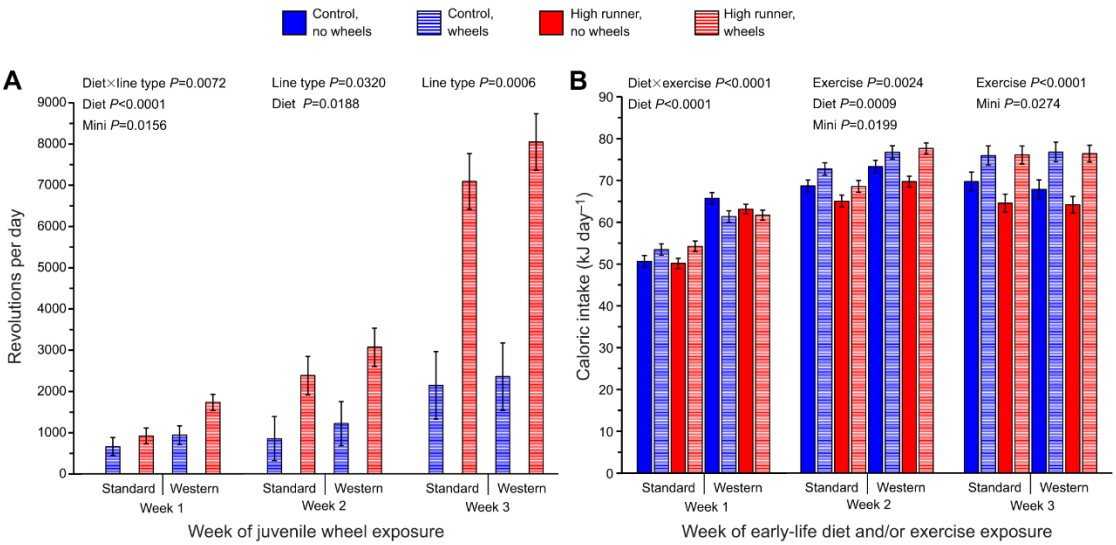


Fig. 3.2. Weekly revolutions per day and caloric intake in response to juvenile diet and/or exercise treatment. Data are presented as untransformed least squares means ± s.e.m. (values for mini-muscle versus normal-muscle mice are not shown). Shown above each week are the significant main effects and interactions (2-tailed ANCOVAs $P \leq 0.05$, not adjusted for multiple comparisons). Full statistical results are in Table S1. A. Weekly juvenile wheel running for half of the mice during the 3 weeks of early-life exposure ($N$=88). B. Weekly mass-adjusted juvenile caloric intake during the 3 weeks of early-life exposure ($N$=165).

**Figure 3.3**

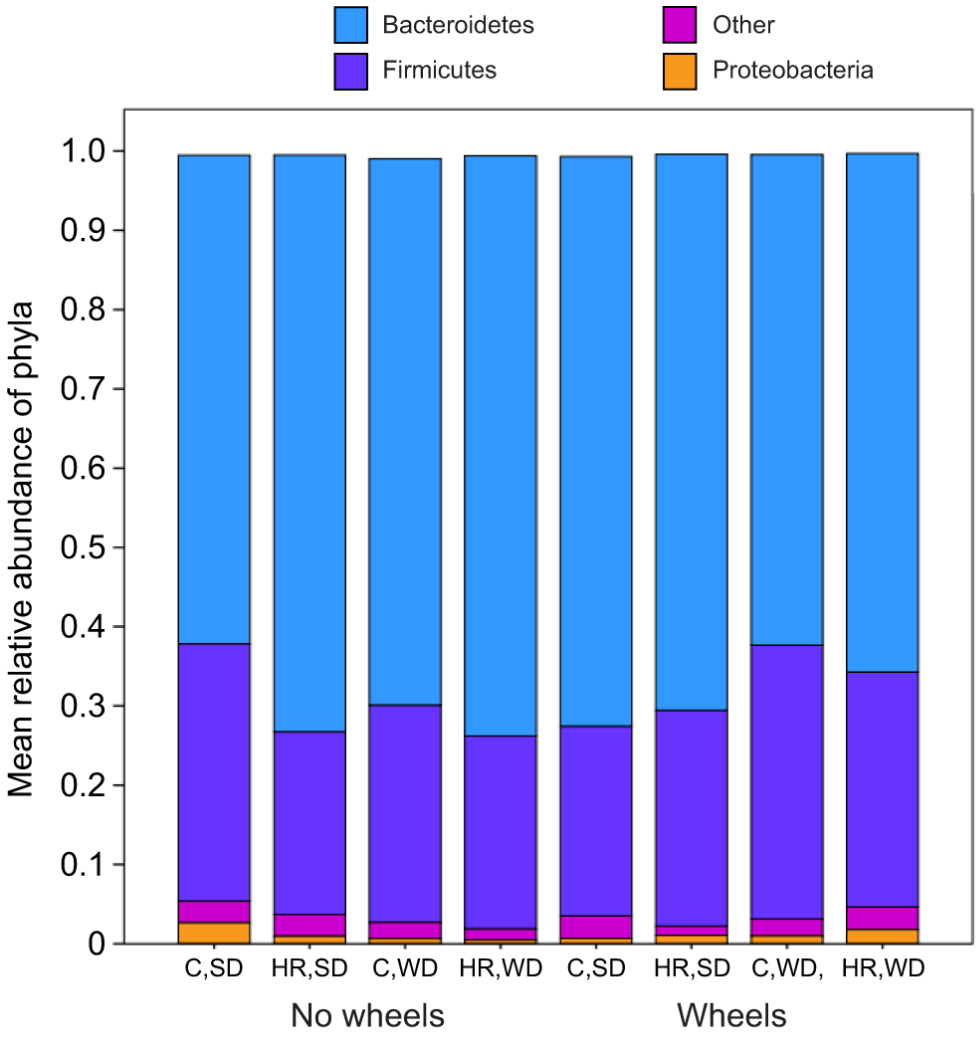


Fig. 3.3. Community composition of the adult gut microbiome for all experimental mice (*N*=149). Bars represent the mean relative abundance of the three main phyla found in greater than 1% of the population, separated by treatment group. C, control; HR, high running; SD, standard diet; WD, Western diet.

**Figure 3.4**

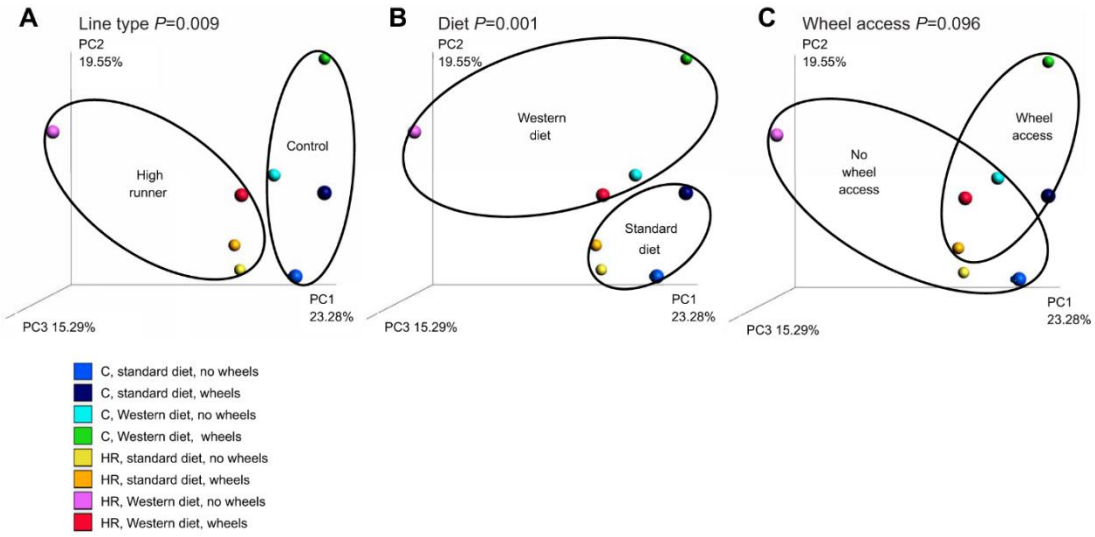


Fig. 3.4. Community membership of the adult gut microbiome Principal Coordinate Analysis (PCoA) using unweighted UniFrac distances. **A.** Clustering of mice by High Runner (*N*=72) and Control (*N*=77) lines of mice (PERMANOVA, $F_{1,147}$ = 1.56, $R^2$ = 0.010, *P*=0.009). **B.** Clustering of mice by Western diet (*N*=77) and Standard diet (*N*=72) (PERMANOVA, $F_{1,147}$ = 2.72, $R^2$ = 0.018 *P*=0.001). **C.** Clustering of mice by wheel access (*N*=75) and no wheel access (*N*=74) (PERMANOVA, $F_{1,147}$ = 1.24, $R^2$ = 0.008 *P*=0.096). Results of statistical analyses are shown in Table 1.

**Figure 3.5**

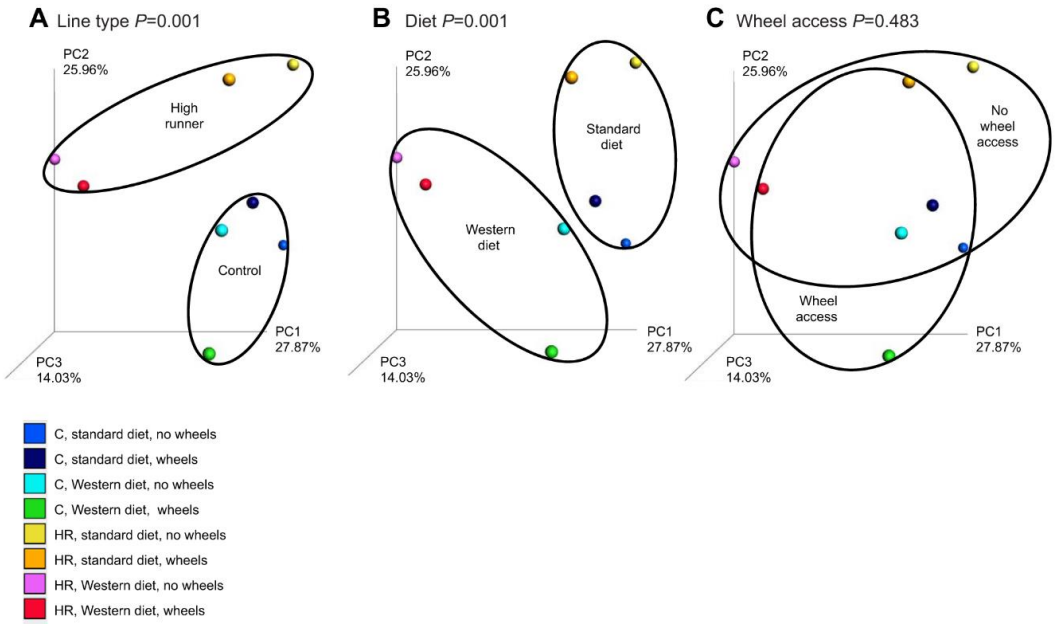


Fig. 3.5. Community membership of the adult gut microbiome PCoA using a Hellinger distance matrix. **A.** Clustering of mice by High Runner (*N*=72) and Control (*N*=77) lines of mice (PERMANOVA, $F_{1,147} = 2.31$, $R^2 = 0.015$, *P*=0.001). **B.** Clustering of mice by Western diet (*N*=77) and standard diet (*N*=72) (PERMANOVA, $F_{1,147} = 2.85$, $R^2 = 0.019$, *P*=0.001). **C.** Clustering of mice by wheel access (*N*=75) and no wheel access (*N*=74) (PERMANOVA, $F_{1,147} = 0.99$, $R^2 = 0.007$, *P*=0.483). Results of statistical analyses are shown in Table 2.

## Figure 3.6

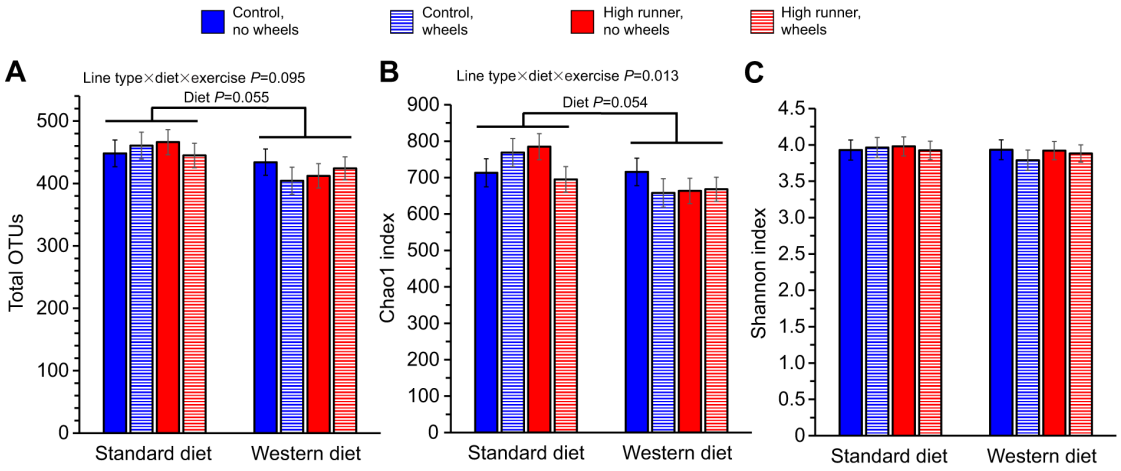
Control, no wheels
Control, wheels
High runner, no wheels
High runner, wheels
A
Line type×diet×exercise P=0.095
Diet P=0.055
Total OTUs
0
100
200
300
400
500
Standard diet
Western diet
B
Line type×diet×exercise P=0.013
Diet P=0.054
Chao1 index
0
100
200
300
400
500
600
700
800
900
Standard diet
Western diet
C
Shannon index
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Standard diet
Western diet

Fig. 3.6. Alpha diversity metrics of the adult gut microbiome (*N*=149 mice). Data are presented as untransformed least squares means ± s.e.m. **(A).** Total operational taxonomic units (OTUs) when the OTU table was rarified to an even number of reads per sample. The three-way interaction between juvenile diet, exercise, and linetype on fecal bacterial richness was significant (2-tailed ANOVA interaction, $F_{1,128} = 2.83$, $P=0.095$, not adjusted for multiple comparisons). Early life exposure to Western diet tended to have a lasting impact on gut microbiome diversity by reducing the total OTUs (2-tailed ANOVA, $F_{1,6} = 5.67$, $P=0.055$, not adjusted for multiple comparisons). **(B).** Chao1 Index. The three-way interaction between Western diet, exercise, and linetype was statistically significant (2-tailed ANOVA interaction, $F_{1,128} = 6.39$, $P=0.013$, not adjusted for multiple comparisons). Early life exposure to Western diet tended to have a lasting impact on the gut microbiome by reducing adult gut community richness (2-tailed ANOVA, $F_{1,6} = 5.68$, $P=0.054$, not adjusted for multiple comparisons). **(C).** The Shannon Index was not significantly affected by any experimental factor.

**Figure 3.7**

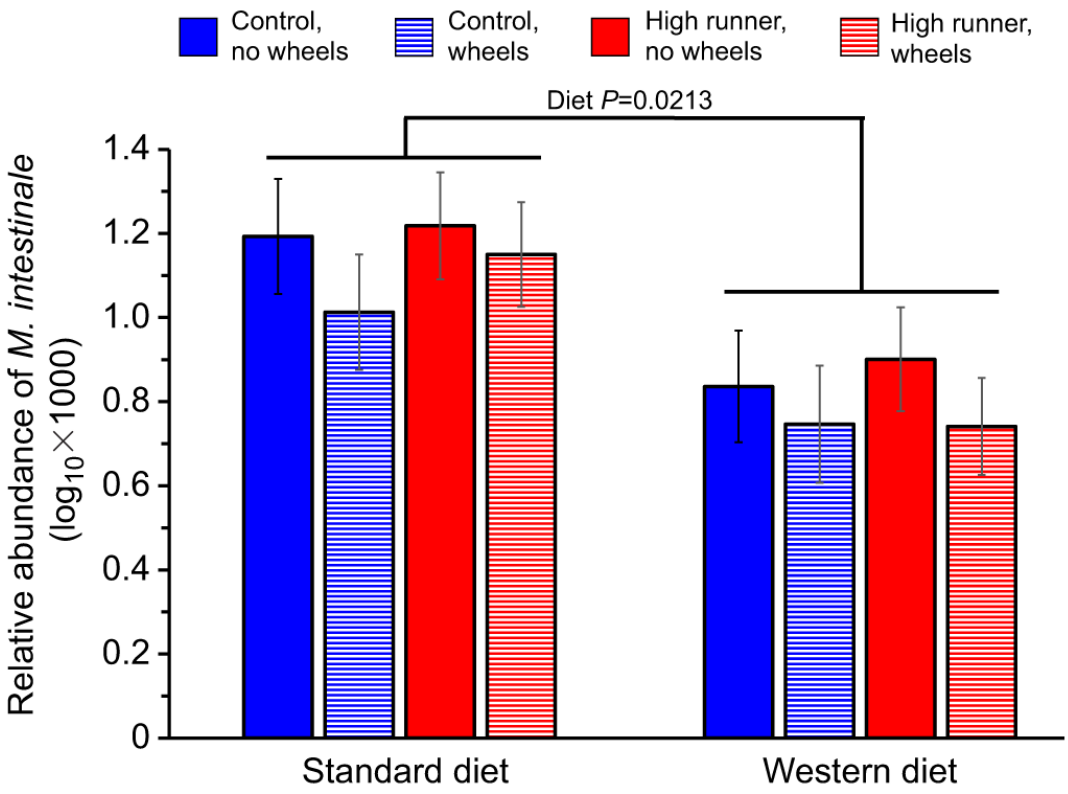


Fig. 3.7. Relative abundance of the species *Muribaculum intestinale* (*N*=149 mice). Data are presented as transformed least squares means ± s.e.m. Mice with juvenile exposure to Western diet had a significantly lower relative abundance of the species *M. intestinale.* (2-tailed ANOVA, $F_{1,128}$ = 19.2; FDR adjusted *P*=0.0213).

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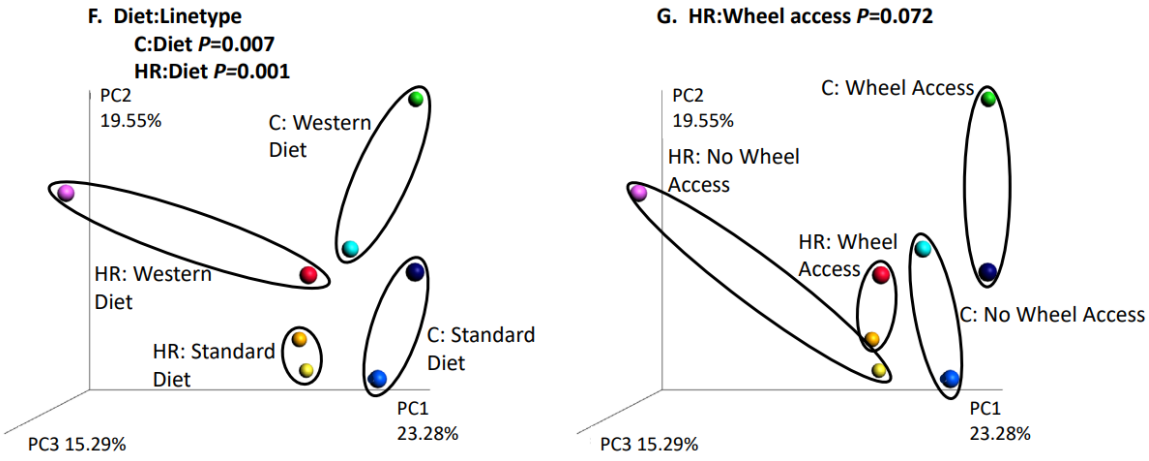
# Appendices

## Appendix 3.1

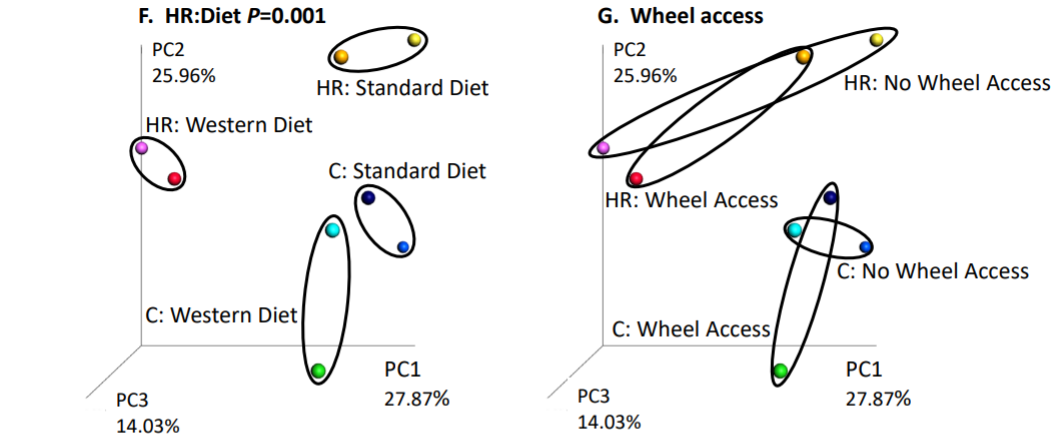



H.

| | Figure | Sum of Squares | D. F. | F | $R^2$ | P |
|---|---|---|---|---|---|---|
| **Linetype** | **4A** | **0.213** | **1, 147** | **1.560** | **0.010** | **0.009** |
| **Diet** | **4B** | **0.369** | **1, 147** | **2.719** | **0.018** | **0.001** |
| Wheel Access | 4C | 0.170 | 1, 147 | 1.243 | 0.008 | 0.096 |
| **C:Diet** | **4B, Sup. F** | **0.225** | **1, 75** | **1.644** | **0.021** | **0.007** |
| **HR:Diet** | **4B, Sup. F** | **0.328** | **1, 70** | **2.462** | **0.034** | **0.001** |
| C:Wheel Access | 4C, Sup. G | 0.116 | 1, 75 | 0.838 | 0.011 | 0.832 |
| HR:Wheel Access | 4C, Sup. G | 0.176 | 1, 70 | 1.304 | 0.018 | 0.072 |

Appendix 3.1. F. Community membership of the adult gut microbiome Principal Coordinate Analysis using unweighted UniFrac distances. Clustering of mice by HR:WD (N=38), HR:SD (N=34), C:WD (N=39), and C:SD (N=38). G. Clustering of mice by HR:Wheel access (N=38), HR:No wheel access (N=34), C:Wheel access (N=37), H:No wheel access (N=40). H. Statistical results.

## Appendix 3.2




H.

| | Figure | Sum of Squares | D. F. | F | $R^2$ | P |
|---|---|---|---|---|---|---|
| **Linetype** | **5A** | **1.150** | **1, 147** | **2.310** | **0.015** | **0.001** |
| **Diet** | **5B** | **1.414** | **1, 147** | **2.851** | **0.019** | **0.001** |
| Wheel Access | 5C | 0.497 | 1, 147 | 0.989 | 0.007 | 0.483 |
| C:Diet | 5B, Sup. F | 0.534 | 1, 75 | 1.043 | 0.014 | 0.384 |
| **HR:Diet** | **5B, Sup. F** | **1.753** | **1, 70** | **3.783** | **0.051** | **0.001** |
| C:Wheel Access | 5C, Sup. G | 0.385 | 1, 75 | 0.749 | 0.010 | 0.843 |
| HR:Wheel Access | 5C, Sup. G | 0.458 | 1, 70 | 0.951 | 0.013 | 0.518 |

Appendix 3.2.F. Community membership of the adult gut microbiome Principal Coordinate Analysis using a Hellinger distance matrix. Clustering of mice by HR:WD (N=38), HR:SD (N=34), C:WD (N=39), and C:SD (N=38). G. Clustering of mice by HR:Wheel access (N=38), HR:No wheel access (N=34), C:Wheel access (N=37), C:No wheel access (N=40). H. Statistical results.

**Appendix 3.3**

| Early-life Trait | N | D.F. | $P_{linetype}$ | $P_{exercise}$ | $P_{diet}$ | $P_{diet\ x\ linetype}$ | $P_{diet\ x\ exercise}$ | $P_{exercise\ x\ linetype}$ | $P_{diet\ x\ linetype\ x\ exercise}$ | $P_{body\ mass}$ | $P_{mini\text{-}muscle}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Week 1 Revolutions/day | 88 | 6. 76 | 0.0854 | NA | **<0.0001** | **0.0072** | NA | NA | NA | NA | **0.0156** |
| Week 2 Revolutions/day | 88 | 6, 76 | **0.0320** | NA | **0.0188** | 0.4765 | NA | NA | NA | NA | 0.9060 |
| Week 3 Revolutions/day | 88 | 6, 80 | **0.0006** | NA | 0.2848 | 0.4950 | NA | NA | NA | NA | **0.3800** |
| Week 1 Caloric Intake | 165 | 6, 143 | 0.7158 | 0.7553 | **<0.0001** | 0.2983 | **<0.0001** | 0.2399 | 0.5121 | **<0.0001** | 0.9206 |
| Week 2 Caloric Intake | 165 | 6, 137 | 0.0658 | **0.0024** | **0.0009** | 0.2030 | 0.2941 | 0.3391 | 0.1514 | **<0.0001** | **0.0199** |
| Week 3 Caloric Intake | 164 | 6, 136 | 0.3158 | **<0.0001** | 0.8676 | 0.8806 | 0.3842 | 0.0881 | 0.5950 | **<0.0001** | **0.0274** |

Appendix 3.3. P values from analyses of juvenile wheel running and caloric intake. Tests for main and interactive effects on juvenile wheel running and caloric intake. For wheel running, a measure of wheel freeness was included but was not significant (results not shown); for caloric intake, body mass was included as a covariate. Significance levels (P values; bold indicates P<0.05, two-tailed, unadjusted for multiple comparisons).

# CHAPTER 4

Oral antibiotics reduce voluntary exercise behavior in athletic mice

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**Abstract**

The gut microbiome can affect various aspects of both behavior and physiology, including exercise ability, but effects on voluntary exercise have rarely been studied. We studied females from a selection experiment in which 4 replicate High Runner (HR) lines of mice are bred for voluntary exercise and compared with 4 non-selected control (C) lines. HR and C mice differ in several traits that likely interact with the gut microbiome, including higher daily running distance, body temperatures when running, spontaneous physical activity when housed without wheels, and food consumption. After two weeks of wheel access to reach a stable plateau in daily running, mice were administered broad-spectrum antibiotics for 10 days. Antibiotic treatment caused a significant reduction in daily wheel-running distance in the HR mice (−21%) but not in the C mice. Antibiotics did not affect body mass or food consumption in either HR or C mice, and we did not observe sickness behavior. Wheel running by HR mice did not recover during the 12 days following cessation of antibiotics. The decreased wheel-running in HR but not C mice, with no apparent negative side effects of antibiotics, suggests that the HR microbiome is an important component of their high-running phenotype.

## 1. Introduction

Mammals have evolved in a world dominated by bacteria, and their gut microbiome has become an essential component of the host's body (McFall-Ngai et al., 2013; Milani et al., 2020). The gut microbiome is crucial for numerous aspects of host biology, including digestion, metabolism, and immune function (Dominguez-Bello et al., 2019; Gilbert et al., 2018; Kohl and Carey, 2016). Considerable evidence indicates that mammalian hosts and their gut microbiomes have coevolved (Ley et al., 2008; Zaneveld et al., 2008), but the microbiome is also influenced by numerous environmental factors on both acute and chronic time scales (Koskella and Bergelson, 2020).

Numerous studies demonstrate that environmental alterations to the normal gut microbiome can affect the behavior and physiology of mammalian hosts (Cryan and Dinan, 2014; Fontaine and Kohl, 2020). These effects on the host may occur through multiple mechanisms, including afferent and efferent signals between the gastrointestinal tract and the brain (via the vagus nerve), that may be influenced by the gut microbiome (Fülling et al., 2019). In addition, bacterial-derived metabolites can be important energy sources for the host (Krautkramer et al., 2021; Rowland et al., 2018). Furthermore, gut microbes can influence the host through their effect on hormonal and immunological pathways (Clarke et al., 2014; Eshleman and Alenghat, 2021).

The influence of gut microbes on behavior has been demonstrated primarily by studies of microbiome depletion via antibiotic treatment and

transplants into germ-free mice (Bercik et al., 2011; Leclercq et al., 2017; Luo et al., 2018; Marin et al., 2017; Sudo et al., 2004; Tochitani et al., 2016). For example, BALB/c mice have lower levels of exploratory behavior than NIH Swiss Webster mice under both specific-pathogen free conditions and in the germ-free state (Bercik et al., 2011). Reciprocal transplants into germ-free BALB/c and NIH Swiss mice resulted in "transplanted behavior." Germ-free NIH Swiss mice colonized with BALB/c microbiota had decreased exploratory behavior in a step-down test, and germ-free BALB/c mice colonized with NIH Swiss microbiota had increased exploratory behavior, compared to baseline (Bercik et al., 2011).

Voluntary exercise behavior, defined as locomotion that is not motivated by any external factor or required for survival (Garland et al., 2011), plays a key role in mammalian health (Hawley et al., 2014; Heinonen et al., 2014; Silverman and Deuster, 2014). Although many studies have shown that exercise can affect the gut microbiome (Campbell and Wisniewski, 2017; Mailing et al., 2019; Mohr et al., 2020), how the microbiome affects exercise behavior is poorly understood (Crowson and McClave, 2020; Hughes, 2020; Oyanagi et al., 2018). The expression of voluntary exercise, as with all voluntary behaviors, will depend on intrinsic motivational state, but may also be limited by physical abilities (e.g. see Garland et al., 2011; Good et al., 2015; Jaromin et al., 2019; Kelly et al., 2014; Lightfoot et al., 2018).

One way that the gut microbiome might affect motivation is by altering signals from the mammalian gastrointestinal tract to the brain (Fülling et al., 2019). The gut microbiome has also been suggested to regulate reward processes in the brain (García-Cabrerizo et al., 2021). Therefore, given that wheel-running can be a self-rewarding, motivated behavior in rodents (Garland et al., 2011; Novak et al., 2012; Sherwin, 1998), changes in the gut microbiome community have the potential to impact motivation to exercise.

One mechanism by which the microbiome might affect exercise ability is through bacterial-derived metabolites (Bindels and Delzenne, 2013; Grosicki et al., 2018; Ticinesi et al., 2017). Bacterial symbionts produce short-chain fatty acids (SCFAs) from the fermentation of carbohydrates in the gastrointestinal tract, which can travel through circulation and affect skeletal muscle performance (Przewłócka et al., 2020). Butyrate, one of the three most abundant SCFAs, is associated with increased epithelial cell wall integrity and increased glucose uptake in skeletal muscle (Ticinesi et al., 2019). In addition to altering the gut microbiome community, antibiotics have been shown to decrease luminal butyrate concentration and decrease serum glucose levels in C57BL/6 mice, resulting in a shift to glucose instead of butyrate as the gut epithelial cell energy source (Zarrinpar et al., 2018). Alterations in host energy substrate availability could affect exercise behavior. Furthermore, as noted in the Discussion, two studies have demonstrated that antibiotic treatment affects

treadmill endurance capacity and muscle physiology in mice (Nay et al., 2019; Okamoto et al., 2019).

In rodent models, voluntary exercise is generally measured as voluntary wheel-running behavior (Garland et al., 2011). Although a few studies have examined the effects of antibiotics on mouse activity in an open-field arena (Ceylani et al., 2018; Diaz Heijtz et al., 2011), this type of test has little to do with voluntary exercise behavior or spontaneous physical activity in home cages (Careau et al., 2012; Novak et al., 2012; Zombeck et al., 2011). To our knowledge, only one study has tested effects of the microbiome on voluntary wheel running. Male C57BL/6 N mice were given 1 week of antibiotic treatment, followed by 7 weeks of a high-fat diet, then transplanted with cecal contents from either sedentary mice or mice with 12 weeks of wheel access, and finally provided with 7 days of wheel access (Oyanagi et al., 2018). Mice transplanted with microbiomes from mice with exercise exposure had higher levels of wheel running compared to the mice colonized with microbiomes from sedentary mice.

The purpose of the present study was to test for effects of eliminating the gut microbiome on voluntary exercise behavior. To increase statistical power (see below), we used mice from an ongoing artificial selection experiment that breeds for high voluntary wheel running over multiple generations. Mice from the 4 replicate High Runner (HR) and 4 non-selected Control (C) lines have several key physiological and behavioral differences, suggesting their gut microbes are likely to be different under baseline conditions and might also respond differently

to antibiotics. Over 89 generations of selective breeding, the HR mice have evolved to run ~3-fold more revolutions per day than C mice (Cadney et al., 2021a; Cadney et al., 2021b; Careau et al., 2013; Copes et al., 2015; McNamara et al., 2021; Swallow et al., 1998). In addition to increased physical activity, HR mice have higher food consumption (for their body size), higher body temperature when active, higher activity levels when housed without wheels, and altered hormone levels (Copes et al., 2015; Malisch et al., 2009; Swallow et al., 2009), all of which could affect the gut environment acutely, as well as alter the selective regime experienced by symbiotic microbes.

As the HR mice run near their physiological limits (Claghorn et al., 2017; Girard et al., 2001; Rezende et al., 2005), dysbiosis of their gut microbiome and possible associated reduction in energy substrate availability has the potential to adversely affect wheel-running behavior. In addition, HR mice appear to have higher motivation for wheel running than C mice (e.g. see Belke and Garland, 2007; Garland et al., 2016; Rhodes et al., 2005; Saul et al., 2017), which could be affected by antibiotic treatment. In a previous study, we found differences in the gut microbiome between adult HR and C mice (McNamara et al., 2021). As discussed in our previous paper, the HR microbiome might have coevolved after many generations of selective breeding and/or it might reflect acute effects of exercise. In either case, we hypothesized that reduction of the gut microbiome would have a larger adverse effect on the amount of wheel running in HR mice than in C mice.

## 2. Materials and methods

All procedures were approved by the Institutional Animal Care and Use Committee of the University of California, Riverside.

### 2.1 Experimental design

Mice were sampled from generation 89 of an ongoing experiment in which 4 replicate High Runner (HR) lines are selectively bred for voluntary wheel-running behavior and compared with 4 replicate non-selected Control (C) lines. Briefly, the selection experiment began in 1993 with 224 outbred, genetically variable Hsd:ICR strain mice (Swallow et al., 1998). Mice were randomly mated for 2 generations and then randomly assigned into 4 replicate HR lines bred for high wheel running and 4 replicate C lines. Each generation, the highest-running HR female and male from each of 10 families are chosen as breeders, based on the average revolutions on days 5 and 6 of a 6-day period of wheel access as young adults (Swallow et al., 1998). The wheels are Wahman-type activity wheels with 1.12 m-circumference, 10-cm wide running surface of 10-mm metal mesh, with clear Plexiglas walls. Breeders in the C lines are chosen without regard to wheel running. Mice are paired outside their family but within their line, and sibling matings are never allowed. Photoperiod is 12:12 L:D and temperature is ~22 °C. All mice receive Standard Laboratory Rodent Diet (SD) from Harlan Teklad (W-8604), which contains 24.3% kJ from protein, 4% kJ from fat, and 40.2% from

carbohydrate. Pregnant dams are given Harlan Teklad Lab mouse breeder diet [S-2335] 7004 through weaning.

For the present experiment, 12-16 females (sampled from as many different families as possible) from each of the 4 replicate HR and 4 C lines were weaned at 21 days of age. We chose female mice, rather than both sexes, to avoid aggressive behavior in males when cohoused (Kappel et al., 2017). Mice were housed individually for < 1 week, then randomly cohoused in groups of four (including mixing of the HR and C lines in an attempt to homogenize the gut microbiome, as mice are coprophagic (Laukens et al., 2016)). Each week, mice were again randomized into clean cages, and this occurred three times (Fig. 1).

At ~7 weeks of age, mice were weighed and placed into individual cages with wheels (same as used in the routine selective breeding protocol) for two weeks to allow daily running distances to reach a stable plateau (Copes et al., 2015; Thompson et al., 2018). Numerous previous studies in the HR mice have shown that the wheel running plateau remains for several weeks (Dumke et al., 2001; Meek et al., 2012; Swallow et al., 1999). After two weeks, mice were weighed, fecal samples were collected, and all mice were given a broad-spectrum antibiotic cocktail in the drinking water to greatly reduce the gut microbiome. The antibiotic cocktail contained 1 g/L ampicillin, 1 g/L neomycin, 125 mg/L vancomycin, and 2.5 g/L Splenda to increase cocktail palatability (similar to Ichinohe et al., 2011; Rakoff-Nahoum et al., 2004; Reikvam et al., 2011). The antibiotic treatment lasted for 10 days; body mass, antibiotic water

consumption, and food consumption were measured at the start and end of treatment. After completion of the antibiotic treatment, 23 mice were randomly removed for a separate experiment, another fecal sample was collected, and the mice were given sterile water for 3 days to washout the antibiotics. Mice were then provided with tap water for 9 days to allow the gut microbiome to naturally "recover" (either from environmental sources or through bacteria present in the gut). Wheel access continued for 12 days after the cessation of antibiotic treatment (Fig. 1).

**2.2 Wheel running**

As noted above, mice were housed individually in home cages with attached wheels. Sensors attached to the wheel record the daily number of revolutions in each 1-minute interval during a 23 hr measurement period (Swallow et al., 1998). Following our previous studies, we calculated the total number of revolutions per day (distance), the number of 1-minute intervals with at least one revolution (duration of activity), the mean running speed (revolutions/interval), and the maximum number of revolutions observed for any 1-minute interval. Before and after housing mice with wheels, we measured wheel freeness by recording the number of revolutions per wheel until it reaches a stop after accelerating each wheel to a constant speed (Copes et al., 2015).

### 2.3 Spontaneous physical activity

As in previous studies, each home cage was fitted with an infrared sensor (Talon TL-Xpress-A; Crow Electronics, Fort Lee, NJ) connected to a digital I/O board (ICS 2313; ICS Electronics, Pleasanton, CA), and a Macintosh computer with custom software (Copes et al., 2015). The sensors have an ~90° field of view with a reset time of 1-2 seconds if no motion is detected. The sensor software takes readings ~3 times per second, recording movement detection as a “1” and no movement detection as a “0”. For each minute a mean value (0-1) is computed, and the software saves mean values every 10 min. To calculate the total home-cage activity, or "spontaneous physical activity" (Acosta et al., 2015; Garland et al., 2011), we summed the total activity recorded in a 23 hr measurement period. We also tallied the number of 1-minute intervals during which motion was detected (duration of activity) and used this to estimate the mean intensity of activity, i.e., the average amount of activity per minute when any home-cage activity was occurring (Copes et al., 2015). We also measured sensor sensitivity with a heated curling iron passed back and forth in front of the sensor 10 times over a period of 10 s.

### 2.4 Food and water consumption

Average daily water consumption during the initial wheel access (seven days) and during antibiotic treatment (ten days) was calculated by weighing the water bottle at the start and end of the period. We had 4 control water bottles to

account for water bottle leakage. Average daily food consumption was calculated in a similar fashion to water consumption, by weighing food hoppers with due allowance for wastage (Koteja et al., 2003).

### 2.5 Fecal sampling and plating

To confirm reduction of the gut microbiome, we determined the number of colony-forming units (CFUs) for a subset of mice from a baseline sample (N = 38) collected after two weeks of wheel access and a post-antibiotic treatment fecal sample from the same subset of mice. Mice were placed into clean, but not completely aseptic, individual cages until defecation. Fecal pellet mass was determined by weighing sterile tubes before and after collection of the pellet. Pellets were suspended in 500 µL sterile phosphate-buffered saline (PBS) using a BeadBeater for 30 s at 1400 rpm. 5 µL of the fecal suspension was plated on Luria Bertani media in a serial dilution to $10^{-6}$ (two per mouse) and incubated both aerobically and anaerobically at 37 °C. After a 24 hr incubation, the colonies were counted and the colony-forming units calculated by dividing the number of colonies per mL plated by the total dilution factor.

### 2.6 Statistical analyses

We used linear mixed models to determine the effects of genetic background (High Runner versus Control linetype), antibiotic treatment (pre-antibiotic versus post-antibiotic), and their interaction on wheel running, spontaneous physical

activity, body mass, water consumption, and food consumption. Following numerous previous studies of these mice, the effect of linetype was tested relative to the variance among replicate lines, which are a nested random effect within linetype, with 1 and 6 d.f. (SAS Procedure Mixed with repeated measures, SAS Institute, Cary, NC, USA) (e.g., Acosta et al., 2015; Swallow et al., 1998). The effect of antibiotics and the antibiotic × linetype interaction were also tested with 1 and 6 d.f. Outliers were removed when standardized residuals were greater than approximately 3 and we accepted nominal statistical significance at $p<0.05$. Data are presented as untransformed least squares means and standard errors. Such covariates as body mass, wheel freeness (results not shown), and home-cage sensor sensitivity (results not shown) were included in the models where appropriate. For food and water consumption statistical tests were run both with and without the amount of wheel running as a covariate (e.g., see Copes et al., 2015; Hiramatsu and Garland, 2018).

A measure of effect size (in this case, Pearson's r) was calculated for all main and interactive effects. We chose Pearson's r because of (1) it's ease of use (in this case requiring only an F-statistic and d.f.) and (2) it's ease of interpretation (r family effect sizes are measures of the strength of association). For a brief primer on effect sizes and why to include them, see Rosenthal (1994), Sullivan and Feinn (2012), and Lakens (2013).

## 3. Results

### 3.1 Depletion of the gut microbiome

Prior to antibiotic treatment, plates averaged $4.18 \times 10^6$ aerobic colony-forming units, with no statistical difference between High Runner (N = 18) and Control (N = 20) mice (ANOVA, $F_{1,6}$ =0.32, $p$ = 0.5905). Ten days of antibiotic treatment reduced the colonies to non-detectible amounts for both linetypes. Anaerobic plates were not usable due to technical issues.

### 3.2 Wheel running

In a repeated-measures analysis, once a wheel-running plateau was attained (Fig. 2A: days 11–13), HR mice ran ~3.5-fold more than C mice, then antibiotic treatment reduced the number of revolutions run per day (days 22–24) in only the HR mice (Fig. 3A: linetype × antibiotic treatment interaction $F_{1,6}$ =15.60, $p$ = 0.0075; differences of least squares means $p$ = 0.0023 for HR mice and $p$ = 0.2071 for C mice). Antibiotic treatment reduced the number of minutes mice ran per day (Fig. 3B) for both linetypes ($F_{1,6}$ =60.79, $p$ = 0.0002; differences of least squares means $p$ = 0.0018 for HR mice and $p$ = 0.0012 for C mice). The linetype × antibiotic interaction approached significance for average running speed and had a large effect size (Fig. 3C: $F_{1,6}$ =4.92, $p$ = 0.0683, r =0.6712), with a slight tendency for a decreased running speed in the HR lines, and increased running speed in the C lines (differences of least squares means $p$ = 0.1925 for HR mice

and $p$ = 0.1209 for C mice). However, HR mice continued to run faster than C mice, both before and during antibiotics (Fig. 3C and 3D).

After antibiotic treatment stopped, mice remained in home cages with wheel access and regular food and drinking water for 12 days (Fig. 2B). Halting antibiotics had no statistical effect on the daily running distance, number of minutes mice ran per day, average speed, or maximum running speed (Fig. 4). In particular, the daily wheel-running distance of the HR mice did not recover to levels before antibiotics (Fig. 4A: differences of least squares means $p$ = 0.0004 for HR mice before antibiotics versus after recovery). During the recovery period, the HR mice ran ~2.7-fold more revolutions per day than C mice (Fig. 4A), which was mainly related to their faster running (Fig. 4C and 4D).

### 3.3 Spontaneous physical activity measured in home cages

In a repeated-measures analysis using the same experimental days before (days 11–13) and during (days 22–24) antibiotic treatment as above, antibiotic treatment tended to decrease total cage activity in C mice, although the interaction did not reach significance (Appendix 4.1: $F_{1,6}$ =3.55, $p$ = 0.1084, r =0.6097; differences of least squares means $p$ = 0.0576 for C mice and $p$ = 0.4914 for HR mice). This decrease in total cage activity for C mice was partially explained by a trend for reduced activity duration in C mice but not in HR mice (Appendix 4.1: $F_{1,6}$ =3.79, $p$ = 0.0996, r =0.6222; differences of least squares means $p$ = 0.0063 and $p$ = 0.5649, respectively). Antibiotics tended to increase

mean intensity of activity (total/minutes) in HR mice, but not in C mice (Appendix 4.1: $F_{1,6}$= 2.85, $p$ = 0.1425; differences of least squares means $p$ = 0.7159 for C mice and $p$ = 0.1166 for HR mice). Data were not available for the period of recovery from antibiotics.

### 3.4 Body mass

Consistent with previous studies (Kelly et al., 2017; Meek et al., 2009; Thompson et al., 2018), HR females tended to weigh less than C females for the duration of the experiment (Fig. 5), but the difference never reached statistical significance (all p > 0.2000). Separate repeated-measures analysis of mass before versus after 10 days of antibiotics indicated mice were larger after antibiotic treatment ($F_{1,6}$ =22.68, $p$ = 0.0031). Analysis of mass after 10 days of antibiotics versus after 12 days of recovery indicated no significant difference ($F_{1,6}$ = 0.01, $p$ = 0.9321).

### 3.5 Food and water consumption

With body mass as a covariate, antibiotics had no effect on food consumption for C mice, but HR mice consumed less food during antibiotic treatment (days 15–24) than pre-antibiotics (days 8-14) (Fig. 6A: linetype × antibiotic treatment interaction $F_{1,6}$ = 6.24, $p$ = 0.0467). HR mice also consumed significantly more food than C mice during both the pre-antibiotic and antibiotic periods ($F_{1,6}$ = 9.15, $p$ = 0.0232). When the amount of wheel running (revs/day) was added as a covariate, it was a highly significant predictor of food consumption (Fig. 6B, $F_{1,166}$

=61.23, $p < 0.0001$) and the effects of linetype ($p = 0.9365$), as well as the linetype × antibiotic interaction, became non-significant ($p = 0.2854$).

Accounting for body mass, both linetypes drank significantly more of the antibiotic water during experimental days 15-24 compared to regular water during days 8-14 ($F_{1,6} = 24.76$, $p$=0.0025; Fig. 7A). With wheel running as an additional covariate (Fig. 7B, $F_{1,166}$ =11.38, $p = 0.0009$), the effect of antibiotics was still highly significant ($F_{1,6}$ =33.46, $p = 0.0012$).

**4. Discussion**

The antibiotic treatment we administered greatly reduced the gut microbiome in both High Runner and Control lines, based on aerobic plating. Although we were not able to show the effects of antibiotics on anaerobic bacteria, previous antibiotic cocktails that included ampicillin, streptomycin, and neomycin in combination with other antibiotics have shown a successful decrease or depletion of anaerobic colonies (Carvalho et al., 2012; Castro-Mejía et al., 2018; Reikvam et al., 2011). Ten days of antibiotic treatment significantly reduced daily wheel-running distance in selectively bred HR lines of mice, but not in their non-selected C counterparts (Fig. 3). Moreover, mice from the HR lines did not recover to pre-antibiotic treatment levels of wheel running during 12 days of recovery (Fig. 4). Based on body mass, food consumption, and behavioral observations, antibiotic treatment did not appear to cause sickness behavior (Fig. 5, 6).

### 4.1 Antibiotic treatment alters wheel-running behavior

Broad-spectrum antibiotic treatment significantly reduced the distance of daily wheel running by the HR lines (-21%), but had no statistical effect on C lines (Fig. 3A). At a more granular level, antibiotics significantly reduced the duration of daily running in both HR and C lines (Fig. 3B), but in the latter the average running speed increased slightly (Fig. 3C), such that daily running distance was not significantly affected. Mice from the HR lines did not recover to pre-antibiotic treatment levels of wheel running during 12 days of recovery (Fig. 4).

Reducing the gut microbiome could have altered the motivation and/or ability for sustained, aerobically supported exercise, both of which are higher in HR mice relative to C mice (e.g. see Cadney et al., 2021b; Hiramatsu et al., 2017; Meek et al., 2009; Singleton and Garland, 2019). Previous research has shown that antibiotic treatment can affect exercise ability in mice. Three weeks of antibiotic treatment in male mice of the inbred C57BL/6 strain decreased treadmill endurance capacity, as measured by time to exhaustion during a submaximal test, with no significant effect on body mass, food consumption or water consumption (Nay et al., 2019). Antibiotics also decreased fatigue resistance in an *ex vivo* contractile test of extensor digitorum longus muscle and glycogen content of the gastrocnemius. Eleven days of natural reseeding with soiled bedding from the control group normalized muscle performance and glycogen levels. These results demonstrate the importance of bacterial species

on host optimal skeletal muscle function. In a separate study of male C57BL/6J mice (Okamoto et al., 2019), two weeks of antibiotic treatment reduced treadmill running time, decreased tibialis anterior muscle mass, reduced fecal bacterial density, decreased fecal short-chain fatty acid (SCFA) concentration, and decreased concentration of acetate in plasma. Given that acetate is the dominant SCFA in circulation, the authors speculated it could have the largest influence on exercise tolerance, as any change in acetate concentration would change the potential energy source for muscles during exercise. Continuous administration of acetate for one week increased treadmill running time after the initial impairment of endurance capacity caused by two weeks of antibiotic treatment. Future studies of the HR mice could introduce SCFAs into circulation to test whether this would counter the negative effects of microbiome ablation on wheel running and/or exercise ability.

As noted in the Introduction, the microbiome has been shown to affect various aspects of behavior, and some of the effects likely occur through changes in motivation. More specifically, the gut microbiome has been suggested to play a role in reward circuits (García-Cabrerizo et al., 2021). For example, ileum infusion of antibiotics for 25 days in piglets decreased concentrations of hypothalamic (1.13-fold) and circulating dopamine (1.18-fold) (Gao et al., 2018). The mechanism(s) for antibiotic treatment to modulate brain function and behavior may be in part from influence of antibiotics upon amino acids being absorbed from the gut. Once in circulation, large neutral amino acid

transporters import essential amino acids across the blood-brain barrier to where they become precursors to serotonin and catecholamines, including dopamine (Zaragozá, 2020). A variety of lines of evidence indicate that the motivation/reward system of the HR mice is altered (e.g. see Belke and Garland, 2007; Garland et al., 2016; Rhodes et al., 2005; Saul et al., 2017). Several studies indicate alterations in dopamine signaling in the HR mice (Rhodes et al., 2005; Saul et al., 2017), which could suggest that reduction of the gut microbiome by antibiotic treatment may have altered dopamine signaling in a way that reduced motivation for wheel running. Future studies of the HR mice could use pharmacological manipulation of reward pathways (e.g. Keeney et al., 2012; Rhodes and Garland, 2003; Rhodes et al., 2005) in an attempt to counteract the negative effect of antibiotic treatment on wheel running.

Separating motivation from ability when examining voluntary exercise is challenging. Our data for daily wheel-running distance can be separated into components that estimate the duration of running versus average running speed. We speculate that duration of activity is more affected by motivation, whereas average (and maximum) running speed is more reflective of ability (see also Kay, 2017; Swallow et al., 2009). Antibiotic administration had stronger negative effects on the duration of activity than on speed (Fig. 3). These results suggest that future studies should explore possible effects of microbiome manipulation on brain motivation and reward systems.

### 4.2 Lack of recovery in wheel-running after cessation of antibiotics

Wheel-running behavior of HR mice did not recover to pre-antibiotic levels even 12 days after cessation of antibiotic treatment (Fig. 4). However, it is important to note that we did not attempt to reseed the gut microbiome community after cessation of antibiotics. In a small pilot study with 7-week old female inbred C57BL/6 strain mice, 10 days of antibiotic treatment caused an immediate reduction in the richness of the gut microbiome and altered the overall community structure (Laubitz et al., 2021). The gut microbiome was then allowed to naturally recover with no reseeding of the community. Twenty days after the cessation of antibiotic treatment, 31 genera primarily belonging to the Firmicutes phylum had gone extinct, although gut microbiome diversity and community structure had recovered. In a separate study, “humanized” germ-free mice (transplanted with human microbes) were given daily streptomycin treatment for 5 days (Ng et al., 2019). During the first day of antibiotic treatment there was an initial drastic drop in the aerobic and anaerobic colony-forming units and a decrease in the number of observed species. The initial drop in diversity began to recover during days 2−5 of the antibiotic treatment. Nine days after the cessation of antibiotics the gut microbiome diversity failed to recover to pre-treatment levels, largely driven by a decrease in the Bacteroidetes phylum, while the CFUs returned to normal levels during day 2 of the antibiotic treatment (Ng et al., 2019).

Given the results of Laubitz et al. (2021) and Ng et al. (2019), and in the absence of reseeding, if wheel-running behavior were to recover, then it likely would have taken longer than the 12 days we allowed. In future experiments, we plan to re-seed the HR microbiome with soiled bedding from HR and/or C mice that had not been administered antibiotics, and also allow a longer period of time for possible recovery. In addition, transplant experiments will be used to further explore the contribution of the HR microbiome to their high motivation and/or ability for exercise.

### 4.3 High variability in wheel-running behavior

Mice in general, including HR and C mice, have high day-to-day variation in wheel running behavior (e.g., see Fig. 4 in Acosta et al., 2017). Indeed, this variability has been the subject of previous papers (e.g., see Biro et al., 2018; Eisenmann et al., 2009). In the present study of females, some variation would be related to the estrus cycle, but that should not account for consistent drops on days 6 and 10 that occurred for both HR and C mice (Fig. 2). The cause of those drops is unknown, but could be related to elevated noise levels in the vivarium and increases in humidity when cages are cleaned in a room down the hall. Importantly, the wheel-testing rooms were always left undisturbed, except when we entered to download data once each day.

At first glance, Fig. 2 also suggests that variability seems to have decreased in both HR and C lines when antibiotics were administered. However,

whereas Figure 2 shows all of the days of data for wheel running, we only used days 11-13 before antibiotics, days 22-24 during antibiotics, and days 34-36 during recovery for statistical comparisons. Considering only those days, the variability does not appear much different across the three treatment phases; moreover, the mixed model analyses provided no clear evidence for differences in variability in relation to treatment phase.

### 4.4 Absence of sickness behavior

Protocols in which a broad-spectrum antibiotic cocktail was given to mice in water bottles have sometimes been shown to cause health problems, even within five days (Knoop et al., 2016; Reikvam et al., 2011). For example, BALB/c mice refused to consume the antibiotic cocktail after 4 days, which resulted in a weight loss of greater than 20% compared to baseline (Reikvam et al., 2011). In the present study, antibiotics (with Splenda) did not cause reduced water consumption. In addition, we observed no decrease in body mass (Fig. 5). In fact, body mass increased with antibiotic treatment in both linetypes (Fig. 5). Given that the mice we used in our experiment were only 9 weeks old when antibiotic treatment began, the mice were likely still growing xxlowercase fig. (e.g., see Fig. 4 in Bronikowski et al., 2006), or at least adding fat mass. Furthermore, we observed no decrease in food consumption when analyzed with wheel running as a covariate (Fig. 6B). Finally, daily checks, which included checking for diarrheal symptoms and for any mice clearly unwell or lying prone,

did not reveal any obvious signs of sickness behavior (e.g., see Downs et al., 2012).

### 4.5 Food consumption

Antibiotics decreased food consumption in HR but not C mice (Fig. 6A), similar to the effect on daily wheel-running distance (Fig. 3A). Decreased voluntary exercise
would reduce energy requirements for HR mice (e.g., see Copes et al., 2015; Hiramatsu and Garland, 2018). When the amount of wheel running (revs/day) was used as a covariate, there was no apparent change in food consumption of HR mice before and during antibiotic treatment and no difference between the HR and C lines (Fig. 6B). Together, these results suggest that both the higher food consumption of HR mice in general (e.g., see Copes et al., 2015) and their decreased food consumption during antibiotic treatment are a function of their wheel-running behavior.

### 4.6 Water consumption

With body mass as a covariate, both HR and C mice had a significant increase in water consumption during antibiotic treatment when compared to pre-antibiotic values (Fig. 7). Adding the number of revolutions run per day as a covariate did not change this pattern. One possible explanation for an increase in water consumption during antibiotic treatment is that mice liked the taste of the Splenda

in the antibiotic cocktail, leading them to consume more of the antibiotic cocktail as compared with ordinary tap water. One study using the same artificially selected mouse model found that both HR and C mice always drank more water with Splenda (and with other sweeteners) as compared with plain tap water (Thompson et al., 2018). However, antibiotic-induced kidney injury could also explain the increased water consumption while on antibiotic treatment (Sinha Ray et al., 2016; Yang et al., 2019).

### 4.7 Home-cage activity

The effects of antibiotic treatment on home-cage activity are somewhat puzzling, as only the Control mice seem to have been affected. More specifically, C mice on antibiotics had reduced home-cage activity, caused by a reduction in the number of active minutes in their home cage (Appendix 4.1). These results suggest that if antibiotics are affecting motivation to run (see Section 4.1 above), then they may also be affecting motivation for physical activity in general. But this interpretation fails to explain the lack of an antibiotic effect on home-cage activity in the HR mice.

### 4.8 Limitations

Constraints on available resources did not allow us to include HR and C groups housed with wheel and provided with water supplemented with artificial sweetener but not antibiotics. Such groups would have served as an additional

"control" for the effects of artificial sweeteners per se. However, previously we showed that artificial sweeteners alone did not affect wheel running in either HR or C mice (Thompson et al., 2018). Therefore, we are confident that the effects observed in the present study can be attributed to antibiotics.

We were also unable to sequence the gut microbiome community, which is highly sensitive to both antibiotic and exercise treatments (Blaser, 2016; Mach and Fuster-Botella, 2017; Rosa et al., 2018). Culturing the majority of bacterial species in the gut microbiome is a challenge (see review Lagkouvardos et al., 2017), but we were able to show that 10 days of treatment with a broad-spectrum antibiotic cocktail eliminated aerobic CFUs under these particular conditions. We could not demonstrate the effectiveness of the antibiotic cocktail on the anaerobic community due to technical issues with the plating. As the gut microbiome is primarily composed of anaerobic bacteria, plating of these CFUs would be important in future studies. We were also unable to plate samples after the 12-day recovery period, which we would expect to recover to near baseline levels. Nevertheless, we were able to show a significant change in the wheel-running of HR mice caused by a broad-spectrum antibiotic cocktail. Here, we did not attempt to determine the mechanistic basis of the apparent effect of gut microbiome depletion on wheel running. Future studies involving reseeding of the microbiome, in addition to transplantation of HR and C microbes, are warranted. Also of interest would be studies of standard inbred strains to test for covariation between wheel-running behavior and the microbiome complement

(e.g. see Careau et al., 2012; Carmody et al., 2015; Garland et al., 2016; Kay et al., 2019; Org et al., 2015).

**Figure 4.1**

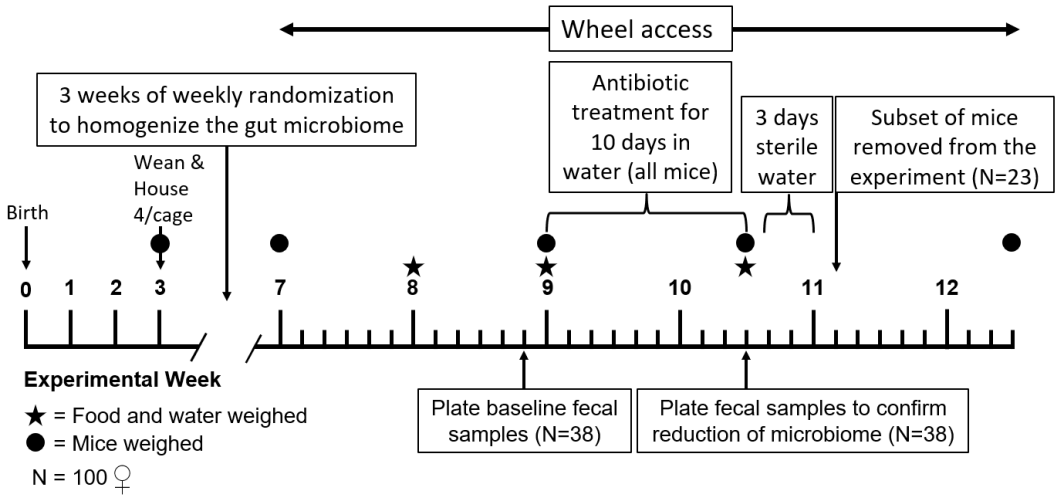



Fig. 4.1 Experimental timeline.

**Figure 4.2**

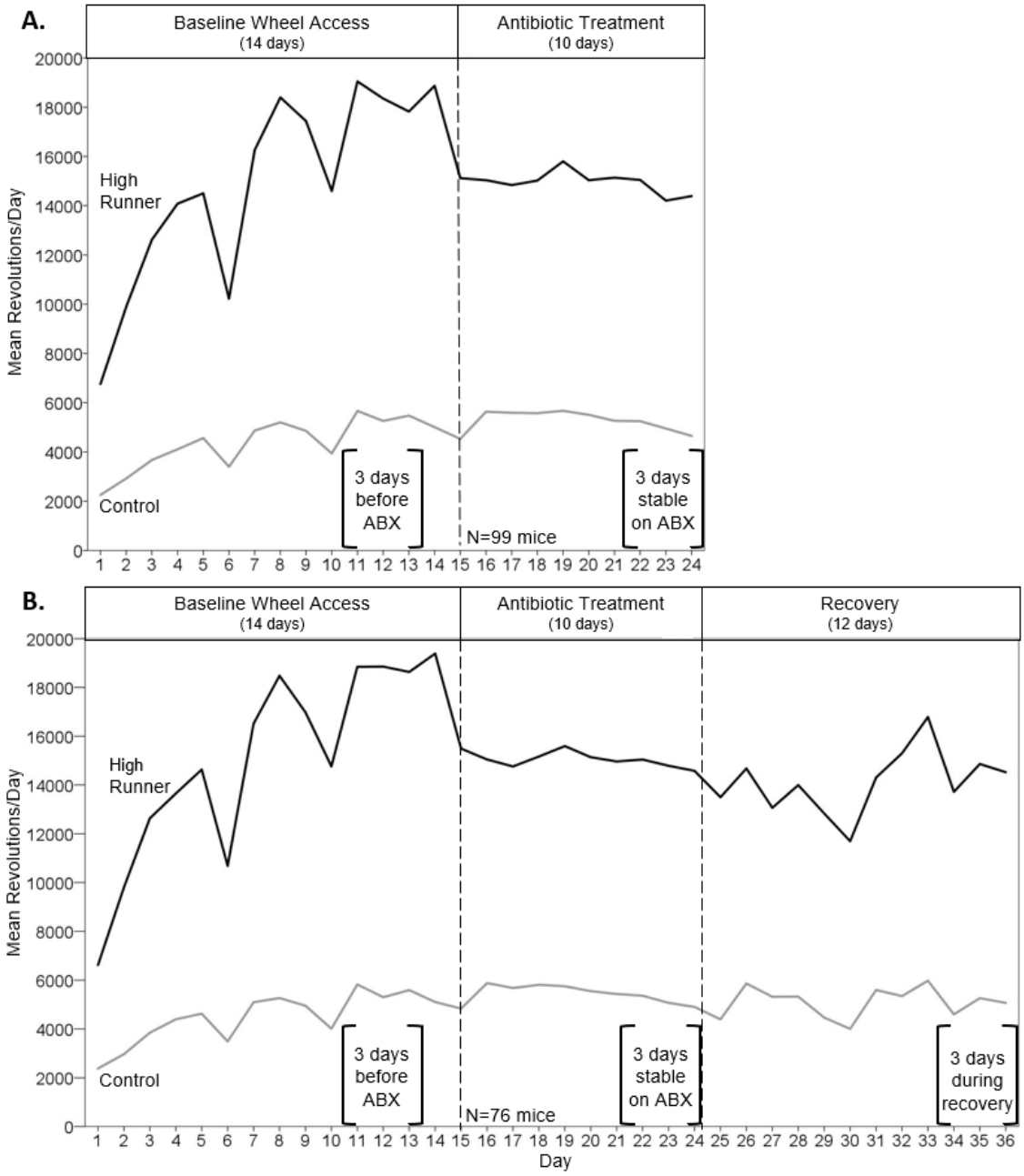

A.
Baseline Wheel Access
(14 days)
Antibiotic Treatment
(10 days)
Mean Revolutions/Day
High Runner
Control
3 days before ABX
3 days stable on ABX
N=99 mice
B.
Baseline Wheel Access
(14 days)
Antibiotic Treatment
(10 days)
Recovery
(12 days)
Mean Revolutions/Day
High Runner
Control
3 days before ABX
3 days stable on ABX
3 days during recovery
N=76 mice
Day

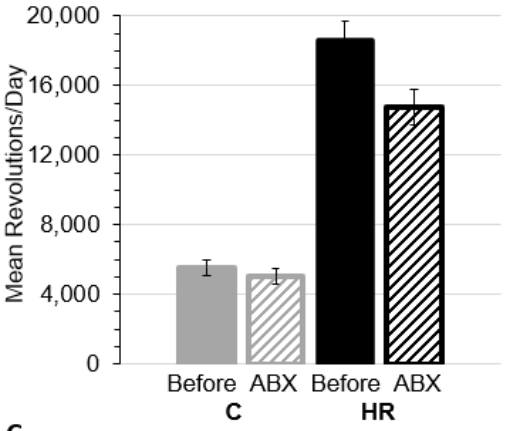
Fig. 4.2. Average daily wheel running (revolutions per 23 h) across the course of the experiment. Values were calculated by taking the simple means of the 4 HR lines and the 4 C lines for each individual day, and then averaging those values to obtain the average for the HR and C lines. Data were truncated on days 8, 14, and 25 (~20 h) when the water bottles were being changed. Brackets indicate days that were used in statistical analyses. See Appendix 4.2 for wheel running mean revolutions per day for each of the 4 HR and 4 C lines. **A.** N = 99 mice, through day 24 of the experiment. Days 11-13 before antibiotics were compared with days 22-24 during antibiotics. **B.** N = 76 mice, excluding a random subset that were removed on day 28 for a separate experiment. The microbiome was allowed to naturally recover after antibiotic treatment (days 25-36). Days 34-36 were used to indicate wheel running in recovery.

**Figure 4.3**

**A.**

**Type 3 Tests of Fixed Effects**

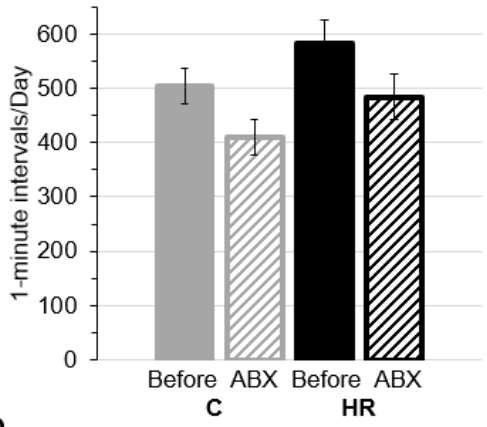
| Effect | d.f. | F | P | r |
|---|---|---|---|---|
| Linetype | 1, 6 | 111.64 | **<.0001** | 0.9742 |
| Antibiotics | 1, 6 | 26.84 | **0.0020** | 0.9040 |
| Linetype × Antibiotics | 1, 6 | 15.60 | **0.0075** | 0.8498 |




**B.**

**Type 3 Tests of Fixed Effects**

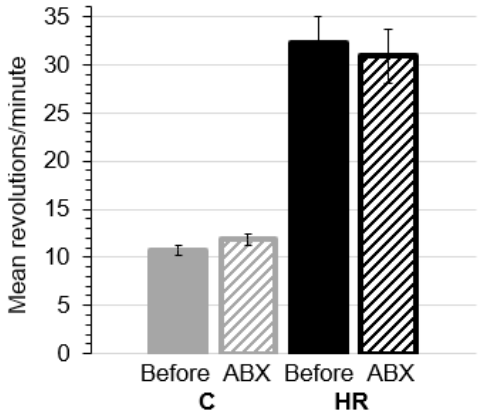
| Effect | d.f. | F | P | r |
|---|---|---|---|---|
| Linetype | 1, 6 | 2.12 | 0.1956 | 0.5110 |
| Antibiotics | 1, 6 | 60.79 | **0.0002** | 0.9540 |
| Linetype × Antibiotics | 1, 6 | 0.06 | 0.8188 | 0.0995 |




**C.**

**Type 3 Tests of Fixed Effects**

| Effect | d.f. | F | P | r |
|---|---|---|---|---|
| Linetype | 1, 6 | 53.07 | **0.0003** | 0.9479 |
| Antibiotics | 1, 6 | 0.05 | 0.8232 | 0.0909 |
| Linetype × Antibiotics | 1, 6 | 4.92 | 0.0683 | 0.6712 |




**D.**

**Type 3 Tests of Fixed Effects**

| Effect | d.f. | F | P | r |
|---|---|---|---|---|
| Linetype | 1, 6 | 55.98 | **0.0003** | 0.9504 |
| Antibiotics | 1, 6 | 2.54 | 0.1623 | 0.5454 |
| Linetype × Antibiotics | 1, 6 | 0.86 | 0.3900 | 0.3541 |

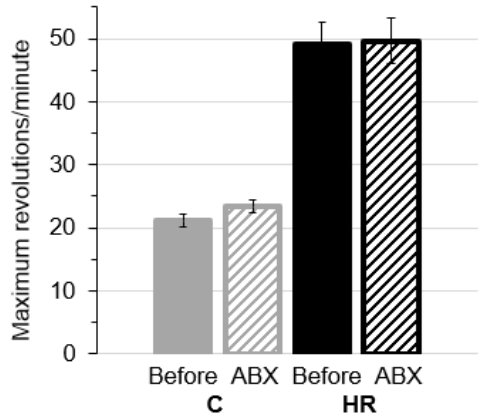


Fig. 4.3. Wheel running before (averages for days 11–13) and during (averages for days 22–24) antibiotics (ABX; see Figure 2). Data are presented as least squares means ± standard errors and type 3 tests of fixed effects from repeated-measures analyses using values for individual days in SAS Procedure Mixed. N = 92 mice and 543 observations after removal of statistical outliers (see Methods). Pearson's r is a measure of effect size (see Methods). **A.** Revolutions per day. Antibiotic treatment reduced the revolutions per day (days 22–24) in only the HR mice (linetype × antibiotic treatment interaction, $p = 0.0075$; differences of least squares means $p = 0.0023$ for HR mice and $p = 0.2071$ for C mice). **B.** Number of 1-minute intervals with at least one revolution. Antibiotic treatment reduced the number of minutes mice run per day for both linetypes ($p = 0.0002$; differences of least squares means $p = 0.0018$ for HR mice and $p = 0.0012$ for C mice). **C.** Revolutions per minute. Antibiotics had no statistical effect on average speed. **D.** Maximum revolutions per minute. Antibiotics had no statistical effect on maximum running speed.

## Figure 4.4

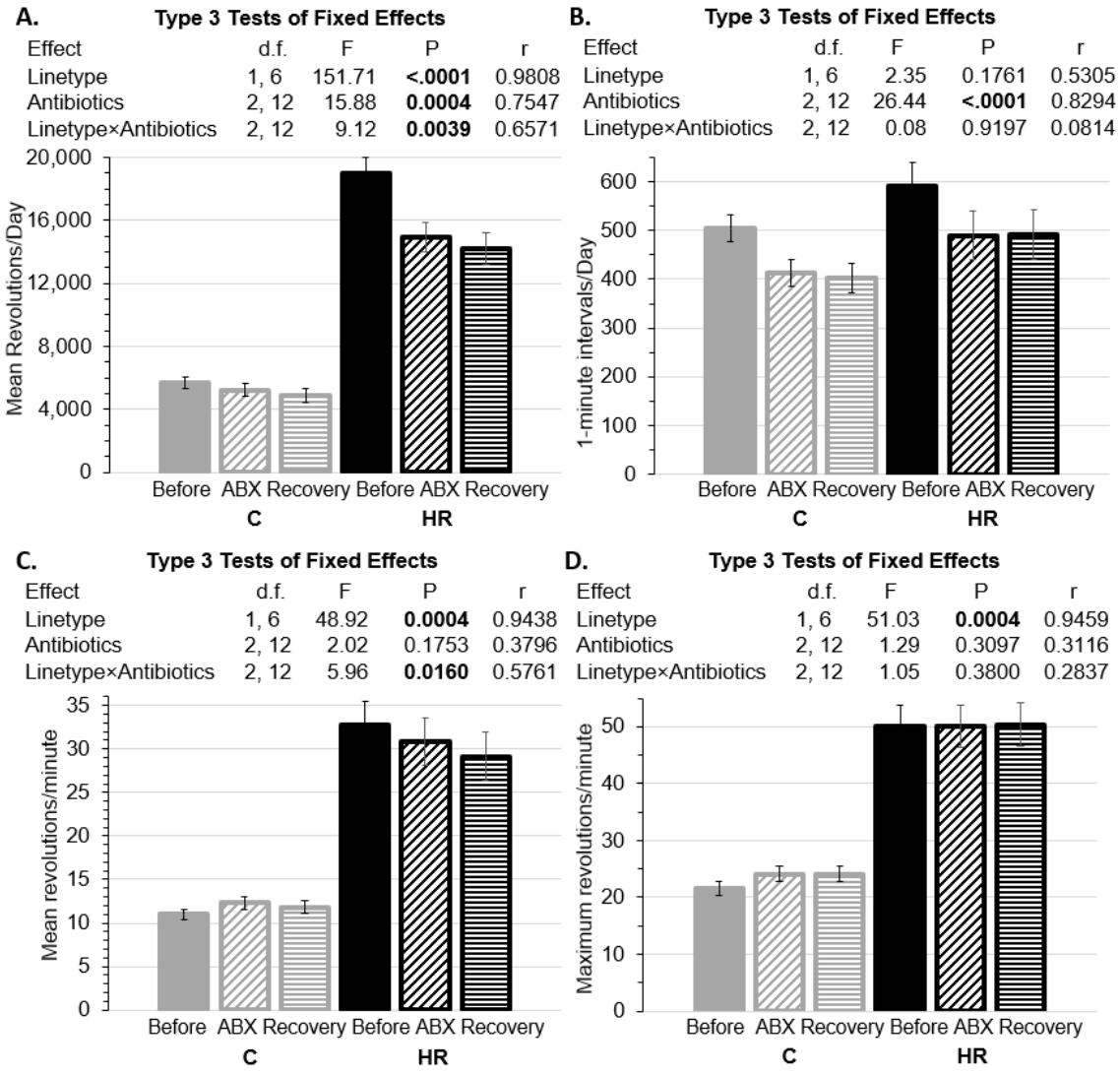

A.
Type 3 Tests of Fixed Effects
Effect d.f. F P r
Linetype 1, 6 151.71 <.0001 0.9808
Antibiotics 2, 12 15.88 0.0004 0.7547
Linetype×Antibiotics 2, 12 9.12 0.0039 0.6571
Mean Revolutions/Day
20,000
16,000
12,000
8,000
4,000
0
Before ABX Recovery Before ABX Recovery
C
HR
B.
Type 3 Tests of Fixed Effects
Effect d.f. F P r
Linetype 1, 6 2.35 0.1761 0.5305
Antibiotics 2, 12 26.44 <.0001 0.8294
Linetype×Antibiotics 2, 12 0.08 0.9197 0.0814
1-minute intervals/Day
600
500
400
300
200
100
0
Before ABX Recovery Before ABX Recovery
C
HR
C.
Type 3 Tests of Fixed Effects
Effect d.f. F P r
Linetype 1, 6 48.92 0.0004 0.9438
Antibiotics 2, 12 2.02 0.1753 0.3796
Linetype×Antibiotics 2, 12 5.96 0.0160 0.5761
Mean revolutions/minute
35
30
25
20
15
10
5
0
Before ABX Recovery Before ABX Recovery
C
HR
D.
Type 3 Tests of Fixed Effects
Effect d.f. F P r
Linetype 1, 6 51.03 0.0004 0.9459
Antibiotics 2, 12 1.29 0.3097 0.3116
Linetype×Antibiotics 2, 12 1.05 0.3800 0.2837
Maximum revolutions/minute
50
40
30
20
10
0
Before ABX Recovery Before ABX Recovery
C
HR

Fig. 4.4. Wheel running before antibiotics (averages for days 11–13), during antibiotics (averages for days 22–24), and after recovery (days 34–36) (see Figure 2). Data are presented as least squares means ± standard error from repeated-measures analyses using values for individual days in SAS Procedure Mixed. N = 73 mice and 639 observations after removal of statistical outliers (see Methods). Pearson's r is a measure of effect size (see Methods). **A.** Revolution per day. **B.** Number of 1-minute intervals with at least one revolution. **C.** Revolutions per minute. **D.** Maximum revolutions per minute. Overall, mice did not recover to pre-treatment levels of running (see Figures 2 and 3) within the time frame of this experiment.

**Figure 4.5**

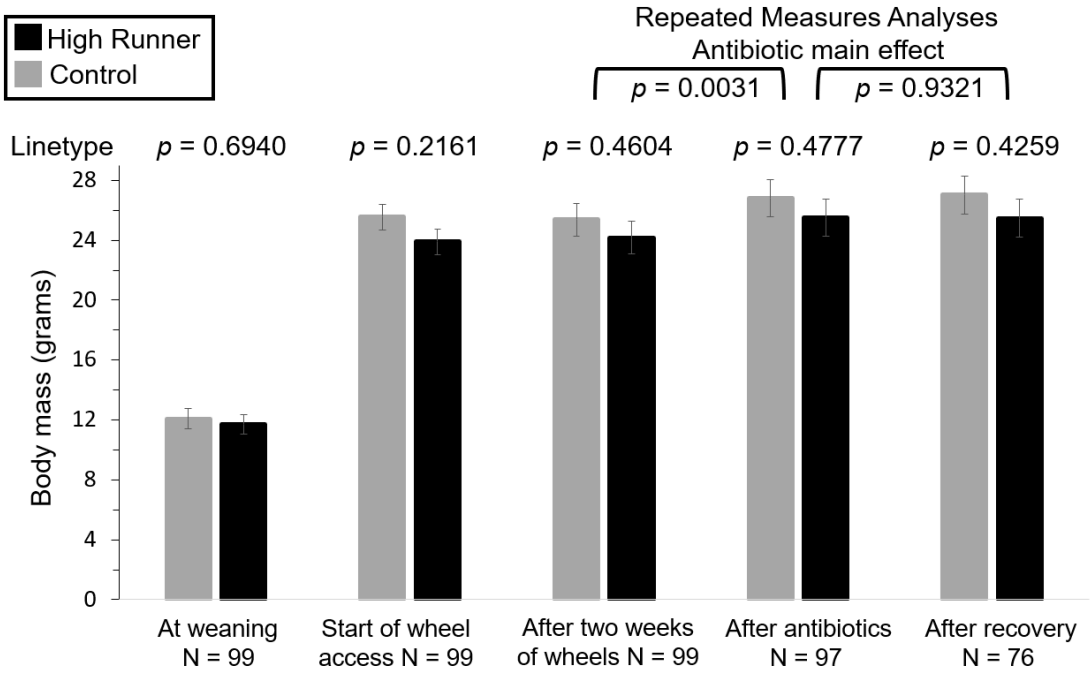



Fig. 4.5. Body mass measured at various timepoints. Analyses were first done separately for each time point (not repeated-measures) and values in the figure are least squares means ± standard errors from those analyses. *p*-values above each pair of bars are for the linetype comparison at each time point. In addition, separate repeated-measures analysis of mass before versus after 10 days of antibiotics indicated mice were larger after antibiotic treatment (treatment $p = 0.0031$, linetype $p = 0.4646$, interaction $p = 0.8329$). A similar analysis of mass after antibiotics versus after 12 days of recovery indicated no effect of recovery (treatment $p = 0.9321$, linetype $p = 0.4780$, interaction $p = 0.6612$).

**Figure 4.6**

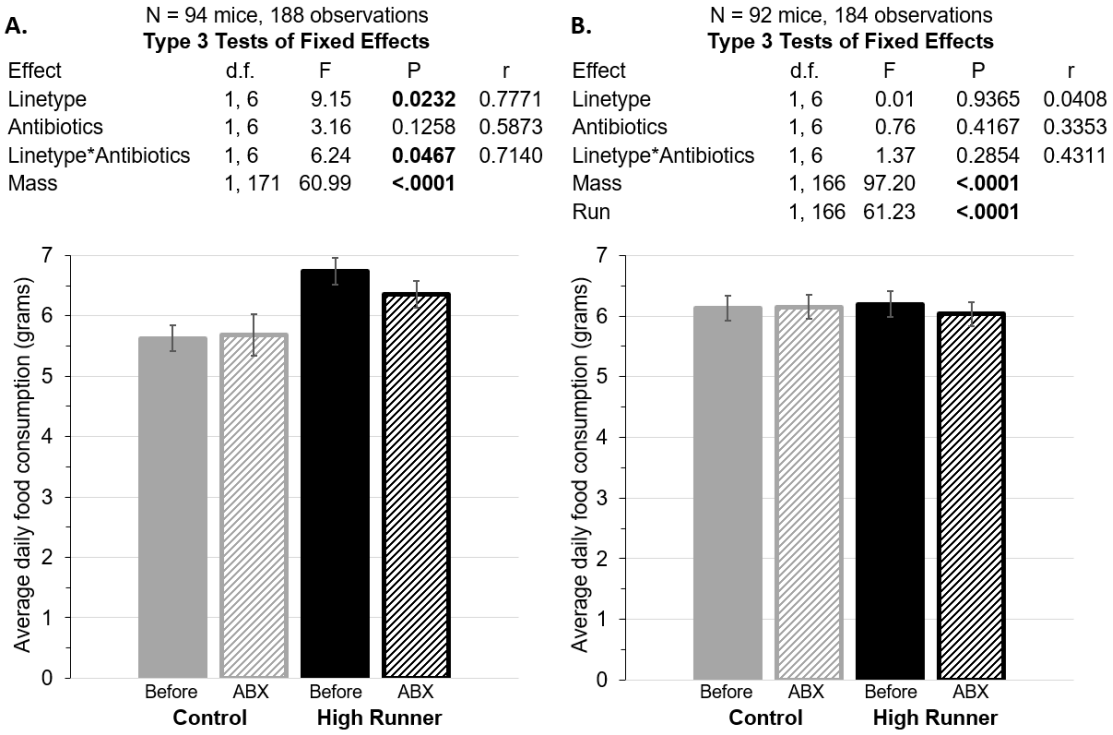



Fig. 4.6. Average daily food consumption during the baseline period (days 8–14) compared to during the antibiotic (ABX) treatment period (days 15–24), with body mass as a covariate. Data are presented as least squares mean ± standard errors from repeated-measures analyses in SAS Procedure Mixed. Pearson's r is a measure of effect size (see Methods). **A.** Antibiotics decreased food consumption in the HR mice (repeated-measures analysis interaction $p$ = 0.0467). **B.** Food consumption with wheel running as an additional covariate. The interaction between linetype and antibiotics is no longer significant.

**Figure 4.7**

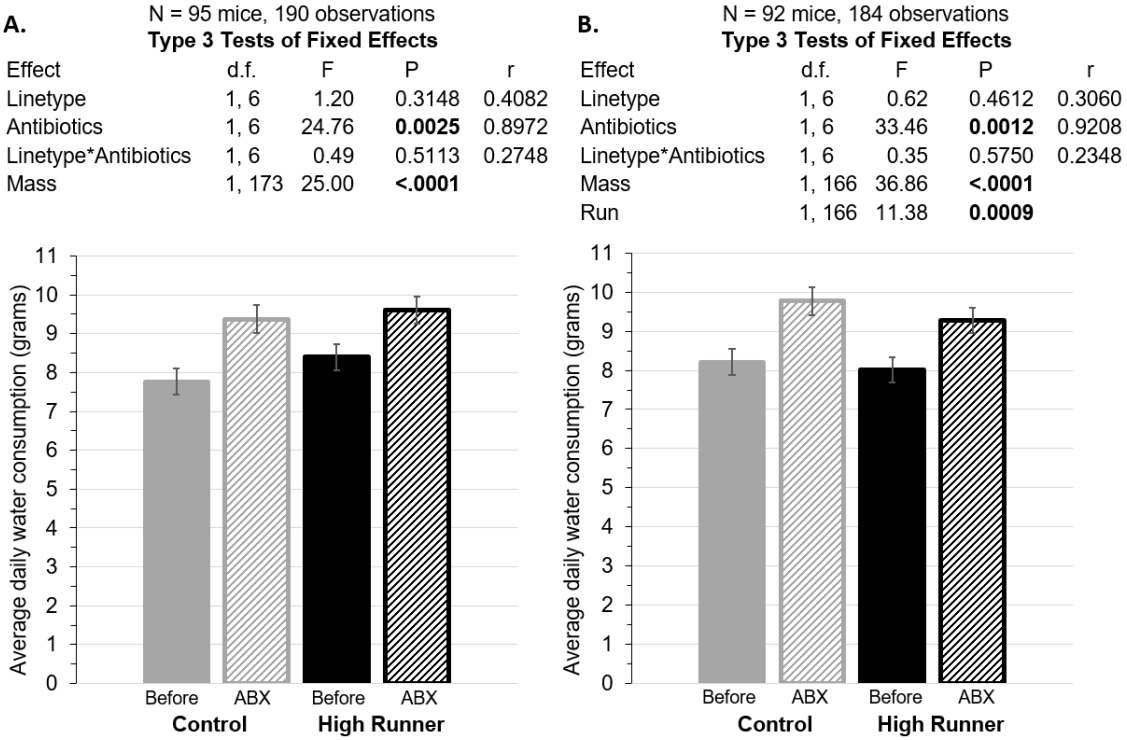



Fig. 4.7. Average daily water consumption during the baseline period (days 8–14) compared to during the antibiotic (ABX) treatment period (days 15-24), with body mass as a covariate. Data are presented as least squares means ± standard errors from repeated-measures analyses in SAS Procedure Mixed. Pearson's r is a measure of effect size (see Methods). **A.** Water consumption increased while on antibiotics. **B.** When included as an additional covariate, wheel running was a significant positive predictor, but water consumption was still higher while on antibiotics. This result differs from food consumption (Figure 6).

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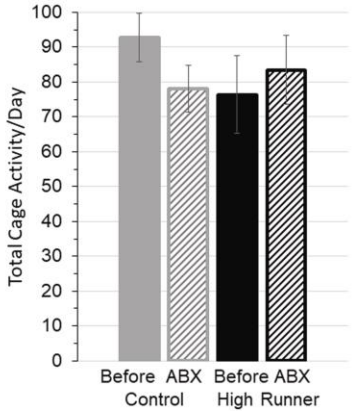
# Appendices

## Appendix 4.1

**A.** N = 70 mice, 388 observations

**Type 3 Tests of Fixed Effects**

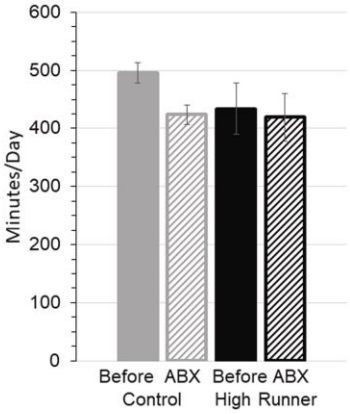
| Effect | d.f. | F | P | r |
|---|---|---|---|---|
| Linetype | 1, 6 | 0.24 | 0.6401 | 0.1961 |
| Antibiotics | 1, 6 | 0.43 | 0.5377 | 0.2586 |
| Linetype*Antibiotics | 1, 6 | 3.55 | 0.1084 | 0.6097 |




**B.** N = 70 mice, 388 observations

**Type 3 Tests of Fixed Effects**

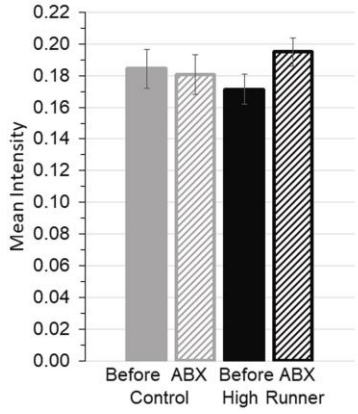
| Effect | d.f. | F | P | r |
|---|---|---|---|---|
| Linetype | 1, 6 | 0.61 | 0.4660 | 0.3038 |
| Antibiotics | 1, 6 | 8.57 | **0.0264** | 0.7669 |
| Linetype*Antibiotics | 1, 6 | 3.79 | 0.0996 | 0.6222 |




**C.** N = 70 mice, 388 observations

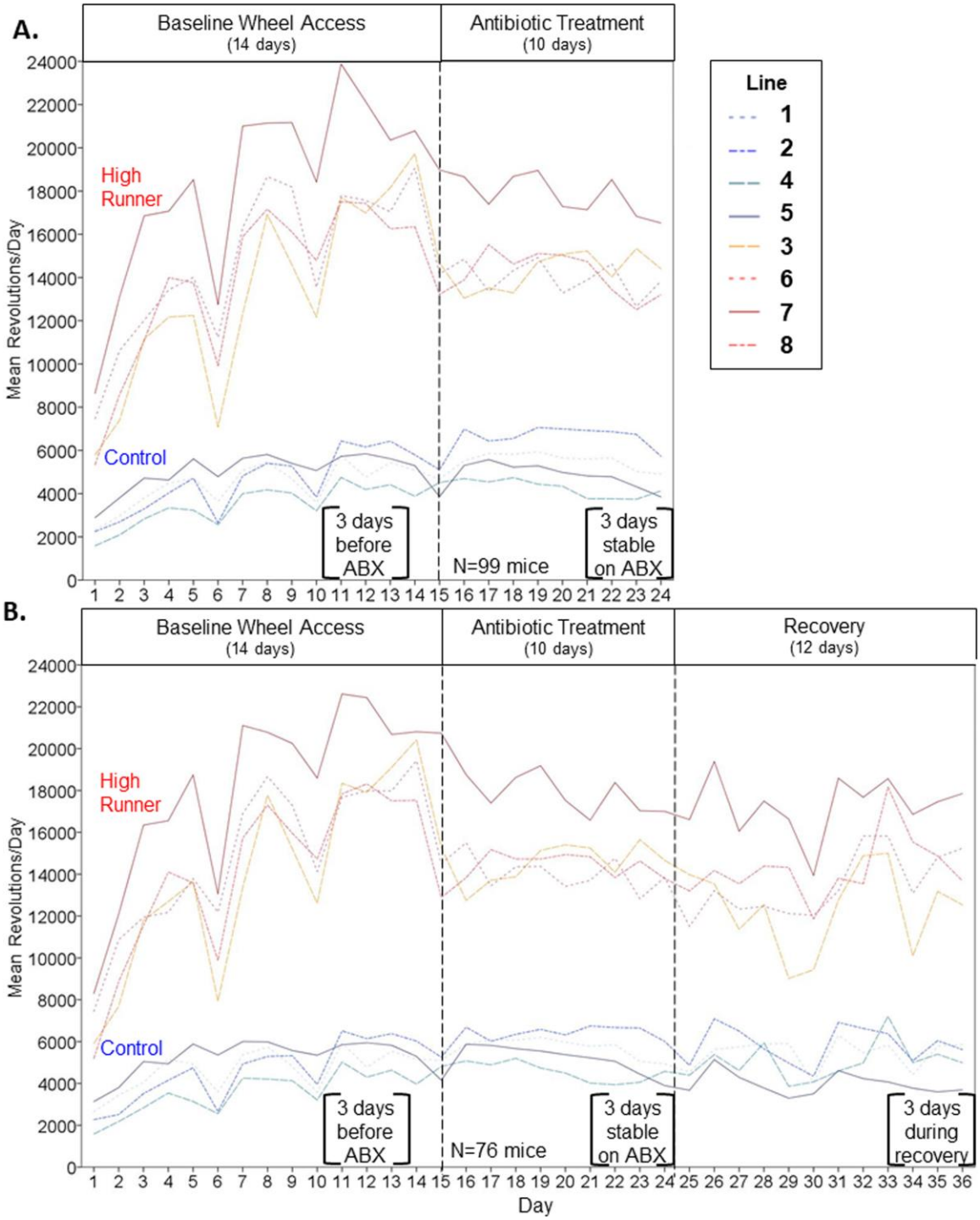
**Type 3 Tests of Fixed Effects**

| Effect | d.f. | F | P | r |
|---|---|---|---|---|
| Linetype | 1, 6 | 0.00 | 0.9598 | 0.0000 |
| Antibiotics | 1, 6 | 1.50 | 0.2670 | 0.4472 |
| Linetype*Antibiotics | 1, 6 | 2.85 | 0.1425 | 0.5675 |



Appendix 4.1. Home-cage activity before (averages for days 11–13) and during (averages for days 22–24) antibiotics (ABX). Note that all mice had wheel access during the entire period. Due to equipment failures, data were not available for the period of recovery from antibiotics. Data are presented as least squares means ± standard errors from repeated-measures analyses of individual daily values in SAS Procedure Mixed. Pearson's r is a measure of effect size (see Methods). A. Although the interaction did not reach statistical significance, C mice on antibiotics tended to have decreased total cage activity during antibiotic treatment (differences of least squares means $p = 0.0576$ for C mice and $p = 0.4914$ for HR mice). B. Although the interaction did not reach statistical significance, antibiotics reduced activity duration in C mice but not in HR mice (differences of least squares means $p = 0.0063$ and $p = 0.5649$, respectively). C. Antibiotics tended to increase mean intensity of activity (total/minutes) in HR mice, but not in C mice (differences of least squares means $p = 0.7159$ for C mice and $p = 0.1166$ for HR mice).

## Appendix 4.2


A.
Baseline Wheel Access
(14 days)
Antibiotic Treatment
(10 days)
Mean Revolutions/Day
High Runner
Control
3 days before ABX
N=99 mice
3 days stable on ABX
Line
1
2
4
5
3
6
7
8
B.
Baseline Wheel Access
(14 days)
Antibiotic Treatment
(10 days)
Recovery
(12 days)
Mean Revolutions/Day
High Runner
Control
3 days before ABX
N=76 mice
3 days stable on ABX
3 days during recovery
Day

Appendix 4.2. Wheel running mean revolutions per day (23 h) for each of the 4 HR and 4 C lines. Data were truncated on days 8, 14, and 25 (~20 h) when the water bottles were being changed. Boxes indicate days that were used in statistical analyses. A. N = 99 mice, through day 24 of the experiment. Days 11–13 before antibiotics were compared with days 22–24 during antibiotics. B. N = 76 mice, excluding a subset that were removed on day 28 for a separate experiment. The microbiome was allowed to naturally recover after antibiotic treatment (days 25–36). Days 34–36 were used to indicate wheel running in recovery.

## Concluding remarks

My dissertation has highlighted the essential role of the gut microbiome for the host. Almost three decades of selective breeding for high voluntary wheel-running behavior has resulted in a differentiated gut microbiome community in the High Runner (HR) lines of mice. These results suggest that the HR mice may be under selection to harbor microbes capable of extracting more energy for sustained physical activity and/or microbes that influence motivation to exercise. I also demonstrate that diet and exercise during a critical developmental period in early life can have lasting effects on the adult gut microbiome. Finally, I highlight the potential negative impacts of antibiotic treatment on host behavior. Specifically, the highly athletic HR mice ran significantly less than usual after depletion of their gut microbiome with antibiotics, suggesting that having the "correct" microbiome is essential for their exceptional exercise performance.

### 1. Chapter 1

Beginning at weaning, mice from the HR lines have elevated activity levels and food consumption, and we know the gut microbiome is highly sensitive to diet and physical activity. Therefore, in this study I sampled feces at weaning, in an attempt to minimizing the impact of activity levels. Weanling mice in our colony were in previous contact with only their mothers and siblings, as well as environmental microbes from the air (Thompson et al. 2021), food, water, and bedding (Ericsson et al. 2018; Pellizzon and Ricci 2020), and, in general, from

being housed in a non-barrier facility (Oriá et al. 2018; Ericsson and Franklin 2021).

Juvenile HR and C mice did not differ in bacterial alpha diversity (within-sample diversity). Within the two selection treatments, replicate HR and C lines had unique microbiomes based on separate beta diversity (between or among experimental groups) distance matrices. I also found that HR mice had a higher relative abundance of the family *Clostridiacae*, a family previously associated with exercise in the literature. In addition, mice homozygous for the mini-muscle allele (characterized primarily by an ~50% reduction in their triceps surae mass) had different gut microbiome communities based on beta diversity distance matrices and higher relative abundance of the genus *Clostridium* compared to normal-muscle mice.

**2. Chapter 2**

The mice used in Chapter 1 were included in a training-effects study, beginning as adults (Chapter 2). I found that 4 weeks of voluntary exercise on wheels increased food and water consumption, increased lean mass while reducing fat mass, increased heart mass, and reduced both reproductive and subdermal fat pad masses. I detected no statistically significant differences between HR and C mice for organ masses, and no interactive effects between linetype and wheel access for body mass, body composition, or organ masses. With body mass as a covariate, mini-muscle mice drank more and had larger reproductive fat pads,

livers, and brains, as compare with normal-muscled individuals. Overall, results show that voluntary wheel running for only 4 weeks can change morphological phenotypes of adult female mice, even those that do not engage in high levels of exercise.

## 3. Chapter 3

### 3.1 Early-life Western diet affects gut bacterial diversity of adults

In Chapter 3, I found that early-life diet and exercise can have lasting impacts on the adult gut microbiome. We know that developmental windows during early life are times of high sensitivity to environmental conditions, with effects that can persist into adulthood (Garland et al. 2017). The gut microbiome is also highly sensitive to environmental conditions during early life (Mika and Fleshner 2016), but very few studies have tested for long-lasting effects of early-life exposures after a substantial washout period. I discovered that early-life Western-diet, exercise, and linetype had an interactive effect on alpha diversity; in addition, Western diet decreased alpha diversity in all groups. Juvenile Western diet also decreased the relative abundance of the species *Muribaculum intestinale*.

The Western diet used in the present study differs from the standard chow not only with respect to the percentage of kilojoules that come from fat and carbohydrates, but also the percentage of kilojoules that come from protein (see Table 1 in Meek et al. 2010). These two diets may also differ in fiber content. Therefore, in future studies, it would be important to determine which of these

differences is responsible for the effects observed in my study and in other studies of the High Runner mice that have used these diets (Meek et al. 2010, 2012; Acosta et al. 2017; Hiramatsu et al. 2017; Cadney et al. 2021; McNamara et al. 2021).

Western diet has been previously associated with decreased bacterial diversity in both rodents and human beings (Turnbaugh et al. 2008; Leamy et al. 2014; Pindjakova et al. 2017; Beilharz et al. 2018; Becker et al. 2020). Fecal samples from individuals in rural communities in Malawi, villages in Venezuela, and cities in the United States demonstrated that Western populations had lower gut bacterial diversity compared to the other groups (Yatsunenko et al. 2012). In a separate study, Hazda hunter-gatherers in Tanzania had higher gut bacterial diversity and had microbes that are not found in Western populations compared to an Italian urban population (Schnorr et al. 2014). Recent papers have suggested that we could “rewild” the gut microbiome of Western populations (Dominguez-Bello et al. 2018; Sonnenburg and Sonnenburg 2019), but as noted by Carmody et al. (2021), we do not know if the Hazda microbiome of today is an accurate representation of ancestral microbiomes, or if these microbiome communities actually promote host health. Additionally, studies demonstrating that higher diversity is beneficial are lacking. Nonetheless, microbial communities that are rich in species are thought to be less susceptible and more resilient to invaders due to their ability to limit resources to any newly acquired species (see modeling papers Lozupone et al. 2012; Dubinkina et al. 2019).

### 3.2 Gut bacterial diversity affects fitness

Evidence regarding the effect of gut microbiome diversity on host fitness in wild species, studied in the wild, is conflicting. For example, body mass (a measure of nestling performance in a wild population of the Great tit) was negatively associated with alpha diversity, but nestling survival was not (Davidson et al. 2021). In a wild population of Sychelles warblers, gut alpha diversity and microbiome community membership was not associated with individual body condition or survival during the next breeding season. However, increased abundance of opportunistic pathogens within the genus *Mycobacterium* was associated with higher mortality in the following breeding season (Worsley et al. 2021). In contrast, in wild house mice, higher gut microbiome diversity was correlated with decreased intensity of nematode and mite infections, and increased number of viral infections (Weldon et al. 2015). In addition, body mass was positively correlated with alpha diversity.

### 3.3 Higher relative abundance of the family *Clostridiaceae* in HR mice

Interestingly, in two of my chapters, HR mice had higher relative abundance of the bacterial family *Clostridiaceae.* In Chapter 1, weanling female HR mice had significantly higher relative abundance of *Clostridiaceae*, as compared with females from the Control lines. In Chapter 3, adult male HR mice tended to have higher relative abundance of this taxon. This family is common in the murine gut

microbiome and has been previously associated with exercise treatment. In a study of male C57BL/6 mice with low-fat or high-fat diet in combination with exercise or no exercise treatment, diet and exercise had an interactive effect on the relative abundance of *Clostridiaceae* (Evans et al. 2014). Specifically, mice on a high-fat diet with exercise had a greater relative abundance compared to mice on a low-fat diet with exercise.

In a separate study, adult female rats from lines selectively bred for high versus low endurance-running capacity were ovariectomized and then fed a high-fat diet with or without wheel access for 11 weeks (Liu et al. 2015). In both lines, 11 weeks of exercise while on a high-fat diet significantly increased the relative abundance of *Clostridiaceae*, as compared with sedentary rats. Interestingly, in the pooled sample of HCR and LCR mice that had wheels, the relative abundance of *Clostridiaceae* was negatively correlated with both food intake and running distance (Fig 2C. and Fig 3C. in Liu et al. 2015). The relative abundance of *Clostridiaceae* has also been shown to be under genetic control and also affected by diet manipulations in an 8 progenitor strains mouse model (O'Connor et al. 2014).

**4. Chapter 4**

In Chapter 4, I provide evidence for an essential role of the gut microbiome in the High Runner phenotype. Broad-spectrum antibiotics can eliminate pathogens that cause infections, but also wipe out the normal gut microbiome community,

and hence disrupt normal physiological functioning as well as allow for proliferation of usually low-abundant microbes or new invaders (Tipton et al. 2019). Additionally, antibiotic usage in athletes may be more detrimental to health and performance as compared with effects in non-athletes. Moreover, in some cases, athletes have increased frequency of antibiotic usage as compared with non-athletes (Alaranta et al. 2006). Additionally, specific types of antibiotics have been associated with negative effects that could influence athletic performance. For example, fluoroquinolones have been associated with tendon injuries (Khaliq and Zhanel 2003; Fayock et al. 2014) and macrolides have been associated with cardiac arrhythmias (Fayock et al. 2014).

I found that ten days of antibiotic treatment reduced wheel-running behavior (average revolutions per day) in the HR lines of mice, but not in the Control lines, suggesting the gut microbiome plays an essential role in the highly athletic HR phenotype. These results can have translational relevance, as manipulation of the microbiome might be a way to increase voluntary exercise behavior.

Perhaps surprisingly, the HR mice did not recover wheel-running behavior during the 12 days after antibiotic treatment. We did not attempt to reseed the gut microbiome. Therefore, recovery would have depended on residual gut bacteria in the gut not eliminated by the antibiotic treatment and/or environmental sources of bacteria. In future studies, it would be important to monitor the

microbiome to determine if and when it recovers and whether the microbial composition is the same as prior to antibiotic treatment.

Another possibility is that the gut microbiome did recover to some extent during the 12 days after antibiotic treatment, but that simultaneously, some bacteria had been translocated into host tissue. This latter possibility is supported by studies that have demonstrated the ability of antibiotics to alter intestinal tight junction barrier health (Knoop et al. 2016; Feng et al. 2019; Kester et al. 2020). Bacteria translocated into host tissue have the potential to adversely affect host physiology (O'Boyle et al. 1998; Vaishnavi 2013; Knoop et al. 2016).

In addition to transplanting the HR microbiome into C and other strains of mice to see if we can increase voluntary exercise behavior, future studies should examine the effects of short-chain fatty acid supplementation on exercise behavior and/or performance. Matsumoto et al. (2008) demonstrated that 5 weeks of voluntary exercise in rodents increased cecal butyrate concentration compared to sedentary controls. Interestingly, 12 weeks of dietary supplementation with butyrate in diet-induced obese C57BL/6J mice increased spontaneous physical activity in the home-cage measured during the active period (Gao et al. 2009). In a separate study, male C57BL/6J mice with two weeks of antibiotics had reduced treadmill running time compared to Controls, and when male mice in a separate group were given two weeks of antibiotics followed by continuous acetate infusion, endurance run time was increased

compared to a saline-infused group (Okamoto et al. 2019). Interestingly, when the same experiment was repeated with butyrate, they found no significant improvement in exercise performance.

**5. Final thoughts**

My findings in this dissertation add to our understanding of the importance of the gut microbiome for the High Runner mouse phenotype. These results have translational relevance to human health, as biomedical transplantations of the gut microbiome are becoming increasingly common (Gupta et al. 2016; Wilson et al. 2019; Leong et al. 2020; Ser et al. 2021). We know that transplantation of "exercised" microbiomes can elicit a significant change in host function/behavior in rodent studies (Allen et al. 2018; Lai et al. 2018; Okamoto et al. 2019; Zoll et al. 2020). In addition, fecal microbiota transplants have been suggested as a conservation technique in wild mammalian species (Guo et al. 2020).

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