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Early-Life Effects of Diet, Exercise, and Maternal Environment on Adult Activity Levels in Mice Selectively Bred for High Voluntary Wheel-Running Behavior

A Dissertation submitted in partial satisfaction
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by

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Acknowledgments

This dissertation is dedicated to Grandma Mary, who regrettably passed away before I could show her the degree she kept asking about.

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Chapter 1

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Previously Published Materials

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ABSTRACT OF THE DISSERTATION

Early-Life Effects of Diet, Exercise, and Maternal Environment on Adult Activity Levels in Mice Selectively Bred for High Voluntary Wheel-Running Behavior

by

Marcell D. Cadney

Doctor of Philosophy, Graduate Program in Evolution, Ecology, and Organismal Biology
University of California, Riverside, December 2021
Dr. Theodore Garland, Jr., Chairman

Unhealthy diets and a lack of exercise in children are leading to metabolic disorders in adults. This dissertation characterized effects of early-life experiences (phenotypic plasticity) on adult activity levels by altering the diet and/or exercise of juvenile mice. Subjects were from a selection experiment in which four replicate lines of High Runner (HR) mice evolved to run 2.5-3-fold more wheel revolutions per day than those from four non-selected Control (C) lines. Expectations were that 1) early-life experiences would have long-lasting effects into adulthood, 2) early-life factors would have interactive effects, and 3) genotype-by-environment interactions would occur).

Chapter 1 considered effects of early-life high fat, high sugar diet and/or exercise on adult physical activity. Juvenile mice were exposed to 3 weeks of Western diet (WD) and/or wheel access, beginning at weaning, followed by an 8-week washout period (~6 human years). In adults, early exercise increased wheel running of C but not HR mice, decreased anxiety-like behavior and heart

ventricle mass, but increased fasted blood glucose levels, triceps surae, subdermal fat pad, and brain masses. In contrast, early-life WD increased adult wheel running of HR but not C mice.

Chapter 2 exposed mice to similar early-life treatments, except WD was replaced with fructose in drinking water to assess its potential to suppress activity levels acutely and into adulthood after a 23-week washout period. One HR and one C line were used. As in Chapter 1, early exercise increased adult running, but also decreased adult fat mass.

Early-life fructose increased fat mass acutely but had inconsistent effects on wheel running and cage activity, with no long-lasting effects into adulthood.

Chapter 3 was a cross-fostering experiment designed to test the importance of the early postnatal maternal environment in the development of the HR phenotype. Fostering C pups to HR dams did not increase the wheel running of C adults, nor did fostering HR pups to C dams suppress running of HR adults.

These studies demonstrate that early-life experiences can have both acute and long-lasting effects on various traits, including physical activity, and that those effects interact with genetic background.

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Introduction

"Early-life" is defined by any period of development preceding sexual maturity, during which time various tissues and organs develop (Garland, Jr. et al. 2017). Effects that alter development in a way that influences adult characteristics are, therefore, called "early-life effects," and may include effects caused by modifications to the sperm and egg from which the adult developed. Early-life effects that persist into adulthood are especially likely when developmental milestones (called "critical periods" of development) are the target of genetic or environmental factors. Effects may be transitory or long-lasting, often depending on the affecting factors, the affected critical period, and sex.

Some early-life effects are well documented. Variation in environmental temperature, for example, has predictable effects on the egg size and clutch size of temperate birds, which in turn affects early survival of offspring (Perrins 1996; Pendlebury and Bryant 2005). Broadly speaking, perturbations to the early developmental environment can have direct consequences for offspring survival and reproduction. Therefore, variation in the early-life environment has important implications for both health and evolution.

Animal locomotion is an important behavior for many ecological and evolutionary reasons: escaping predators, hunting prey, foraging, migration/dispersal, and finding mates. Thus, understanding the sources of variation in physical activity may be helpful in explaining various aspects of animal life history, behavioral ecology, and evolution. Consequently,

experimental evolution and artificial selection experiments have been routinely used to understand the biological nuances of how complex traits evolve.

In humans, early-life experiences have been implicated in effects on adult physical activity. Famine during gestation, for example, has resulted in male offspring becoming less physically active (Lussana et al. 2008). Rodent studies offer a better means for understanding early-life effects, as their generation times are shorter compared to humans and many aspects of their early environment are ideal for experimental change (e.g., temperature, diet, stress).

The High Runner Mouse Model

Each chapter of this dissertation uses laboratory mice from an ongoing selection experiment for high voluntary wheel-running behavior (Swallow et al. 1998). The founder population consisted of 224 outbred Hsd:ICR strain (HarlanSprague-Dawley; Indianapolis, Indiana, USA) laboratory house mice (*Mus domesticus*). After 2 initial generations of random breeding, mice were randomly assigned into eight replicate lines. Lines designated 3, 6, 7, and 8 were selectively bred for high voluntary wheel running behavior, while lines 1, 2, 4, and 5 were bred randomly (regardless of wheel running). Each generation, approximately 560 mice (males and females from all 8 lines) were wheel tested as young adults (~6 to 8 weeks of age) for 6 days in standard housing cages attached by a tunnel to Wahman-type running wheels (1.12 meter circumference; Lafayette Instruments, Lafayette, IN), in which mice had *ad libitum* access to

standard mouse food (Harlan Teklad Laboratory Rodent Diet 8604) and tap water. During wheel testing, revolutions were recorded every minute for approximately 23 hours per day. Wheel running was quantified as the total number of wheel revolutions on days 5 and 6 of a 6-day test. Selected (HR) lines were subjected to within-family selection – breeders were paired based on wheel running, where the highest runner of each family was mated to another family's highest runner (within the same line). Sibling mating was not allowed.

By generation 25, HR lines had reach a selection limit (Careau et al. 2013), where HR lines ran 2.5 to 3-fold more revolutions per day than C lines. Multiple correlated responses to selection have been reported. High Runner mice have evolved greater endurance during forced treadmill exercise, greater maximal exercise-induced oxygen consumption, a narrower stance width during treadmill running, smaller and leaner bodies, elevated circulating levels of corticosterone, larger hearts, larger brains, and altered brain reward systems (Swallow et al. 2001; Meek et al. 2009; Claghorn et al. 2016, 2017; Thompson et al. 2018; Singleton and Garland 2019). In general, these and other correlated traits are associated with either the ability or motivation to voluntarily engage in sustained physical activity.

More recently, the Garland lab has investigated the effects of early-life diet and exercise on adult wheel running and associated traits in the HR mouse model. In one study, half of the mice were given wheel access during the three-week early-life period between weaning and sexual maturity (Acosta et al. 2015).

After 52 days of a washout period, early-life wheel access significantly increased adult wheel running for both HR and C mice over a one-week test period (home cage activity was not affected). Early-life wheel access also reduced body mass throughout the experiment, increased caloric intake in HR lines, and increased circulating levels of the hormone leptin in C lines but decreased it HR lines. In another study, pregnant dams were fed a "Western" diet (WD, high in fat and sugar) or standard diet (SD) until the weaning of their pups (Hiramatsu et al. 2017). Maternal exposure to WD had numerous early-life effects on offspring, including grand-daughter effects on wheel running in two HR lines (Hiramatsu et al. 2017).

In summary, previous work in the Garland lab supports the hypothesis that the early-life environment (at least diet and exercise) can have lasting (sometimes positive) effects on adult physical activity levels, body mass and composition, and related traits. In this dissertation, I conducted a number of related early-life studies using the HR mice to examine some of the genetic and environmental factors that influence adult activity levels. This work has important implications for public health and health education in human populations.

In Chapter 1, I exposed male juvenile mice to Western diet and/or access to an exercise wheel for three weeks following weaning. After an 8-week washout period, adult testing of physical activity (voluntary exercise and spontaneous physical activity), anxiety-like behavior, aerobic capacity, body mass and composition, and preference for Western diet began. The experiment

concluded with measurements of organ masses and circulating levels of corticosterone, leptin, and adiponectin. This was the Garland Lab's first test of whether early-life effects involving both exercise and diet could interact on adult traits.

In Chapter 2, I similarly exposed female juvenile mice to fructose-water and/or access to an exercise wheel for three weeks following weaning. After a 23-week washout period, adult testing of physical activity and many other traits measured in Chapter 1 began. One cohort was dissected without adult wheel testing, while another cohort was dissected shortly after adult wheel testing, allowing for a comparison of training effects. Here, we test the hypothesis that fructose acutely reduces physical activity levels and that the effect may persist into adulthood as an early-life effect. We also test whether simultaneous exercise could protect against the potential negative effects of early-life fructose consumption.

In Chapter 3, I performed the Garland Lab's first cross-fostering of HR and C mice. Four experimental groups were made at birth: HR pups raised by HR dams, HR pups raised by C dams, C pups raised by C dams, and C pups raised by HR dams. The effect of cross-fostering was determined by the comparison of the crossed groups (e.g., HR pups raised by C dams) with the in-fostered groups (e.g., HR pups raised by HR dams). This experiment tested the hypothesis that the HR phenotype is in part developed by the early postnatal maternal environment via indirect genetic effects passed from mother to offspring.

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CHAPTER 1

Effects of Early-Life Exposure to Western Diet and Voluntary Exercise on Adult Activity Levels, Exercise Physiology, and Associated Traits in Selectively Bred High Runner Mice

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Abstract

Exercise behavior is under partial genetic control, but it is also affected by numerous environmental factors, potentially including early-life experiences whose effects persist into adulthood. We studied genetic and early-life environmental effects on wheel-running behavior in a mouse model that includes four replicate high runner (HR) lines selectively bred for increased voluntary wheel running as young adults and four non-selected control (C) lines. In a full factorial design, mice from each line were granted wheel access or not and administered either standard or Western diet (WD) from weaning (3 weeks old) to 6 weeks of age (sexual maturity). In addition to acute effects, after a washout period of 8 weeks (~6 human years) in which all mice had standard diet and no wheel access, we found both beneficial and detrimental effects of these early-life exposures. During the first week of treatments, WD increased distance run by 29% in C mice and 48% in HR mice (significant Diet × Linetype interaction), but diet effects disappeared by the third week. Across the three weeks of juvenile treatment, WD significantly increased fat mass (with lean mass as a covariate). Tested as adults, early-life exercise increased wheel running of C mice but not HR mice in the first week. Early-life exercise also reduced adult anxiety-like behavior and increased adult fasted blood glucose levels, triceps surae mass, subdermal fat pad mass, and brain mass, but decreased heart ventricle mass. Using fat mass as a covariate, early-life exercise treatment increased adult leptin concentration. In contrast, early-life WD increased adult wheel running of HR

mice but not C mice. Early-life WD also increased adult lean mass and adult preference for Western diet in all groups. Surprisingly, early-life treatment had no significant effect on adult body fat or maximal aerobic capacity (VO_2max). No previous study has tested for combined or interactive effects of early-life WD and exercise. Our results demonstrate that both factors can have long-lasting effects on adult voluntary exercise and related phenotypes, and that these effects are modulated by genetic background. Overall, the long-lasting effects of early-life exercise were more pervasive than those of WD, suggesting critical opportunities for health intervention in childhood habits, as well as possible threats from modern challenges. These results may be relevant for understanding potential effects of activity reductions and dietary changes associated with the obesity epidemic and COVID-19 pandemic.

1. Introduction

Human obesity and most of its negative health consequences result from complex interactions among diet, physical activity, environmental exposures, sex, genetic predisposition, and sociocultural factors (Eisenmann 2004, 2006; Ross and Desai 2005; Adam and Epel 2007; Papas et al. 2007; Choh et al. 2009; Coccurello et al. 2009; McAllister et al. 2009; Sisson et al. 2009; Stein et al. 2009; Kirk et al. 2010; North et al. 2010; Graff et al. 2011; Patterson and Abizaid 2013; Yang and Huffman 2013; Rohde et al. 2019). Of these, declining physical activity and "Western" diets (high in fat & simple sugars) are seen as key

contributors to increasing rates of obesity and its comorbidities in all age groups across multiple populations (Cornier et al. 2008; Westerterp and Speakman 2008; Steinberger et al. 2009; Flegal et al. 2010; Halpern et al. 2010). Conversely, a "good" diet (e.g., relatively low in sugar and fat [but see Esposito et al. (2010); O'Neill and Raggi (2020)]) and higher activity levels promote physical fitness and cardiovascular health while lowering the risk for obesity, Type 2 diabetes, and their comorbidities (Blair and Morris 2009; Hills et al. 2011; McGuire and Ross 2011; Janssen et al. 2013; Lee et al. 2017). Importantly, not only voluntary exercise (VE) but also incidental or spontaneous physical activity (SPA) has beneficial effects in both animal models and humans (e.g., Levine 2007; Levine et al. 2008; Novak et al. 2009; Garland, Jr. et al. 2011; McGuire and Ross 2011; Teske et al. 2014; Lightfoot et al. 2018).

Like obesity itself, human physical activity and diet choices are products of both genes and numerous environmental effects acting throughout an individual's development (Trost et al. 2002; Lightfoot et al. 2018). Many key environmental factors are experienced early in life, including *in utero* (Garland, Jr. et al. 2017). Such ontogenetic factors can strongly contribute to the development of various unhealthy adult behaviors, habits, diseases, and disorders (Dishman et al. 1985; Sallis and Hovell 1990; Twisk 2001; Barker 2003; de Boo and Harding 2006; Nathanielsz 2006; Hanson and Gluckman 2008; Gardner and Rhodes 2009; Langley-Evans 2009; Metges 2009; Ozanne and Siddle 2011; Blondin et al. 2016). Unfortunately, interventions to improve diet or increase physical activity

often have little impact on children's activity (Wilkin 2011; Roberto et al. 2015; but see Gomersall et al. 2016; Reilly 2011), and have not halted the increasing prevalence of obesity in children (Harris et al. 2009).

Human studies suggest that experiences occurring from conception to sexual maturity can alter adult levels of voluntary exercise and/or SPA. For example, adults who were exposed to the Dutch Famine *in utero* have increased adiposity and more atherogenic lipid profiles that may be related to decreased physical activity (Lussana et al. 2008; Stein et al. 2009). However, mechanisms by which early-life experiences affect physical activity are poorly understood (Dishman et al. 1985; Twisk 2001; Jung et al. 2006; Lussana et al. 2008; Andersen et al. 2009; Downing et al. 2011; Koeneman et al. 2011; Li et al. 2013; Baker et al. 2015), in part because existing human studies are mostly correlational, cross-sectional (Trost et al. 2002), or of questionable methodological quality (e.g., use of questionnaires to gauge physical activity: Hallal et al. 2006; Stein et al. 2009; Koeneman et al. 2011).

Numerous rodent studies establish that *in utero* and other early-life exposures to calorie-restricted, low-protein, high-fat, or high-carbohydrate diets can affect obesity-related measures in adults, such as body composition, appetite, dietary preferences, reward signaling, and the microbiome (Frazier et al. 2008; Teegarden et al. 2009; Vucetic et al. 2010, 2012; Ozanne and Siddle 2011; Sun et al. 2013; Desai et al. 2014; McNamara et al. 2021). Far fewer studies address maternal or early-life effects on adult voluntary exercise or SPA

(Breier et al. 2006; Dai et al. 2012; Sun et al. 2013; Hiramatsu et al. 2017; see also Donovan et al. 2013 on sheep).

Here, we aimed to identify possible long-lasting effects of juvenile exposure to Western diet (WD) and exercise. We used mice from a well-characterized experimental evolution animal model: four replicate High Runner (HR) lines that were selectively bred for 76 generations for increased voluntary wheel-running behavior on days 5 and 6 of a 6-day wheel exposure as young adults and four non-selected control (C) lines (Swallow et al. 1998; Careau et al. 2013). On average, mice from the HR lines run ~3-fold greater distance per day compared with the C lines, for both sexes (Swallow et al. 1998). A number of other phenotypes have evolved in association with wheel-running behavior in the HR lines, including greater SPA when wheels are not provided, increased running endurance during forced treadmill exercise, increased maximal aerobic capacity during forced exercise (VO_2max), reduced body fat, larger hearts, altered dopamine signaling in the brain, altered endocannabinoid signaling, higher baseline circulating corticosterone levels, and lower circulating leptin levels (Rhodes et al. 2005; Rhodes and Kawecki 2009; Swallow et al. 2009; Garland, Jr. et al. 2011, 2016; Keeney et al. 2012; Wallace and Garland, Jr. 2016; Thompson et al. 2017; Kay et al. 2019).

Previously, we found that access to Western diet beginning at weaning and continuing through adulthood had a large stimulatory effect (~50–75%) on daily wheel-running distance of both juvenile and adult male HR mice, with no

statistically significant effect on C mice (Meek et al. 2010; only males tested). These dramatic results for voluntary exercise were unprecedented for rodents, although in adult rats a high-fat diet can increase VO_2max and endurance capacity (Simi et al. 1991). A subsequent study of HR and C mice found that early-life wheel access for just 3 weeks, starting at weaning, increased adult wheel running in males of both linetypes, with no effect on SPA and contrasting effects on adult circulating leptin levels in HR and C mice (Acosta et al. 2015). All early-life-exercise mice had significantly reduced adult body mass and a trend for reduced visceral fat pad mass (Acosta et al. 2015). Taken together, these previous results demonstrate that early-life effects on adult physical activity can be genotype-dependent.

In the present study, we addressed three specific questions. (1) Do WD and wheel access given to juveniles have acute/immediate effects on juvenile physical activity, caloric intake, and body composition? (2) Do WD and wheel access given to juveniles have long-lasting effects on adult physical activity and related phenotypes? (3) Do early-life diet or exercise interact with genetic backgrounds to influence adult phenotypes? From what has been reported in similar previous studies (Meek et al. 2010; Acosta et al. 2015), we expected that WD and/or exercise would have substantial effects when applied during the juvenile period, and we hypothesized that those effects would carry over into adulthood, potentially affecting locomotor behavior (Garland, Jr. et al. 2017). To address these questions, we used HR and C male mice from all replicate lines of

generation 76 of the HR animal model. During the juvenile period (3-6 weeks post birth), we administered either standard diet or WD and altered voluntary exercise by granting wheel access or not. We tested juveniles at 6 weeks for SPA, caloric intake, and body composition. After a washout period of eight weeks (equivalent to ~6 human years), we then tested adults for VE, SPA, and related traits.

2. Materials and Methods

2.1. Experimental mice

Starting in 1993, four replicate lines of house mice were bred in an ongoing selection experiment for high voluntary wheel running (HR lines), based on the number of wheel revolutions on days five and six of six days of access to Wahman-type activity wheels (1.12-meter circumference) as young adults (Swallow et al. 1998). The experiment began with a population of 224 mice from the outbred Hsd:ICR strain, which was randomly mated for two generations before being randomly partitioned into eight lines. Four of these were bred randomly as Control (C) lines to the four HR lines. The current experiment used a subset of mice from generation 76. All experiments followed University of California Riverside IACUC guidelines.

2.2. Early-life diet and exercise manipulation

In this experiment, 196 male mice (half HR and half C) were weaned individually into standard cages at 3 weeks of age and placed in one of four treatment groups for 3 weeks until sexual maturity at 6 weeks of age (Fig. 1.1.). Only males were studied due to resource constraint (e.g., the number of wheels available) and to avoid complications caused by possible estrus-cycle effects on some of the outcome variables. Half of the mice from each line were given "Western" diet (WD: Harlan Teklad TD.88137, 42% kJ from fat [anhydrous milk], 42.7% kJ from carbohydrates [sucrose and cornstarch], 15.2% kJ from protein [casein], 34.1% added sucrose by weight) and the other half were given standard diet (SD: Teklad Rodent Diet W-8604, 14% kJ from fat, 54% kJ from carbohydrates, and 32% kJ from protein, no added sugars [less than ~9% naturally occurring sugars by weight, mostly from grains]). According to Teklad/Envigo, the protein sources are very different for these two diets, so the amino acid profiles may be different as well, but both should meet all of the estimated requirements for amino acids for rodents (Derek Martin in email of 8 Feb. 2021). See Meek et al. (2010) for more nutritional information on the Western and standard mouse chow.

Within each diet group, half of the animals from each line occupied cages attached to activity wheels (see above). Body mass, food consumption, and body composition were measured every seven days after weaning until mice were 6 weeks old. Photoperiod was 12:12, with lights on at 07:00 h.

2.3. Adult testing

Beginning at 6 weeks of age, all mice were individually housed with SD and without wheel access for an additional 8 weeks. At 14 weeks of age, testing of VO_2max, open-field behavior, WD and sucrose preference began. At 18 weeks of age, blood lipid levels were measured, and all adult mice were given access to wheels for 2 weeks to measure VE. Adult SPA was again measured for the subset of mice that did not have access to wheels as juveniles. Mice were euthanized and tissues collected at approximately 20 weeks of age.

For each day of wheel testing (3 weeks as juveniles and 2 weeks as adults), we recorded revolutions in each 1-minute interval over a period of 23 h. This allowed computation of the total distance run, the number of 1-minute intervals with at least one revolution (a measure of the daily running duration), and the mean running speed (distance/intervals), as well as the maximum speed in any 1-minute interval (Copes et al. 2015). We also computed weekly means for these measures. Wheel freeness (an inverse measure of how difficult it is to turn the wheel) was used as a covariate in all analyses of wheel running (e.g., Copes et al. 2015).

2.3.1. Home-cage activity

SPA was measured as home-cage activity. Mice were housed in home cages fitted with a passive infrared sensor (Talon TL-Xpress-A; Crow Electronics, Fort Lee,

New Jersey, USA), protected within wire mesh, as in previous studies (Acosta et al. 2015; Copes et al. 2015). The sensors were connected to a computer with custom activity-recording software (developed by M. A. Chappell) via a digital I/O board (ICS 2313; ICS Electronics, Pleasanton, CA, USA). The sensors recorded activity three times per second and a mean value between 0 (no movement detected) and 1 (movement detected) was calculated for each minute over 23 hour-periods of measurement. All analyses of SPA data used a measure of sensor sensitivity as a covariate (Acosta et al. 2015; Copes et al. 2015). Juvenile and adult SPA could only be measured in mice that were not given wheel access during the juvenile treatment stage.

2.3.2. Body composition

Whole-animal fat, lean, free water, and total water masses were measured by restraining each mouse within a translucent tube before insertion into an EchoMRI-100 (Echo Medical Systems, Houston, TX, USA) for scanning. This procedure lasted approximately 1-2 min per mouse and did not require sedation or anesthesia. Body composition was analyzed at weaning, after each of the three weeks of juvenile exposure, and again at 9 weeks of age, prior to adult wheel testing.

2.3.3. Caloric intake

Food consumption was measured by the change in food hopper weight each week during early-life treatment (when both SD and WD were used) and during adult wheel testing (when all mice received SD), taking care to note

wasted or shredded food (Koteja et al. 2003). Weekly food consumption was converted from grams to caloric intake, taking the caloric content of SD and WD into account (14 and 19 kJ per gram, respectively; Meek et al. 2010).

2.3.4. Maximal aerobic capacity

To measure VO_2max, mice were subjected to forced exercise within a 900 mL enclosed mouse wheel approximately 15 cm in diameter (Dlugosz et al. 2009; Claghorn et al. 2017). Each mouse was run for approximately 5 min. while researchers manually accelerated the wheel. Duplicate trials were conducted, allowing a day of rest between each trial. Air was pumped into the enclosed metabolic chamber at a rate of 2000 mL per min, and the concentration of O_2 in excurrent air was measured by an oxygen analyzer (S-3A Applied Electrochemistry, Inc., Sunnyvale, CA). We then calculated oxygen consumption as the difference in O_2 concentration between reference air (pumped through the wheel before the mouse was placed inside) and sampled air (while the mouse was inside). Following previous studies (Claghorn et al. 2017; Hiramatsu et al. 2017), VO_2max was taken as the highest minute of oxygen consumption during either trial, as calculated with LabHelper software (Warthog Systems, www.warthog.ucr.edu). Trials that did not evidence a plateau in O_2 with increasing wheel speed were excluded.

Researchers subjectively assessed mouse cooperation during each trial and tiredness after each trial to assess the quality of the trial. Cooperation was

scored between 1, least cooperative (the mouse made repeated attempts to breathe through the chamber aperture and/or resisted moving in the direction of motion) and 5, most cooperative (the mouse was least interested in the aperture and focused mostly on running with the direction of rotation). Tiredness was scored between 1, least exhausted after the trial, to 3, most exhausted. Tiredness (1-3 scale) was scored based on long it took for the mouse to begin moving about the respiration chamber after the end of forced exercise (1 = 1 s or less; 3 = 5 s or more). The identity of the investigator scoring each trial was recorded, so that potential differences in scoring could be accounted for. Trials were excluded from analyses if cooperation scores were less than 3 or tiredness scores were less than 2, as values this low likely correspond to trials in which VO_2max would not be attained.

2.3.5. Preference for Western diet and sucrose solution

At approximately 17 weeks of age, adult mice were presented in their home cage with partitioned food hoppers containing WD and SD for 24 h (13:00–13:00 h). Consumption of each diet was measured as the change in hopper weight across the test period, with due allowance for spillage and wasting (Koteja et al. 2003). The next day, mice were given a similar choice between water and sucrose-water (13:00–13:00 h) (10.5% sucrose; Fisher Scientific Certified ACS Grade). WD preference was calculated by caloric conversion: WD kJ / (WD kJ + SD kJ) (Meek et al. 2010; Acosta et al. 2017). Sucrose preference was

calculated similarly as volume of sucrose-water / (volume of sucrose-water + volume of water).

2.3.6. Open-field behavior

Between 15 and 16 weeks of age, mice were tested for open-field behavior (Fuss et al. 2009) on reversed photoperiod (at approximately 07:00–18:00 h). Each mouse was placed in the center of an arena (1 m^2) surrounded by 0.5 m black Trovicel walls under low-light conditions (Bronikowski et al. 2001). The mouse was recorded for 5 min with a Logitech HD C525 Webcam placed above the arena. Videos were assessed with TopScan LITE software (Clever Sys Inc., Reston, VA). The amount of urine (scored as 0, 1, 2) and the number of fecal pellets left by the mouse during the test were recorded. The arena was cleaned with warm, soapy water and dried between each trial. Analyses of time spent near the arena walls were done on each min of the trials, using time of day as a covariate.

2.3.7. Blood lipid profile

At 17 weeks of age, food was removed at 07:00 h and blood samples were taken from the submandibular vein using sterile, single-use lancets under isoflurane anesthesia at 13:00–19:00 h (Golde et al. 2005). Total cholesterol (TC), high-density lipoprotein (HDL) cholesterol, triglycerides, and glucose (GLU) in whole blood, as well as TC/HDL (total cholesterol/HDL cholesterol) ratio and

estimates of low-density lipoprotein (LDL) and non-HDL cholesterol were measured and calculated by an Abbott Cholestech LDX™ Analyzer. Time of day and bleed delay time (elapsed time from disturbing the mouse to collecting a blood sample) were used as covariates in statistical analyses.

2.3.8. Dissections and plasma hormone concentrations

Mice were removed from wheel access for one day then euthanized via decapitation. Trunk blood was immediately collected into heparinized containers, and organs (brain, heart ventricles, spleen, liver, triceps surae muscles, and fat pads) were dissected and weighed to 0.0001 g. Plasma samples were prepared and preserved as instructed by the assay kits described below.

Terminal plasma leptin was measured in duplicate using a Crystal Chem enzyme-linked immunosorbent assay (ELISA) kit (Mouse Leptin Assay Catalog #90030) without dilution. Absorbances were read at 450 nm in an EPOCH2 microplate reader using GEN5 2.07 reading software (microplate and reading software: BioTek Instruments, Winooski, VT, USA). Terminal plasma adiponectin was measured in duplicate with an AssayPro ELISA kit (Mouse Adiponectin ACRP30 Catalog #EMA 2500-1), using a 400-fold dilution. Prior to dissection, plasma was collected from the caudal vein at 17 weeks of age (Fig. 1.1.) specifically to measure corticosterone concentration in duplicate with an Arbor Assays ELISA kit (Corticosterone EIA kit Catalog #K014-H1), using a 100-fold dilution. All assay plates were read in triplicate.

2.3.9. Statistical analysis

Data were analyzed as mixed models in SAS 9.1.3 (SAS Institute, Cary, NC, USA) Procedure Mixed, with REML estimation and Type III Tests of Fixed Effects. Depending on the trait analyzed, body mass, age, wheel freeness, and home-cage sensor sensitivity were used as covariates. Linetype (HR vs. C), diet, and wheel access were fixed effects, while replicate lines were a random effect nested within linetype. Effects of linetype, diet, and wheel access, as well as their interactions, were tested with 1 and 6 degrees of freedom. Modeling the aforementioned random effects occasionally resulted in covariance parameter estimates of zero for some of the interactive random terms. In these cases, the interactive random terms were removed, starting with higher-order terms. The line within linetype random term was never removed. Once analyses returned non-zero covariance parameter estimates, outliers were then removed when the standardized residual exceeded ~3.

An additional fixed effect, termed mini-muscle, was included in all statistical analyses. The original base population of the outbred strain had a mini-muscle phenotype, which is caused by a Mendelian recessive allele that is a novel intronic single nucleotide polymorphism in the *Myosin heavy polypeptide 4* gene (Kelly et al. 2013). The allele started at a frequency of ~7% in the base population and was under positive selection in the HR lines, eventually going to fixation in one HR line while remaining polymorphic in another (Garland, Jr. et al. 2002; Syme et al. 2005; Hannon et al. 2008; Hillis et al. 2020). The mini-muscle

phenotype is characterized by a 50% reduction in the triceps surae muscle and total hindlimb muscle mass – due largely to reduced type IIb muscle fibers (Guderley et al. 2006; Talmadge et al. 2014). The effect of mini-muscle phenotype is reported in tables of statistical results but, for simplicity and as it is not a primary focus of the present study, not mentioned in the Results. In Discussion, we summarize results of the mini-muscle phenotype.

Leptin and adiponectin concentrations were analyzed with covariates of fat mass (as measured by MRI). To remove outliers from plasma hormone data, we tested for variation due to multiple reads of the assay plates and variation between duplicates. SAS Procedure Mixed was used with Mouse ID as the independent variable and sample duplicate nested within Mouse ID and read triplicate nested within sample duplicate as random effects. Corticosterone samples with bleed delay time of 3 minutes or more were excluded from analysis (n = 10). As described above, outliers were removed when the standardized residual exceeded ~3.

To investigate any possible effects of litter sex ratio at weaning, we used sex ratio as a covariate in many relevant preliminary tests (e.g., juvenile and adult wheel running distance, body mass and composition, maximal aerobic capacity, etc.). The effect of sex ratio was never statistically significant (results not shown), so final models did not include it as a covariate.

3. Results

Juvenile exposure to a Western diet and/or voluntary exercise had numerous effects on juvenile and adult wheel-running behavior, body mass and fat composition, and other behavioral and physiological traits, some in an interactive genotype-by-environment manner.

Note: Supplemental File 1.1 is a spreadsheet that summarizes p-values for main and interactive effects of linetype and treatment on various traits (most of which are reported in this chapter). Subsequent sheets contain more detailed results (degrees of freedom, least squares means, standard errors, etc.) for each trait, which can be found by navigating to the sheet corresponding to the "index" number indicated in column A of the RESULTS sheet.

3.1. Juvenile wheel running and spontaneous physical activity (during treatment)

During the first week of treatment, WD increased the distance (i.e., number of revolutions) run by 29% in C mice and by 48% in HR mice (Fig. 1.2.A; diet $p < 0.0001$; diet × linetype $p = 0.0072$). During the second week, mice on WD ran 25% more revolutions than those on SD (diet $p = 0.0188$) and HR mice ran 160% more than C mice (linetype $p = 0.0320$), with no interaction. Diet effects disappeared completely by the third week, and HR mice ran more than 3-fold more revolutions per day than C mice ($p = 0.0006$). See Fig. 1.2.B–D and Supplemental File 1.1 for results on duration, average speed, and maximum speed.

Home-cage activity was only recorded for mice housed without wheels, and only during the third week of exposures. Diet did not affect juvenile SPA ($p = 0.5734$); however, HR mice were significantly more active in the home-cage than C mice ($p = 0.0036$) (Supplemental File 1.1).

3.2. Adult wheel running and spontaneous physical activity

In the first week of adult testing, HR mice ran 3.5-fold more distance than C mice (Fig. 1.3.; $p = 0.0008$). Early-life exercise increased wheel running distance among C mice, but not HR mice (exercise × linetype $p = 0.0512$). Early-life WD increased distance for HR mice, but not for C mice (diet × linetype $p = 0.0091$). In the second week, HR mice ran 3.3-fold more than C mice ($p < 0.0001$; Supplemental File 1.1) and early-life WD exposure significantly increased average daily running distance of HR mice ($p = 0.0186$), but not C ($p = 0.8914$; diet × linetype interaction $p = 0.0534$).

During the first week, daily running duration, mean speed, and maximum speed averaged greater for HR mice (all $p \leq 0.0251$). However, significant diet × linetype interactions occurred ($p = 0.0146$, 0.0414, and 0.0761; Fig. 1.3.B, C, and D, respectively), whereby early-life WD decreased these measures among C mice but increased them among HR mice. Early-life wheel access increased mean and maximum speeds, but not running duration (all $p \leq 0.0086$). See Supplemental File 1.1 for results from the second week of adult wheel running.

As noted above, only mice that did not have wheel access during the juvenile period were measured. SPA of adult mice housed with wheel access was unaffected by juvenile diet ($p = 0.4535$) and did not differ between HR and C mice ($p = 0.2218$), with no diet × linetype interaction ($p = 0.3496$ for first week; results for week two in Supplemental File 1.1).

3.3. Body mass and composition

3.3.1. Body mass

Body mass increased throughout the experiment for all four experimental groups for both HR and C lines, until finally declining during the two weeks of adult wheel testing for all groups (Fig. 1.4.A, B). The change in body mass from the end of juvenile exposures (9 weeks of age) to the start of adult wheel testing (18 weeks of age) was not affected by juvenile treatments, with or without body mass at 9 weeks of age as a covariate. The change in body mass across the two weeks of adult wheel testing was influenced by a three-way interaction of juvenile exercise, juvenile diet, and linetype ($p = 0.0059$). Mice from the HR lines were significantly smaller than C mice at all time points, including at weaning (all $p \leq 0.0020$).

3.3.2. Lean mass

Body lean mass increased throughout the experiment (Fig. 1.4.C, D). Adult lean mass did not change significantly across two weeks of wheel testing. Early-life WD increased lean mass a week after treatment ended ($p = 0.0383$),

and this effect persisted through washout into adulthood ($p = 0.0316$). Access to wheels during the juvenile period increased lean mass after the third week of treatments ($p = 0.0104$). HR mice were consistently leaner than C mice during early life and into adulthood (all $p < 0.05$, Supplemental File 1.1).

3.3.3. Fat mass

During each week of juvenile treatment, WD significantly increased body fat in all groups (Fig. 1.4.E, F; all three $p \leq 0.0001$, with lean mass as a covariate), with C mice on WD having the sharpest rise (compared to HR mice on WD) in body fat after 3 weeks of juvenile treatment (diet × linetype $p = 0.0099$). The apparent increase in relative body fat between 9 and 18 weeks of age was not statistically significant for any group, with no significant effect of linetype or early-life treatment. After two weeks of adult wheel testing, relative body fat was significantly reduced in all groups (all $p \leq 0.0138$). At the end of adult wheel testing, relative fat mass had a three-way interaction (exercise × diet × linetype $p = 0.0224$).

3.4. Caloric intake

During the first week of early-life treatment, WD increased mean daily caloric intake by ~21% (Fig. 1.5.; diet $p < 0.0001$). Among SD mice, those with wheels consumed significantly more calories than the sedentary group; however, for WD mice, those with wheel access consumed significantly fewer calories than

the sedentary group (exercise × diet $p < 0.0001$). During the second week of treatment, both exercise ($p = 0.0024$) and diet ($p = 0.0009$) increased caloric intake, with no interactions. During the third week of treatment, mice with wheels consumed more ($p < 0.0001$), and the effect was somewhat greater for HR mice (diet × linetype $p = 0.0881$).

In the first week of adult testing, the early-life treatments did not affect caloric intake. However, in the second week of adult testing caloric intake was decreased by early-life exercise, and the effect was greater among HR mice (Fig. 1.6.; exercise × linetype $p = 0.0126$). HR mice consumed more calories than C mice during both the first (Fig. 1.6.; $p = 0.0276$) and second ($p = 0.0178$) weeks of adult wheel testing.

3.5. Maximum aerobic capacity

With body mass as a covariate, HR mice had greater VO_2max than C mice (Fig. 1.7.; $p = 0.0012$). Neither early-life exercise, nor diet had any effect on VO_2max.

3.6. Preference for Western diet and sucrose-water

All experimental groups preferred WD to SD (see Supplemental File 1.1). Early-life WD increased adult preference for Western diet in all groups, and a three-way interaction indicated that the magnitude of this effect depended on linetype and early-life exercise (see Supplemental File 1.1; $p = 0.0578$). All

groups preferred sucrose-water to regular water, but this preference was not affected by linetype or either early-life treatment.

3.7. Open-field behavior

Early-life exercise reduced the time spent near the arena walls during each of the five minutes of the open-field test for all groups (all $p < 0.05$). Summing across all five minutes also showed a 3-way interaction (exercise × diet × linetype $P = 0.0432$), indicating that the magnitude of the effect varied somewhat among groups. We found no statistical effects on distance moved, except for a linetype effect during minute 5 ($p = 0.0494$; HR > C by ~12%) and an exercise × diet interaction for minute 3 ($p = 0.0412$). We found no statistical effects on open-field defecation or defecation plus urination (Supplemental File 1.1).

3.8. Blood lipid profiles

Using bleed delay time and time of day as covariates, adult levels of triglyceride and total cholesterol were unaffected by early-life treatments. Fasted blood glucose levels were increased by early-life exercise (Supplemental File 1.1; $p = 0.0140$).

3.9. Circulating levels of leptin, adiponectin, and corticosterone

HR mice had approximately 17% greater corticosterone concentration than C mice (Supplemental File 1.1; $p = 0.0068$). Among mice exposed to an early-life SD, early-life exercise decreased corticosterone concentration for C mice but increased it for HR (Supplemental File 1.1; exercise × diet × linetype = 0.0259). Using fat mass as a covariate, early-life exercise treatment increased adult leptin concentration (Supplemental File 1.1; $p = 0.0173$), while adiponectin concentration was not affected by linetype or either early-life treatment.

3.10. Organ masses

Early-life exercise increased adult triceps surae, subdermal fat pad, and brain mass, but decreased heart ventricle mass (Fig. 1.8.; all $p \leq 0.0170$). Reproductive fat pad and spleen masses were unaffected by either early-life treatment or genetic background. The hearts and brains of HR mice were heavier than those of C mice (all $p \leq 0.0270$).

4. Discussion

Physical activity should be a cornerstone of effective prevention and treatment strategies for obesity and its comorbidities (Ryan and Diabetes Prevention Program Research Group 2003; Hill and Wyatt 2005; Janiszewski and Ross 2007, 2009; Church and Blair 2009; Swift et al. 2014; Kirwan et al. 2017). Given the risk of overweight children developing into unhealthy,

overweight adults (Malina 2001; Morrison et al. 2007; Singh et al. 2008; The et al. 2010; Lloyd et al. 2012), it is important to attempt to identify early-life factors that might have lasting, positive effects on adult physical activity (Bahls et al. 2014). Implementation of such early-life factors could be much more efficient than waiting until metabolic, cardiovascular, and other disorders appear years later (Vickers et al. 2003; Warden et al. 2007).

The present study is one of the few to examine the possibility of lingering early-life effects of diet and/or physical activity after a substantial washout period with no additional treatment(s). In a full factorial experiment using mice, we tested whether Western diet and/or access to wheels during the juvenile period would: (1) affect juvenile physical activity, caloric intake or body composition; (2) have long-lasting effects into adulthood, following a substantial washout period; and (3) have interactive effects with each other and/or with genetic background. To our knowledge, no other experimental studies have integrated all three factors. We found a number of acute effects of diet and exercise manipulation in juvenile male mice as well as many longer-lasting effects in adults. In addition, some early-life effects interacted with genetic linetype (selectively bred High Runner vs. non-selected Control lines of mice). Our results have implications for understanding pre-pubertal environmental effects in humans, including the complex interactions that result in human obesity and its negative health consequences.

4.1 Immediate and long-lasting effects of early-life Western diet and exercise

4.1.1 Wheel running

Western diet (WD) increased voluntary exercise (total distance run) during weeks 4 and 5 of the initial period of juvenile exposure (Fig. 1.2.) and also when adults were retested (Fig. 1.3.), mainly through increased running duration. This effect was greater for HR mice, and in a previous study was observed only in HR mice (Meek et al. 2010). Together, these results suggest that inherently athletic individuals and/or those who are more physically active might gain a greater benefit from high-energy diets.

Juvenile exercise increased adult running distance for C mice only (Fig. 1.3.: exercise × linetype $p = 0.0512$). In contrast, Acosta et al. (2015) found that early-life wheel access increased distance run in both HR and C mice. This difference might relate to previously an observed seasonal variation in wheel running (see Fig. S4 in Careau et al. 2013), as the mice in Acosta et al. (2015) were weaned in June whereas ours were weaned in October. In both studies, early-life wheel effects on adult running distance were transient in that they became statistically nonsignificant after the first week of adult testing (Supplemental File 1.1).

Although the positive effects of juvenile Western diet and exercise that we observed lasted for only one week, this translates to ~9 months for humans (Demetrius 2006; Flurkey et al. 2007). As these sorts of early-life environmental factors can be manipulated relatively easily (e.g., through school cafeterias or

youth sports programs), they may be an efficacious tool in the fight against the obesity epidemic and adult sedentary behavior, although they likely need to be combined with reinforcers during adulthood (Dalle Grave et al. 2011; Gardner et al. 2012; Strohacker et al. 2014).

4.1.2 Body mass and composition

During the juvenile period, male mice given access to wheels did not show the expected reduction in body mass, compared to juveniles without wheel access (e.g. for male mice see Hayes and Williams 1996; Swallow et al. 1999; Acosta et al. 2015). In fact, after three weeks of early-life exposure, mice with wheels had *increased* body mass compared to those without wheels ($p = 0.0415$); this difference was related to increased absolute lean mass ($p = 0.0104$) and accompanied by slightly reduced body fat ($p = 0.0936$ or $p = 0.0552$ with lean mass as a covariate).

At the start of adult wheel testing, early-life wheel access had no significant effect on body mass (consistent with Acosta et al. 2015), lean mass or fat mass (see Fig. 1.4. and Supplemental File 1.1), although HR mice weighed significantly less and had lower lean mass as compared with C mice. To our knowledge, no other study has reported effects of early-life exercise on adult body mass after a substantial washout period.

All treatment groups lost body mass across the two weeks of adult wheel testing, but the magnitude of these reductions differed among groups (exercise ×

diet × linetype interaction p = 0.0059). With respect to body composition, none of the eight experimental groups lost a statistically significant amount of lean mass, whereas all of them lost significant fat mass (see Supplemental File 1.1). Consistent with a previous study that used a 6-day wheel test, HR lines lost less fat mass than C mice during adult wheel testing (see Supplemental File 1.1) (Hiramatsu and Garland 2018), indicating that the regulation of body weight when faced with acute exercise varies with genetic background.

Although WD consistently increased fat mass during the three weeks of early-life exposure, simultaneous exercise partially protected mice from this increase (though it was not eliminated completely [(exercise x diet p = 0.0170 for Week 1, p = 0.5136 for Week 2, and p = 0.0355 for Week 3)]). This effect mostly faded by the end of the 8-week washout, during which all mice received SD. Early-life WD increased adult lean mass (p = 0.0316), but the effect disappeared after two weeks of adult exercise (p = 0.0960).

4.1.3 Caloric intake

We expected that body mass-adjusted caloric intake would increase with wheel access (Swallow et al. 2001; Acosta et al. 2015; Copes et al. 2015). Surprisingly, in the first week of juvenile treatments, early-life wheel access decreased caloric intake for mice feeding on WD and increased it for mice feeding on SD (Fig. 1.5.). During this first week, mice are experiencing multiple environmental changes (novel exposures), including not only diet and exercise opportunity, but also single housing, which affects social behavior and thermoregulation, so their normal homeostatic mechanisms (both

physiological and neurobiological) may not yet have adjusted. During the second and third weeks, however, wheel access had the expected positive effect on caloric intake and did not interact with diet or linetype. In any case, the neural and endocrine mechanisms that underlie homeostatic responses to novel (acute) versus longer-term (chronic) environmental changes deserve further study.

During the first week of adult wheel testing, HR mice had greater mass-adjusted caloric intake than C mice, consistent with previous studies (e.g., Acosta et al. 2015; Copes et al. 2015; Hiramatsu and Garland 2018), with no statistical effects of either early-life WD ($p = 0.0929$) or exercise (Fig. 1.6.). During the second week of adult testing, an effect of early-life exercise emerged, in which caloric intake was decreased in HR mice, but not in C mice (exercise × linetype $p = 0.0126$). When we added distance run as a covariate, to account for the greater running by HR mice, the interaction disappeared, and the effect of early-life exercise was negative for both HR and C mice (Supplemental File 1.1). Given that early-life exercise increased adult wheel running in both HR and C mice, and that HR mice ran much more than C, this effect suggests an alteration in digestive or metabolic efficiency, or perhaps in the regulation of body weight, although the loss in body mass across the two weeks of adult wheel access did not show any statistically significant interactive effects (Supplemental File 1.1). In any case, early-life exercise exposure combined with adult exercise had a dampening effect on caloric intake, which could have important implications for human health (Chin et al. 2016; Golbidi et al. 2017; Obert et al. 2017).

4.1.4 Western diet and sucrose preference

As adults, almost all mice preferred the WD to standard diet when given 24-hour access to both diets, as is commonly reported (e.g., Acosta et al. 2017). On average, however, those given WD as juveniles had a significantly stronger preference for WD than those given juvenile SD (Supplemental File 1.1). Although WD is high in sugar and fat, we did not find any statistically significant effects on adult sucrose preference, suggesting that familiarity with the taste of the fatty component of WD may be driving the diet preference. Additionally, a three-way interaction effect on WD preference was found (exercise × diet × linetype $p = 0.0578$), indicating some effect of genetic background. Although few rodent studies are available (e.g., Teegarden et al. 2009), various human studies indicate that early-life factors can affect adult dietary preferences (e.g., Lussana et al. 2008; Paglia 2019).

4.1.5 Open-field behavior

Early-life wheel access generally reduced adult anxiety-like behavior (i.e., less time spent near walls in the open field), although the strength of the effect varied among groups (exercise × diet × linetype $p = 0.0432$). In principle, effects on anxiety-like behavior might be mediated by plasma corticosterone levels (Korte 2001; McEwen et al. 2015). As noted in the next section, adult plasma corticosterone levels also showed a 3-way interaction (exercise × diet × linetype $p = 0.0259$). The patterns for these 3-way interactions were somewhat different, but the correlation of the eight group means for time spent near walls (over all 5 minutes, squared) and plasma corticosterone (log-transformed) was positive

(Pearson's r = 0.546, p = 0.1615), suggesting that corticosterone might have played some role in mediating effects of early-life wheel access on anxiety-like behavior. The reduction in anxiety-like behaviors cannot be attributed to effects of early-life wheel access on activity levels because distance moved in the open-field test was not affected in a way that paralleled time spent near walls. An interesting direction for future studies would be to evaluate a possible role of the neuropeptide corticotropin-releasing hormone, which regulates corticosterone secretion, strongly influences anxiety-like behavior, and has been suggested to mediate effects of experience (stress) during the juvenile period on adult phenotypes (Burke and Miczek 2014; Syed et al. 2017; Zhang et al. 2017).

4.1.6 Leptin, adiponectin, corticosterone, and blood glucose

Leptin is produced by white adipose tissue and, among other functions, regulates body weight by increasing energy expenditure and inhibiting food intake (Zhang et al. 2005; Triantafyllou et al. 2016; Zhang and Chua 2017), along with a host of other effects, including an important role in glucose metabolism (Meek and Morton 2016). As expected from numerous previous studies, including those involving the HR mice (Girard et al. 2007; Acosta et al. 2015; Hiramatsu et al. 2017), body fat was a strong positive predictor of adult levels of circulating leptin in the present study. In addition, early-life wheel access increased fat-adjusted leptin levels in both linetypes, whereas Acosta et al (2015) found that early-life wheel access interacted with linetype (no wheels: HR>C;

wheels: HR<C). The discrepancy in these results may be explainable by Acosta et al.'s. use of visceral fat pad mass and our use of body fat measured by MRI as covariates: dissected visceral fat pads are only a subset of a mouse's leptin-producing adipocytes; however, not all body fat measured via MRI produces leptin (Cinti 2012). It is also worth noting that leptin regulation in mice can be influenced by age (Ahren et al. 1997); we collected terminal serum samples when mice were 20 weeks old, whereas Acosta et al. (2015) took samples at ~15 weeks.

Although some previous studies have reported higher plasma adiponectin levels in HR mice as compared with C lines (Vaanholt et al. 2007, 2008; Garland, Jr. et al. 2017), we did not find any statistically significant effects on adiponectin. However, adult plasma corticosterone showed a 3-way interaction (exercise × diet × linetype $p = 0.0259$), as well as a main effect of linetype ($p = 0.0068$). The selected HR lines always have elevated corticosterone levels, as has generally been found in previous studies (e.g., Malisch et al. 2007, 2008, 2016; Downs et al. 2012; but see Vaanholt et al. 2007; Hiramatsu et al. 2017). In addition, HR mice exposed to early-life wheel access and SD had much higher levels of corticosterone than any other group (Supplemental File 1.1).

To our knowledge, no previous studies have tested for long-lasting effects of early-life exercise on adult blood glucose concentrations. We observed higher glucose levels in mice given juvenile wheel access than in those housed without wheels. Perhaps this effect is related to the effects observed when humans and rodents are cycled between standard and high-fat diets (e.g., Strohacker et al. 2009). It is also possible that early-life exercise has affected glucose tolerance (Oelkrug et

al. 2020); however, the current study did not include a glucose tolerance test, which may have been useful in addressing these questions. In any case, the early-life exercise effects on leptin noted above may be involved in the effects on blood glucose (Meek and Morton 2016).

4.1.7 Organ masses

Numerous studies spanning many decades show that providing laboratory rodents with chronic wheel access leads to changes in the size of internal organs relative to body size, but few have tested for long-lasting effects of juvenile exercise alone. In a previous study of male HR and C mice, Acosta et al. (2015) found no effects of early-life wheel access on visceral fat pads, heart ventricles, liver, or spleen masses. Some of our results are similar, but others differ. Heart ventricle and liver masses were affected by multiple interactions among diet, exercise, and linetype (see Fig. 1.8.A, C). These effects could be either beneficial or detrimental, depending on the cause. For example, an increase in liver mass may be detrimental if caused primarily by WD (Fig. 1.8.D), which often causes cirrhosis or non-alcoholic fatty liver disease (Zhou et al. 2014; Maurice and Manousou 2018, respectively).

An apparently novel finding was the positive effect of early-life exercise on calf-muscle mass for all groups (Fig. 1.8.B). This type of effect, if it occurs in humans, has obvious relevance for human health from early-life to advanced age (Westcott 2012; McLeod et al. 2016; Orsso et al. 2019).

Another notable effect of early-life exercise was an increased brain mass in all groups. Several recent studies have shown that chronic exercise can increase brain mass in rodents, even within one week (Sumiyoshi et al. 2014). To our knowledge, however, this is the first report of a juvenile effect on brain mass that persists into adulthood after a substantial washout period. Exercise is known to upregulate growth factors, including brain-derived neurotrophic factor, which may have an even larger effect during a developmental period (e.g., see Park and Höke 2014 for rodents and Negaresh et al. 2018 for humans).

4.2 Differences between High Runner and non-selected Control lines

The current study reaffirms many previously reported differences between the HR and C linetypes (Acosta et al. 2015; Garland, Jr. et al. 2016; Wallace and Garland, Jr. 2016; Hiramatsu et al. 2017). Average daily wheel-running distance during juvenile testing was higher for HR than C mice, due mainly to greater average running speeds, consistent with many previous studies (e.g., Swallow et al. 1999; Meek et al. 2010; Acosta et al. 2015) (Fig. 1.2.A).

In addition, as adults, HR mice were smaller in total body mass and had leaner bodies, and (with body mass as a covariate), they had greater maximal aerobic capacity, heavier hearts and brains, and elevated circulating levels of corticosterone. Other differences, including several that involved interactions with early-life diet and/or exercise exposure, are mentioned above.

4.3 Characteristics of mini-muscle individuals

As described above (see *2.3.9. Statistical analysis*), the mini-muscle phenotype and underlying gene were an unexpected discovery in the High Runner mouse selection experiment (Garland, Jr. et al. 2002; Kelly et al. 2013). In addition to their greatly reduced hindlimb muscle mass, the present study confirmed several previously reported differences between mini- and normal-muscled individuals, including heavier heart ventricles, livers, and greater VO_2max (Rezende 2006; Kolb et al. 2010; Hiramatsu et al. 2017; Kelly et al. 2017; Kay et al. 2019). In addition, we found some new differences, including that mini-muscle individuals spent more time near the walls in an open-field behavior test (suggesting elevated anxiety) and increased total body fat. Future studies will be required to determine the molecular and cellular pathways through which the mini-muscle gene causes such a wide range of pleiotropic effects.

4.4. Limitations and Concluding Remarks

One limitation of the present study is that all mice were given two weeks of adult wheel access, followed by one day with the wheels removed, prior to dissection. Therefore, organ masses and circulating concentrations of adiponectin and leptin would have been affected by early-life effects, two weeks of training effects (physical conditioning), plus any acute "rebound" effects that might have occurred over ~24 hours (e.g., see Dumke et al. 2001). Any of these effects could have obscured the others. In future experiments, we intend to have

multiple cohorts of subjects, such as one that is sampled prior to adult wheel testing.

Another limitation is that we studied only males. Importantly, nutrient intake in early life has been shown to have sex-specific effects on the regulation of physical activity (i.e., wheel running) in mice (Leszczynski et al. 2019). In an early-life experiment on the same mouse model as the current study, Hiramatsu et al. (2017) observed sex differences as well (see their Fig. 1.8. and online supplemental Fig. 1.4.). Moreover, given that many aspects of physical activity (including both motivation and ability), exercise physiology, body composition, and hormone profiles are sexually dimorphic in both mice (Wells 2007; Lightfoot 2008; Palmer and Clegg 2015; Klinker et al. 2017; van Keulen et al. 2020) and humans (Buffa et al. 2001; Malisch et al. 2007; Ingvorsen et al. 2017; Rosenfeld 2017; Zore et al. 2018), future studies should include both sexes.

Considering only statistically significant main effects ($p < 0.05$), we observed 11 effects of early-life exercise but only three effects of early-life Western diet on adult traits, which suggests that the former may be a more effective target for interventions with human youth. Evidence concerning whether activity levels of children can be increased in particular areas without compensation in others is the subject of ongoing debate over a proposed homeostatic compensatory mechanism known as an “activity-stat” (Rowland 1998; Wilkin 2011; Gomersall et al. 2016). In any case, studies of declining physical activity in children suggest that sedentary behaviors begin displacing

physical activity habits much earlier than is generally believed and that interventions should begin prior to initial school admission (Reilly 2011, 2016).

Our results may also be relevant to the current COVID-19 pandemic, wherein the lack of regular physical activity caused by lockdowns and other restrictions on mobility (and likely effects on diet) is becoming a widespread epidemiological issue (Mattioli et al. 2020). In particular, children are poised to develop simultaneous lifestyle habits of physical inactivity and high consumption of calorie-dense foods in quarantine that may have lasting effects into adulthood, such as overweight and obesity (Rundle et al. 2020). (Of course, SARS-CoV-2 infection itself may be viewed as an early-life environmental factor with potentially long-lasting adverse effects.) Changes in lifestyle habits related to diet and exercise appear to differ by country. For example, Di Renzo et al. (2020) found increased physical exercise and consumption of Mediterranean diet in Italy during the pandemic, whereas Sidor and Rzymski (2020) found increased consumption of fast foods (which are usually high-fat, high-sugar) in Poland. In a survey of adults in Australia, 48.9% of respondents reported reduced daily physical activity as a result of pandemic confinement (Stanton et al. 2020). In another survey conducted on young people (aged 10 to 19 years) in a number of South American and European countries, respondents reported a significant increase in the intake of foods high in fat and sugar since the pandemic began (Ruiz-Roso et al. 2020). Because the pandemic is fairly recent, long-term effects of these lifestyle changes are yet unknown; however, we predict that the COVID-

19 pandemic will have long-lasting effects on physical activity and food preferences as today's affected children become adults. Given the acute maladies associated with physical inactivity and the virus itself (Dunton et al. 2020; Woods et al. 2020), it seems especially important to implement interventions that may engender better lifestyle habits in children, who now experience the dietary and physical activity challenges that come with school closures and reduced access to recreational activities (Rundle et al. 2020; Wang et al. 2020).

Conflict of interest statement

The authors declare no conflict of interest.

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Figure Legends

Figure 1.1. Experimental timeline of events starting with the first births of generation 76 mice. Of these mice, 192 males were used for the present experiment. Three-week-long early-life diet and exercise manipulation began immediately after weaning and was followed by an 8-week washout period, during which mice were individually housed without wheels and given standard diet. At the end of washout, all mice were tested. Note that corticosterone was measured in plasma taken before wheel running, whereas leptin and adiponectin were measured in terminal blood samples collected at dissections. WD = Western diet. Home-cage activity is an indicator of spontaneous physical activity (SPA).

Figure 1.2. Juvenile wheel running of male mice during the 3-week treatment period, shown as least squares means and standard errors with accompanying p-values from SAS Procedure Mixed. Note the data shown are from the experimental group given access to wheels (see Fig. 1.1.). A) Mean wheel revolutions per day (circumference 1.12 meters), B) duration of daily running, C) mean revolutions per minute, D) maximum revolutions per minute. Above each week (set of four bars) of measurements are tables of main and interactive effects ($p < 0.05$ in bold font). See Supplemental File 1.1 for additional statistical details.

Figure 1.3. Adult wheel running during days 1-7 of a 2-week testing period. Values are least squares means and standard errors and accompanying p-values from SAS Procedure Mixed. A) Mean wheel revolutions per day (circumference 1.12 meters), B) duration of daily running, C) mean revolutions per minute, D) maximum revolutions per minute. Above each measurement are tables of main and interactive effects ($p < 0.05$ in bold font). SD = standard diet; WD = Western diet; E = exercise; D = diet; LT = linetype. Values for the second week of adult testing can be seen in Supplemental File 1.1.

Figure 1.4. Total body mass, lean mass, and fat mass. Values are least squares means and standard errors from SAS Procedure Mixed. Note that body composition (lean and fat mass) was not measured during weaning (week 3); however, HR mice at weaning weighed 12.61% less than C mice ($p = 0.0278$). Fat mass was analyzed using lean mass as a covariate. See Supplemental File 1.1 for additional statistical details. SD = standard diet, WD = Western diet, Sedentary = housed without wheels as juveniles, Exercise = housed with wheels as juveniles.

Figure 1.5. Weekly mass-adjusted juvenile caloric intake in response to juvenile diet and/or exercise treatment. Values are least squares means and standard errors and accompanying p-values from SAS Procedure Mixed. Above each week are tables of main and interactive effects ($p < 0.05$ in bold font). See Supplemental File 1.1 for additional statistical details.

Figure 1.6. Adult caloric intake with body mass as a covariate. Values are least squares means and standard errors and accompanying p-values from SAS Procedure Mixed. Above each week are tables of main and interactive effects ($p < 0.05$ in bold font). See Supplemental File 1.1 for additional statistical details.

Figure 1.7. Maximal oxygen consumption (VO_2max) measured during forced exercise (see Methods 2.3.4). Body mass was a significant predictor of VO_2max ($p < 0.0001$), mice from High Runner lines had higher VO_2max than those from non-selected Control lines ($p = 0.0019$), and individuals with the mini-muscle phenotype (not shown with a separate symbol) had higher values than normal-muscled mice ($p = 0.0271$), with no effect of either early-life treatment and no interactions (see Supplemental File 1.1 for additional statistical details).

Figure 1.8. Body-mass adjusted heart ventricles, triceps surae, brain, liver, and subdermal fat pad masses. Values are mass-adjusted least squares means and standard errors and accompanying p-values from SAS Procedure Mixed with body mass as a covariate. See Supplemental File 1.1 for additional statistical details.

Figure 1.1.

	4 Control Lines	4 High Runner Lines
Standard diet (4% kJ from fat)	**C**, Standard diet, Wheels	**HR**, Standard diet, Wheels
	C, Standard diet, No wheels	**HR**, Standard diet, No wheels
Western diet (42% kJ from fat)	**C**, Western diet, Wheels	**HR**, Western diet, Wheels
	C, Western diet, No wheels	**HR**, Western diet, No wheels

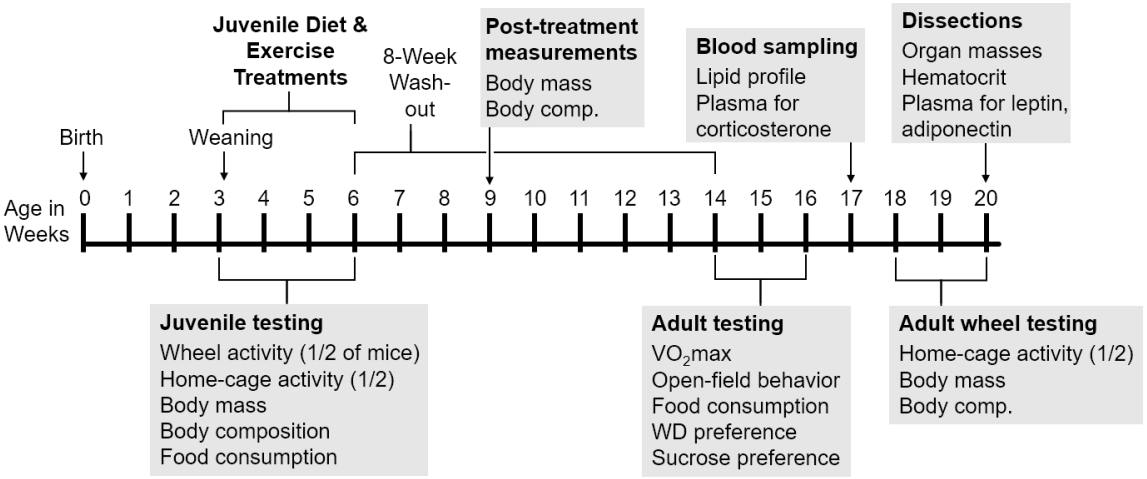


Figure 1.2.

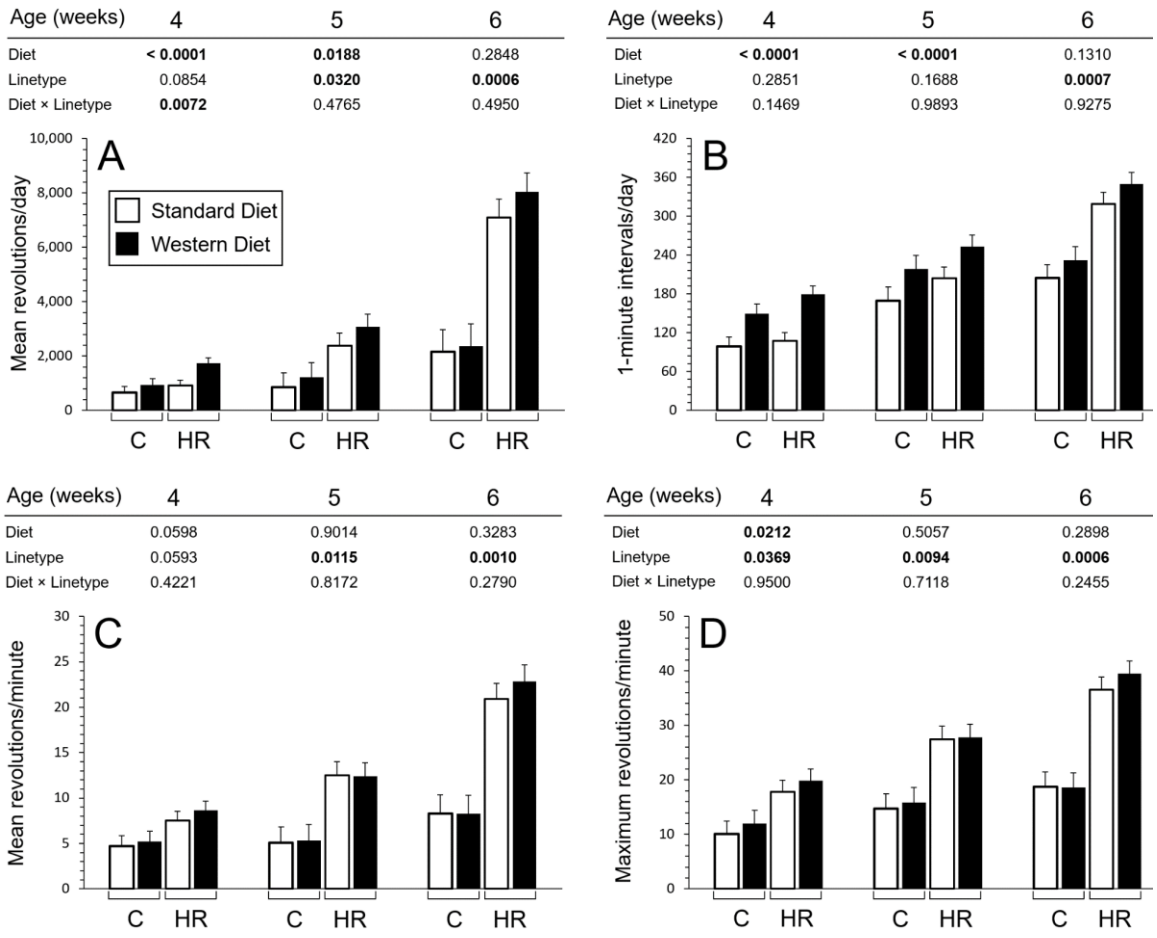

Age (weeks) 4 5 6
Diet < 0.0001 0.0188 0.2848
Linetype 0.0854 0.0320 0.0006
Diet × Linetype 0.0072 0.4765 0.4950
A
Mean revolutions/day
10,000
8,000
6,000
4,000
2,000
0
Standard Diet
Western Diet
C HR C HR C HR
Age (weeks) 4 5 6
Diet < 0.0001 < 0.0001 0.1310
Linetype 0.2851 0.1688 0.0007
Diet × Linetype 0.1469 0.9893 0.9275
B
1-minute intervals/day
420
360
300
240
180
120
60
0
C HR C HR C HR
Age (weeks) 4 5 6
Diet 0.0598 0.9014 0.3283
Linetype 0.0593 0.0115 0.0010
Diet × Linetype 0.4221 0.8172 0.2790
C
Mean revolutions/minute
30
25
20
15
10
5
0
C HR C HR C HR
Age (weeks) 4 5 6
Diet 0.0212 0.5057 0.2898
Linetype 0.0369 0.0094 0.0006
Diet × Linetype 0.9500 0.7118 0.2455
D
Maximum revolutions/minute
50
40
30
20
10
0
C HR C HR C HR

Figure 1.3.

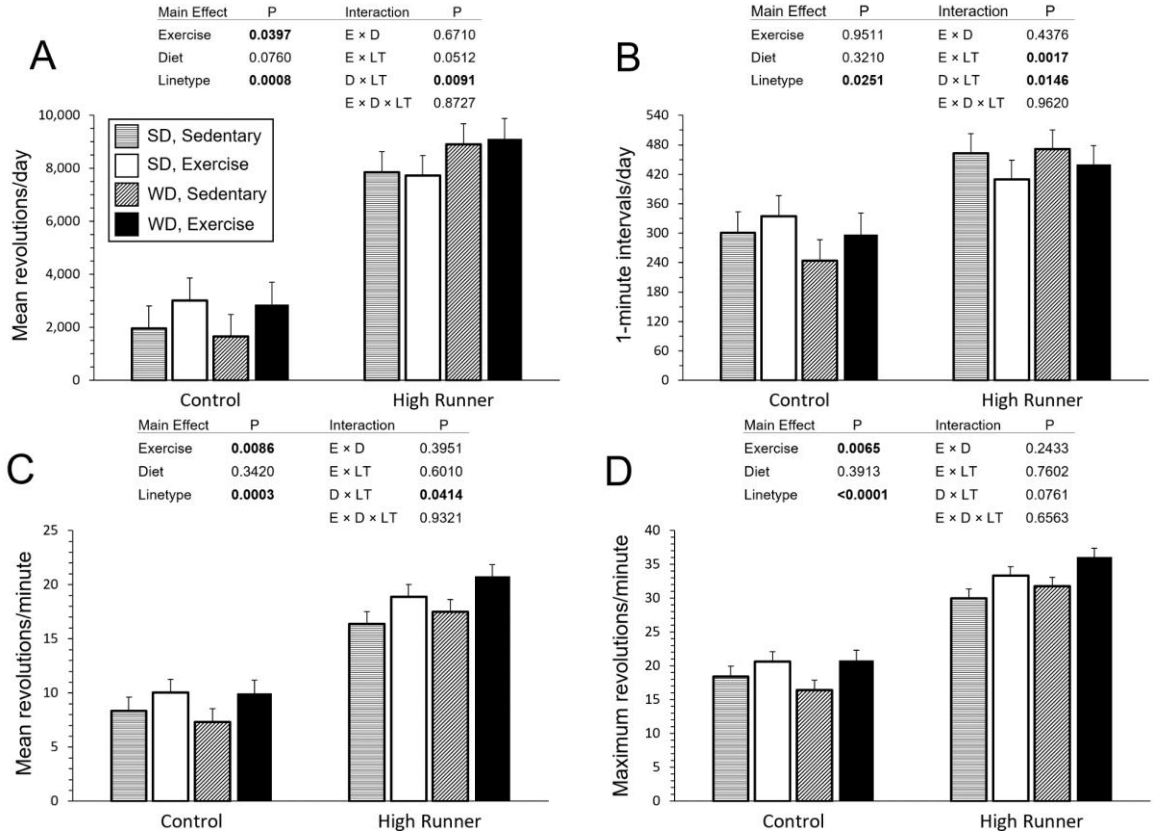

A
Main Effect P
Exercise 0.0397
Diet 0.0760
Linetype 0.0008
Interaction P
E × D 0.6710
E × LT 0.0512
D × LT 0.0091
E × D × LT 0.8727
SD, Sedentary
SD, Exercise
WD, Sedentary
WD, Exercise
Mean revolutions/day
10,000
8,000
6,000
4,000
2,000
0
Control
High Runner
B
Main Effect P
Exercise 0.9511
Diet 0.3210
Linetype 0.0251
Interaction P
E × D 0.4376
E × LT 0.0017
D × LT 0.0146
E × D × LT 0.9620
1-minute intervals/day
540
480
420
360
300
240
180
120
60
0
Control
High Runner
C
Main Effect P
Exercise 0.0086
Diet 0.3420
Linetype 0.0003
Interaction P
E × D 0.3951
E × LT 0.6010
D × LT 0.0414
E × D × LT 0.9321
Mean revolutions/minute
25
20
15
10
5
0
Control
High Runner
D
Main Effect P
Exercise 0.0065
Diet 0.3913
Linetype <0.0001
Interaction P
E × D 0.2433
E × LT 0.7602
D × LT 0.0761
E × D × LT 0.6563
Maximum revolutions/minute
40
35
30
25
20
15
10
5
0
Control
High Runner

Figure 1.4.

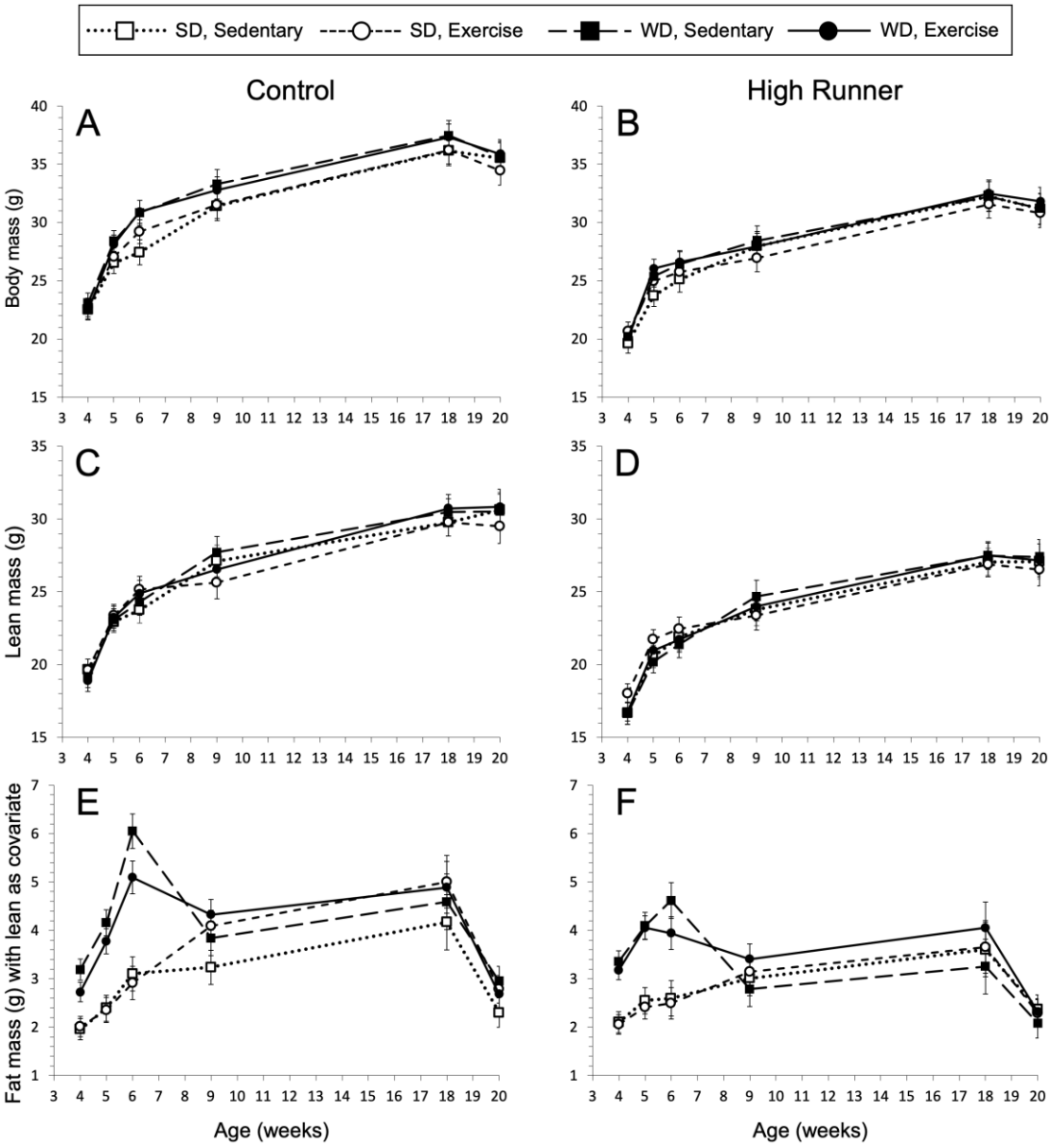

SD, Sedentary
SD, Exercise
WD, Sedentary
WD, Exercise
Control
High Runner
A
B
C
D
E
F
Body mass (g)
Lean mass (g)
Fat mass (g) with lean as covariate
Age (weeks)
Age (weeks)

Figure 1.5.

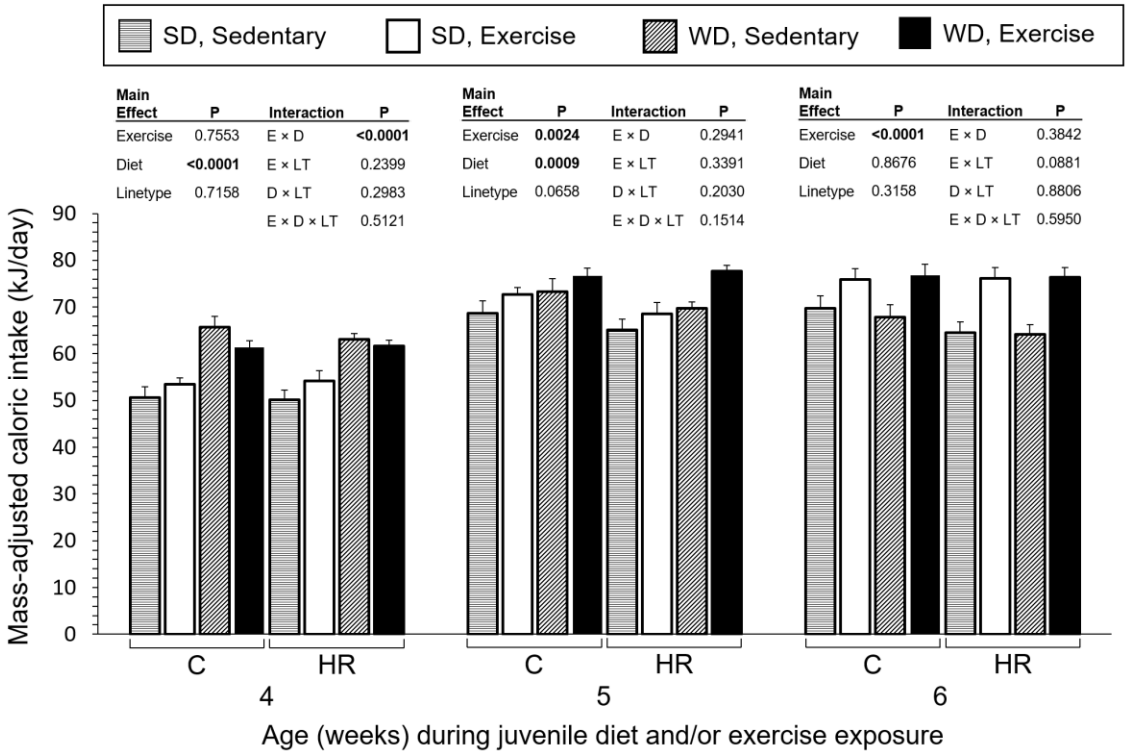

SD, Sedentary
SD, Exercise
WD, Sedentary
WD, Exercise
Main Effect P Interaction P
Exercise 0.7553 E × D <0.0001
Diet <0.0001 E × LT 0.2399
Linetype 0.7158 D × LT 0.2983
E × D × LT 0.5121
Main Effect P Interaction P
Exercise 0.0024 E × D 0.2941
Diet 0.0009 E × LT 0.3391
Linetype 0.0658 D × LT 0.2030
E × D × LT 0.1514
Main Effect P Interaction P
Exercise <0.0001 E × D 0.3842
Diet 0.8676 E × LT 0.0881
Linetype 0.3158 D × LT 0.8806
E × D × LT 0.5950
Mass-adjusted caloric intake (kJ/day)
0
10
20
30
40
50
60
70
80
90
C
HR
C
HR
C
HR
4
5
6
Age (weeks) during juvenile diet and/or exercise exposure

Figure 1.6.

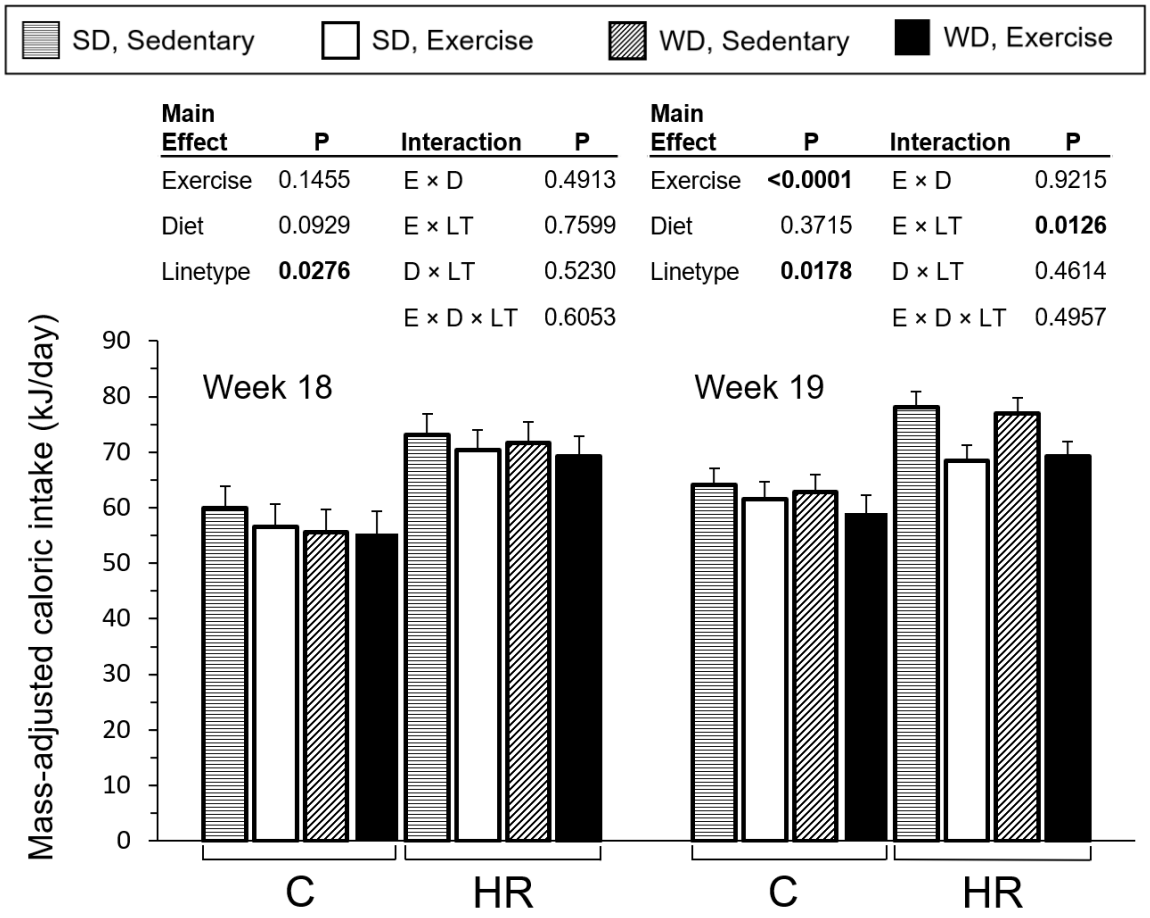

SD, Sedentary
SD, Exercise
WD, Sedentary
WD, Exercise
Main Effect P Interaction P
Exercise 0.1455 E × D 0.4913
Diet 0.0929 E × LT 0.7599
Linetype 0.0276 D × LT 0.5230
E × D × LT 0.6053
Main Effect P Interaction P
Exercise <0.0001 E × D 0.9215
Diet 0.3715 E × LT 0.0126
Linetype 0.0178 D × LT 0.4614
E × D × LT 0.4957
Mass-adjusted caloric intake (kJ/day)
0
10
20
30
40
50
60
70
80
90
Week 18
Week 19
C
HR
C
HR

Figure 1.7.

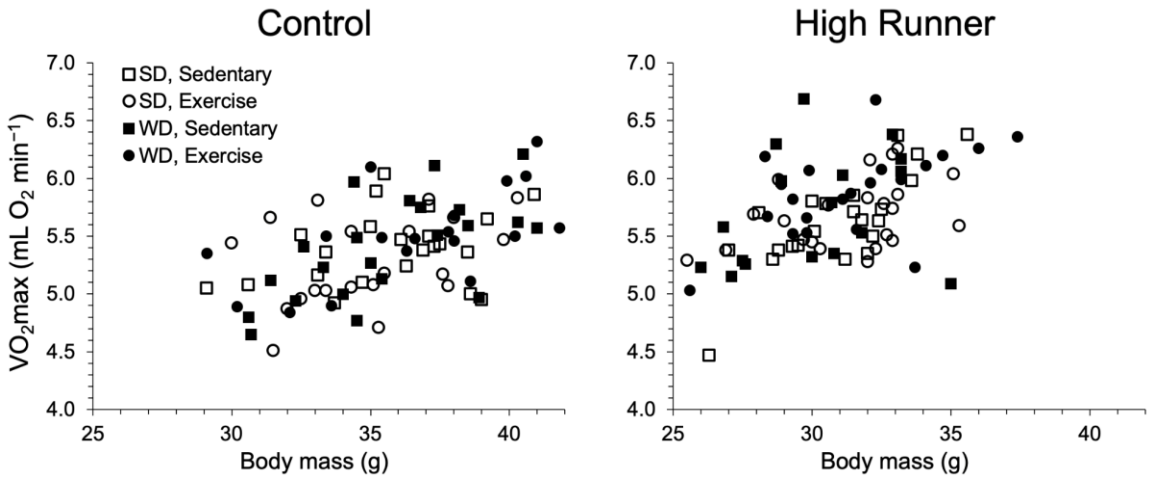
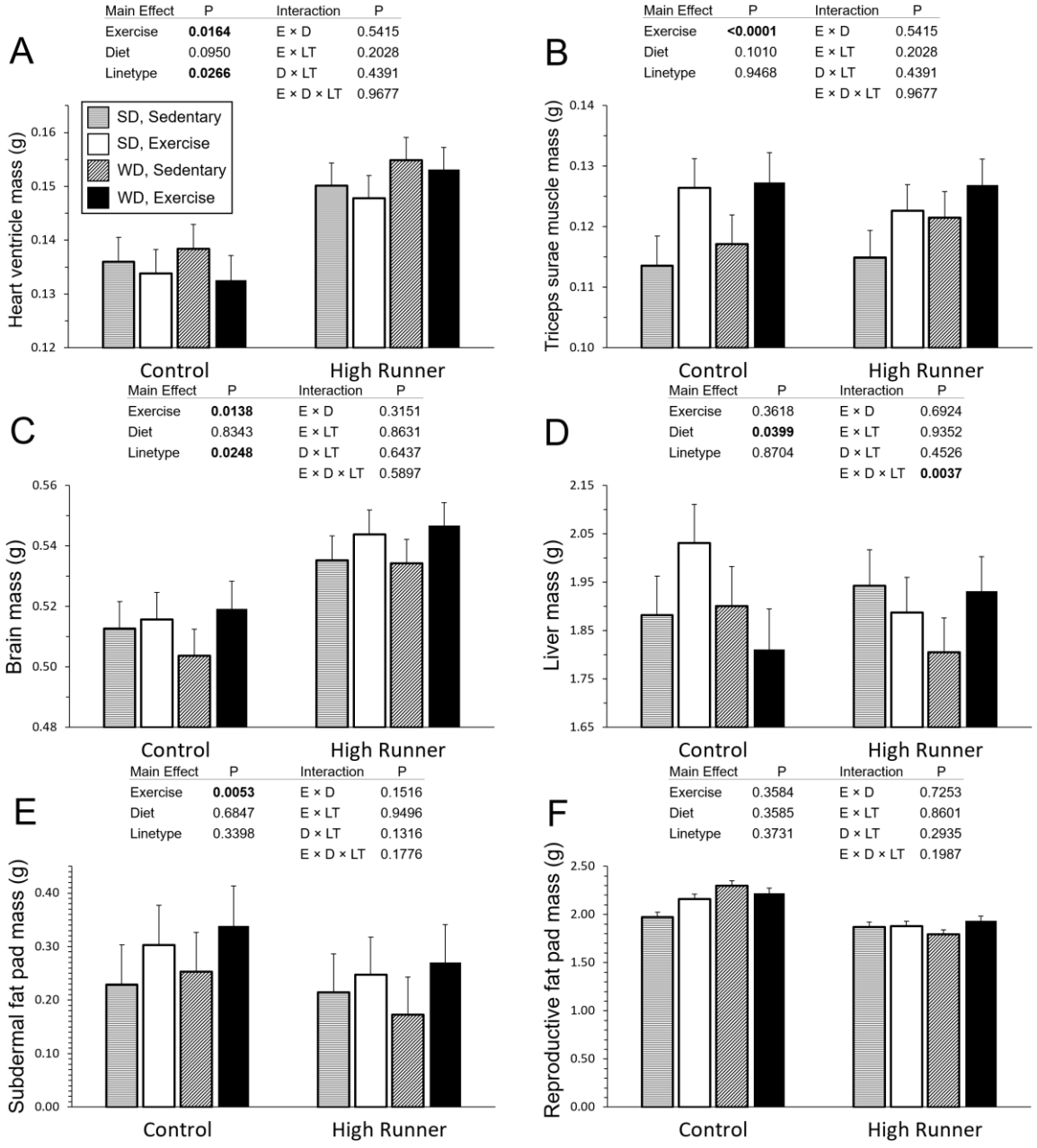

Control
High Runner
SD, Sedentary
SD, Exercise
WD, Sedentary
WD, Exercise
VO2max (mL O2 min−1)
Body mass (g)
Body mass (g)
7.0
6.5
6.0
5.5
5.0
4.5
4.0
25
30
35
40

Figure 1.8.


A
Main Effect P
Exercise 0.0164
Diet 0.0950
Linetype 0.0266
Interaction P
E × D 0.5415
E × LT 0.2028
D × LT 0.4391
E × D × LT 0.9677
SD, Sedentary
SD, Exercise
WD, Sedentary
WD, Exercise
Heart ventricle mass (g)
0.16
0.15
0.14
0.13
0.12
Control
High Runner
B
Main Effect P
Exercise <0.0001
Diet 0.1010
Linetype 0.9468
Interaction P
E × D 0.5415
E × LT 0.2028
D × LT 0.4391
E × D × LT 0.9677
Triceps surae muscle mass (g)
0.14
0.13
0.12
0.11
0.10
Control
High Runner
C
Main Effect P
Exercise 0.0138
Diet 0.8343
Linetype 0.0248
Interaction P
E × D 0.3151
E × LT 0.8631
D × LT 0.6437
E × D × LT 0.5897
Brain mass (g)
0.56
0.54
0.52
0.50
0.48
Control
High Runner
D
Main Effect P
Exercise 0.3618
Diet 0.0399
Linetype 0.8704
Interaction P
E × D 0.6924
E × LT 0.9352
D × LT 0.4526
E × D × LT 0.0037
Liver mass (g)
2.15
2.05
1.95
1.85
1.75
1.65
Control
High Runner
E
Main Effect P
Exercise 0.0053
Diet 0.6847
Linetype 0.3398
Interaction P
E × D 0.1516
E × LT 0.9496
D × LT 0.1316
E × D × LT 0.1776
Subdermal fat pad mass (g)
0.40
0.30
0.20
0.10
0.00
Control
High Runner
F
Main Effect P
Exercise 0.3584
Diet 0.3585
Linetype 0.3731
Interaction P
E × D 0.7253
E × LT 0.8601
D × LT 0.2935
E × D × LT 0.1987
Reproductive fat pad mass (g)
2.50
2.00
1.50
1.00
0.50
0.00
Control
High Runner

CHAPTER 2

Effects of Early-Life Exposure to Fructose and Voluntary Exercise on Adult Activity Levels, Body Composition, Exercise Physiology, and Associated Traits in Mice

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Abstract

Among-individual differences in locomotor behavior are caused by genes, environmental factors, and gene-by-environment interactions. Some environmental factors acting early in life can have long-lasting effects, resulting in the "programming" of adult behavior and physiology. For example, recent studies have shown that early-life exercise opportunity or a "Western" diet (high in fat and sugar) can affect adult physical activity and related traits. We tested for effects of early-life fructose supplementation and/or wheel access in a line of selectively bred High Runner (HR) mice and a non-selected Control (C) line. Exposures began at weaning and continued for 21 days to sexual maturity, followed by a 23-week washout period (equivalent to ~17 human years). Fructose increased body mass during the juvenile period, had no statistical effect on juvenile wheel running, but increased home-cage activity during the third week of exposure, for both HR and C mice. Wheel access reduced body fat of all groups when measured at the end of the exposure period; in addition, mice with fructose but without wheels had notably higher body fat than other groups at the end of juvenile exposures. Adult maximal aerobic capacity (VO_2max) was affected by an exercise × fructose interaction: interestingly, mice with early life wheel access and fructose had reduced VO_2max. When tested as adults, early-life fructose had no effect on wheel running, but increased home-cage activity during the first week of wheel testing, for both HR and C mice. In addition, and consistent with previous studies, early-life wheel access promoted adult wheel

running. Early-life fructose did not have a main effect on adult mass or body composition; however, early-life fructose, exercise, and genetic line interactively affected adult fat mass, as well as the response of lean and fat mass to two weeks of adult wheel access. Organ masses experienced both main and interactive effects; for example, early-life exposure to exercise decreased reproductive fat pad mass. Overall, these results show that early-life exercise opportunity can have long-lasting positive effects, while effects of early-life fructose are relatively minimal. Results have implications for public policy and for understanding sources of variation in the movement ecology of wild animals.

1. Introduction

Compared with fat and other sugars, fructose metabolism involves specific intestinal transporters (GLUT5) and distinct inter-organ trafficking mechanisms. These aspects of fructose metabolism allow rapid energy assimilation and efficient energy storage, which are of particular benefit to animals that must accumulate energy prior to migration or hibernation (Johnson et al. 2020). Fructose metabolism may also benefit humans during times of food scarcity; however, excessive and/or continuous consumption of fructose can lead to insulin resistance and non-alcoholic fatty liver disease (NAFLD), especially in the absence of regular physical activity and the presence of consistent food availability (Pereira et al. 2017; Tappy 2018). As compared with other sugars and carbohydrates, fructose may present metabolic challenges because it bypasses a key regulatory step in glycolysis and is rapidly metabolized in the liver, potentially leading to such metabolic ailments as lacticaemia, hyperlipidemia, and hyperuricemia (Mayes 1993), especially in modern societies.

As fructose became available in the marketplace in the early 1970s, it immediately saw a meteoric rise to prominence among sweeteners in the United States, owing to its low cost in manufacturing, relative sweetness, usefulness in sweetened beverages, and improved shelf-life, among other properties (Hanover and White 1993; Vos et al. 2008; White 2013). At present, ~10% of caloric intake is derived from fructose in the United States, with children consuming the most fructose, primarily in the form of sugar-sweetened beverages (SSBs) (Vos et al.

2008; Kit et al. 2013). The overconsumption of fructose is of epidemiological interest for its contribution towards the development of obesity and other diseases, such as type 2 diabetes, NAFLD, certain cancers, and cardiovascular and kidney disease (Febbraio and Karin 2021). Additionally, some research on laboratory mice suggests fructose may even reduce physical activity (Rendeiro et al. 2015). If true, then the overconsumption of fructose may represent a unique metabolic predicament in that it affects two lifestyle risk factors for obesity – physical inactivity and unhealthy eating behavior. Furthermore, "flavor learning" (which takes place during the early-life period [Beauchamp and Mennella 2009]), may play an important role in the development of obesogenic feeding patterns (Bachman et al. 2006; Paglia 2019). Taken together, this evidence suggests that early-life consumption of SSBs might have lasting effects on adult activity levels and food consumption habits, which in turn may lead to further dysregulation.

Early-life overconsumption of fructose or, more generally, of a Western diet (high in fat and sugar), does not occur in isolation from other risk factors for adult obesity and related diseases. Rather, in Western societies, children and adolescents often also experience a lack of physical activity and a high amount of sedentary behavior. On the other hand, early-life exercise might counteract negative effects of fructose, as exercise during this period has been shown to have a number of positive effects on adult activity levels and caloric intake (see Chapter 1).

Although some early-life studies in rodent models have demonstrated effects of high-fructose intake on adult memory (Noble and Kanoski 2016; Noble et al. 2019), the microbiome (Noble et al. 2017), thermoregulation (Alzamendi et al. 2021), and the development of arterial hypertension (Nüsken et al. 2020), no previous study has examined effects on adult physical activity and related traits. Moreover, the possibility that early-life exercise might serve as a countermeasure to adverse effects of fructose overconsumption has not been studied.

Here, we used a unique mouse model, which includes four replicate High Runner (HR) mouse lines that have been selectively bred for high voluntary wheel-running on days 5 and 6 of a 6-day running period as young adults for more than 80 generations and compared it with one of the non-selected Control (C) mouse lines. HR lines run 2.5–3× times more revolutions per day when given access to wheel and are more active in their home-cage without wheels. HR lines show changes in other relevant traits as well, including increased heart mass, increased maximal aerobic capacity, altered levels of circulating corticosterone, adiponectin, and leptin, and an altered brain reward system (Girard et al. 2007; Meek et al. 2012; Garland, Jr. et al. 2016; Cadney et al. 2021).

Previously, the HR lines (but not C lines) have been shown to increase wheel running while given Western diet continuously from weaning through adulthood (Meek et al. 2010). In contrast, when restricted to the period from weaning to sexual maturity (three weeks), followed by eight weeks of washout

period, Western diet increased adult wheel running in both HR and C lines (see Chapter 1). However, the source of dietary sugar in the Western diet mouse chow used in those studies (Harlan Teklad TD.88137) is sucrose rather than fructose (Meek et al. 2010). Sugar addiction may be therapeutically weakened by regular physical exercise, which increases production of brain-derived neurotrophic factor (BDNF) and affects the dopaminergic system (Johnson et al. 2003; Sleiman et al. 2016). Exercise may also act as a competing reward in the brain (Thompson et al. 2018 and references therein).

Therefore, the purpose of the present study was to test for effects of fructose specifically on physical activity levels in mice selectively bred for wheel running behavior. Accordingly, we administered HR and C mice 30% w/v fructose-water and/or access to exercise wheels during the three-week early-life period between weaning and sexual maturity, similar to Chapter 1 (see Fig. 2.1.). After a 23-week washout period (equivalent to ~17 human years), adult testing of wheel-running, cage activity, sucrose-preference, and maximal aerobic capacity began. Then, organ masses were measured in two separate cohorts: adults with and without two-weeks of adult wheel access. We hypothesized that: 1) early-life exercise would affect adult traits, as previously demonstrated in both the HR and C mice (see Chapter 1); 2) early-life fructose would suppress adult activity levels; and 3) early-life effects would interact with genetic background (i.e., effects would differ between of HR and C mice). Our hypotheses are related to the

overarching idea that adult health is “programmable” by innate genetic variation as well as variation in environmental conditions

2. Materials and Methods

2.1. Experimental mice

Starting in 1993, four replicate lines of house mice were bred in an ongoing selection experiment for high voluntary wheel running (HR lines), based on wheel revolutions on days five and six of six days of access to Wahman-type activity wheels (1.12-meter circumference) as young adults (Swallow et al. 1998a). The experiment began with a population of 224 mice from the outbred Hsd:ICR strain, which was randomly mated for two generations before being randomly partitioned into eight lines. Four of these were bred randomly as Control (C) lines to the four HR lines. The current experiment used female mice from only HR line 8 and C line 2 of the selection experiment. All experiments have followed university IACUC guidelines.

2.2. Early-life diet and exercise manipulation

In this experiment, 196 female mice were weaned individually into standard clear plastic cages (27 × 17 × 12.5 cm) at 3 weeks of age and placed in one of four treatment groups for 3 weeks until sexual maturity at 6 weeks of age (see Fig. 2.1. and Table 2.1.). Mice were given 30% fructose w/v (commonly used in mouse models: e.g., Dotimas et al. 2016; Cho et al. 2017; Tripathi et al.

2017) in standard cages, with half of the cages attached to activity wheels. Spontaneous physical activity (SPA) was measured for all mice, using infrared sensors placed in home cages (see below). Body mass, food consumption, and body composition were measured during exposure (see Fig. 2.1. for a full account of these measurements). Photoperiod was 12:12, with lights on at 07:00 PST.

2.3. Adult testing

Beginning at 6 weeks of age, all mice remained individually housed with SD and without wheel access for an additional 23 weeks. At 29 weeks of age, testing of VO_2max, open-field behavior, sucrose preference began in two cohorts. Cohort 1 was dissected at week 31 without having received access to wheels for adult testing. During weeks 32-34, cohort 2 received wheel access for 2 weeks to measure VE. Adult SPA was again measured for the during wheel testing. Cohort 2 was dissected at week 34, immediately following wheel testing. Dissected tissue samples from cohorts 1 and 2 allowed for separate analyses of mice with and without adult access to exercise wheels.

2.3.1. Home-cage activity

During wheel testing and through the washout period, mice were housed in home cages fitted with a passive infrared sensors (Talon TL-Xpress-A; Crow Electronics, Fort Lee, New Jersey, USA), protected within wire mesh, as in

previous studies (Acosta et al. 2015; Copes et al. 2015). The sensors were connected to a computer with custom activity recording software (developed by M. A. Chappell) via a digital I/O board (ICS 2313; ICS Electronics, Pleasanton, California, USA). A mean value between 0 and 1 was calculated for each minute over 23 hours. Analyses of SPA data used a measure of sensor sensitivity as a covariate (Acosta et al. 2015; Copes et al. 2015). During washout, mice were taken from their co-housed cages and put into HCA home cages for 3 days so that individual measurements of activity could be obtained.

2.3.2. Body composition

Whole-animal fat, lean, free water, and total water masses were measured by restraining each mouse within a translucent tube before insertion into an EchoMRI-100 (Echo Medical Systems, Houston, TX) for scanning. Composition was analyzed throughout the experiment (see Fig. 2.1.).

2.3.3. Total Caloric Intake

Food consumption was measured by the difference in food hopper weight each week during early life treatment and adult wheel testing (only SD), taking care to note wasted or shredded food (Koteja et al. 2003). Weekly chow consumption was converted from grams to caloric intake, taking the caloric content of SD into account (14 kJ per g: Meek et al. 2010). Where mice were given fructose-water, those calories were added to obtain caloric totals.

2.3.4. Maximal aerobic capacity

To measure VO_2max, mice were subjected to forced exercise within a 900 mL enclosed mouse wheel approximately 15 cm in diameter (Dlugosz et al. 2009; Claghorn et al. 2017). Each mouse was run for approximately 5 minutes. Duplicate trials were conducted, allowing a day of rest between each trial. Air was pumped into the enclosed metabolic chamber at a rate of 2,000 mL per min. using a mass-flow controller. The concentration of O_2 in dried, CO_2-free excurrent air was measured by an oxygen analyzer (S-3A Applied Electrochemistry, Inc., Sunnyvale, CA). After instantaneous correction (Bartholomew et al. 1981), VO_2max was taken as the highest minute of oxygen consumption during either trial, as calculated with LabHelper software (Warthog Systems, www.warthog.ucr.edu).

Researchers subjectively assessed quality during and tiredness after each trial. Trial quality (following Swallow et al. 1998b; Claghorn et al. 2017) was scored between 1, being least cooperative (the mouse resisted moving in the direction of rotational motion), and 5, being most cooperative (the mouse consistently ran with the direction of rotational motion). Trial tiredness was determined by how quickly the mouse recovered from the trial, where a score of 1 indicates spontaneous locomotion within the chamber 1 second or less after the end of each trial and a score of 3 indicating movement after 5 or more seconds. Researcher also recorded their initials so that differences in scoring the mice between researchers could be considered.

In analysis of VO_2max, age and body mass were significant predictors and were used as covariates. However, trial quality and tiredness were not significant predictors and were excluded from the model. Trial quality and tiredness were also analyzed as dependent variables and the identity of the researcher making subjected score was used as a random effect in all analyses of VO_2max and subjective scores. See Methods for more details on the statistics used.

2.3.5. Preference for sucrose solution

At weeks 29 and 30, adult mice were presented with water bottles containing a sucrose-water solution (10.5% sucrose; Fisher Scientific Certified ACS Grade) and regular water. Fluid consumption, with due allowance for spillage and evaporation, was measured after mice had 24 hours (10:00-10:00 h) of choice.

2.3.6. Fecal droppings in a novel environment

Mice were tested for open-field behavior (Fuss et al. 2010) from approximately 07:00 h to 18:00 h. Each mouse was placed in the center of the arena (1 m × 1 m) under low-light conditions (Bronikowski et al. 2001). The mouse was recorded for 5 minutes with a Logitech HD C525 Webcam placed one meter above the arena. Unfortunately, video recordings were lost before they could be analyzed. However, the amount of urine (scored as 0, 1, 2) and

the number of fecal pellets left by the mouse during the test were recorded. The arena was cleaned with warm, soapy water between each test.

2.3.7. Dissections

Animals were euthanized and organs were dissected and weighed to 0.0001 g (heart ventricles, triceps surae muscles, brains, liver, spleen, cecum, and subdermal and reproductive fat pads). Organs were preserved at -80°C. Cohort 2 samples were taken from mice that received access to wheels for adult exercise, while cohort 1 samples were taken from mice that did not (Fig. 2.1.).

2.4. Statistical analysis

Data were analyzed as covariance models in SAS 9.1.3 (SAS Institute, Cary, NC, USA) Procedure Mixed, with REML estimation and type III tests of fixed effects. Depending on the trait analyzed, body mass, age, wheel freeness, and home-cage sensor sensitivity were used as covariates. Line (selected line 8 vs. control line 2), early-life fluid type (fructose-water vs. water), and wheel access (exercise vs sedentary) exposures were fixed effects – except in analyses of traits measured prior to or during early-life exposure (e.g., weaning mass, juvenile running distance). Effects of line, fructose, and exercise, as well as their interactions, were tested. Dam ID, nested within line was used as a random effect. Outliers were determined at ~3 standard errors from the mean and removed.

3. Results

Note: Supplemental Files 2.1 and 2.2 are spreadsheets that summarize p-values for main and interactive effects of linetype and treatment on various traits (most of which are reported in this chapter). Subsequent sheets contain more detailed results (degrees of freedom, least squares means, standard errors, etc.) for each trait, which can be found by navigating to the sheet corresponding to the "index" number indicated in column A of the RESULTS sheet.

3.1. Juvenile wheel running

During the first week of early-life treatments, fructose reduced average and maximum wheel-running speeds among HR mice, but not C (Fig. 2.2.C, D) – although the line × fructose interaction was significant only for average speed, an examination of the differences of least-squares means shows a significant reduction in both average and maximum speeds among HR mice and no significant differences among the C lines (see Supplemental File 2.1). During the third week, fructose increased wheel-running duration (Fig. 2.2.B). Despite effects on its wheel-running components, running distance itself was not significantly affected by fructose (Fig. 2.2.A). Average daily wheel-running distance gradually diverged between HR and C lines (HR > C) during the three weeks after weaning, with the weekly HR/C ratio increasing from 1.27 to 1.91 to 2.45, respectively (Fig. 2.2.; $p = 0.2163$, $p < 0.0001$, and $p < 0.0001$, respectively). Average and maximum speed diverged similarly.

3.2. Juvenile home-cage activity

Home-cage activity was recorded only for the third week of juvenile treatments. Fructose increased activity in the home-cage ($p = 0.0464$), wheel access decreased activity ($p < 0.0001$), and HR mice were more active than C ($p = 0.0009$), with no interactions of main effects (Supplemental File 2.1).

3.3. Adult wheel running

Early-life exposure to fructose did not significantly affect adult wheel running (Fig. 2.3.). Early-life exposure to exercise generally increased adult wheel running (Fig. 2.3.); However, the effects on wheel-running distance and maximum speed was gone by the second week of testing (see Supplemental File 2.1). HR mice ran more than C mice on all measures of wheel running across both weeks of testing (Fig. 2.3., Supplemental File 2.1). Additionally, line and exercise had an interactive effect on duration across both weeks of adult testing (line × exercise $p = 0.0066$ on week 1 and $p = 0.0011$ on week 2), wherein exercise increased duration in C mice, but HR mice (Fig. 2.3.).

3.4. Adult home-cage activity

During washout (Fig. 2.1.), adult home-cage activity differed between HR and C mice, similarly to juvenile measurement (all $p < 0.0001$). Early-life exposure to fructose increased activity in the home-cage during washout on (week 23 $p = 0.0098$), and again during the first week of adult wheel testing for

cohort 2 (week 32 $p = 0.0372$). Early-life exposure to exercise decreased activity in the home-cage during the first week of adult wheel testing (week 32 $p < 0.0001$).

3.5. Body, fat, and lean mass

3.5.1. Body mass

Fructose predictably increased body mass compared to the water group at the ends of weeks 4-6 (all $p \leq 0.0182$) and into washout, where it increased body mass at weeks 16 ($p = 0.0083$) and 19 ($p = 0.0419$), but the effect was gone after week 19. Wheel access did not affect body mass during the juvenile period. Early-life exposure to exercise temporarily decreased body mass during washout at week 19 ($p = 0.0282$), an effect which returned after two and three weeks of adult wheel testing (week 33 $p = 0.0014$, week 34 $p = 0.0021$). At weaning, HR mice weighed less than C mice ($p = 0.0404$), and this pattern continued at the ends of weeks 5 ($p = 0.0199$), and 6 ($p = 0.0001$) (Fig. 2.4.A, B). Throughout washout, HR mice continued to weigh less than C mice, but the effect was significant only on weeks 16 ($p = 0.0008$), 19 ($p = 0.0344$), and 27 ($p = 0.0141$). Similarly, the effect persisted through adult wheel testing. At the end of juvenile treatment (week 6), line and exercise interacted such that HR mice were smaller than C mice, except in the exercise group (line × exercise $p = 0.0135$). At the end of adult wheel testing (week 34), early-life exercise reduced body mass in C mice, but not HR (line × exercise $p = 0.0413$). The change in body mass when

given two weeks of adult wheel access had a 2-way (fructose × line $p = 0.0146$) and 3-way (exercise × fructose × line $p = 0.0680$) interaction. All groups lost body mass, except for C mice given juvenile fructose and no exercise opportunity (Fig. 2.5.).

3.5.2. Lean mass

Early-life exposure to fructose increased lean mass throughout the washout period on weeks 11 ($p = 0.0018$), 23 ($p = 0.0156$), and 27 ($p = 0.0374$). Early-life exposure to exercise reduced lean mass throughout the washout period and the effect was only non-significant at week 23 ($p = 0.0667$). After two weeks of adult wheel testing, early-life exposure to exercise decreased lean mass (week 33 $p = 0.0035$, week 34 $p = 0.0073$). Wheel access decreased lean mass in C mice but increased it in HR (line × exercise $p = 0.0086$). During washout, early-life exercise decreased lean mass in C mice, but not HR mice (week 19 line × exercise $p = 0.0319$). After the washout period and immediately prior to wheel testing, HR mice had less lean mass than C mice (week 32 $p = 0.0380$) and the effect persisted through adult wheel testing. After two weeks of adult wheel testing, early-life exercise decreased lean mass in C mice, but not HR mice (week 34 line × exercise $p = 0.0177$)

3.5.3. Fat mass

Repeated-measures ANOVA of fat mass indicated statistically significant interactions for exercise × fructose × line × trial ($p = 0.0146$), fructose × line × trial ($p = 0.0429$), and line × trial ($p = 0.0038$) across the washout period (see Fig. 2.1. and Supplemental File 2.1). Therefore, we examined each week separately.

Fructose did not affect fat mass as a main effect but was involved in three- and two-way interactions. Mice from the sedentary, fructose group had increased body fat compared to other groups after 3 weeks of early-life treatment (exercise × fructose $p = 0.0160$). Mice from the water, sedentary group had increased body fat in C and decreased body fat in HR (exercise × fructose × line $p = 0.0075$) at week 32. Early-life exposure to exercise generally decreased fat mass. During juvenile exposure, the effect was never statistically significant, but it was during adult wheel testing at weeks 33 ($p = 0.0215$) and 34 ($p = 0.0280$). HR mice had less fat mass than C mice from weaning ($p = 0.0212$) throughout the experiment (Fig. 2.4.E).

In analyses where lean mass was used as a covariate (Fig. 2.4.G, H), mice from the sedentary, fructose group had increased body fat compared to other groups after 3 weeks of early-life treatment (exercise × fructose $p = 0.0254$). HR mice from the fructose group, but not the water group had reduced body (fructose × line $p = 0.0280$) at week 11. Mice from the water, sedentary group had increased body fat in C and decreased body fat in HR (exercise × fructose × line $p = 0.0320$) at week 32. Early-life exposure to exercise generally

decreased fat mass. The effect was significant only at the end of juvenile treatment ($p < 0.0001$), and at weeks 19 ($p = 0.0357$) and 32 ($p = 0.0227$).

HR mice generally had less fat mass than C mice throughout the experiment, but the effect was not significant during early-life treatment and at weeks 23 and 32 (Fig. 2.4.G, H).

3.6. Food consumption

Mice that had fructose in their water consumed substantially less chow (each juvenile week $p < 0.0001$). The difference in food consumption was ~38% on the first week of juvenile treatments and ~50% on the second and third weeks. During the first of juvenile exposure, HR mice consumed more chow than C mice ($p = 0.0003$). Across the second and third weeks of juvenile exposure, fructose and wheel access had an interactive effect on food consumption (exercise × fructose $p = 0.0072$ for week 2 and $p = 0.0116$ for week 3), where access to wheels significantly increased food consumption in the water group, but not the fructose group (Fig. 2.6.).

During washout (week 23), food consumption had a main effect of early-life exercise ($p < 0.0001$), which reduced caloric intake of standard mouse chow in all groups, as well as a three-way interaction (exercise × fructose × line $p = 0.0302$).

During the first week of adult wheel testing, as would be expected, HR mice consumed more chow than did C mice ($p < 0.0001$), mice with early-life

wheel access consumed more chow ($p = 0.0132$) in three of four groups, with a significant three-way interaction (exercise × fructose × line $p = 0.0389$). During the second week of adult testing, only the effect off genetic line remained statistically significant (main effect of line $p = 0.0013$; main effect of exercise $p = 0.1554$; exercise × fructose × line $p = 0.0747$).

3.7. Maximal aerobic capacity

When cohorts were analyzed together (with age and body mass as covariates), VO_2max was affected by a two-way interaction – early life wheel access and fructose reduced adult VO_2max (exercise × fructose $p = 0.0144$). Trial quality did not differ across any group; however, trial tiredness was affected by line, with C mice being more tired after a trial than HR mice (Fig. 2.7.; $p < 0.0001$).

3.8. Preference for sucrose-water

Preference for sucrose-water was not affected by line or by either early-life treatment.

3.9. Fecal and urine elimination in a novel environment

The number of fecal pellet droppings and urine pools left by a mouse during a 5-min open-field trial were not affected by line, either early-life treatment or interactive effects. Cohorts 1 and 2 were analyzed separately, as well as

combined with cohort as a random effect. We also analyzed sum of the fecal and urine scores and again individually. No difference was observed in any combination of model and dependent variable.

3.10. Organ masses

Organ masses were taken during dissections for both cohorts.

3.10.1. Organ masses for cohort 1

With log body mass as a covariate, early-life exposure to exercise increased heart mass in the water group and decreased it in the fructose group (Fig. 2.8.A; exercise × fructose $p = 0.0250$) for both HR and C mice. In addition, HR mice had larger hearts than C in all groups (linetype $p = 0.0035$). Liver mass was not affected by early-life treatments but was larger in HR than C mice (Fig. 2.8.D; $p = 0.0027$). Early-life exposure to exercise decreased reproductive fat pad mass (Fig. 2.8.H; $p = 0.0205$). Triceps surae muscle, spleen, cecum, and subdermal fat pad masses did not have main effects of line or early-life treatments, though it is worth mentioning the interactive effects on brain mass (Fig. 2.8.C): early-life exercise increased brain mass in C but not HR (exercise × line $p = 0.0702$) and early-life fructose reduced brain mass in C but not HR (fructose × line $p = 0.0563$).

3.10.2. Organ masses for both cohorts combined

Organ masses were analyzed in four-way analyses, adding cohort as a main effect to test for potential training effects caused by two weeks of wheel access. In each case, wheel-running distance was never a significant predictor of organ mass when used as a covariate, so it was removed from the final models (see Supplemental File 2.2 for results of four-way ANOVAs with and without wheel running distance as a covariate).

With body mass as a covariate, heart ventricle mass was affected by line ($p < 0.0001$), cohort ($p < 0.0001$), and a line cohort interaction ($p = 0.0176$): HR mice had larger hearts, wheel access increased heart mass, and the training effect was greater in HR mice.

Cohort and line interactively ($p = 0.0018$) affected subdermal fat pads: adult wheel access reduced fat pad mass in C mice but increased it in HR mice. Cohort and line interactively ($p = 0.0443$) affected reproductive fat pads: adult wheel access reduced fat pad mass in C mice but not in HR mice.

Liver mass was increased by adult wheel access ($p < 0.0001$), larger in HR mice ($p = 0.0005$), and also affected by an exercise × fructose interaction ($p = 0.0490$). Triceps surae muscle mass was larger in HR mice ($p = 0.0496$) and was also affected by an exercise × fructose ($p = 0.0402$) and a cohort × fructose × line interaction ($p = 0.0236$). Adult wheel access had a consistent effect of increasing cecum mass ($p < 0.0001$), and two 3-way interactions were also statistically significant (Supplemental File 2.2).

4. Discussion

In the present study, mice from a High Runner (HR) line selectively bred for voluntary wheel running and from a non-selected Control (C) line were administered fructose and/or access to exercise wheels during the 3-week period between weaning and sexual maturity. Numerous acute effects were detected during the treatment period, including obesogenic effects of fructose and fat-reducing effects of wheel access. When mice were tested as adults (after a 23-week washout period), early-life fructose had no detectable effect on daily wheel-running distance (or any of its components), but increased home-cage activity by a small amount (~4%). As predicted, some effects on adult traits were interactive, including an increase in adult wheel-running duration in C (but not HR) mice during both weeks of adult wheel testing. In addition, we found some early-life effects as well as training effects on organ masses. Overall, we found that early-life exercise was responsible for the majority of effects on adult traits; fructose produced acute variable effects on activity levels; and HR and C mice were affected differentially by early-life treatments, in both the short- and long-term.

4.1. Effects of early-life wheel access on adult physical activity

Similar to our two previous studies of similar design, early exercise had long-lasting effects on adult wheel running – however, where exercise previously increased wheel running of only C mice in one study (see Chapter 1), we found a

consistent effect of increasing adult wheel running in both HR and C lines, as in another study (Acosta et al. 2015). The discrepancy may be attributable to the use of only one of the four possible selected HR lines. Alternatively, this and our previous early-life studies took place across different times of year, and wheel running shows strong seasonal variation in HR and C mice (Careau et al. 2013).

Previous early-life studies on the HR model have altered diet by use of a commercially available Western diet. Compared with the effects of early-life Western diet, which increased adult wheel running in HR but not C lines after a washout period of 8 weeks (see Chapter 1), early-life fructose had no effect on adult wheel running, suggesting that the stimulative effect on wheel running previously reported may be driven by the total fat content or other dietary components of the mouse Western diet (Meek et al. 2010; Cadney et al. 2021). Interestingly, others have reported a reduction in physical activity associated with high-fat diets. For instance, Bjursell et al. (2008) fed mice a Western or standard diet for 72 hours, during which time locomotor activity (SPA, measured by infrared sensors) was acutely lower in the Western diet group (Bjursell et al. 2008).

4.2. Effects of fructose on physical activity

We opted to use fructose rather than sucrose (the sugar component in the Teklad Western diet for rodents) as used in our previous studies (Acosta et al. 2015, 2017; Hiramatsu et al. 2017; Cadney et al. 2021) because we wanted our

results to be more comparable to human populations experiencing childhood overconsumption of high-fructose corn syrup. In addition, we wanted to partially repeat a previous study that found fructose to acutely reduce activity levels in mice. Specifically, Rendeiro et al. (2015) used two isocaloric diets containing either fructose or glucose at 18% of total metabolizable energy, where the diets replaced all sucrose and a fraction of cornstarch with either glucose or fructose. Mice received treatment diets for 11 weeks and then home-cage activity was measured via video tracking over 5 days. The fructose diet was gradually obesogenic over the course of the experiment, despite no statistical differences in food consumption (grams/body weight). The effect of fructose on body mass was attributed to reduced physical activity in the home cage, where the energetic expenditure of activity was estimated at 1.95 kcal/day for the fructose group and 2.44 kcal/day for the glucose group (Rendeiro et al. 2015).

We found mixed evidence regarding the acute effects of fructose on physical activity. Total home-cage activity (which we measured only during the third week of juvenile exposures) was significantly increased by fructose ($p = 0.0464$) in the analysis of all mice (Supplemental File 2.1), but the effect was not significant when we considered only the mice without wheels ($p = 0.3740$). In none of the 3 weeks did we detect an effect on average daily running distance. During the first week of early-life exposure, fructose decreased average wheel-running speed in both HR and C mice ($p = 0.0212$) and maximum speed only in HR mice (fructose × line $p = 0.0324$); however, fructose increased the duration of

wheel running ($p = 0.0400$) during the third week. Comparison of our results with those of Rendeiro et al. (2015) is not straightforward, in part because we did not attempt to impose equivalent caloric intake among groups.

4.3. Possible protective effects of early-life exercise

Exercise is reported to curb dyslipidemia in a study on healthy human subjects fed a high-fructose diet (Egli et al. 2013). More generally, adequate regular physical activity is known to prevent and help reverse obesity, type 2 diabetes, and other metabolic ailments (Blair and Morris 2009; Church and Blair 2009; King et al. 2009; Jakicic and Davis 2011; Swift et al. 2014; Grazioli et al. 2017; Ruegsegger and Booth 2018). Therefore, we predicted that early-life exercise might blunt any adverse effects of early-life fructose. Indeed, what might be interpreted as a "protective" exercise × fructose interaction was observed for juvenile body fat mass. Specifically, with or without lean mass as a covariate, mice given fructose and with no opportunity for voluntary exercise had significantly greater body fat than other groups at the end of the 3-week exposure period.

4.4. Unfamiliar early-life conditions as stressors

Although acutely obesogenic, fructose did not have a lasting effect on adult body mass (after the washout period). Previous studies have established that HR mice have evolved to be smaller and leaner than C mice (e.g., see

Girard et al. 2007; Meek et al. 2010; Hiramatsu and Garland, Jr. 2018). We also found these differences for mice housed without either early-life wheels or fructose. Interestingly, however, when HR and C mice were exposed to either early-life fructose, wheel access, or both, differences in adult body fat were not apparent. We speculate that these effects may reflect differential responses to early-life "stress," with stress being caused generally by conditions that are unfamiliar in the evolutionary history of house mice since being brought into a laboratory setting. Specifically, early-life exercise and/or overnutrition may trigger thrifty fat storing (Neel 1962) in HR mice, or adaptive fat loss in C mice.

In an early-life stress study using mice, Yam et al. (2017) induced stress by limiting nesting and bedding material for 1 week after birth. Plasma leptin levels and leptin mRNA expression in white adipose tissue were measured 9 days (short-term) and 180 days (long-term) after birth – both were significantly reduced by early-life stress. Then mice were fed a Western diet 6−14 weeks after birth, which resulted in an obese phenotype in mice that had experienced early-life stress (Yam et al. 2017). If our early-life treatments similarly affected leptin homeostasis (in Chapter 1, we found that early-life exercise increased adult leptin concentration), it is possible that HR lines, which evolved significantly lower circulating leptin levels and body fat than C lines (Girard et al. 2007), might have responded to early stress differentially. Further studies of the altered behavioral and physiological responses to early-life "stress" in HR mice are needed.

4.5. Effects on the response to adult wheel access

When we provided adult mice with exercise wheels for 2 weeks, nearly all groups lost body mass and fat mass. This general pattern has been reported previously for both sexes of HR and C mice given 6 days of wheel access (Hiramatsu and Garland 2018). In the present study, the sole exception were C mice from the early-life sedentary fructose group, who actually gained body mass (Fig. 2.5.), attributable primarily to a relatively large increase in lean mass, accompanied by less of a drop in fat mass than seen in the other groups. Notably, this group in particular represents the "unhealthy" combination of genetic and experimental factors: C mice (fatter and predisposed for less physical activity than HR mice); fed excessive amounts of simple carbohydrates as juveniles; without any access to exercise wheels. That this "unhealthy" group had an aberrant response to adult exercise after such a long washout period is startling and suggests permanent alterations to some aspects of their exercise physiology and metabolism may have occurred. Future studies would be required to determine the biochemical and molecular mechanisms that might underlie such hypothesized changes. We can, however, suggest that any such effects were not mediated by either VO_2max or wheel-running behavior (but see Claghorn et al. 2017), given that they were not affected by early-life fructose.

When analyzed as a 4-way model of exercise, fructose, line, and cohort, we tested for possible training effects of 2 weeks of adult wheel running. We observed a cohort x line interaction for heart mass: HR mice that were wheel

tested had greater ventricle mass than all other groups. The amount of wheel running over the 2 weeks was not significant when added as a covariate (Supplemental File 2.2), suggesting that the HR mice have increased adaptive plasticity, rather than just following a "more pain, more gain" pattern (Swallow 2005; Garland and Kelly 2006; Middleton et al. 2008; Kelly et al. 2017). We also observed a cohort x line interaction for both reproductive and subdermal fat pads: C mice that were wheel tested had smaller fat pads, but that was not true for HR mice. Cecum mass showed two different 3-way interactions that were not simple to interpret. Additionally, adult exercise increased cecum mass for all groups, which may be an effect of increased food consumption caused by wheel running.

4.6. Limitations

Numerous studies have targeted fructose and other added simple carbohydrates as likely candidates that drive burgeoning trends of obesity in Western societies by establishing a link between sugar consumption and obesity, dyslipidemia, and insulin resistance (Mayes 1993; Basciano et al. 2005). To address the concern these effects may be driven by excess calories, some researchers have suggested using isocaloric diets to focus on hypotheses related to the potential causal impacts of sugar (e.g., see Hydes et al. 2021; Sigala and Stanhope 2021). In the present study, however, we gave mice *ad libitum* access to fructose-water, which means effects reported here may be the result of hypercaloric dietary conditions, rather than effects of fructose *per se*.

We found the present study design an acceptable compromise because we wanted to maximize the likelihood of observing early-life effects, which we believed could be best achieved with an *ad libitum* diet. Moreover, given that there were no early-life effects of *ad libitum* fructose on adult activity levels, we believe that repeating the experiment with an isocaloric fructose diet is unlikely to provide evidence for such early-life effects after a similar washout period.

4.7. Concluding Remarks

High-fructose corn syrup is chemically similar to sucrose (i.e., common table sugar). However, fructose and sucrose are metabolized differently – fructose is metabolized primarily in the liver and leads to *de novo* lipogenesis and elevated triglyceride synthesis. Compared to glucose, fructose metabolism has relatively few regulatory steps and does not trigger an insulin or leptin response upon uptake. Whether the unique metabolism of fructose has been a causative agent in the historic surge in the rate of obesity is controversial (Bray et al. 2004; Lustig 2013; Febbraio and Karin 2021). As compared with sucrose or other simple carbohydrates studied in comparable ways (e.g., other dietary components controlled, isocaloric intake), consumption of fructose, *per se*, has not been shown to have adverse effects on metabolic health in either human or rodent studies (Stanhope 2016; Prinz 2019; Bier 2020). As previously mentioned, in many studies of fructose consumption, researchers fail to control for the quantity of fructose consumed and, consequently, their results are more a

reflection of sugar overconsumption than fructose-induced metabolic vulnerability (Forshee et al. 2007; Tappy and Lê 2010).

Conflict of interest statement

The authors declare no conflict of interest.

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Table 2.1. 2×2 fully crossed experimental design used to investigate the potential interactive effects of fructose-water and access to exercise for three weeks during the juvenile period, from weaning to sexual maturity. This was followed by a 23-week washout period (see Fig. 2.1.). Total N = 192.

	Control Line 2	High Runner Line 8
Water	C, Water, No wheels	HR, Water, No wheels
	C, Water, Wheels	HR, Water, Wheels
Fructose-water (30% w/v)	C, Fructose, No wheels	HR, Fructose, No wheels
	C, Fructose, Wheels	HR, Fructose, Wheels

Figure Legends

Figure 2.1. Experimental timeline of events starting with the first births of generation 84 mice. Of these mice, 192 females (representing 36 families) were weaned and housed individually for the duration of the experiment. Early-life diet and exercise manipulation began immediately after weaning, lasted three weeks, and was followed by a 23-week washout period (equivalent to ~17 years for humans), during which mice were housed 4/cage without wheels and given a standard diet and regular drinking water (mice were individually housed during periodic washout measurements). At the end of washout, all mice were tested in two cohorts. Only Cohort 2 received wheel testing (two weeks) so that two sets of dissected organ tissues could be collected – one with and one without having had recent adult exercise that may have caused training effects.

Figure 2.2. Juvenile wheel running of female mice during three weeks of early-life treatment, shown as least-squares means, standard errors, and accompanying P values from type 3 tests of fixed effects from SAS Procedure Mixed. These data are only from mice in the experimental exercise group (Table 2.1.). Shown are mean values per day (circumference 1.12 m) for each week. White bars are mice from the early-life water treatment group and black bars the early-life fructose treatment group.

Figure 2.3. Adult wheel running during days 1-7 of a 2-week testing period. Values are least-squares means, standard errors, and accompanying P values from type 3 tests of fixed effects from SAS Procedure Mixed. A) Mean wheel revolutions per day, B) duration of daily running, C) mean revolutions per minute, D) maximum revolutions per minute. Values for days 8-13 can be seen in Supplemental File 2.1.

Figure 2.4. Total body mass, lean mass, and fat mass. Values are least-squares means and standard errors from SAS Procedure Mixed. Note that body mass and composition were also measured at weaning (week 3) but are not shown here (see Results); HR mice at weaning weighed 10.3% less than C mice ($p = 0.0404$). Fat mass was analyzed with and without lean mass as a covariate. See Supplemental File 2.1 for additional statistical details.

Figure 2.5. Change in body mass across two weeks of adult wheel access. Measurements were taken immediately before and after wheel testing (see Fig. 2.1.). Values are least squares means from SAS Procedure Mixed. See Supplemental File 2.1 for statistical details.
*Fat mass with lean mass as a covariate.

Figure 2.6. Weekly mass-adjusted juvenile caloric intake in response to juvenile fructose and/or exercise treatment. Values are least squares means and standard errors from SAS Procedure Mixed. See Supplemental File 2.1 for additional statistical details.

Figure 2.7. Maximum oxygen consumption (VO_2max) measured during forced exercise (see Chapter 2 Methods). Body mass was a significant predictor of VO_2max ($p = 0.0005$), with no significant main effects.

Figure 2.8. Body-mass adjusted heart ventricles, triceps surae, brain, liver, spleen, and cecum, and fat pad masses for cohort 1. Values are least-squares means, standard errors, and accompanying P values from type 3 tests of fixed effects from SAS Procedure Mixed. All traits were analyzed with body mass as a covariate (and with log body mass when the dependent variable was log-transformed [A, F]). See Supplemental File 2.1 for additional statistical details

Figure 2.1.

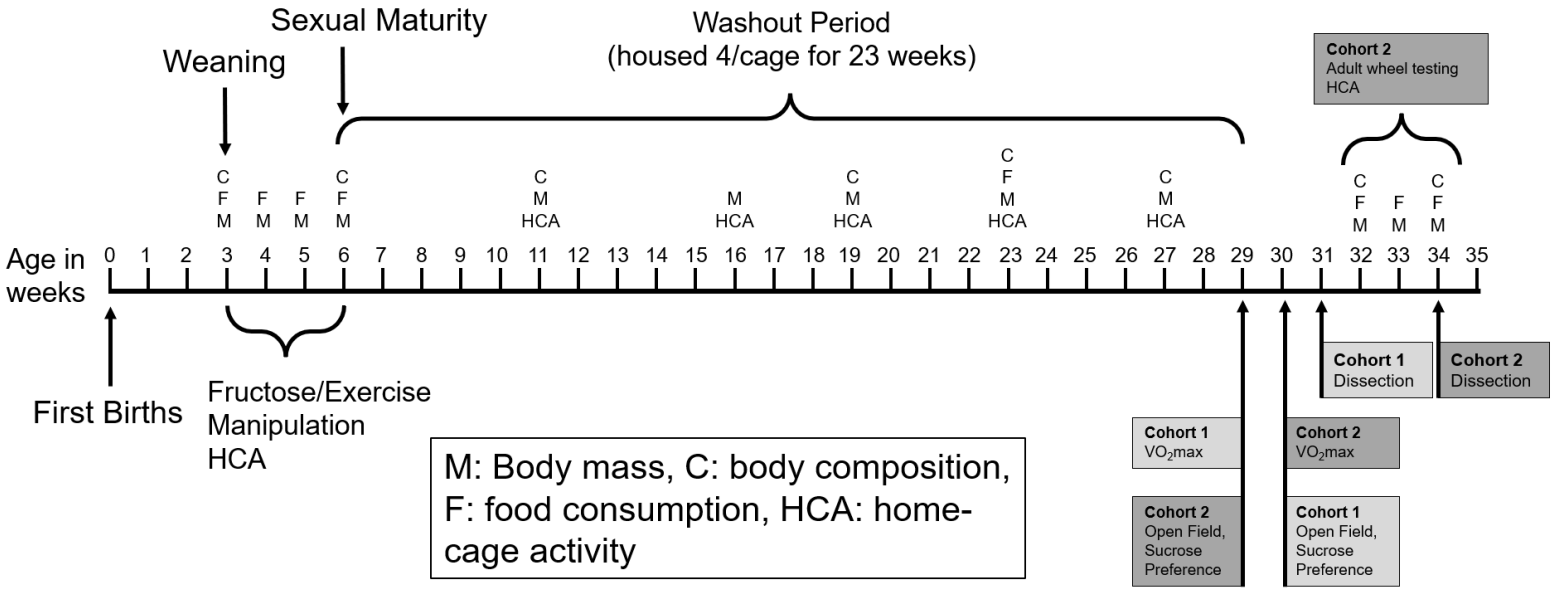

Sexual Maturity
Weaning
Washout Period
(housed 4/cage for 23 weeks)
Cohort 2
Adult wheel testing
HCA
C F M
F M
F M
C F M
C M HCA
M HCA
C M HCA
C F M HCA
C M HCA
C F M
F M
C F M
Age in weeks
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35
First Births
Fructose/Exercise Manipulation HCA
M: Body mass, C: body composition, F: food consumption, HCA: home-cage activity
Cohort 1 Dissection
Cohort 2 Dissection
Cohort 1 VO2max
Cohort 2 VO2max
Cohort 2 Open Field, Sucrose Preference
Cohort 1 Open Field, Sucrose Preference

Figure 2.2.

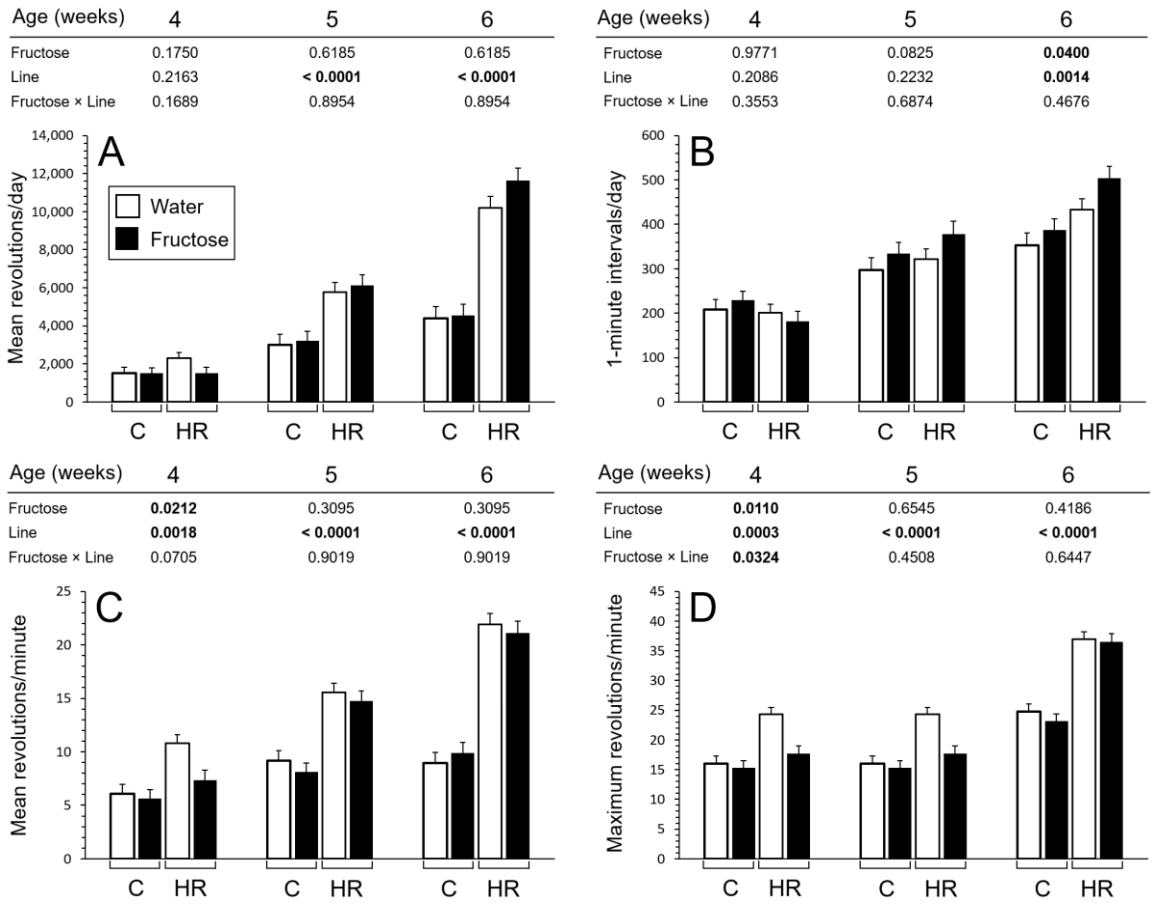

Age (weeks) 4 5 6
Fructose 0.1750 0.6185 0.6185
Line 0.2163 < 0.0001 < 0.0001
Fructose × Line 0.1689 0.8954 0.8954
A
Water
Fructose
Mean revolutions/day
14,000
12,000
10,000
8,000
6,000
4,000
2,000
0
C HR C HR C HR
Age (weeks) 4 5 6
Fructose 0.9771 0.0825 0.0400
Line 0.2086 0.2232 0.0014
Fructose × Line 0.3553 0.6874 0.4676
B
1-minute intervals/day
600
500
400
300
200
100
0
C HR C HR C HR
Age (weeks) 4 5 6
Fructose 0.0212 0.3095 0.3095
Line 0.0018 < 0.0001 < 0.0001
Fructose × Line 0.0705 0.9019 0.9019
C
Mean revolutions/minute
25
20
15
10
5
0
C HR C HR C HR
Age (weeks) 4 5 6
Fructose 0.0110 0.6545 0.4186
Line 0.0003 < 0.0001 < 0.0001
Fructose × Line 0.0324 0.4508 0.6447
D
Maximum revolutions/minute
45
40
35
30
25
20
15
10
5
0
C HR C HR C HR

Figure 2.3.

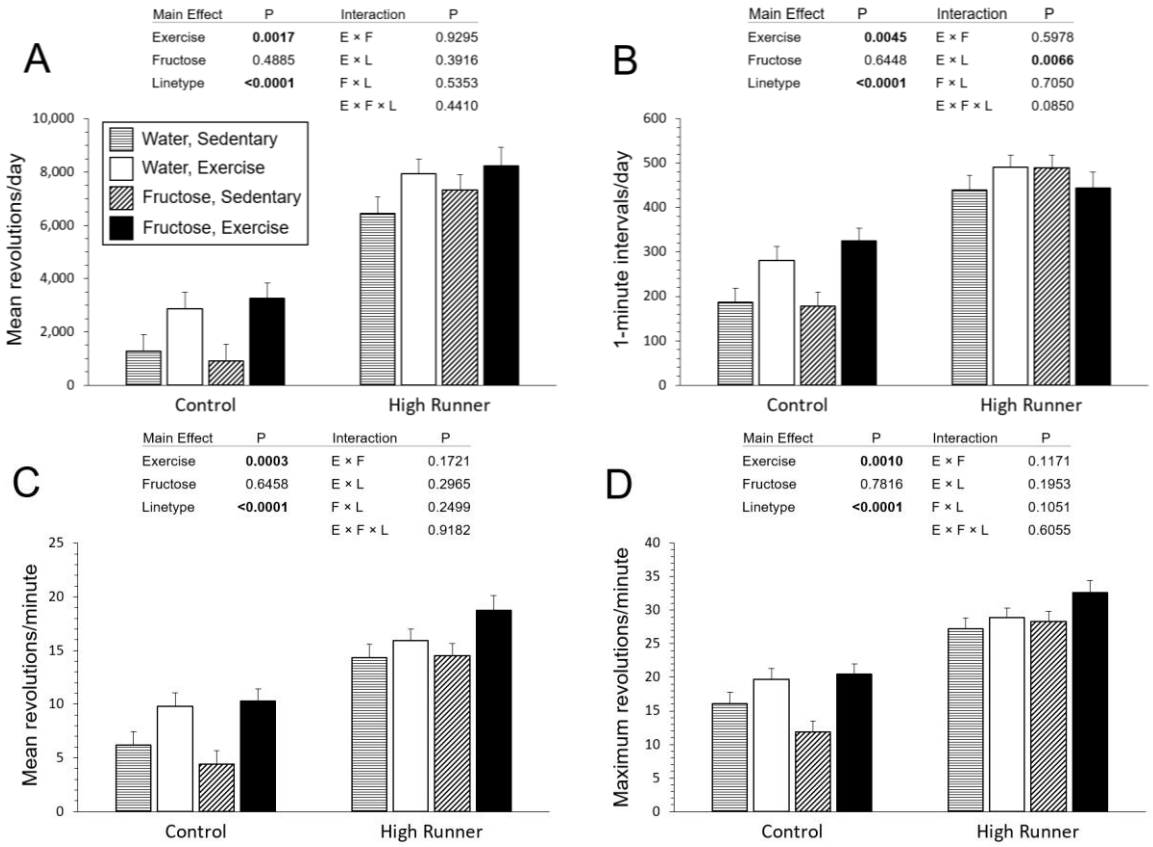

A
Main Effect P
Exercise 0.0017
Fructose 0.4885
Linetype <0.0001
Interaction P
E × F 0.9295
E × L 0.3916
F × L 0.5353
E × F × L 0.4410
Water, Sedentary
Water, Exercise
Fructose, Sedentary
Fructose, Exercise
Mean revolutions/day
10,000
8,000
6,000
4,000
2,000
0
Control
High Runner
B
Main Effect P
Exercise 0.0045
Fructose 0.6448
Linetype <0.0001
Interaction P
E × F 0.5978
E × L 0.0066
F × L 0.7050
E × F × L 0.0850
1-minute intervals/day
600
500
400
300
200
100
0
Control
High Runner
C
Main Effect P
Exercise 0.0003
Fructose 0.6458
Linetype <0.0001
Interaction P
E × F 0.1721
E × L 0.2965
F × L 0.2499
E × F × L 0.9182
Mean revolutions/minute
25
20
15
10
5
0
Control
High Runner
D
Main Effect P
Exercise 0.0010
Fructose 0.7816
Linetype <0.0001
Interaction P
E × F 0.1171
E × L 0.1953
F × L 0.1051
E × F × L 0.6055
Maximum revolutions/minute
40
35
30
25
20
15
10
5
0
Control
High Runner

Figure 2.4.

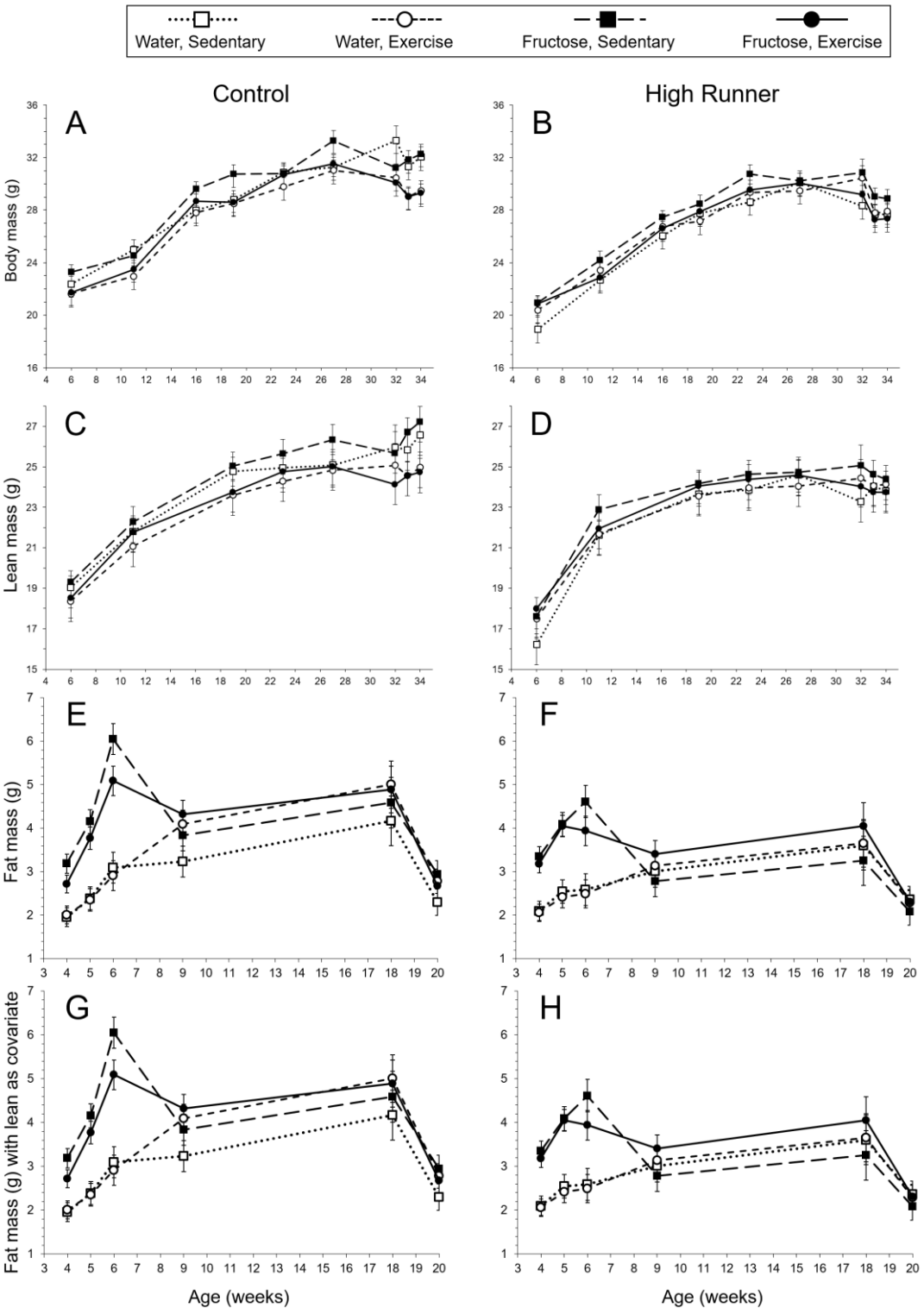

Water, Sedentary
Water, Exercise
Fructose, Sedentary
Fructose, Exercise
Control
High Runner
A
B
C
D
E
F
G
H
Body mass (g)
Lean mass (g)
Fat mass (g)
Fat mass (g) with lean as covariate
Age (weeks)
Age (weeks)

Figure 2.5.

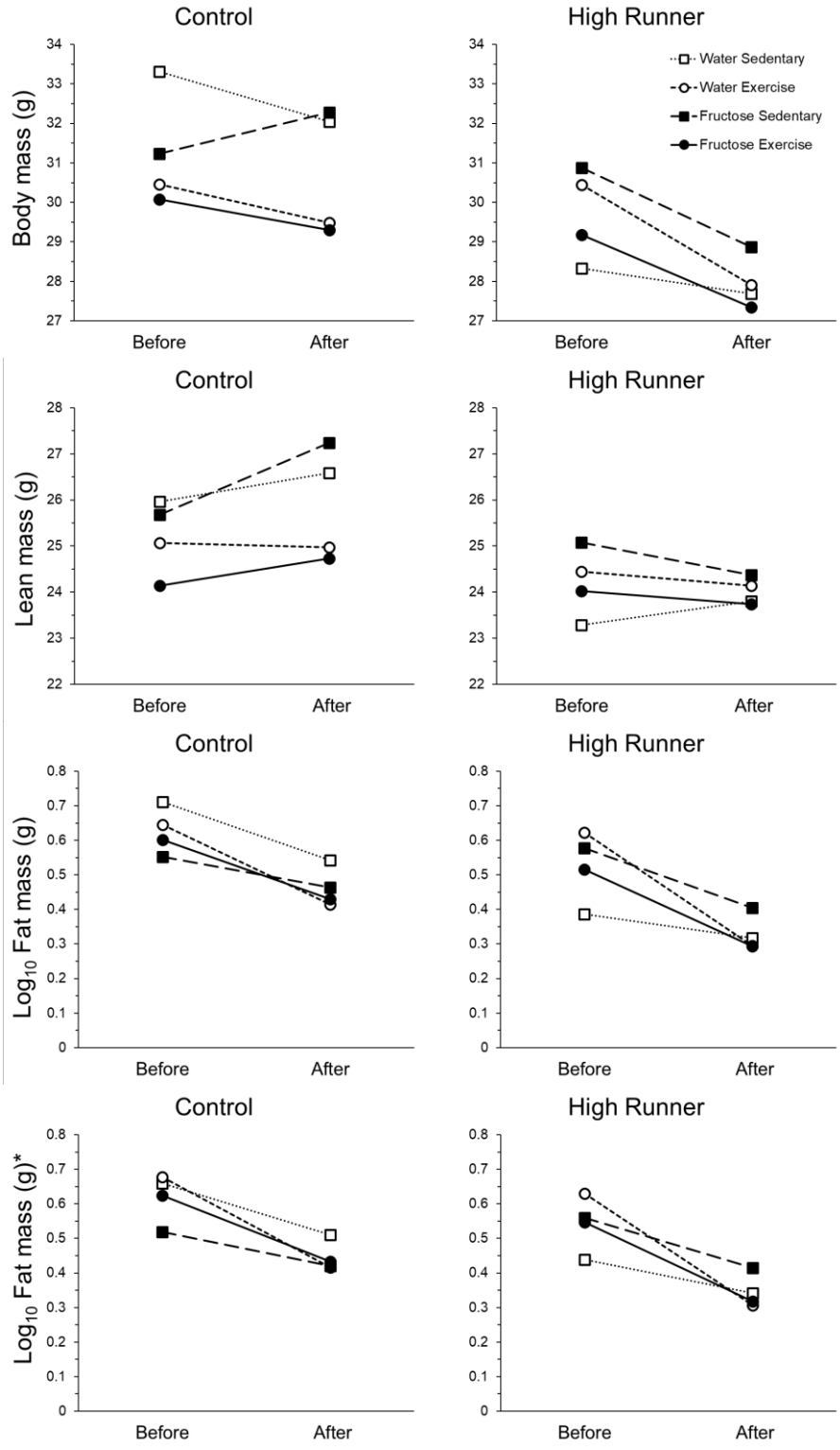

Control
High Runner
Water Sedentary
Water Exercise
Fructose Sedentary
Fructose Exercise
Body mass (g)
Lean mass (g)
Log10 Fat mass (g)
Log10 Fat mass (g)*
Before
After

Figure 2.6.

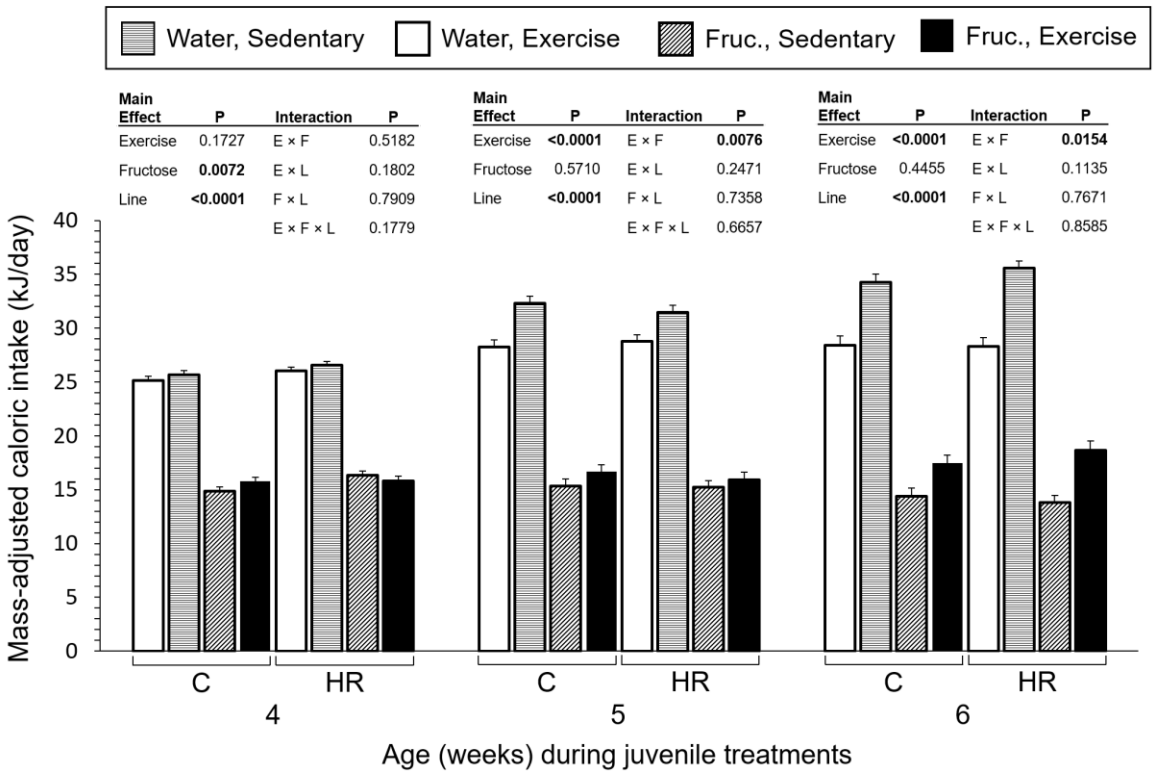

Water, Sedentary
Water, Exercise
Fruc., Sedentary
Fruc., Exercise
Main Effect P Interaction P
Exercise 0.1727 E × F 0.5182
Fructose 0.0072 E × L 0.1802
Line <0.0001 F × L 0.7909
E × F × L 0.1779
Main Effect P Interaction P
Exercise <0.0001 E × F 0.0076
Fructose 0.5710 E × L 0.2471
Line <0.0001 F × L 0.7358
E × F × L 0.6657
Main Effect P Interaction P
Exercise <0.0001 E × F 0.0154
Fructose 0.4455 E × L 0.1135
Line <0.0001 F × L 0.7671
E × F × L 0.8585
Mass-adjusted caloric intake (kJ/day)
0
5
10
15
20
25
30
35
40
C
HR
4
5
6
Age (weeks) during juvenile treatments

Figure 2.7.

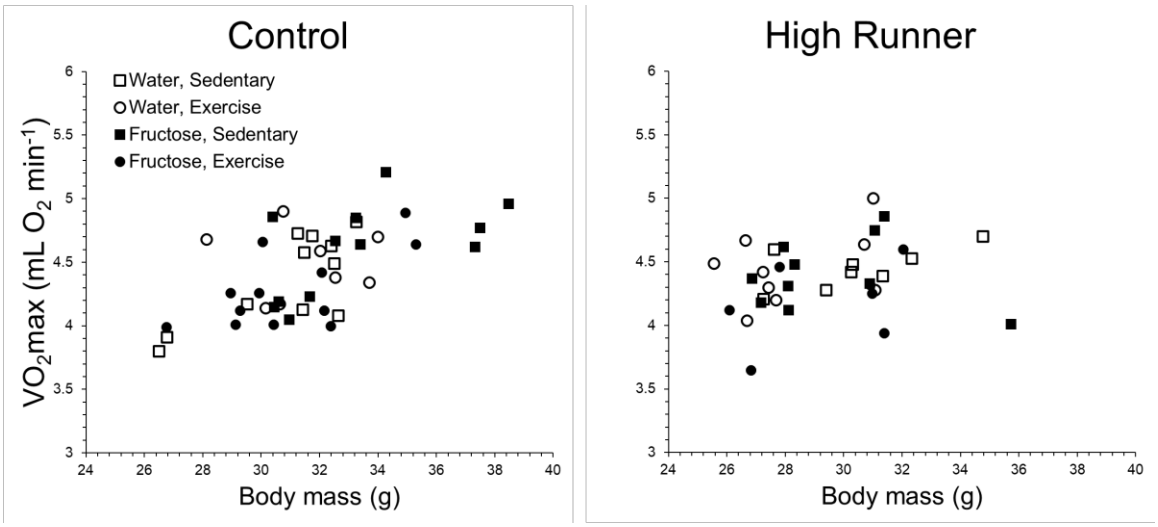
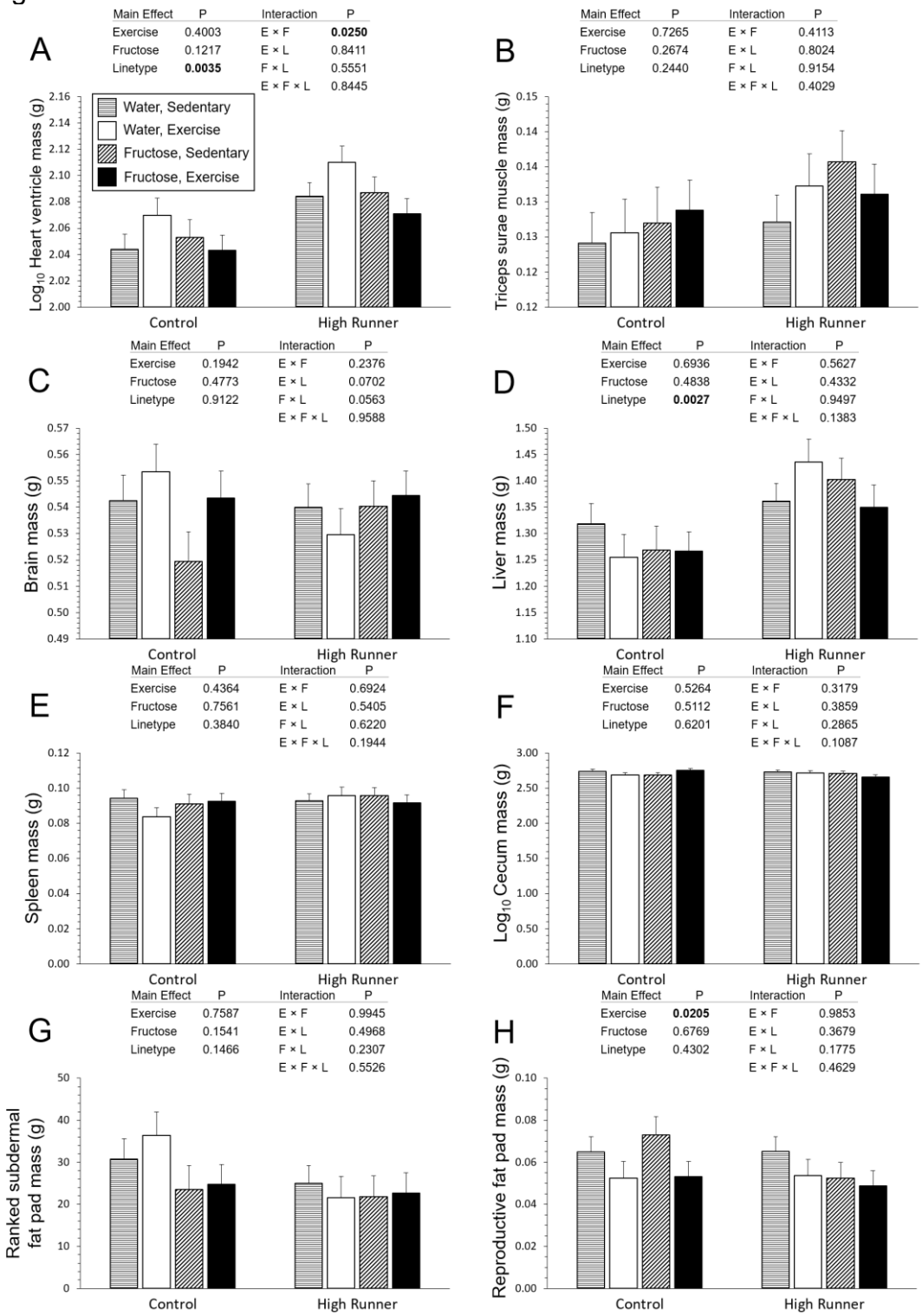

Control
Water, Sedentary
Water, Exercise
Fructose, Sedentary
Fructose, Exercise
VO2max (mL O2 min-1)
Body mass (g)
High Runner
Body mass (g)

Figure 2.8.


A
Main Effect P
Exercise 0.4003
Fructose 0.1217
Linetype 0.0035
Interaction P
E × F 0.0250
E × L 0.8411
F × L 0.5551
E × F × L 0.8445
Water, Sedentary
Water, Exercise
Fructose, Sedentary
Fructose, Exercise
Log10 Heart ventricle mass (g)
Control
High Runner
B
Main Effect P
Exercise 0.7265
Fructose 0.2674
Linetype 0.2440
Interaction P
E × F 0.4113
E × L 0.8024
F × L 0.9154
E × F × L 0.4029
Triceps surae muscle mass (g)
C
Main Effect P
Exercise 0.1942
Fructose 0.4773
Linetype 0.9122
Interaction P
E × F 0.2376
E × L 0.0702
F × L 0.0563
E × F × L 0.9588
Brain mass (g)
D
Main Effect P
Exercise 0.6936
Fructose 0.4838
Linetype 0.0027
Interaction P
E × F 0.5627
E × L 0.4332
F × L 0.9497
E × F × L 0.1383
Liver mass (g)
E
Main Effect P
Exercise 0.4364
Fructose 0.7561
Linetype 0.3840
Interaction P
E × F 0.6924
E × L 0.5405
F × L 0.6220
E × F × L 0.1944
Spleen mass (g)
F
Main Effect P
Exercise 0.5264
Fructose 0.5112
Linetype 0.6201
Interaction P
E × F 0.3179
E × L 0.3859
F × L 0.2865
E × F × L 0.1087
Log10 Cecum mass (g)
G
Main Effect P
Exercise 0.7587
Fructose 0.1541
Linetype 0.1466
Interaction P
E × F 0.9945
E × L 0.4968
F × L 0.2307
E × F × L 0.5526
Ranked subdermal fat pad mass (g)
H
Main Effect P
Exercise 0.0205
Fructose 0.6769
Linetype 0.4302
Interaction P
E × F 0.9853
E × L 0.3679
F × L 0.1775
E × F × L 0.4629
Reproductive fat pad mass (g)

CHAPTER 3

Cross-fostering Selectively Bred High Runner Mice Affects Adult Body Mass but Not Voluntary Exercise

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Abstract

While nursing, mammals progress through critical developmental periods for the cardiovascular, musculoskeletal, and central nervous systems. The suckling period in mammals is therefore especially vulnerable to environmental factors that may affect the "developmental programming" of many complex traits. As a result, various aspects of maternal behavior and physiology can influence offspring in ways that have lasting effects into adulthood. Several recent studies of animal models have shown that maternal effects can partially program adult activity behaviors, which has important implications for health and locomotor performance. Here, we used cross-fostering to test for possible maternal effects on adult wheel-running behavior (voluntary exercise), maximal aerobic capacity during forced exercise (VO_2max), body mass and composition, and organ masses. Subjects were from a line of mice that has been selectively bred for ~90 generations for high voluntary wheel-running behavior (High Runner; HR) and a non-selected Control (C) line. Adult HR mice run ~3-fold the daily distances of C mice and have evolved other differences associated with exercise capacity, including elevated VO_2max, reduced body mass and fat mass, and larger hearts. At birth, we fostered offspring to create 4 experimental groups: C pups to other C dams (in-foster), HR pups to other HR dams (in-foster), C pups to HR dams (cross-foster), HR pups to C dams (cross-foster). Thus, all pups were fostered to a different mother. Mice were weaned 3 weeks later, and adult testing began at ~6 weeks of age. At weaning, pups raised by HR dams were smaller than those

raised by C dams for both sexes and as expected, HR pups raised by HR dams weighed less than C pups raised by C dams. As adults, mice raised by HR dams continued to have reduced body masses. As expected, adult HR mice ran approximately 3-fold more than their C counterparts and females ran more than males. However, cross-fostering did not statistically affect any aspect of wheel-running behavior (distance, duration, speed). Similarly, with body mass as a covariate, HR mice had higher VO_2max than C mice, and males had higher VO_2max than females, but cross-fostering had no effect. With body mass as a covariate, cross-fostering had variable effects on adult organ masses in a sex-specific manner. Overall, our results indicate that development of the adult High Runner phenotype does not require rearing by an HR dam, suggesting that high adult activity in humans may be independent of high maternal activity.

1. Introduction

Physical activity is essential for both the development and maintenance of physical and mental health (e.g., Manley 1996; Callaghan 2004; Sharma et al. 2006; Ginsburg 2007; Pedersen and Fischer 2007; King et al. 2009). The many contributors to the levels of adult physical activity can be broadly classified as genetic versus environmental effects. Numerous studies of both humans and rodent models have demonstrated an important genetic component to physical activity, including levels of voluntary exercise (Lightfoot et al. 2018). Among the many environmental factors (e.g., the built environment for humans) that can

influence adult activity levels, those experienced early in life, especially during critical periods, can have long-lasting effects into adulthood (Garland, Jr. et al. 2017; Ferguson et al. 2019).

In mammals, mothers are a key component of the early-life environment, as they provide nutrition, care, and protection during critical periods of growth and development. Thus, variation in aspects of the mammalian maternal environment, including care *per se* (e.g., grooming), thermoregulation, and feeding via lactation (e.g., frequency of nursing bouts, milk nutritional content) has the potential to affect offspring development, with potentially persistent effects (e.g., Fish et al. 2004; Weaver et al. 2004; Chen et al. 2009; Carter et al. 2012). For example, maternal licking/grooming behavior in rats epigenetically induces altered behavior and stress responses in offspring later in life (Meaney and Szyf 2005).

A growing number of studies show that adult physical activity can, to some extent, be "developmentally programmed" by maternal effects (e.g., Breier et al. 2006; Dai et al. 2012; Donovan et al. 2013; Eclarinal et al. 2016; Zhu et al. 2016; Hiramatsu et al. 2017). These studies are routinely done by using cross-fostering experiments, which are a powerful method for elucidating maternal effects in mammals, as well as possible gene × environment interactions. This approach has been applied many times using rodent models (Francis et al. 1999; Bartolomucci et al. 2004; Fish et al. 2004; van der Veen et al. 2008; Hager et al. 2009; McCarty 2017) and has been used to investigate maternal differences

between genetically distinct strains of rodents (e.g., Defries 1964; Kessler et al. 2011; Cohen et al. 2015), including those resulting from artificial selection. For example, Barrenha and Chester (2012) cross-fostered mice selectively bred for high- and low-alcohol preference. When fostered to low-alcohol preferring dams, high-alcohol preferring mice had a significant reduction in alcohol consumption and preference across a 28-day period with 24-hour access (Barrenha and Chester 2012).

Although cross-fostering is a common approach within rodent models, to our knowledge only two cross-fostering studies have examined possible maternal effects on adult voluntary exercise or spontaneous physical activity (SPA). For example, when mice were fostered into small-litter families (resulting in postnatal overnutrition), adults had greater adiposity and female mice, specifically, had reduced SPA (Li et al. 2013). In a study using an obese mouse model (viable yellow agouti A^{vy}/a), newborn mice were cross-fostered between A^{vy}/a and wildtype (*a/a)* dams, which resulted in reduced adult SPA in *a/a* females born to A^{vy}/a dams and fostered to *a/a* dams (Baker et al. 2015).

In the current study, we used a cross-fostering approach to test for possible maternal effects on adult physical activity in a unique animal model, which includes four replicate selectively bred High Runner (HR) lines of mice and their four non-selected Control (C) lines (Swallow et al. 1998a). Mice in the HR lines have been bred for voluntary exercise on wheels on days 5 and 6 of a 6-day running period as young adults for >90 generations. Since reaching apparent

selection limits between generations 17 and 27 (Careau et al. 2013), HR lines have continued running ~3-fold more revolutions per day than C mice. This wide differential in the amount of voluntary exercise should increase statistical power to detect maternal effects on activity levels if they exist.

Given that HR and C mice differ in many additional traits, including circulating levels of multiple hormones (Malisch et al. 2007; Vaanholt et al. 2007; Meek et al. 2012; Garland, Jr. et al. 2016) and reduced body fat in the HR mice (Girard et al. 2007), we reasoned that differences in maternal care might broadly contribute to the adult differences between HR and C mice (we discuss our results in the context of potential coadaptation of maternal effects below). The plausibility of maternal effects on adult HR activity levels is further supported by recent studies that have demonstrated the importance of early-life factors in the development of the HR phenotype. For instance, when given wheel access during the juvenile period (3 weeks between weaning and sexual maturity), followed by a washout period of 7.5 weeks (no wheel access), adult mice ran greater distances on wheels, demonstrating that activity levels could be modulated not only in HR lines, but in C lines as well (Acosta et al. 2015). When fed a Western diet during the same early-life period and after a similar washout period, adult wheel running was increased in HR lines, but not C, indicating that the early-life "programmability" of adult activity levels may depend on genetic background (Cadney et al. 2021). In another study, Western diet fed to HR and C dams before mating until the weaning of their pups had no overall effect on the

adult wheel running of their offspring (Hiramatsu et al. 2017). Taken together, the foregoing studies suggest that both the magnitude and direction of early-life effects on adult levels of activity in HR and C mice depend on the timing and nature of the early-life factor.

In the present study, we used one representative HR line and one C line. At birth, we cross-fostered C pups to HR dams and *vice versa*, as well as C pups to C dams and HR pups to HR dams (in-foster: see Table 3.1.). We hypothesized that cross-fostering C pups to HR dams would result in higher adult wheel running and supportive traits (e.g., VO_2max, heart mass). Conversely, we expected the cross-fostering of HR pups to C dams would decrease their adult wheel running, for lack of hypothetical developmental stimuli otherwise provided by HR dams.

2. Methods and Methods

2.1. Experimental mice

Starting in 1993, four replicate lines of house mice were bred in an ongoing selection experiment for high voluntary wheel running (HR lines), based on the number of wheel revolutions on days five and six of six days of access to Wahman-type activity wheels (1.12-meter circumference) as young adults (Swallow et al. 1998a). The experiment began with a population of 224 mice from the outbred Hsd:ICR strain, which was randomly mated for two generations before being randomly partitioned into eight lines. Four of these were bred

randomly as Control (C) lines to the four HR lines. The current experiment used a subset of virgin male and female mice from generation 89 to produce the experimental focal mice of generation 90. For the purposes of maintaining comparability with previous generations of mice, all generation 89 males and females had previous access to a running wheel for a six-day period, as described above. All mice were fed standard mouse chow (Teklad Rodent Diet W-8604) and regular drinking water. Pregnant dams were given a breeder diet (Teklad S-2235 Mouse Breeder Sterilizable Diet 7004) through weaning. All experiments were approved by the University of California, Riverside IACUC.

To keep the number of required cross-fostering litters manageable, here we used one C line (Line 4) and one HR line (Line 7), chosen because they represented extremes in body mass among their respective linetypes. We reasoned that differences in dam body size could lead to differential cross-fostering effects, e.g., on the mass of their pups at weaning. Thus, by using lines with different body masses, they could serve as a type of positive control for offspring body masses at weaning. The wheel running behavior of these lines was representative of their respective linetypes (Table 3.2.). The dams used in the present study were typical of other line 4 and 7 breeders of the same generation in terms of both wheel running and body mass (Table 3.2.). Moreover, we intentionally avoided the use of HR lines 3 and 6, which express the mini-muscle phenotype, a simple Mendelian recessive allele characterized by massive alterations to skeletal muscle (Syme et al. 2005; Kelly et al. 2013).

2.2. Cross-fostering

Figure 3.1 presents the experimental timeline. Mice from generation 89 were sampled randomly to create a total of 60 line 4 and 60 line 7 mating pairs, with the constraint that sample size per foster group (Table 3.1.) was equal. We created such a large number of pairings because only pups born on the same day were to be used for fostering (Dohm et al. 1996, 2001). Only mice that were wheel-tested (Table 3.2.) were used for mating and fostering of pups to allow for the possibility that wheel-running itself may be important the maternal environment of HR dams. For logistical reasons, mating pairs were performed in two batches, one week apart (Fig. 3.1. shows one batch).

At birth, litters were standardized to eight pups from an average of ~10 (Girard et al. 2002; Hiramatsu et al. 2017) to avoid competition that might favor either HR or C pups, or other unforeseen litter effects. As sex could not be determined at birth, litter sex ratio could not be controlled (see Results). Fostering only occurred between litters born within 24 hours of one another. During the 48 hours after fostering, fostered pups were checked three times daily and none were rejected by their foster mother.

As births occurred, entire litters were fostered to another dam (no pup was returned to its biological mother). Thus, we did not include a "control" group for the effects of fostering *per se*. This design was chosen to maximize the sample size in experimental groups sufficient to address our specific hypotheses (see Introduction), given logistical constraints on total sample size. We wanted to

determine whether rearing by an HR dam might be necessary for some proportion of high-running phenotypic variance. We also wanted to know whether rearing by an HR dam might confer some aspect of the HR phenotype, such as higher adult voluntary wheel-running behavior.

2.3. Adult testing of fostered offspring

Physical activity is typically partitioned into voluntary exercise and spontaneous physical activity (SPA: Garland, Jr. et al. 2011b), and both components can have important effects on many aspects of health (Deslandes et al. 2009; Carek et al. 2011; Dinas et al. 2011; Garber et al. 2011; Jakicic and Davis 2011; Grazioli et al. 2017; Mokhtari-Zaer et al. 2018; Ruegsegger and Booth 2018). Accordingly, we measured both wheel-running behavior and home-cage activity as indicators of voluntary exercise and SPA, respectively (Garland, Jr. et al. 2011b).

At 7 weeks of age, all mice were individually housed with food and access to running wheels. For each day of wheel testing (6 days total), we recorded revolutions in each 1-minute interval over a period of 23 h. This allowed computation of the total distance run, the number of 1-minute intervals with at least one revolution (a measure of the daily running duration), and the average running speed (distance/intervals), as well as the maximum speed in any 1-minute interval (Copes et al. 2015). We also computed average values for days 5 and 6, as these are the values used for the selection protocol (Swallow et al.

1998a). Wheel freeness (an inverse measure of how difficult it is to turn the wheel) was used as a covariate in all analyses of wheel running (e.g., Copes et al. 2015).

During wheel testing, home cages were fitted with passive infrared sensors (Talon TL-Xpress-A; Crow Electronics, Fort Lee, New Jersey, USA), protected within wire mesh, as in previous studies (Acosta et al. 2015; Copes et al. 2015). The sensors were connected to a computer with custom activity-recording software (developed by M. A. Chappell) via a digital I/O board (ICS 2313; ICS Electronics, Pleasanton, CA, USA). The sensors recorded activity three times per second and a mean value between 0 (no movement detected) and 1 (movement detected) was calculated for each minute over 23 hour-periods of measurement. All analyses of SPA data used a measure of sensor sensitivity as a covariate (Acosta et al. 2015; Copes et al. 2015).

2.4. Body composition

Whole-animal fat and lean masses were measured by restraining each mouse within a translucent tube before insertion into an EchoMRI-100 (Echo Medical Systems, Houston, TX, USA) for scanning. This procedure lasted approximately 1-2 min per mouse and did not require sedation or anesthesia. Body composition was measured on mice approximately one hour before the start of wheel testing and within one hour of being taken off wheels.

2.5. Maximal aerobic capacity (VO_2max)

To measure VO_2max, mice were subjected to forced exercise within an enclosed wheel metabolic chamber approximately 15 cm in diameter, with an effective volume of 900 mL (Dlugosz et al. 2009; Claghorn et al. 2017); this method yields estimates of VO_2max that are statistically indistinguishable from treadmill procedures (Dlugosz et al. 2013). Air was pumped into the enclosed metabolic chamber at a rate of 2,000 mL per min (with instantaneous corrections applied), and the concentration of O_2 in excurrent air was passed through H_2O and CO_2 scrubbers (Drierite and indicating soda lime) and measured by an oxygen analyzer (S-3A Applied Electrochemistry, Inc., Sunnyvale, CA), the second channel of which was used to record ambient O_2 concentration.

Mice were weighed and then placed into the metabolic chamber. After an initial 1-2 minutes of acclimation, mice were induced to run for approximately 5 min while researchers manually accelerated the wheel until VO_2 plateaued for at least 2 min or mice stopped running. Duplicate trials were conducted, allowing a day of rest between trials, and VO_2max was taken as the highest minute of oxygen consumption during either trial, as calculated with LabHelper software (Warthog Systems, www.warthog.ucr.edu).

Similar to previous studies (Swallow et al. 1998b; Claghorn et al. 2017), we subjectively assessed trial quality, as well as tiredness at the end of each trial. Trial quality was scored between 1, least cooperative (the mouse resisted running by grasping at the pneumatic apparatus or bracing against the direction

of motion) and 5, most cooperative (the mouse consistently ran with the direction of rotation). Tiredness at the end of each trial was scored on a scale of 1 (least exhausted) to 3 (most exhausted). This scale was based on how long it took for the mouse to begin moving about the chamber, with a score of 1 being 1 second or less and a score of 3 being 5 seconds or more). Trials where both quality and tiredness scores were less than 2 were excluded from analyses of quality, tiredness, and VO_2max (more stringent exclusion criteria, which further reduced sample size, did not alter the final statistical results). The investigator scoring each trial was recorded and used as a random effect in statistical analyses.

Trial quality and tiredness during the higher of the two VO_2max trials (i.e., corresponding to VO_2max) were analyzed as dependent variables (Swallow et al. 1998b; Claghorn et al. 2017), with age used as a covariate. For analyses of VO_2max, body mass and age were used as covariates, but trial quality and tiredness were not significant predictors and were not included in the final model.

2.6. Dissections

At seven weeks of age, mice were removed from wheel access for one day, then euthanized. Brains, heart ventricles, spleen, liver, left triceps surae muscle, and reproductive fat pads (Cinti 2012) were dissected and weighed to 0.0001 g (some organs are reported in mg for ease of viewing).

2.7. Statistical analysis

Data were analyzed as mixed models in SAS 9.1.3 (SAS Institute, Cary, NC, USA) Procedure Mixed, with REML estimation and Type III Tests of Fixed Effects. Line (selected line 7 vs. non-selected line 4) and foster line were fixed effects. To adjust for possible litter effects that would violate the statistical assumption of independence (see Festing 2006), dam ID (n = 28) was used as a random effect nested within line × foster line for all analyses of foster offspring, which partitioned litter variance from residual error variance (see Golub and Sobin 2020). Depending on the trait analyzed, body mass, age, wheel freeness, and/or home-cage sensor sensitivity were used as covariates. We inspected graphs of traits in relation to covariates (e.g., weaning body mass versus dam body mass) to make sure the covariate effect was not confounded with group effects, and that we did not have any obvious group × covariate interactions. (We did not include interactions between main effects and covariates in our statistical models because we had no *a priori* hypotheses concerning such interactions.) Body mass was excluded as a covariate for analyses of brain mass because we observed an anomalous negative relationship. Lean mass was excluded as a covariate for analyses of fat mass for the same reason.

We analyzed the sexes both separately and combined. We emphasize the former analyses because many sex differences have been reported previously for the traits studied, including specifically in the HR and C lines of mice (e.g., see Swallow et al. 1998a; Wells 2007; Bartling et al. 2017; Hiramatsu

et al. 2017; Thompson et al. 2017), and because we had no hypotheses about sex-specific effects of cross-fostering. In all figures we show least squares means and standard errors from separate-sex analyses, along with the four p-values for differences of least squares means between the in-fostered and cross-fostered groups (i.e., the effect of cross-fostering between the HR and C lines by sex). For completeness, we present combined-sex analyses and associated least squares means in Supplemental File 3.1. Supplemental material can be referenced for main effects, as well as interactions (line × foster line, line × sex, foster line × sex, line × foster line × sex). In all analyses, outliers were removed when the standardized residuals exceeded ~3.

Statistical significance was judged at $p \leq 0.05$. However, excluding the results of combined-sex analyses, nuisance variables (such as age and wheel freeness), and body mass when used as a covariate, Supplemental File 3.1 includes 360 p-values for the two *a priori* contrasts of primary interest, as well as main effects of line, foster line, and their interaction. Of these 360 p-values, 88 were nominally significant at $p < 0.05$. If all null hypotheses were in fact true, then one would expect 18 p-values (0.05×360) to be < 0.05 by chance alone. In addition, these tests include a substantial amount of nonindependence because the same individuals were measured for all traits, some traits were correlated (e.g., wheel running on successive days), and many tests were interrelated (e.g., body mass and fat mass). Therefore, to compensate for nonindependence in multiple related tests, we used the positive False Discovery Rate (pFDR)

procedure as implemented in PROC MULTTEST in SAS version 9.4 (SAS, Cary, NC). Based on this procedure, an adjusted critical value of 0.012 would be appropriate for controlling the false discovery rate at a 5% Type I error rate. All p-values reported in the text and tables are raw values (i.e., not adjusted for multiple comparisons), so the reader should keep this in mind.

3. Results

Note: Supplemental File 3.1 is a spreadsheet that summarizes p-values for main and interactive effects of linetype and treatment on various traits (most of which are reported in this chapter). Subsequent sheets contain more detailed results (degrees of freedom, least squares means, standard errors, etc.) for each trait, which can be found by navigating to the sheet corresponding to the "index" number indicated in column A of the RESULTS sheet.

3.1. Characteristics of mothers

When wheel-tested at 6-8 weeks of age as part of the routine selection protocol (see Methods and Swallow et al. 1998a), mice from C Line 4 ran near the bottom of the range for the C lines, whereas HR Line 7 mice ran near the top of the range for the HR lines (Table 3.2.). As expected, dams from the C line used in the present study (Line 4) were the largest (at weaning) of any C line and those from the HR line used here (Line 7) were the smallest (Table 3.2.).

3.2. Litter size at birth and sex ratio of fostered litters as covariates

Neither the litter size at birth nor the sex ratio of fostered litters could be controlled in the experimental design, so they were used as covariates in preliminary analyses, but subsequently removed because they were not significant predictors, with three exceptions (see Supplemental File 3.1). Sex ratio (determined at weaning) had a statistically significant (1) negative effect on weaning mass in both combined- and separate-sex analyses, (2) positive effect on growth rate from weaning to sexual maturity in combined-sex analyses, and (3) negative effect on the change in body mass across six days of wheel access for male mice only (Supplemental File 3.1).

3.3. Body mass, growth rate, and body composition

At weaning, pups raised by HR dams were significantly smaller than those raised by C dams for both sexes (Fig. 3.2.A, Supplemental File 3.1). In addition, HR pups raised by HR dams weighed significantly less than C pups raised by C dams (Supplemental File 3.1). However, when dam mass was used as a covariate, least squares means for pups raised by HR dams were larger than those raised by C dams, and HR pups raised by HR dams no longer weighed less than C pups raised by C dams (Fig. 3.2.B). In other words, for their body size, HR dams weaned larger pups.

As adults during experimental week 10 (measurement of VO_2max), mice raised by HR dams still weighed less than those raised by C dams, although the

effect was not significant for female HR pups (Supplemental File 3.1). At the beginning of experimental week 11 (just prior to adult wheel testing), mice raised by HR dams were still smaller than those raised by C dams (Fig. 3.3.A). For lean mass, being raised by an HR dam only had a significant effect (negative) for female C mice (Fig. 3.3.B). For fat mass the effect of cross-fostering was not significant for either sex (Fig. 3.3.C).

Immediately following wheel testing, mice raised by HR dams continued to have less total body mass. The change in body, lean, and fat mass across wheel testing was taken as the difference between measurements (i.e., after − before). Cross-fostering did not significantly affect the change in body mass or lean mass, but HR mice did lose more total mass and lean mass across wheel testing than did C mice (Supplemental File 3.1).

3.4. Maximal aerobic capacity (VO_2max)

Cross-fostering did not statistically affect VO_2max for either sex (Fig. 3.4.), but in the combined-sex analysis mice from the HR line had higher values than C ($p < 0.0001$) and males had higher values than females ($p = 0.0496$), with no significant interactions. Body mass a significant predictor of VO_2max (Supplemental File 3.1).

Trial quality was not affected by line, sex, or cross-fostering. Trial tiredness (rank-transformed to achieve normality of residuals) differed by line, especially for females. C mice were tired for longer after trials than HR mice ($p =$

0.0138). Additionally, female C pups raised by HR dams had increased tiredness, as compared with those raised by C dams (Supplemental File 3.1; p = 0.0470).

3.5. Wheel-running behavior

In no case did cross-fostering have a statistically significant effect on any measure of adult wheel-running behavior (Fig. 3.5.). As expected, however, mice from the HR line ran significantly more revolutions on days 5 and 6 than those from the C line (females ran 4.5-fold and males 5.7-fold more). Mice from the HR line also ran for more minutes per day, at higher average speeds, and attained higher maximum speeds on days 5 and 6, and on all six days analyzed separately (Supplemental File 3.1, Fig. 3.5.).

3.6. Home-cage activity

Cross-fostering did not affect home-cage activity (total minutes or average intensity) on days 5 and 6 of adult wheel testing (Supplemental File 3.1). For females, HR mice had greater total home-cage activity than did C mice (Supplemental File 3.1; p = 0.0416).

3.7. Organ masses

With body mass as a covariate, cross-fostering had variable effects on adult organ masses, and all such effects were sex-specific (Fig. 3.6.). Some

differences between the HR line and C line were also observed, as well as sex differences (Supplemental File 3.1).

Cross-fostering increased muscle mass among female C mice, although the effect was not statistically significant (Fig. 3.6.B; $p = 0.0504$). Male mice had greater triceps surae muscle mass than female mice (sex $p < 0.0001$). Cross-fostering increased brain mass among female HR mice (Fig. 3.6.C; $p = 0.0476$). Cross-fostering significantly reduced male reproductive fat mass among C mice (Fig. 3.6.E; $p = 0.0032$). HR mice had significantly smaller male reproductive fat masses than C mice (Supplemental File 3.1; $p = 0.0003$). Cross-fostering increased spleen mass in male HR mice (Fig. 3.6.F; $p = 0.0117$). Spleen mass differed between HR and C lines among both sexes (Supplemental File 3.1; female $p < 0.0001$; male $p = 0.0147$).

Cross-fostering did not affect heart ventricle mass (Fig. 3.6.A), but HR mice had larger ventricles than C mice in both sexes (Supplemental File 3.1; female $p = 0.0010$; male $p < 0.0001$). Cross-fostering did not significantly affect liver mass (Fig. 3.6.D), but males had larger livers than females (sex $p < 0.0001$). For males, HR mice had significantly smaller livers than C mice (Supplemental File 3.1; sex × line $p < 0.0001$).

4. Discussion

4.1. Coadaptation of maternal effects on adult voluntary exercise

The artificial selection protocol used to produce the HR lines has resulted in changes in allele frequencies for numerous genes that appear related to both their motivation and ability for wheel running (e.g., see Xu and Garland, Jr. 2017; Hillis et al. 2020; Nguyen et al. 2020). In addition, selection may have caused changes in allele frequencies for genes that are expressed primarily or exclusively in mothers up to the time of weaning their pups, causing phenotypic effects in the mothers that also have effects on wheel running of those offspring when they are adults (e.g., see Wolf and Brodie 1998; Wolf et al. 1998). Indeed, significant divergence in wheel running between HR and C mice occurs within a few days after weaning (e.g., see Fig. 1.2. of Chapter 1), which is 3-5 weeks prior to normative adult wheel testing in the selection experiment. In other words, maternal traits that promote wheel running by their offspring may have evolved in response to the HR selection regime (Dohm et al. 2001; Careau et al. 2013; Kusuyama et al. 2020). Such maternal effects are one example of early-life effects and several studies have specifically demonstrated early-life effects on adult physical activity, including in the HR mice (Le Pape and Lassalle 1984; Bartolomucci et al. 2004; Acosta et al. 2015; Garland, Jr. et al. 2017; Cadney et al. 2021). In general, cross-fostering can affect various other behaviors, such as emotionality (Malkesman et al. 2008; Lu et al. 2009; Plyusnina et al. 2009).

In the present study, we used cross-fostering to test whether the adult HR phenotype is influenced by the maternal environment. We recorded body mass at weaning and then, in adults, we measured maximal aerobic capacity (VO_2max: an important determinant of endurance exercise capacity, which is elevated in HR mice), body mass (reduced in HR mice), body fat (reduced in HR mice), voluntary wheel running, home-cage activity (both elevated in HR mice), and organ masses (several of which are altered in HR mice). Although we detected statistically significant effects of cross-fostering on mice at weaning and as adults (body mass, composition, and organ masses), we did not detect any effects on wheel running, home-cage activity or VO_2max. Thus, we find no evidence for coadaptation of maternal effects (via the early postnatal environment) on adult voluntary exercise during the evolution of the HR mice. However, future studies (e.g., using embryo transplants) will be required to test for possible pre-natal maternal influences.

4.2. Effects of cross-fostering on body mass and composition

Obesity of human (e.g., Sewell et al. 2006; Lindell et al. 2018) and rodent (De Sousa 2021; Han et al. 2021) mothers is associated with a host of adverse health consequences in their adolescent and adult offspring, including increased risk of obesity, type 2 diabetes, non-alcoholic fatty liver disease, and altered behavior. In rodents, for example, Desai et al (2014) used a rat model involving a high-fat (HF) maternal diet during pregnancy and/or lactation, which resulted in

hyperglycemia and increased systolic blood pressure in offspring. Their HF dams had elevated plasma corticosterone levels (Desai et al. 2014), which, interestingly, is a characteristic of HR mice in general (e.g., see Malisch et al. 2007; Garland, Jr. et al. 2016; Singleton and Garland 2019). In another rodent study, Miranda et al. (2017) cross-fostered rat offspring with an obese phenotype to non-obese control dams, resulting in "rescued" body weight and food intake, among other health factors. In obese offspring raised by obese dams, males developed a wide range of metabolic disturbances, including hyperglycemia and hyperinsulinemia (Miranda et al. 2017).

Although C mice are not generally viewed as obese under standard housing conditions and on standard chow, they do have more body fat than HR mice (Swallow et al. 2001). Therefore, we presumed cross-fostering effects on offspring body mass and/or composition would be apparent at weaning. As expected, pups raised by HR dams were smaller at weaning (Fig. 3.2.A), an effect that persisted into adulthood (Fig. 3.3.A).

The cross-fostering effects on body mass and composition (Fig. 3.2., 3.3.) might be mediated by the body mass and composition of the dams. Consistent with this idea, body mass at weaning was smaller for HR dams as compared with C dams (Table 3.2.), but we were not able to measure body composition of either dams or their weaned offspring. However, another study in our lab did examine body composition of nursing dams from generation 84 (N. E. Schwartz et al. unpublished results). In a one-way ANCOVA with age as a covariate, nursing

line 7 dams had significantly reduced body mass, lean mass, and fat mass than those from line 4 (all $p < 0.0001$). Average body masses of those dams (line 7, 29.58 g; line 4, 41.31 g) were similar to those used in the current study (Table 3.2.).

As a statistical test of dam body mass mediation of cross-fostering effects on weaning mass, we repeated the analyses shown in Fig. 3.2.A with dam mass as a covariate and found that the least squares (adjusted) means for pup mass were then significantly larger for those raised by HR dams (Fig. 3.2.B). Previous studies of unmanipulated litters in earlier generations of the selection experiment (i.e., HR and C dams raising their own pups) generally did not find statistically significant differences in body mass at weaning between the complete set of four HR lines and four C lines (Swallow et al. 1999; Girard et al. 2002; Keeney 2011), but studies of later generations have reported significantly smaller body mass at weaning for HR mice, especially for males (Hiramatsu et al. 2017 at generation 73; Cadney et al. 2021 at generation 76), although not significant differences in lean or fat mass at weaning (Hiramatsu et al. 2017).

4.3. Sex-specific cross-fostering effects

Sex-specific cross-fostering effects have been reported for a range of traits in rodents (e.g., Bester-Meredith and Marler 2001; Li et al. 2013; Baker et al. 2015; Zhu et al. 2016). Indeed, early-life effects in general often interact with sex (e.g., see Whitaker et al. 2012 and references therein). In the present study,

sex-specific cross-fostering effects were observed for weaning mass (with dam mass as a covariate), body mass and lean mass before and after adult wheel testing, brain mass, spleen mass, and reproductive fat mass (see Supplemental File 3.1). Main effects of sex were nearly ubiquitous, as would be expected from numerous previous studies of, for example, adult wheel running, body mass, and composition (Hiramatsu and Garland 2018). Taken together, these results emphasize the importance of including both sexes in studies of early-life effects in general (e.g., see Hiramatsu et al. 2017; Conner et al. 2020 for mice; Ross and Desai 2005; Tao et al. 2019 for human famine studies).

4.4. Limitations of the Present Study

Differences among the four replicate HR lines and also among the four non-selected C lines have been documented for a variety of traits (e.g., see Koteja et al. 2003; Malisch et al. 2007; Dlugosz et al. 2009; Garland, Jr. et al. 2011a; Careau et al. 2013; Hiramatsu et al. 2017). Therefore, ideally, a cross-fostering study would have included all eight lines, but such a study would require a very large number of litters to include all possible combinations. To keep the number of litters within our logistical capacities, we used only one C line and one HR line (the reasons for using these particular lines are explained in the Methods). Our results might have been different if we had used different lines, e.g., ones that did not differ so much in body size (Table 3.2.).

The purpose of the present study was to test the specific hypotheses outlined in the Introduction concerning cross-fostering HR and C mice. We have used the term "cross-fostering" to mean the fostering of pups *between* HR and C dams. As we were not specifically interested in the effects of cross-fostering *per se* (i.e., between families within a linetype of mouse), we did not include a non-fostered control group. However, cross-fostering in and of itself is a stressful event for both mothers and their offspring, and has a variety of effects (e.g., Bartolomucci et al. 2004; Santangeli et al. 2016). For example, Eisen et al. (1980) reported that fostering of mice among dams within a single inbred strain can influence lactation, with consequences for offspring. If such effects occurred in our study, and if HR and C lines responded differently to cross-fostering stress, then differential effects of maternal care by HR and C mice may have been confounded. No studies of the stress responsiveness of lactating females or of pre-weaning pups are available for these lines, but one study using 40 minutes of restraint stress reported a smaller increase in circulating corticosterone concentrations from baseline for HR mice (Malisch et al. 2007), which could be interpreted as evidence of reduced stress responsiveness.

In the present experiment, all adult mice were tested on wheels for 6 days, followed by one day with the wheels removed, prior to dissection. Therefore, organ masses may have experienced training effects (physical conditioning), as well as acute effects that might have occurred during the intervening ~24-h period between wheel testing and dissections (e.g., see Dumke et al. 2001).

Given that HR mice run much more than C and also have greater phenotypic plasticity in response to a few days of running for some traits (e.g., see Gomes et al. 2009), our ability to detect differential cross-fostering effects on organ masses may have been affected. Nonetheless, some effects were detected (Supplemental File 3.1; Fig. 3.6.).

An interesting avenue for future research would be to test for differences in milk quantity, quality or composition between HR and C dams. Several studies have shown that inbred strains of mice differ in milk properties (Ventrella et al. 2021 and references therein) that may have long-lasting (even multi-generational) effects on offspring (Ozkan et al. 2020). Although physical activity before and during pregnancy has effects on milk properties in mice (Harris et al. 2020), no study has demonstrated effects of altered milk content on adult physical activity levels.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Table 3.1. Four treatment groups were generated by cross-fostering between families of HR (line 7) and C (line 4) mice.

	Foster group	Birth dam	Foster dam	# of Litters
In-foster	C→C	C	C	7
Cross-foster	C→HR	C	HR	7
In-foster	HR→HR	HR	HR	7
Cross-foster	HR→C	HR	C	8

Table 3.2. Characteristics of generation 89 breeder females. Measurements of female breeders from generation 89 were taken as part of the on-going selection experiment. Average daily revolutions on days 5 and 6 (n = 126) and subsequent body mass (n = 134) at the time their pups were weaned are shown as least squares means and standard errors from SAS Procedure Mixed (mass with age as a covariate, wheel running with age and wheel freeness as covariates). Shown in parentheses are the corresponding values for the separate set of breeders used in the present study.

	Line	Revolutions/day	S.E.	Body mass (g)	S.E.
C	**1**	4,868	589	35.3	0.73
	2	4,248	555	35.8	0.70
	4	4,275 (3,552)	752 (855)	40.2 (40.5)	0.98 (0.44)
	5	4,901	718	32.1	0.82
HR	**3**	14,275	641	36.9	0.74
	6	16,525	585	35.7	0.68
	7	16,291 (16,378)	678 (855)	28.5 (28.0)	0.87 (0.44)
	8	16,171	606	33.2	0.70

Figure Legends

Figure 3.1. Experimental timeline, starting with the pairing of generation 89 mice and ending with the dissections of generation 90 focal mice. The timeline represents experimental weeks, not age. Mice were fostered within 24 h of birth and weaned at 3 weeks of age, at the start of experimental week 7. Wheel testing (over a 6-day-period) started when mice were 7 weeks old; dissections occurred at 8 weeks of age. Note that two experimental batches (not indicated on the timeline) were offset by one week for logistical reasons, but all mice followed the outlined procedure (see Methods).
M – body mass measurement; C – body composition measurement.

Figure 3.2. Effects of cross-fostering on body mass at weaning. A) Mice reared by HR dams were smaller at weaning than those reared by C dams. This effect persisted into adulthood (see Fig. 3.3.). B) With dam body mass as a covariate, mice reared by HR dams were larger at weaning than those reared by C dams, especially for females. Shown are least squares means and standard errors from separate-sex analyses; p-values are differences of least squares means from SAS Procedure Mixed. Statistically significant values ($p < 0.05$) are in bold. See Supplemental File 3.1 for complete statistical results, including combined-sex analyses.

Figure 3.3. Effects of cross-fostering on adult body, lean, and fat mass as measured by echo MRI, immediately prior to wheel testing. Similar to effects on body mass at weaning, adults had lower body mass (A) if they were raised by an HR dam (except male HR mice, $p = 0.0818$). For lean mass (B), the only significant effect was that female C mice had lower lean mass when raised by an HR dam. For fat mass (C), there was no significant effect of cross-fostering, though note the same pattern of effects as in adult body mass (A) and weaning (Fig. 3.2.A). Shown are least squares means and standard errors from separate-sex analyses; p-values are differences of least squares means from SAS Procedure Mixed. P-values ($p < 0.05$) are in bold. See Supplemental File 3.1 for complete statistical results, including combined-sex analyses.

Figure 3.4. Adult maximal oxygen consumption (VO_2max) during forced exercise. Body mass (g) was a significant predictor of VO_2max ($p < 0.0001$). Male mice (right plot) had greater VO_2max than female mice (left plot; $p = 0.0496$) and HR mice had greater VO_2max than C mice (female $p = 0.0005$, male $p < 0.0001$), with no statistically significant effect of cross-fostering. C mice are indicated by gray circles and HR mice are indicated by black circles. In-fostered mice are indicated by solid circles and cross-fostered mice are indicated by open circles. See Fig. 3.2 for more detailed explanation of legend. See Supplemental File 3.1 for complete statistical results, including combined-sex analyses.

Figure 3.5. Effects of cross-fostering on adult wheel-running behavior on days 5 and 6 of a 6-day test. Cross-fostering had no statistically significant effect on mean A) revolutions/day (circumference 1.12 meters), B) duration of daily running (min/day), C) speed (revs/min), or D) maximum revolutions in any 1-min interval. However, for all measures, mice from the selectively bred HR line had higher values than those from the non-selected C line, and females had higher values than males. Shown are least squares means and standard errors from separate-sex analyses (see Supplemental File 3.1 for combined-sex analyses); p-values are differences of least squares means from SAS Procedure Mixed. Statistically significant values ($p < 0.05$) are in bold.

Figure 3.6. Effects of cross-fostering on adult body-mass adjusted organ masses. Cross-fostering had relatively few effects on organ masses, and the effects were inconsistent between the sexes. Organ masses are log-transformed for analysis and log body mass was used as a covariate. Triceps surae (B), brain (C) reproductive fat (E), and spleen (F) masses are shown in mg rather than g for better viewing. Shown are least squares means and standard errors from separate-sex analyses (see Supplemental File 3.1 for combined-sex analyses); p-values are differences of least squares means from SAS Procedure Mixed. Statistically significant values ($p < 0.05$) are in bold.

Figure 3.1.

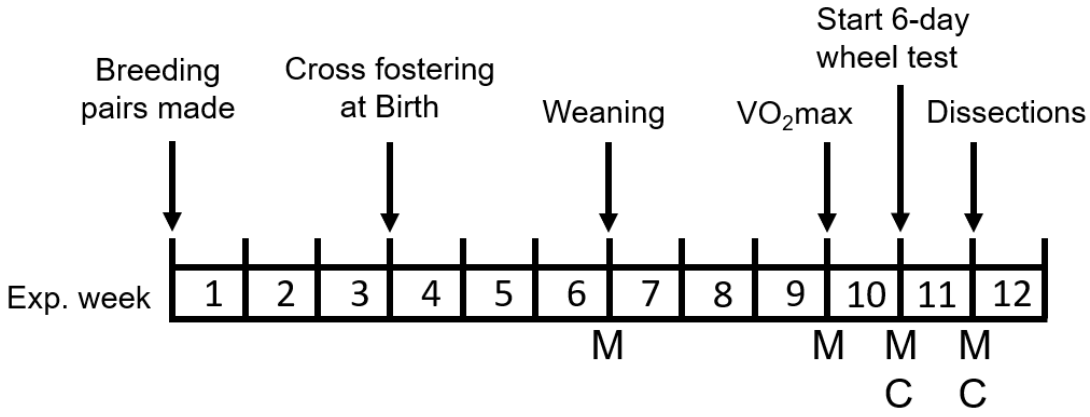

Breeding pairs made
Cross fostering at Birth
Weaning
VO2max
Start 6-day wheel test
Dissections
Exp. week
1
2
3
4
5
6
7
8
9
10
11
12
M
M
M
C
M
C

Figure 3.2.

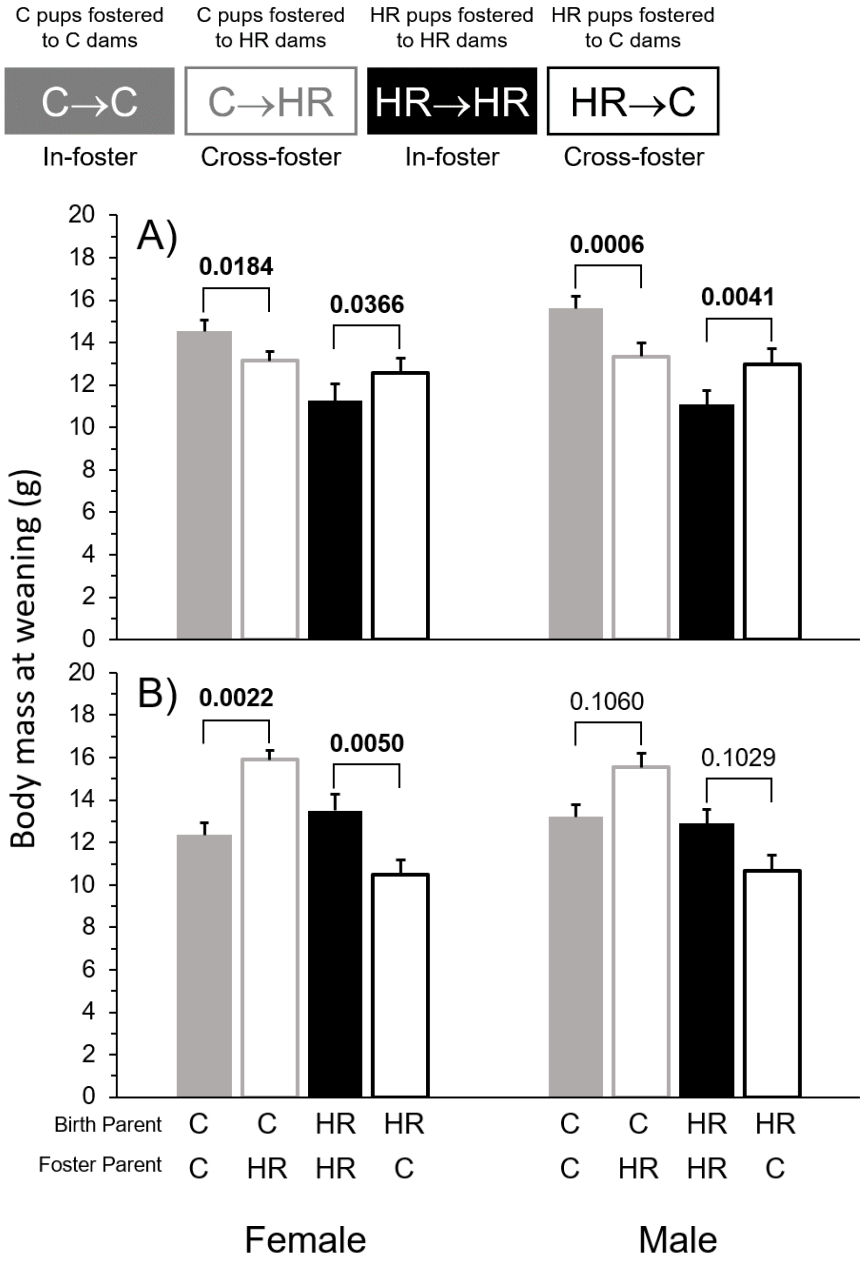

C pups fostered to C dams
C pups fostered to HR dams
HR pups fostered to HR dams
HR pups fostered to C dams
C→C
C→HR
HR→HR
HR→C
In-foster
Cross-foster
In-foster
Cross-foster
A)
B)
0.0184
0.0366
0.0006
0.0041
0.0022
0.0050
0.1060
0.1029
Body mass at weaning (g)
Birth Parent
Foster Parent
C C HR HR
C HR HR C
Female
Male

Figure 3.3.

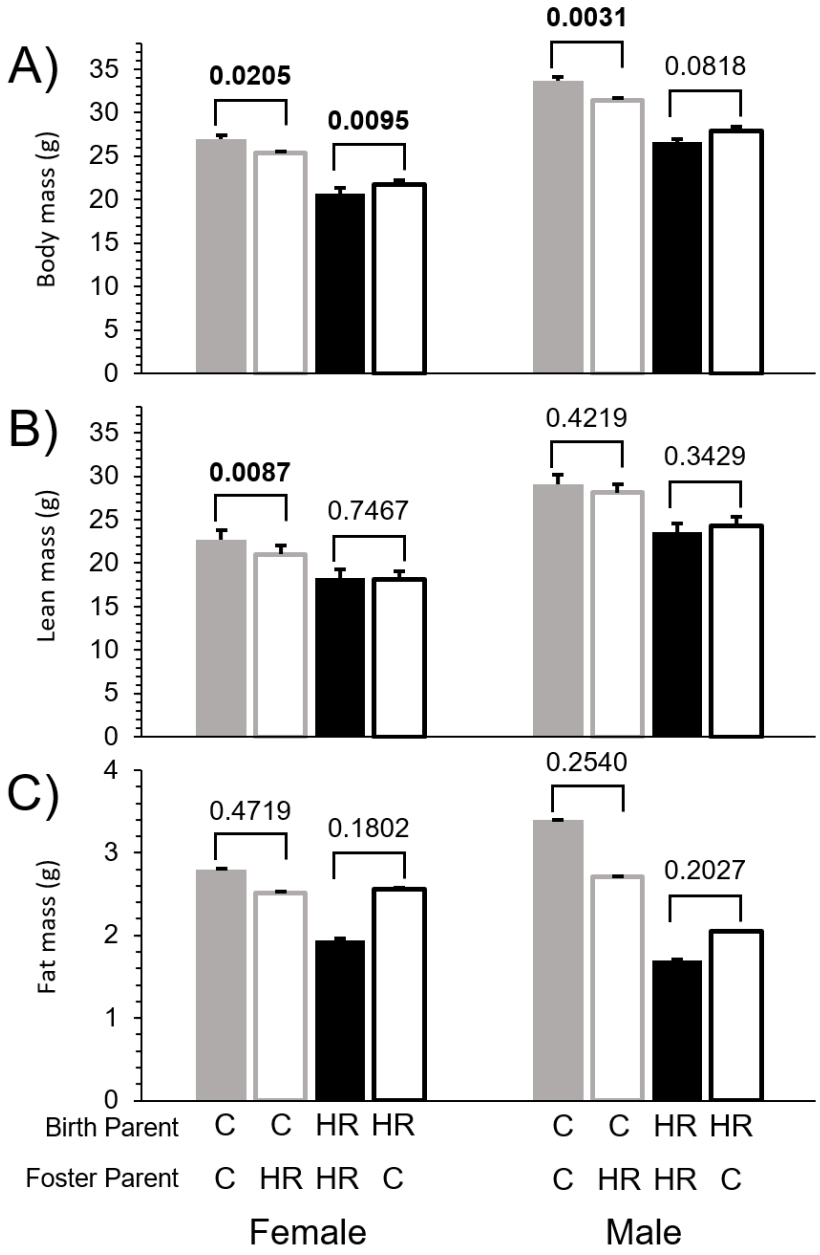

A)
Body mass (g)
0
5
10
15
20
25
30
35
0.0205
0.0095
0.0031
0.0818
B)
Lean mass (g)
0.0087
0.7467
0.4219
0.3429
C)
Fat mass (g)
0
1
2
3
4
0.4719
0.1802
0.2540
0.2027
Birth Parent C C HR HR C C HR HR
Foster Parent C HR HR C C HR HR C
Female
Male

Figure 3.4.

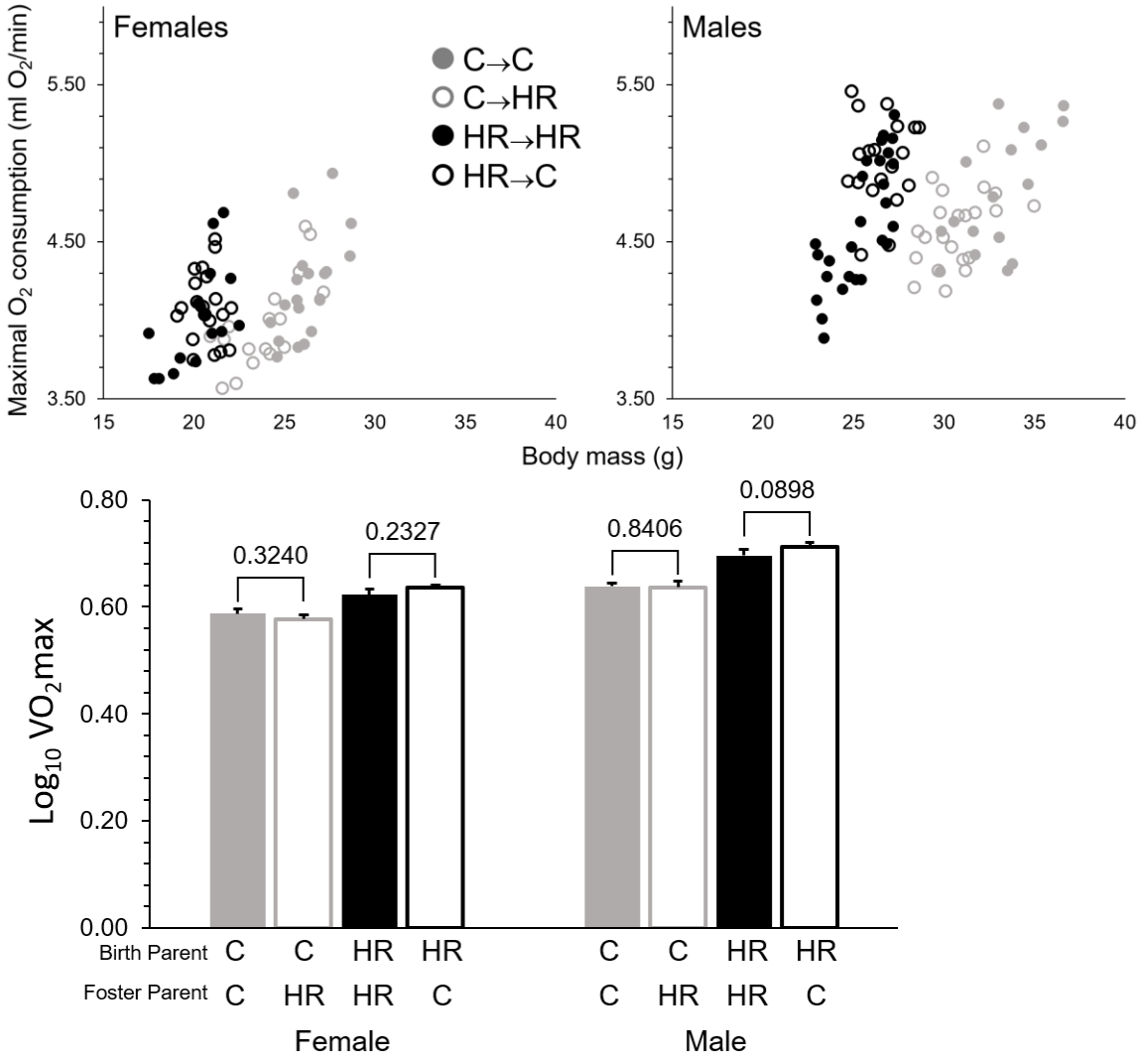

Females
Males
C→C
C→HR
HR→HR
HR→C
Maximal O2 consumption (ml O2/min)
5.50
4.50
3.50
15
20
25
30
35
40
Body mass (g)
0.80
0.60
0.40
0.20
0.00
Log10 VO2max
0.3240
0.2327
0.8406
0.0898
Birth Parent C C HR HR C C HR HR
Foster Parent C HR HR C C HR HR C
Female
Male

Figure 3.5.

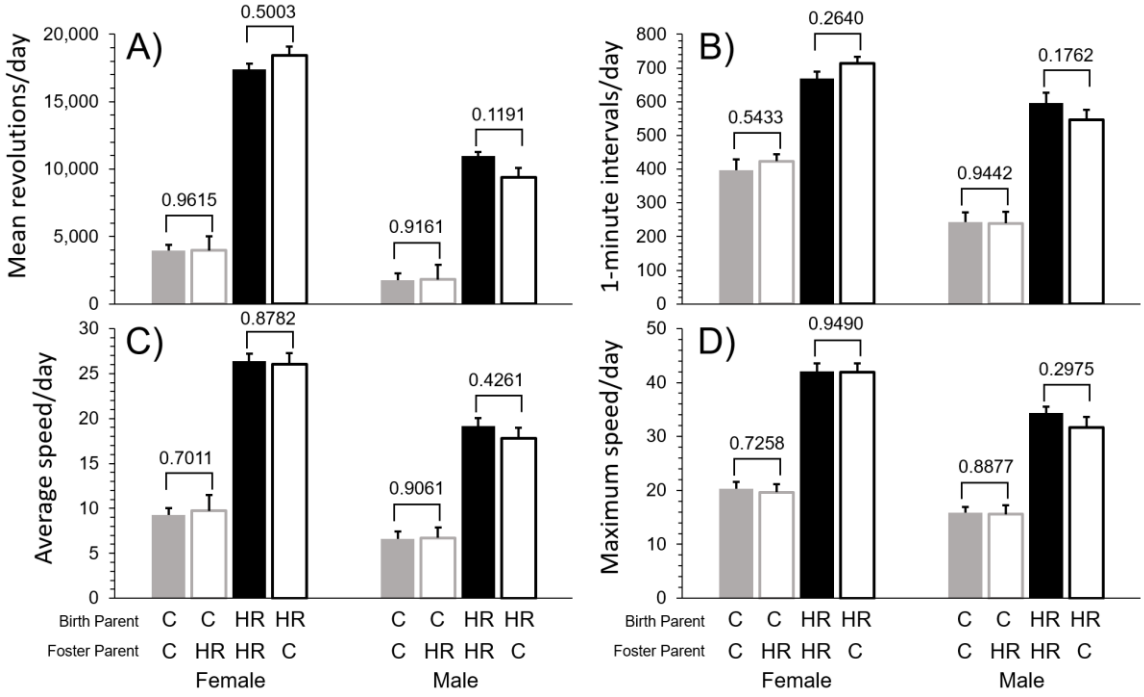
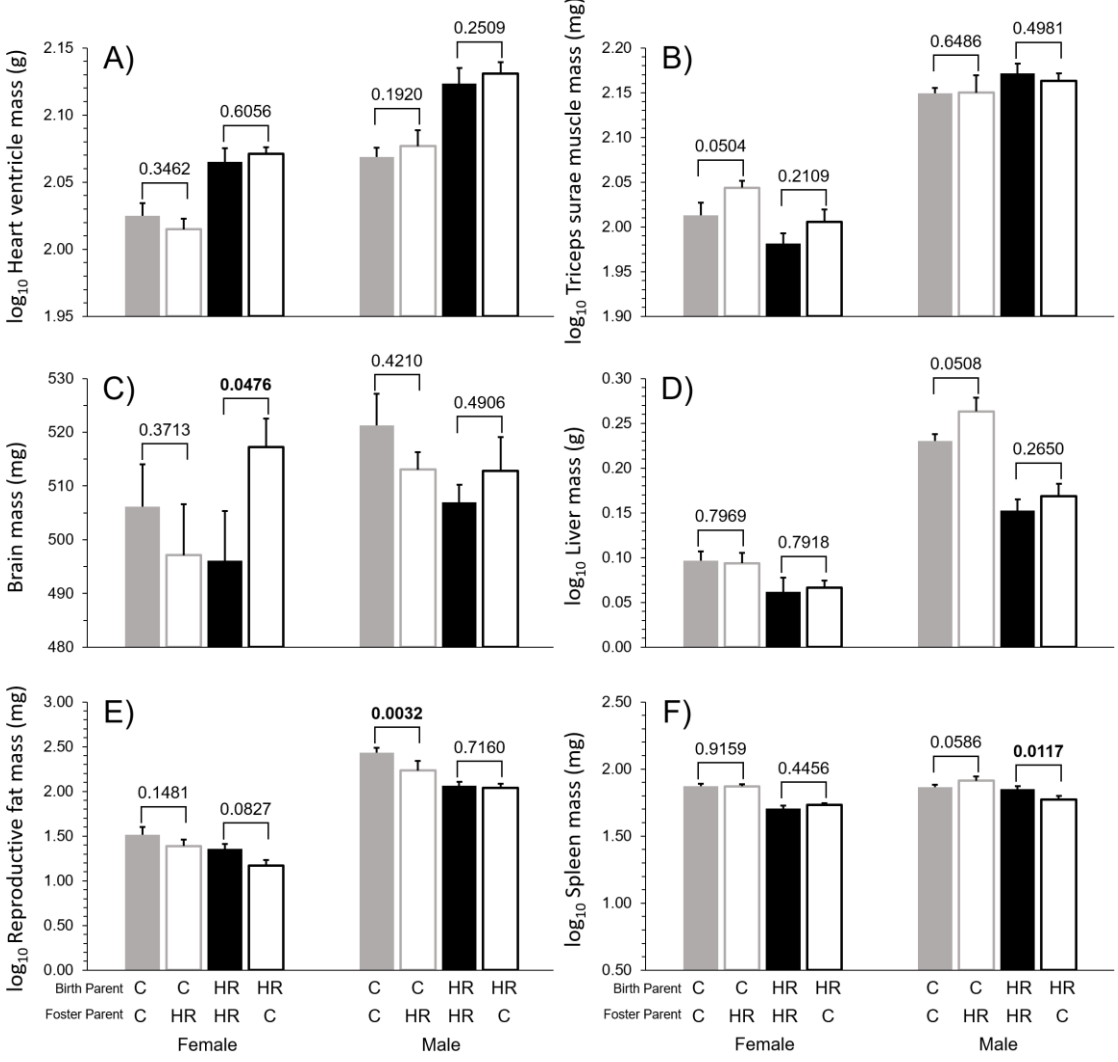

A)
Mean revolutions/day
20,000
15,000
10,000
5,000
0
0.9615
0.5003
0.9161
0.1191
B)
1-minute intervals/day
800
700
600
500
400
300
200
100
0
0.5433
0.2640
0.9442
0.1762
C)
Average speed/day
30
25
20
15
10
5
0
0.7011
0.8782
0.9061
0.4261
D)
Maximum speed/day
50
40
30
20
10
0
0.7258
0.9490
0.8877
0.2975
Birth Parent C C HR HR C C HR HR
Foster Parent C HR HR C C HR HR C
Female
Male

Figure 3.6.


A)
log10 Heart ventricle mass (g)
0.3462
0.6056
0.1920
0.2509
B)
log10 Triceps surae muscle mass (mg)
0.0504
0.2109
0.6486
0.4981
C)
Brain mass (mg)
0.3713
0.0476
0.4210
0.4906
D)
log10 Liver mass (g)
0.7969
0.7918
0.0508
0.2650
E)
log10 Reproductive fat mass (mg)
0.1481
0.0827
0.0032
0.7160
F)
log10 Spleen mass (mg)
0.9159
0.4456
0.0586
0.0117
Birth Parent C C HR HR C C HR HR
Foster Parent C HR HR C C HR HR C
Female
Male

Concluding Remarks

Regular physical exercise and diet are often prescribed as interventions for a host of diseases, mentioned throughout this dissertation. However, only 23% of adults aged 18 – 64 meet recommended physical activity in the U.S. (Blackwell and Clarke 2018) and 39% of adults in the same age group are overweight (WHO). To help us understand why we don't exercise as much as we need to, we used an artificial selection experiment model of mice selectively bred for increased wheel-running behavior. In this long-term experiment, four replicate High-Runner (HR) lines evolved to run 2.5 to 3× more revolutions on wheels per day on days 5 and 6 of a 6-day running test period (Swallow et al. 1998). Selection has continued since 1993 for more than 96 generations. Variation in physical activity is a function of both motivation and ability to exercise. Since a clearer understanding of *why* human populations don't get enough exercise must be related in part to both motivation and ability to exercise, the HR model is therefore an ideal system with which to study a genetically variable population. This dissertation attempted to test the overarching hypothesis that early-life experiences can have long-lasting effects on exercise behavior.

Chapter 1 demonstrates that adult traits, such as adult wheel running, home-cage activity, and anxiety-like behavior, can be affected by early-life exercise and dietary conditions across a moderate washout period (equivalent to

~6 human years). The majority of effects, however, were modulated by early-life access to exercise wheels, rather than feeding on a Western diet.

Chapter 2 followed with the substitution of fructose administered in the drinking water for Western diet administered as chow. The overall design was similar to the one used in Chapter 1, except only HR and one C line. Despite the many claims about possible adverse effects of heightened fructose consumption leading to human obesity in recent decades, early-life consumption of fructose did not have lasting effects on adult activity levels, body mass, or body composition across a lengthy washout period (equivalent to ~17 human years). However, fructose did have the expected obesogenic acute effects during juvenile treatment (similar to Western diet used in Chapter 1), suggesting moderation is warranted. Future studies could further isolate fructose-specific effects by comparison to a glucose diet in appropriate isocaloric supplementation (Rendeiro et al. 2015) and explore possible sex-specific effects (e.g., see grand-daughter effects of maternal Western diet in Fig. 9 of Hiramatsu et al. 2017)

Chapter 3 cross-fostered HR and C mice for the first time, showing that the elevated exercise levels of HR mice are largely independent of the early postnatal maternal environment. However, rearing by HR dams did increase the weaning body mass of C pups (an effect that persisted into early adulthood), and cross-fostering seemed to affect many different organ masses in various directions. Whether *in utero* maternal effects play an important role in "programming" adult activity levels remains to be seen. Moreover, an attempt to

reproduce these results using representatives from all eight lines should be made in the future.

I have made three major contributions in this dissertation. 1) Studies of early-life effects on locomotor activity (Garland, Jr. et al. 2017) are rare and no previous work has examined the adult impact of early exercise in a mammalian model after a substantial washout period. Chapters 1 and 2 both make clear that when Western diet, fructose, and/or exercise were administered in the early-life, exercise played a more important role than diet in "programming" many different adult traits, including physical activity, aerobic capacity, anxiety-like behavior, circulating hormone concentrations, and organ masses. 2) Early-life effects are known to depend on both the timing and nature of the genetic and environmental factors involved. We show that these factors often operate interactively, making it difficult to understand the impact of early experiences using single-factor designs (Meek et al. 2010; Acosta et al. 2015; Hiramatsu et al. 2017) that may operate in a relative vacuum. 3) The HR phenotype is no doubt genetically determined to a large extent, but other factors, such as indirect genetic effects mediated by the maternal environment, may also be important. For the first time, we have used a cross-foster experimental design (in Chapter 3) which showed that the early influence of the postnatal maternal environment was relatively unimportant in the development of the HR phenotype.

This dissertation opens many avenues for future studies. In particular, I am interested in what role epigenetic modifications play in mediating early-life

effects. Early-life diet and/or exercise may act as “triggers” for the developmental programming of epigenetic pathways related to many aspects of health, disease, and overall survival (Hanson and Gluckman 2008). Physical activity is known to modulate epigenetic mechanisms related to many human diseases, including several cancers, cardiovascular disease, type 2 diabetes, and neurodegenerative disease (Grazioli et al. 2017). Many of these effects are modulated by exercise-induced hypomethylation or hypermethylation of specific disease candidate genes, such as BDNF. For example, exercise-induced DNA methylation affects gene networks which are closely related to cancer suppression (Brown 2015). The Garland lab currently has hundreds of tissue samples resulting from this dissertation work, preserved at -80°C for future potential projects, possibly related to the epigenetic effects of acute, chronic, and/or early-life exercise.

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