Voluntary Aerobic Exercise Attenuates Frailty in a Sex-Specific Manner in Older Male and Female C57Bl/6 Mice

By

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Dalhousie University is located in Mi'kma'ki, the ancestral and unceded territory of the Mi'kmaq.

We are all treaty people.

Dedication page

This work is dedicated to my parents Tanya and Mark, my siblings Sharon and Keir, my fiancée Jeff, and the mice in this study

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Abstract

Aerobic exercise is thought to improve overall health and reduce frailty, but whether this is beneficial in both sexes is unclear. We introduced voluntary wheel running to older (~22 months) male and female mice to see if it reduced frailty. While frailty declined in both, mortality was not affected. Exercise did reduce signs of sarcopenia, where exercised females lost fat and gained lean tissue, while males lost total tissue weight; these effects were graded by activity. Exercise protected the heart against age-related declines in systolic and diastolic function, but only in male hearts. Exercise also prevented the age-related dysregulation of the immune system, measured as serum cytokines, but in only females. Aerobic exercise improved the overall health of mice even when introduced late in life, but the mechanisms involved are sex specific.

List of Abbreviations used

FI	Frailty index
DNA	Deoxyribonucleic acid
NF-κB	Nuclear factor kappa B
mTOR	Mammalian target of rapamycin
AMPK	AMP-activated protein kinase
IL	Interleukin
LV	Left ventricular
EF	Ejection fraction
IVCT	Isovolumetric contraction time
E/A	Early wave/Active wave
IVRT	Isovolumetric relaxation time
LVAW;d	Left ventricular anterior wall at diastole
FS	Fractional shortening
TNFα	Tumor necrosis factor alpha
TARC	Thymus and activation related chemokine
IFNγ	Interferon gamma
MCP-1	Monocyte chemoattractant protein 1
SD	Standard deviation
DEXA	Dual-energy X-ray absorptiometry
FRIGHT	Frailty inferred geriatric health timeline
AFRAID	Analysis of frailty and death
MV	Mitral valve
HR	Heart rate
D;s	Diameter at systole
D;d	Diameter at diastole
V;s	Volume at systole
V;d	Volume at diastole
LV mass	Left ventricular mass

LVAW;s	Left ventricular anterior wall at systole
LVPW;s	Left ventricular posterior wall at systole
LVPW;d	Left ventricular posterior wall at diastole
MV E	Mitral valve early wave
MV A	Mitral valve active wave
AET	Aortic ejection time
MPI	Myocardial performance index
km	Kilometers
hr	Hours
mm	Millimeters
m	Meter
g	Grams
N	Newtons
cm	Centimeters
ms	Milliseconds
°C	Degrees Celsius
M	Molar
μL	Microliter
MIP-1α	Macrophage inflammatory protein alpha
MIP-1β	Macrophage inflammatory protein beta
KC	Keratinocyte-derived chemokine
RANTES	Regulated on activation, normal T cell expressed and secreted
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating
SEM	Standard error of the mean

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Chapter 1: Introduction

1.1 General overview

As Canadian demographics shift to older ages, frailty has become a pressing concern. This demographic shift can be seen as the number of adults aged 65 and older will rise from approximately 17% to between 21 to 29% of the population in 2068 (Chagnon et al., 2019). This older population is at heightened risk of age-related health complications including frailty. Although there is no definition of frailty, it is thought of as an increased risk of negative health outcomes, including falls, hospitalization, and even death (Xue, 2011). While frailty can exist at any age, its prevalence increases with age, and it is generally higher in women (Pérez-Zepeda et al., 2021). This is problematic, both for the health of the individual and cost to the health care system (Mondor et al., 2019). It is imperative to explore the biology of frailty both to understand its underlying mechanisms and determine interventions to attenuate frailty.

The biology of frailty is complex, as multiple possible mechanisms have been implicated, including inflammation and sarcopenia (Bisset & Howlett, 2018). Any successful intervention would target one of more of these mechanisms. One proposed frailty intervention is aerobic exercise, which has a long list of beneficial effects on health. Clinical and preclinical studies have shown aerobic exercise can improve cardiovascular health, although there remains some disagreement surrounding the intensity of exercise in older, frailer adults (Nystoriak & Bhatnagar, 2018). It is also important to recognize that both human and mouse research studies of the effects of exercise in aging typically use male subjects (Costello et al., 2014; Graber et al., 2015;

Gomez-Cabrera et al., 2017; Garcia-Valles et al., 2013; Seldeen et al., 2018; Seldeen et al., 2019; Graber et al., 2019). This means that very little is known about how aerobic exercise will impact frailty and cardiovascular health in older females.

One of the mechanisms of frailty that links aging, exercise, and cardiovascular health is chronic inflammation. Serum markers of inflammation, known as cytokines, can be chronically reduced by aerobic exercise in older adults (Zheng et al., 2019), although the effects may differ between the sexes (Gillum et al., 2011) and exercise can even increase acute serum markers of inflammation in some cases (Suzuki, 2019). Chronic inflammation is also a mechanism involved in the development frailty (Bisset & Howlett, 2019) and in the pathogenesis of cardiovascular disease (Katsiari et al., 2019). However, the role of inflammation in the potentially beneficial effects of exercise on frailty for older mice of both sexes is unclear. Another mechanism of frailty is sarcopenia. Changes in body composition, increasing fat tissue and decreasing muscle, are markers of age-associated sarcopenia (Rosenberg, 1997) which could be impacted by aerobic exercise (Manzanares et al., 2019). Yet it is still unclear how body composition is altered when exercise is introduced late into life in preclinical models.

The overall goals of this thesis are to determine whether the introduction of voluntary aerobic exercise to older mice of both sexes can attenuate frailty and improve cardiovascular health and whether chronic inflammation and/or changes in body composition play a role in these effects of exercise in the setting of aging.

1.2 Frailty and aging

1.2.1 Frailty definition and assessment

Frailty is a relatively new concept that, to date, has no consensus definition. Generally, it is described as increased susceptibility to adverse health outcomes like falls, hospitalizations and even death (Xue, 2011). This concept has helped explain the heterogeneity of aging and why chronological age can be separate from biological age.

Along with the lack of a universal definition of frailty, there is no single method of measuring it. Two methods of quantifying frailty are the most common. The *frailty phenotype* approach views frailty as a syndrome caused by a depletion in energy reserves that can be quantified using measures of physical endurance and strength (Fried et al., 2001). *The frailty index* (FI) approach views frailty as an accumulation of sub-clinical deficits across a multitude of bodily systems (Mitnitski et al., 2001). Both the frailty index and the frailty phenotype were first developed in human populations and later translated to mice (Liu et al., 2014; Rockwood et al., 2017; Whitehead et al., 2014), then other rodents (Yorke et al., 2017), non-human primates (Yamada et al., 2018) and dogs (Bartling et al., 2019; Hua et al., 2016). While both tools can predict mortality (Shi et al., 2019), this thesis will use the FI tool for the assessment of mouse frailty.

Both methods of quantifying frailty necessarily measure frailty in slightly different ways. The human frailty phenotype measures five signs of physical frailty; loss of body weight, loss of grip strength, self-reported exhaustion, reduced walking speed and reduced weekly activity (Fried et al., 2001). Baseline levels are used to create quintiles, where scores counted as frail fall in the lowest quintiles (e.g. grip strength is

scored as a deficit if it falls into the lowest 20% of baseline grip strength) (Fried et al., 2001). A person would be considered "frail" if they had three or more of the physical signs and would be considered "pre-frail" if they had one or two of the signs of physical frailty (Fried et al., 2001). Those with no signs are considered "robust". During the translation to mouse models, some signs of physical frailty were modified from the human version of the frailty phenotype. In mice, five signs were modified to four: grip strength, walking speed (on a rotarod), physical activity (voluntary wheel running) and endurance (combined rotarod and wheel running) (Liu et al., 2014). Similar to the human frailty phenotype, a mouse requires three or more signs of physical frailty to be considered frail (Liu et al., 2014).

Another common measure, the FI, measures a more diverse set of bodily systems in addition to physical frailty. Initially the index consisted of 92 items across multiple systems (e.g. mood complaints, tremor, and blood creatinine levels) and they were scored in a binary yes/no (0 or 1) fashion (Mitnitski et al., 2001). The sum of the deficits present was then divided by the total number measured to give a ratio between 0 and 1 (Mitnitski et al., 2001). During the translation to mice, the number of measured deficits was reduced to 31 non-invasive items and the scoring system was altered to 0, 0.5 and 1 (0 is absent, 0.5 is mild and 1 is severe for each deficit) (Whitehead et al., 2014). Both the humans and mouse index share several common traits. For example, both FI tools predict mortality (Rockwood et al., 2017). There is an upper limit to frailty (e.g. 0.54 in humans and 0.44 in mice), and both humans and mice show gradual increase in frailty over time (Rockwood et al., 2017). The deficit model has been more recently used to generate the frailty inferred geriatric health timeline (FRIGHT) and analysis of frailty and death

(AFRAID), frailty clocks that predict biological age and survival respectively (Schultz et al., 2020).

While frailty and aging are not the same, they are interconnected. Though frailty can occur at any age, the frequency and severity increases with chronological age (Hanlon et al., 2018). Interestingly, there is also a sex difference in frailty. Women tend to have higher levels of frailty, yet they also tend to live longer, a phenomenon known as the "morbidity-mortality paradox" (Gordon et al., 2017). Interestingly, this frailty-sex difference is found in other mammals as well (Baumann et al., 2019) suggesting that the unknown underlying cause may have a physiological basis. However, whether the same morbidity-mortality paradox exists in rodent models is less well known. This sex difference in frailty highlights the importance of using both male and female subjects for frailty research, as effects on one sex can not be inferred from effects on the other.

1.2.2 Mechanisms of frailty

As frailty is closely associated with age, they are thought to share many of the same mechanisms (Bisset & Howlett, 2019). The mechanisms of frailty are thought to be similar to the nine "hallmarks of aging" (López-Otín et al., 2013) which are: altered intercellular communication, stem cell exhaustion, cellular senescence, mitochondrial dysfunction, degraded nutrient sensing, loss of proteostasis, epigenetic alterations, telomere attrition, and genomic instability. Others have proposed the seven "pillars of aging" (Figure 1.2.1) which are: loss of proteostasis, decline in metabolism, inflammation, Deoxyribonucleic acid (DNA) damage, hormone dysregulation, epigenetics, senescence, and stem cell exhaustion (Kennedy et al., 2014). It is believed

that these underlying mechanisms can then be compounded by environmental stressors like smoking (Hubbard et al., 2009) or sedentary behavior (del Pozo-Cruz et al., 2017) to accelerate biological age and frailty. As such, any intervention focused on reducing or attenuating frailty would be expected to involve one or more of these mechanisms.

1.2.3 Interventions for frailty

As frailty is a relatively new concept, there are only a handful of studies that focus on frailty interventions in a preclinical setting. Generally, these interventions fall into two categories: pharmaceutical therapies or lifestyle changes.

At present, potential pharmaceutical therapies for frailty are all repurposed medications. This includes the anti-hypertensive drug enalapril, an angiotensin converting enzyme inhibitor that reduces frailty in mice of both sexes (Keller et al., 2019). Enalapril is believed to impact frailty by altering chronic inflammation (Keller et al., 2019). Enalapril acts through the angiotensin type 1 receptor, which involves the downstream nuclear factor-kappa beta (NF-κB), a transcription factor (Dandona et al., 2007). This transcription factor regulates multiple cytokines and other immune responses. Therefore, enalapril helps both reduce pro-inflammatory cytokines and frailty in mouse models, although effects are sex-specific. Enalapril also increases anti-inflammatory cytokines in males (Keller et al., 2019).

Another possible therapy is the drug rapamycin, an antifungal metabolite with many complex mechanisms of action (Blagosklonny, 2019). It shows promise as an anti-aging therapy because it extends the lifespan of both *Drosophila* (Kapahi et al., 2004) and mice (Fok et al., 2014). While the biological impact of rapamycin is still being

investigated, of importance for frailty is its ability to prevent the formation and proliferation of senescent cells (e.g. cells that are non-replicative, accumulate with age, and release pro-inflammatory cytokines). Rapamycin inhibits the mammalian target of rapamycin (mTOR), through an allosteric binding site on mTOR complex 1 (Li et al., 2014). This inhibition reduces the number of senescent cells that can form and is thought to attenuate aging (Pospelova et al., 2012; Iglesias-Bartolome et al., 2012).

Another pharmaceutical therapy of interest is metformin. Metformin is a current therapy for type 2 diabetes, but it can also reduce the proliferation and formation of senescent cells. Metformin can interact with AMP kinase (AMPK), which inhibits the mTOR complex 1 pathway (Zhan et al., 2018). Metformin can also suppress the signal transducer and activator of transcription 3, which is involved in a senescent cell's release of pro-inflammatory cytokines (Hu et al., 2021). In addition to being anti-senescent, metformin's principal mechanism is increasing insulin sensitivity though the Glucose transporter type 4 transporter activity. This increases glycogen synthesis and insulin receptor tyrosine kinase activity (Giannarelli et al., 2003). As altered metabolism is another mechanism of frailty, improved insulin sensitivity could provide a biological avenue for treatment (Espinoza et al., 2019). This intervention has reduced frailty in older adults (Baskaran et al., 2020) and has improved healthspan in mice (Martin-Montalvo et al., 2013), although frailty has not yet been measured in these latter models. The field of pharmaceuticals as tools for the prevention or treatment of frailty is still very young, with many new potential areas for research.

Lifestyle changes are another avenue for the treatment or prevention of frailty. With lower costs than pharmaceuticals, they are relatively simple to deploy either alone

or in combination with drug treatments in mouse models. One option is caloric restriction. Caloric restriction without malnutrition was first published as a method to extend lifespan in 1935 using rats (McCay et al., 1935) and has since been replicated in mice (Weindruch & Walford, 1982) and non-human primates (Colman et al., 2009). Interestingly, one of the proposed reasons that reduction of calories can extend lifespan is epigenetic. Caloric restriction delays the age-related DNA methylation drift (Maegawa et al., 2017), a proposed cause of both aging and frailty. Indeed, caloric restriction has been shown to attenuate frailty in a mouse model (Kane et al., 2016). Interestingly, while all the previous studies reduce the total number of calories consumed, similar results can be seen by using other restrictive diets. One of these diets is intermittent fasting, where nutrients are only eaten in between periods of fasting often of 12 hrs or more (Anton et al., 2018). Intermittent fasting is believed to alter lifespan through multiple mechanisms such as reducing inflammation and switching metabolism to burn free fatty acids over glucose (Anton et al., 2018). In preclinical trials, intermittent fasting has been shown to reduce frailty in older mice (Henderson et al., 2021).

Another restrictive diet option focuses on the type of calories consumed. For example, female mice fed a diet that restricted the amount of methionine eaten, lived longer than their control counterparts (Miller et al., 2005). This was later tested in both sexes with a diet that restricted the amount of branched chain amino acids (Richardson et al., 2021). It was found that while lifelong restriction of branched chain amino acids did extend lifespan and attenuate frailty in male mice, it had no impact in females (Richardson et al., 2021). Another possible intervention is aerobic exercise, which has been shown to improve physical frailty in older male mice (Garber et al., 2015). While

exercise does show promising results for physical frailty in male animals, whether it reduces other aspects of frailty has not yet been investigated and whether it can benefit both sexes is unclear. The next section will outline aerobic exercise programs in mice and highlight what is known about exercise and frailty in mouse models.

1.2 Aerobic exercise

1.3.1 Aerobic exercise definition

Aerobic exercise is a form of physical activity that improves the capacity of the cardiovascular system to both take up and transport oxygen (Abrams et al., 2013). Even moderate intensity aerobic exercise can reduce all-cause mortality and the incidence of cardiovascular disease in clinical studies (Stofan et al., 1998). While in humans this type of exercise can take many forms, in mice aerobic exercise is performed by either swimming, treadmill running or wheel running. The following discussion will focus on wheel running, which is the intervention used in this thesis.

1.3.2 Voluntary wheel running

Voluntary wheel running is a useful way to deploy aerobic exercise in mice due to the ease of use, low cost, and low maintenance. In addition, voluntary wheel running is not as stressful as treadmill running (Svensson et al., 2016) or forced swimming (Reardon, 2019). The typical running wheel is a stainless-steel wheel in a cage that generally houses a single mouse. While voluntary wheel running does not allow for a structured exercise program such as with a mouse treadmill, it does allow observation of more natural running patterns (Smith et al., 2015). It also allows the investigation of

factors that influence voluntary activity. For example, there is evidence that activity levels are impacted by mouse strain (Lerman et al., 2002), sex (Manzanares et al., 2019; Rosenfeld, 2017), and age (Manzanares et al., 2019; Vaanholt et al., 2008). In terms of sex, younger female mice tend to run more than their male counterparts (Manzanares et al., 2019; Rosenfeld, 2017), although this difference tends to decline with age (McMullan et al., 2016). Overall activity levels also decline with age in mice of both sexes. A young mouse can run up to 10 kilometers (km) per day (Manzanares et al., 2019), while an older mouse (e.g. 29 months old) will typically run less than 0.1 km per day (Garcia-Valles et al., 2013). Mouse strain also affects running levels. C57Bl/6 mice are one of the more active strains on a voluntary running wheel (Lerman et al., 2002). Their naturally higher running levels and well-studied genome makes them a useful murine exercise model (Didion & de Villena, 2013).

The running wheel also can illustrate how mice run. Mice tend to run in short bursts or bouts, rather than in long stretches (Goh & Ladiges, 2015). This preference is shared between research animals and wild mice (Meijer & Robbers, 2014). Interestingly, there are also external factors that affect activity. One powerful external influence is time of day. The C57Bl/6 strain used in this study is naturally nocturnal, similar to most mouse strains (Valentinuzzi et al., 1997). Therefore, they do the vast majority of their running at night, in the dark cycle (Valentinuzzi et al., 1997). Curiously, the circadian rhythm of mice is affected by age, and this can be seen in their running schedules (Valentinuzzi et al., 1997). For example, Valentinuzzi and colleagues showed that while adult male mice started running shortly into the dark cycle with little variability, older males started running much later into the dark cycle and with much more variability

(Valentinuzzi et al., 1997). There are also hints that frailty can affect circadian rhythms (Walston et al., 2008), as the interleukin-10 knockout (IL-10KO) mouse, a model of frailty, had increased gene expression of casein kinase 1, which is involved in circadian rhythms (Walston et al., 2008). This study however is exploratory and only involved adult female mice (Walston et al., 2008). While mice tend to run during dark periods, the effects of age, frailty, and sex on running are not well known. A voluntary wheel running intervention can help elucidate the complex relationship between activity levels, sex, and frailty that may occur in older mice.

1.3.3 Frailty and aerobic exercise in mice

There are very few studies which examine the impact of aerobic exercise on frailty in older mice. While they did not study frailty directly, Garcia-Valles and colleagues used a lifelong wheel running exercise program (from 3 months to 20-29 months of age) in male mice (Garcia-Valles et al., 2013). Results showed that running reduced sarcopenia but did not impact lifespan (Garcia-Valles et al., 2013). Another lifelong running program (from 3 months until 28 months of age) used a modified physical frailty index, the "Valencia score" (Gomez-Cabrera et al., 2017). Scores were calculated from a series of physical tests, scored as pass or fail, where the number of failed tests was divided by the total number of tests administered. Voluntary wheel running resulted in lower Valencia scores in the exercised group starting at 17 months of age to the end of the study (Gomez-Cabrera et al., 2017). While both previous studies used lifelong aerobic interventions to prevent frailty, other studies have focused on introducing exercise in late life. One such study used only male mice, aged 28 months, and subjected them to a 4-month exercise intervention (Graber et al., 2015). They showed

that aerobic exercise reversed some signs of physical frailty in older male mice (Graber et al., 2015). Another study used 20-month-old male mice and a 4-month exercise intervention (Schafer et al., 2016). Exercise reduced body fat and improved signs of physical function, although frailty itself was not measured (Schafer et al., 2016). Together, these studies suggest that exercise may benefit frailty, at least in older male mice, although few studies have actually measured frailty.

1.3.4 Aerobic exercise and the effect of sex

Mouse activity levels are influenced by sex. This is most clearly shown based on sex differences in running distances. Young female mice tend to run further than their male counterparts, but with higher daily variability (Bartling et al., 2017; Konhilas et al., 2004; De Bono et al., 2006; Lightfoot et al., 2004). Of note, Bartling and colleagues found that female mice ran greater distances due to higher running speeds, not longer durations (Bartling et al., 2017). They also discovered that sex differences in running decrease with age, such that there is no difference in running parameters between adult (9 month and older) male and female mice (Bartling et al., 2017). One proposed mechanism is hormonal changes with age. This was investigated by Ogawa and colleagues who studied estradiol supplementation in genetically modified C57Bl/6J/129 mice with and without estrogen receptors alpha or beta (Ogawa et al., 2003). They found that estradiol supplementation increased daily wheel running in both female and male mice in all groups except those without estrogen receptor alpha (Ogawa et al., 2003). Another study used ovariectomized C57Bl/6J female mice in the absence or presence of estrogen and progesterone supplementation (Cabelka et al., 2019). Ovariectomy reduced running distances by approximately 58%, which was partially restored by estradiol and

progesterone supplementation, by estradiol alone but not by progesterone alone (Cabelka et al., 2019). This suggests that estrogen may contribute to longer running distances in young female mice although the mechanisms involved are not clear. This may also explain why there are fewer differences in running distance between older male and female mice, as estradiol naturally decreases with age (Yan et al., 2017; Ghimire et al., 2018).

To conclude voluntary activity in mice is influenced by both age and sex. It is possible that sex differences in running distances are impacted by sex hormones. Interestingly, while there are promising signs that voluntary wheel running attenuates physical frailty in older male mice, there is less evidence in female mice and few studies have investigated other aspects of frailty. One of these aspects is changes in body composition, or sarcopenia, which is discussed below.

1.3 Body composition

1.4.1 Effect of age and frailty on body composition.

Aging is characterized by a loss of muscle mass and an increase in fat mass mostly around the abdominal area (Bisset & Howlett, 2019). This age-related loss of muscle is known as sarcopenia (Rosenberg, 1997), and is a distinct process compared to either atrophy (muscle loss due to disuse) or cachexia (muscle wasting caused by disease). Sarcopenia involves muscle loss in terms of reduced muscle fiber size, a decrease in the number of fibers, and a shift in muscle fiber type (Romanick et al., 2013). Sarcopenia has been intrinsically linked to frailty (Bisset & Howlett, 2019; Cesari et al., 2014). For example, sarcopenia causes a loss of muscle function that can be measured in

tests like grip strength or walking speed, which are components of the FI. As with frailty, there is no single cause of sarcopenia, and many of the mechanisms involved are shared across the two conditions.

One contributor to sarcopenia is an age-related loss of appetite, known as the anorexia of aging (Cruz-Jentoft et al., 2017). As energy intake declines, the body will begin to catabolize itself leading to weight loss and sarcopenia (Cruz-Jentoft et al., 2017). In humans this has been linked to loss of taste, slower gastric emptying, and difficulties with chewing or swallowing (Cruz-Jentoft et al., 2017). In mice, this phenomenon is less well understood. In addition, older mice tend to spill food in the cage making it possible to underrepresent actual food intake (Starr & Saito, 2012). While food consumption in relation to aging is relatively unknown in mice, the overall decline in body weight with age has been well described across many strains and in both sexes (Turturro et al., 1999). This decline in weight comes near the end of a mouse's lifespan, generally starting around 2 years of age (Pappas & Nagy, 2019). Fat mass in mice peaks around the 2 year mark while lean mass plateaus around 21 weeks, then both decline near the end of life resulting in a decline in body weight (Pappas & Nagy, 2019). Interestingly, organ weights exhibit the opposite trend. The relative weights of liver, lung, heart, and kidneys all increase with age (Marino, 2012). The spleen can also increase in size, but this is variable (Marino, 2012). As mice age, they can have lower food consumption, lower body weights and higher organ weights, all signs that mice can exhibit sarcopenia. Therefore, an intervention that improves body condition could have a positive effect on the signs of sarcopenia in older mice of both sexes. This in turn could help to reduce frailty in the setting of aging.

1.4.2 Effect of exercise on body composition

In young mice overall weight is not greatly affected by aerobic exercise (Manzanares et al., 2019) although changes in body composition seem to be more pronounced in male mice and with long-term training protocols (Manzanares et al., 2019). In a study where young male mice completed an aerobic exercise training program, weight loss was due to changes in fat tissue and not lean tissue (Kim et al., 2020). Interestingly, their food intake also increased during the intervention (Kim et al., 2020). It is important to recognize however that changes in overall lean mass are not always a good indication of muscle size or function. While male mice that ran for 8 weeks showed no change in lean mass, they did have larger gastrocnemius and extensor digitorum longus muscles as well as improved forelimb grip strength (Kim et al. 2020). Thus, young mice show only moderate changes in overall body composition with exercise, but this may mask changes in individual muscles.

The effects of aerobic exercise on the body composition of aged mice are sex-dependent. For example, McMullan and colleagues used middle-aged male and female mice for a one-year wheel running intervention (McMullan et al., 2016). While exercised females had lower levels of fat than males at baseline, this trend reversed halfway through the intervention. Compared to sedentary controls, the exercised mice of both sexes had less fat at the end of the intervention (McMullan et al., 2016). However, this study used mice aged between 1-2 years and, as mentioned in section 1.4.1, many of the age-related changes in body composition occur after 2 years of age. As mentioned above, some organs such as specific skeletal muscles are impacted by aerobic exercise and

aging. The next section will highlight changes in heart structure and function as a consequence of exercise in the setting of aging.

1.4 Cardiac structure and function

1.5.1 Cardiac health

As exercise and age can both impact signs of cardiac health, we will first discuss measures of heart structure and function. Firstly, cardiac function can be evaluated as both systolic and diastolic function. Measures of left ventricular (LV) systolic function quantifies the heart's ability to contract. An example of such a value would be the LV ejection fraction (EF), a global value describing the percentage of blood that is pumped out of the heart each beat. A healthy young mouse should have an EF of between 55-65% (Vinhas et al., 2013). Another measure linked to systolic function is isovolumetric contraction time (IVCT) (Biering-Sørensen et al., 2015), the time period between the mitral valve closing and the atrial valve opening, during which the pressure within the LV increases. A young wildtype mouse (C57Bl/6 background) has an IVCT of approximately 12 milliseconds (ms) (Reddy et al., 2007). On the other hand, diastolic function is the heart's ability to relax and refill with blood. It is possible to measure diastolic function by analyzing the blood flow through the mitral valve, an opening between the left atrium and LV. An example of a value derived from blood flow is the E/A ratio, from the E (early diastole) and A (late diastole, due to atrial contraction) wave velocities. The E/A ratio has a U-shaped relationship with diastolic dysfunction (Mottram, 2005), where having a ratio much higher than 1 or lower than 1 is a sign of diastolic dysfunction. Small E/A ratios are a sign of restrictive LV relaxation whereas

high E/A ratios are a sign of restrictive filling (Gao et al., 2011). Another measurement of diastolic function is the isovolumetric relaxation time (IVRT), the time period between the atria closing, and the mitral valve opening, where pressure increases within the left ventricle although the volume inside the heart remains the same. In young mice, the IVRT should be approximately 17 ms (Gao et al., 2011).

Cardiac structure combines both the dimensions of the heart along with the tissue composition of the heart. Firstly, the heart's total mass is an important measurement, often normalized to the body weight or tibia length (Yin et al., 1982). The individual chamber dimensions can also be measured. For example, the left ventricular anterior wall thickness at diastole (LVAW;d) in adult healthy mice is approximately 0.7 millimeters (mm) (Vinhas et al., 2013). The tissue composition of the heart is also a sign of cardiac health, which can relate to the stiffness of the ventricular walls. One of the principal components of stiff ventricular tissue is collagen deposition (Wynn, 2008). Collagen is in a constant balance between synthesis and degradation, where procollagen strands bind together in a triple helical structure. This helical structure is stabilized by a modified amino acid, a hydrolyzed proline or hydroxyproline (de Jong et al., 2012). Hydroxyproline is specific for collagen so can reflect the amount of collagen present, and the level of fibrosis (de Jong et al., 2012). Hearts with high levels of fibrosis are stiff, and cannot relax properly, indicative of diastolic dysfunction. Quantification of cardiac health are not limited to these methods, but they serve as a valuable starting point to assess the impact of age and aerobic exercise on the heart.

1.5.2 Aerobic exercise and cardiovascular health

As mentioned in section 1.3.1, aerobic exercise improves cardiovascular function. Interestingly, aerobic capacity is also a marker of cardiovascular health. With age-related deterioration or other pathologies, the heart has impaired cardiac reserve. This reserve refers to the heart's ability to properly increase cardiac output to meet the demands of exercise, resulting in inadequate oxygen delivery to the muscles and reduced exercise capacity (Roh et al., 2016). In male mice, maximal oxygen consumption declines by 28% between the ages of 12 to 24 months (Roh et al., 2016; Schefer & Talan, 1996). This helps show that, while exercise can affect the heart, so can the heart affect exercise.

Before focusing on how exercise can affect the heart in the context of aging, we will first briefly explain cardiac adaptations to aerobic exercise in young mice. One of the most obvious structural adaptations is physiological hypertrophy, where the heart increases in size. This was demonstrated in a study where adult male mice (8-10 weeks old) ran for 4 weeks on a voluntary running wheel (Allen et al., 2001). They showed an increase in heart mass relative to body mass and an increase in cardiac messenger ribonucleic acid markers for hypertrophy (atrial natriuretic factor and brain natriuretic peptide) (Allen et al., 2001). Curiously, while both aerobic and anaerobic training can produce cardiac hypertrophy, the type of the hypertrophy depends on the mode of exercise (Figure 1.5.1). Aerobic training tends to produce eccentric hypertrophy, where new sarcomeres are added in series which increases the chamber volume without impacting the wall thickness (Platt et al., 2015). Anaerobic exercise, on the other hand, produces concentric hypertrophy where the sarcomeres are added in parallel which increases LV wall thickness. The expected result from aerobic voluntary wheel running

would therefore be an enlarged heart without significant changes in wall thickness. This type of eccentric hypertrophy occurred when male mice (6 weeks old) are given access to a running wheel for 6 weeks. They show increased heart size and chamber dilation (increased left ventricular diastolic diameter) but no significant changes in LV wall thickness (Lakin et al., 2018). Many of these effects also show sex-specific differences. Firstly, when both male and female mice had access to a voluntary wheel for 3 weeks, the females showed a marked increase in heart weight, even when accounting for sex differences in distance run (Konhilas et al., 2004). This is important, as all the previous mouse exercise studies used only male mice. Exercise produces physiological adaptations in the heart, although whether these changes occur in older preclinical models of both sexes is unclear.

1.5.3 Effects of age and frailty on cardiac structure and function

Aging causes detrimental cardiac remodeling in mouse hearts, regardless of disease status, though some of these changes are sex specific. With age, there is atrial dilation, an increase in left ventricular wall thickness but decrease in left ventricular chamber size, an increase in fibrotic tissue, loss of myocytes, and increase in epicardial fat (Keller & Howlett, 2016). Though the decrease in LV chamber size is a shared adaptation with physiological concentric hypertrophy, age-related alterations are signs of pathologic hypertrophy. Both exercise-related eccentric and concentric hypertrophy accompany improved cardiac function while age-related pathologic hypertrophy is associated with a decline in heart function. Pathologic hypertrophy can involve both reduced chamber size and thicker walls (concentric hypertrophy), or larger chamber size and thinner walls (eccentric hypertrophy). Age-associated dilated cardiomyopathy is

characterized by dilated chambers and systolic dysfunction (Li et al., 2015). While LV chamber dilation is a similar structural adaptation to eccentric hypertrophy, the pathological changes, unlike those promoted by exercise, are accompanied by a decline in cardiac function. While age can increase the incidence of cardiac remodeling, so can frailty. For example, age-related cardiac hypertrophy is correlated with frailty levels but not age in male mice (Feridooni et al., 2017). These structural changes accompany detrimental functional changes in the heart, as discussed below.

The age-related changes in the heart outlined above generally accompany signs of reduced cardiac function. There is emerging evidence that systolic function moderately declines with age and frailty in mice, but this effect is sex dependent. For example, when mice of both sexes are combined, there is an overall decline in fractional shortening (FS) of 12% from young to older adult ages (Dai et al., 2009). However, this decline occurs mostly in male mice and not in their female counterparts (Keller & Howlett, 2016). Interestingly, measures of diastolic dysfunction tend to show a more significant age-related changes in mice of both sexes and are accompanied by signs of ventricular fibrosis (Dai et al., 2009). As mentioned previously, the IVRT is a parameter of diastolic function. In human populations, prolonged IVRT is correlated with major adverse cardiovascular outcomes (Biering-Sørensen et al., 2015). Increased IVRT has also been linked to frailty in the frail interleukin-10 knockout mouse model, where older animals had significantly higher IVRTs than their wildtype counterparts (Sikka et al., 2013). Curiously, this study combined male and female mice, so did not study sex-differences (Sikka et al., 2013). Combined, this demonstrates that age-related changes in cardiac function include both systolic and diastolic dysfunction and signs of pathologic

hypertrophy. Systolic parameters have shown sex-dependent differences while diastolic function is less well studied. This highlights the importance of using both sexes when investigating the changes in cardiac structure and function in older mice.

1.5.4 Aerobic exercise and aging in remodeling

Interestingly, the remodeling expected from aerobic exercise and from frailty share some similarities. In both cases there is increase in cardiac hypertrophy where both can result in chamber dilation, though one is pathologic, and the other is physiological. Pathological changes accompany declines in cardiac function, while physiological changes can result in enhanced cardiac function. With respect to hypertrophy, there are large variations in rodent responses to aerobic exercise. In old male mice (22 months of age) subjected to a treadmill training program, there were no significant changes in left ventricular mass after 6 weeks of running (Shanmugam et al., 2017). Similarly, while older male mice trained on a treadmill for 12 months had an increase in heart weight relative to young adult controls, they were not different from heart weights of older sedentary mice (Walton et al., 2016). However, when Walton and colleagues looked at myocyte size, they noted that while cell width increased with age, cells were wider in older exercised males compared to sedentary mice (Walton et al., 2016). Recall that though physiologic hypertrophy and pathologic hypertrophy can both cause chamber dilation, pathologic hypertrophy is characterized by fibrotic tissue build up and reduced cardiac function. Whether aerobic exercise can attenuate age-related deterioration (e.g. pathologic hypertrophy, dysfunction and increased fibrosis) in preclinical models is still unclear (Roh et al., 2016). Another note of importance is that neither the work of Walton

and colleagues (2016) or Shanmugam and colleagues (2017) investigated sex-related differences in cardiac adaptations to aerobic exercise as both used only male mice.

To conclude, cardiac health is impacted by aerobic exercise and age in similar fashions from a simple structural standpoint. For example, they both can result in cardiac hypertrophy, but the cellular alterations, and functional repercussions differ between the two. While physiological hypertrophy involves an increased in myocyte volume, pathological hypertrophy involves an increase in fibrosis and reduced cardiac function. However, few studies have investigated this and information on the effects of aerobic exercise on the heart in aging females is an important knowledge gap.

1.5 Chronic inflammation

1.6.1 Link between age and chronic inflammation

One of the principal mechanisms of both aging and frailty is an increase in chronic inflammation. This phenomenon, named "inflammaging" (Franceschi et al., 2006), occurs when individuals experience chronic inflammation with age, often trigged by the innate immune system. This can be measured by examining the balance between pro and anti-inflammatory cytokines. Cytokine is a general term for a secreted protein (Zhang & An, 2007). Cytokines can be classified based on their physiological role (e.g. pro- vs anti-inflammatory) or their origin (e.g. an interleukin is a cytokine produced by a leukocyte to act on another leukocyte) (Zhang & An, 2007). Pro-inflammatory cytokines include: chemokines, interleukin (IL)-6, IL-1β, and tumor necrosis factor alpha (TNFα) among others while anti-inflammatory cytokines include: IL-4, IL-10, and IL-13 (Zhang & An, 2007). With age, pro-inflammatory cytokine levels increase while anti-

inflammatory levels decrease, leading to chronic inflammation (Franceschi et al., 2006). This effect has been demonstrated in male mice, where serum levels of the pro-inflammatory eotaxin, IL-9 and thymus and activation-related chemokine (TARC) were higher in old mice (21 months old) compared to young mice (5 months old) (Jeon et al., 2012). Interestingly, this study also showed that the pro-inflammatory cytokine IL-16 was higher in young mice compared to older mice (Jeon et al., 2012), demonstrating the notion that pro-inflammatory cytokines are elevated in aging may be overly simplistic. This complex relationship between age and inflammation was also demonstrated using a concanavalin-stimulated cellular inflammation model (stimulates murine T-cells) where the production of pro-inflammatory interferon gamma (IFNγ) and IL-2 decreased from 3 months to 15 months of age (Dayan et al., 2000), which is the opposite of the typical inflammaging model. Thus, while aging generally accompanies an increase in low grade chronic inflammation, this is not always the case and further research is necessary to uncover the possible nuances.

1.6.2 Link between frailty and chronic inflammation

As discussed above, there is a link between serum levels of inflammatory markers and aging, but this link also exists between inflammation and frailty. This can be demonstrated using mouse models of frailty that have genetically altered cytokine profiles. For example, the anti-inflammatory IL-10 deficient (IL-10KO) mouse model is considered a frail mouse model (Walston et al., 2008; Ko et al., 2012), although it is actually a model of inflammatory bowel disease (Sturlan et al., 2001). In initial characterization, IL-10KO mice showed hair loss and a reduction in both activity and grip strength compared to wild type animals at 15 months of age (Walston et al., 2008). While

they did not measure frailty per say, the IL-10KO mice showed signs of increased physical frailty without a change in survival (Waltson et al., 2008). Another group used the same IL-10KO model showed that these mice had elevated levels of the pro-inflammatory cytokines IL-6, TNFα, IFNγ and IL-1β (Ko et al., 2012). Another frail mouse model uses the overexpression of pro-inflammatory IL-6. At 28 months of age, the IL-6 overexpressing mice had higher frailty index scores and lower grip strength than their wild type counterparts (Jergovic et al., 2020). Another study showed that IL-6, IFNγ, and IL-9 correlated with frailty only in older female mice, suggesting sex-specific immune aging may occur (Kane et al., 2019). These mouse models of frailty demonstrate the close link between levels of low anti-inflammatory cytokines and high levels of pro-inflammatory cytokines in frailty.

1.6.3 Chronic inflammation and cardiac aging

While previous sections have focused on the body's response to chronic inflammation, the increase in pro-inflammatory cytokines also plays a detrimental role in the age-related decline in cardiac structure and function. In terms of cardiac structure, chronic inflammation plays a role in the development of fibrosis (Thomas & Grisanti, 2020). There is a substantial population of fibroblasts in the heart that, under pathological conditions, proliferate and become myofibroblasts (Thomas & Grisanti, 2020). These myofibroblasts are primarily responsible for secretion of the extracellular matrix proteins including collagen. While this process is initially adaptive to help heal wounds, an accumulation of collagen leads to pathological remodeling (Thomas & Grisanti, 2020). This change from adaptive to age-related pathologic remodeling can involve inflammation. In tissue, pro-inflammatory cytokines can help recruit fibroblasts, increase

collagen synthesis, and increase the release of pro-fibrotic factors (Dobaczewski & Frangogiannis, 2009). Interestingly, most work on the role of cytokines and fibrosis uses disease models with acute cardiac damage (i.e. ischemia or myocardial infraction). Studies of cytokines and their effects on fibrosis in aging are less well understood (Biernacka & Frangogiannis, 2011). One study used the variably aged hearts of male mice (3-30 months old) to analyze both fibrosis and cytokine expression (Cieslik et al., 2011). The older hearts had increased fibrosis, an increase in the pro-inflammatory cytokines IL-13, monocyte chemoattractant protein 1 (MCP-1) and an increase in the anti-inflammatory cytokine IL-4, as well as signs of diastolic dysfunction compared to young male mice (Cieslik et al., 2011). This suggests that high levels of inflammation can lead to detrimental cardiac aging, at least in males. Effects on female hearts are unclear. Together, this helps show the role cytokines can play in cardiac aging but does not explain the reasons behind the increase in cytokines. One of the many biological mechanisms for chronic inflammation is changes in body composition.

1.6.4 Impact of aerobic exercise on chronic inflammation

Aerobic exercise is believed to help reduce chronic inflammation. In humans, metadata shows that IL-6 and TNFα decline with various types of aerobic exercise, while IL-4 levels did not change in older adults (aged 40-95 years old) (Zheng et al., 2019). In mice, the impact of aerobic exercise on cytokine levels was measured during a lifelong aerobic exercise intervention (4 to 26 months of age) (Nilsson et al., 2020). Compared to young control animals, old sedentary mice exhibited increases in both pro and anti-inflammatory cytokines. By contrast, there was no increase in those same cytokines in older exercised mice (Nilsson et al., 2020). Interestingly, this study pooled male and

female mice together and did not measure sex differences. A typical inflammaging model would traditionally show that age reduces anti-inflammatory cytokine levels, however this study showed that they increased instead (Nilsson et al., 2020). Interestingly in terms of pro-inflammatory cytokines, exercise can cause an acute increase in inflammation (Barcellos et al., 2021). In young mice, this acute affect in muscles lasts at least 12 hrs after exercise is finished (Barcellos et al., 2021). There are a few possible theories for exercise-induced acute inflammation. These include the idea that it is a natural mechanism to heal damaged muscle or that the increased inflammation arises from an increase in reactive oxygen species induced by exercise (Barcellos et al., 2021). Thus there is evidence that aerobic exercise has variable effects on inflammation, where it can both increase and decrease systemic inflammation. With few studies focusing on how aerobic exercise impacts systemic inflammation in older animals, more research is needed.

In summary, while there is likely a connection between aerobic exercise, frailty, and chronic inflammation, the link may be more complex than the traditional inflammaging model. Exercise can, in fact increase inflammatory markers in young animals. The changes in inflammation related to age may also be linked to age-related deterioration in heart health although few studies have investigated this.

The overall hypothesis to be tested here is that aerobic exercise will prevent many of the age-related changes in cytokine levels, resulting in lower frailty and improved cardiac health.

1.6 Research goals

The specific objectives of this thesis are:

1. Investigate the impact of voluntary aerobic exercise on frailty and mortality in older mice of both sexes.
2. Observe possible sex-differences in running distances in older mice.
3. Examine changes in body composition resulting from age or aerobic exercise and whether they are correlated to exercise or frailty in older mice of both sexes.
4. Analyze cardiac structure and health in sedentary and exercised mice to determine if aerobic exercise protected hearts against age-related dysfunction.
5. Investigate serum cytokines to determine if aerobic exercise prevents inflammaging in mice of both sexes.

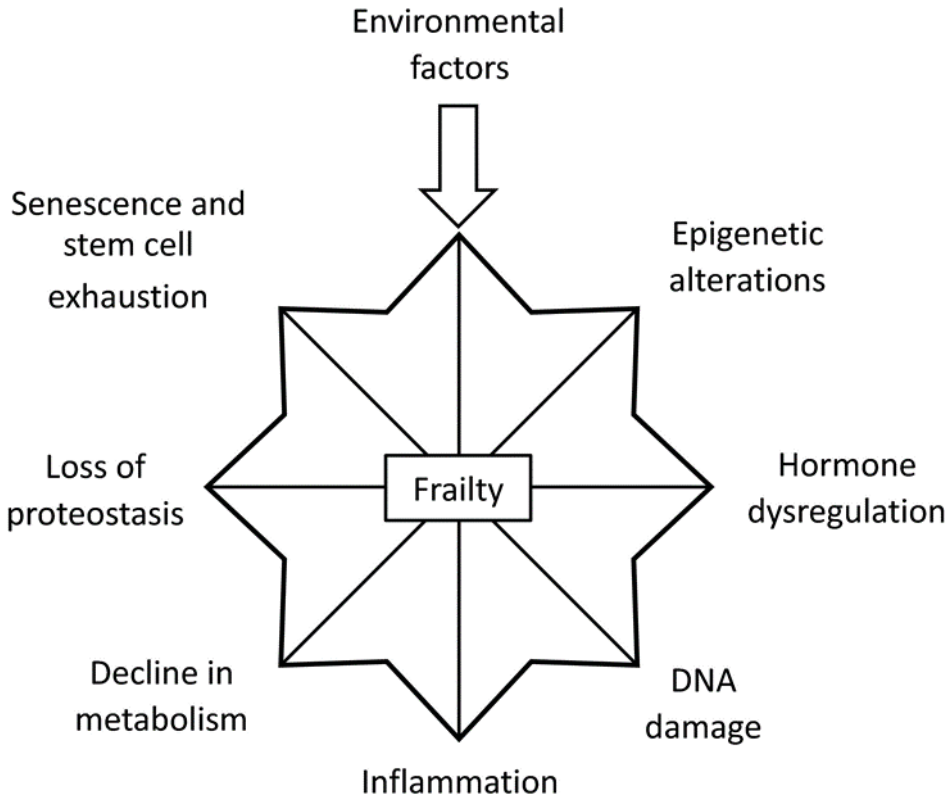

Figure 1.2.1 The 7 pillars of frailty: Used with permission (Bisset and Howlett, 2019).

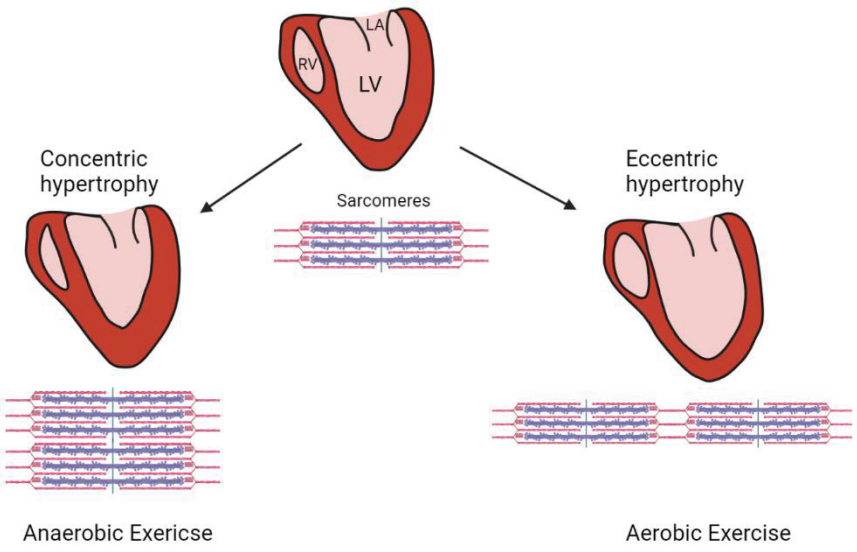

Figure 1.5.1 Structural differences between eccentric and concentric hypertrophy. Created and used with permission from BioRender.

Chapter 2: Methods

2.1 Animals

C57Bl/6 male and female mice were purchased from Charles River (St. Constant, QC, Canada) and aged in the Carlton Animal Care Facility. They were housed in Individually Ventilated Caging systems (Allentown, Inc) at 21 degrees Celsius (°C) with 35% humidity. Mice were aged until they were 21-23 months of age (females n=22, males n=12), when they were transferred to individual cages, with or without a running wheel (details below). Animals were exposed to a 12-hr light-dark cycle with *ad libitum* access to food and water. Mice were fed ProLab RMH3000 (LabDiet, MO), and their food consumption was tracked by weighing the food weekly. All cages had the same mouse houses, bedding, and nesting material and they were cleaned once a week. All experiments were approved by the Dalhousie University Committee on Laboratory Animals and performed in accordance with guidelines published by the Canadian Council on Animal Care (Canadian Council on Animal Care, 1993). A detailed experimental timeline is outlined in Figure 2.1.1.

2.2 Frailty

Mice were assessed for frailty using the mouse clinical frailty index (FI), as described previously by our group (Whitehead et al., 2014; Feridooni et al., 2015). In brief, mice were tested for 31 individual health deficits such as problems with gait, body condition, hearing, vision, and respiration, as outline in Table 2.1.1. This testing was done by placing mice in a clean cage in a quiet room in the animal care facility to acclimatize for five minutes. The mice were weighed, and body surface temperature was

measured in triplicate with an infrared thermometer (InfraScan™, La Crosse Technologies, model IR 101). They were then scored for health deficits, where 0 indicated no deficit, 0.5 a mild deficit and 1 a severe deficit. For body weight and body surface temperature, values were compared to group averages at the beginning of the experiment. Values that differed between 0-1 SD from the group mean were scored as 0, 1-2 SD were scored 0.25, 2-3 SD were 0.5, 3-4 SD were 0.75 and values above 4 SD were scored as 1. Values for all 31 items were then summed and divided by 31 to achieve an FI score between 0 and 1. Mice were allocated into exercise or sedentary groups for each sex based on similar FI scores, so both groups had the same average starting FI. Frailty assessments were completed every two weeks, with an extra assessment at week 13.

FRIGHT (Frailty Inferred Geriatric Health Timeline) and AFRAID (Analysis of Frailty and Death) frailty clocks were calculated using an online algorithm on http://frailtyclocks.sinclairlab.org/. Clocks were calculated using a combination of deficit index scores, mouse's weight, and chronological age.

2.3 Forelimb grip strength

Grip strength was measured at baseline, and endpoint with a strength test device shown in Figure 2.3.1 (Biosed In Vivo Research Instruments, model: BIO-GS3). Mice were held by the base of their tails, lifted vertically, and allowed to grip a metal mesh grid with their forelimbs before being gently pulled up. This was repeated four times, with the mice resting for 10 sec between repetitions. The maximum grip out of the four repetitions was used for analysis.

2.4 Echocardiography

At baseline, midpoint and endpoint cardiac structure and function were measured using echocardiography. Mice were moved to cages without running wheels for at least 1 hours (hr) before echocardiography was performed. Mice were anesthetized with 3% isoflurane in oxygen, then maintained at 1-2% throughout the procedure. They were rested in the supine position on a raised heated platform and hair was removed with a depilatory agent (Nair™). Temperature was monitored using a rectal probe and heart rate was monitored using electrode probes under the paws. The heart was viewed using the Vevo 2100 imaging platform (FUJIFILM VisualSonics, Toronto CA) using an MS 400 (18-30 MHz) probe. The cardiac short axis M-mode was used to determine left ventricular function (Figure 2.4.1A) while apical 4-chamber view was used to measure mitral valve function in terms of blood flow (Figure 2.4.1B) and tissue function (Figure 2.4.1C). Definitions of all echocardiography parameters are outlined in Table 2.4.1 along with calculations used. These images were analyzed with Vevo lab (Version 3.1.1, FUJIFILM VisualSonics, Toronto CA). All measurements and attributed calculations are shown in Table 2.4.1. All animals were allowed to recover overnight before being returned to cages with wheels.

2.5 Body composition

Body composition (*e.g.* fat tissue, total tissue, and non-fat tissue) was measured with a dual energy x-ray absorptiometry (DEXA) scanner, Figure 2.5.1 (Lunar PIXImus, GE Lunar Corp.). Mice were lightly anesthetized using 1-2% isoflurane, which was administered throughout the scanning process. The DEXA scan was done at baseline,

midpoint, and endpoint (13 weeks) immediately after echocardiography. An example of the scan is shown in Figure 2.5.1. Non-fat mass was expressed as % non-fat mass, which was calculated by dividing the non-fat mass (g) by the total tissue mass (g) for each mouse. A similar approach was used to determine % body fat.

2.6 Wheel running

Voluntary wheel running was tracked using cages where the mice had free and constant access to a stainless-steel running wheel, as shown in Figure 2.6.1, for 13 weeks (Clocklab, ActiMetrics ^{TM}, model: ACT-551-MS-SS: circumference 34.56 cm). Wheel rotations were counted using magnetic switches. Wheel running supplemented regular in-cage activity done by both exercise and sedentary groups. Twenty-four-hour activity data were collected on a computer and analyzed with Clocklab Analysis software version 6 (ActiMetics software^{TM}) to measure distance run and bouts. A bout is a period of activity, defined here as a minimum of 5 wheel rotations without more than a 5 minute break between rotations. Using bouts helps remove artifacts caused by mice simply playing with the wheel. An example of the actogram the software produced is shown in Figure 2.6.2.

2.7 Serum collection and analysis

Mice were moved to cages with no running wheels at least 1 hr before blood was collected. For the exercised and sedentary mice, blood was collected directly from the aorta. Blood samples were allowed to clot at room temperature for 30 mins, before centrifugation at 211 G, (4°C) for 12 mins. The supernatant (serum) was collected and frozen at -20°C. Serum cytokines were analyzed with a bead-based multiplex assay

(BioRad, M60009RDPD) following manufacturer's instructions. Cytokine concentrations that fell below the limit of detection were replaced with the lower limit of detection divided by 2, as in our previous study (Keller et al. 2019). Cytokines were divided into pro- and anti-inflammatory cytokines as well as chemokines using previously defined classifications (Nilsson et al., 2019).

2.8 Tissue collection and cardiac fibrosis

At the endpoint, animals were weighed then injected with an overdose of sodium pentobarbital (200 mg/kg, IP). Anesthesia was checked using the paw pinch method to ensure lack of sensation. Hearts were removed, weighed, and divided into apex, ventricles, and atria. They were snap frozen in liquid nitrogen before being stored at -80°C.

Hydroxyproline concentrations were measured in ventricular tissue using a colorimetric assay (Abcam, #ab222941, Cambridge UK) following manufacturer's instructions. Hydroxyproline is a major component of collagen, so was used as a proxy for cardiac fibrosis. To prepare tissue for analysis, frozen tissue was thawed then homogenized in distilled water using a bead homogenizer. Dissolved tissue was decanted and mixed with 100 microliter (µL) concentrated 10 molar (M) sodium hydroxide before being heated at 120°C for 1 hr. The solution was chilled on ice then neutralized with 100 µL of 10 M hydrochloric acid before centrifugation (10 000 g, 5 min). The supernatant was placed in a plastic 96 well plate and heated until only tissue residue remained (65°C). This tissue was oxidized (Chloramine T solution), developed (propriety solution) then

measured at with a plate reader at 560 nanometers. Fibrosis was reported as collagen per wet tissue weight.

2.9 Statistics

Data were expressed as mean ± standard error (SEM) unless otherwise indicated. Data analyses were done with SigmaPlot (version 14, Systat Software, Germany) or Prism (version 8.3, GraphPad Software, San Diego CA). P values less than 0.05 were considered statistically significant.

2.9.1 Activity Statistics

Activity was analyzed using bouts, where a bout was a period of continuous activity with more than 5 wheel rotations and less than 5 minutes of rest between rotations. Bout parameters (bout length, peak rate and bouts per day) were averaged over the course of the study then compared across sex using a Student's T-test. The running time of day was calculated by dividing up the 24 hr day into quartiles and summing the rotations that fell in each quartile. This sum was then divided by the total number of rotations by all animals throughout the entire study. The analysis of each quartile percentage was compared using a Student's T-test between males and females.

2.9.2 Frailty and mortality statistics

Frailty, FRIGHT and AFRAID data were analyzed using a 2 or 3-way repeated measures linear mixed model using time and activity as main factors. This allows for missing data due to mortality. A Fischer LSD post hoc was used. The baseline and endpoint frailty data were compared using a 2-way ANOVA with activity and sex as

main factors. Proportional deficits were analyzed by first codding every deficit as present (1) or absent (0). This allowed for the data to be analyzed using a Fischer's exact test. For proportional deficits, baseline analysis contained only the animals also present at endpoint. Activity parameters and frailty were correlated using a two tailed Pearson's correlation using the activity data from five consecutive days before the FI was measured. Mortality data were analyzed using a LogRank analysis. FRIGHT score and actual age were correlated using a two-tailed Pearson's correlation.

2.9.3 Body composition statistics

Body composition parameters (lean tissue, fat tissue, and total tissue) and forelimb grip strength were compared across time, and between both activity groups and sex using either a 2 or 3 way linear mixed model with a Fischer LSD post hoc, which allowed both for repeated measures no loss of data points due to mortality. Body composition was correlated with either frailty or activity using a two-tailed Pearson's correlation, using the activity data from the five preceding days before body composition was measured. Food consumption was compared across 12 weeks using a 2 way linear mixed model. Food consumption was also normalized to the individual mouse's body weight which was also measured weekly.

2.9.4 Cardiac structure and function statistic

Measures of cardiovascular structure and function were analyzed using a 2 or 3 way linear mixed model with a Fischer LSD post hoc to allow for retention of data and repeated measures analysis. Hydroxyproline concentration was normalized the wet tissue weight and analyzed with a 2-way ANOVA with sex and activity as main factors and a

Holms-Sidak post hoc test. Cardiac parameters were correlated to frailty and activity using a two-tailed Pearson's correlation test, with the activity data from the five prior consecutive days before echocardiography was measured.

2.9.5 Inflammation statistics

Cytokine levels at endpoint were normalized to a pooled average baseline for both male and female mice. Normalized levels for each cytokine were then analyzed using a Student's T-test. Endpoint serum cytokine levels were correlated with activity in both male and female mice using a Pearson's correlation using the activity data measured on the five preceding consecutive days before serum cytokines were measured.

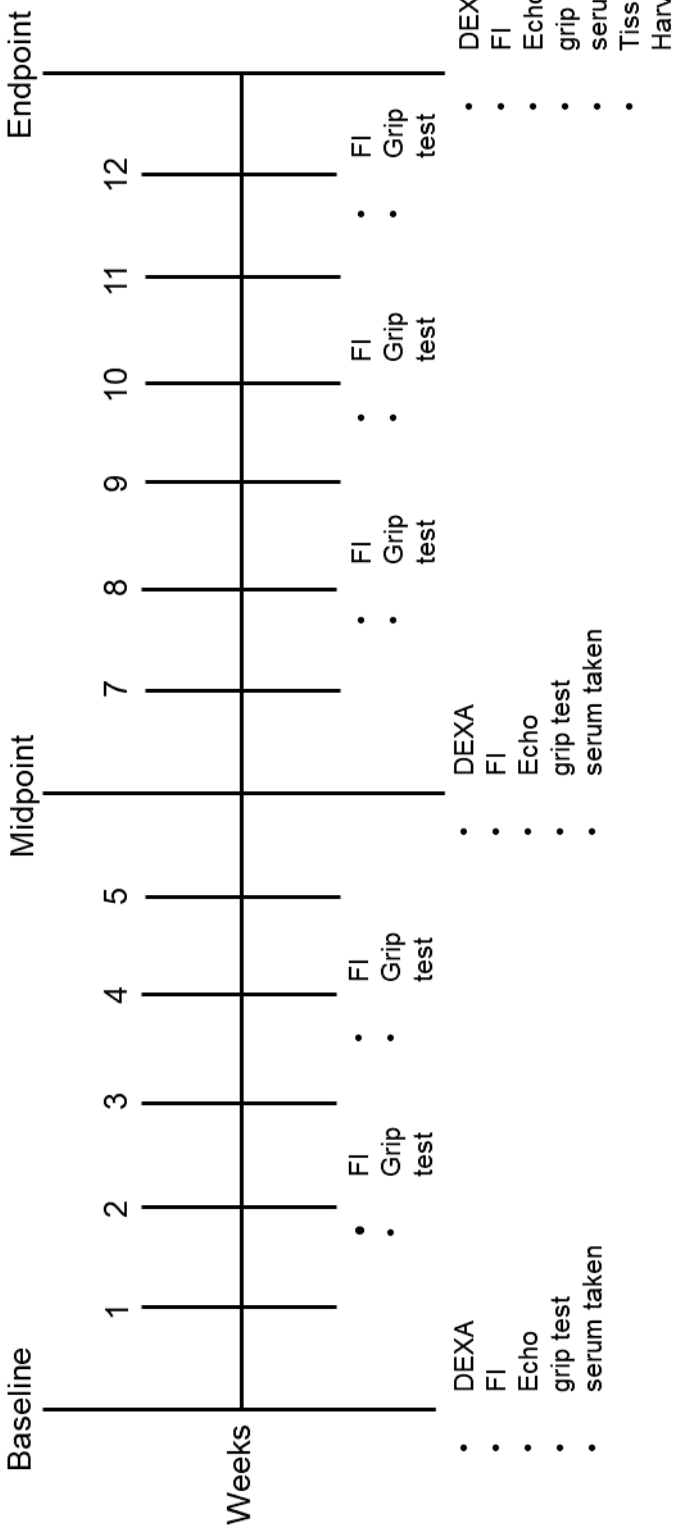

Figure 2.1.1: Experimental Timeline. Mice were singly housed for baseline measurements then put into their exercise cages, or sedentary ones, starting at week 1. Let echo denote echocardiography, DEXA denote dual-energy-x-ray absorptiometry and FI denote frailty index

Table 2.2.1: Mouse Frailty Index: Overview of Deficits

Mouse Biological System	Deficit measured
Skin and coat	Alopecia
	Loss of fur colour
	Dermatitis
	Whisker condition
	Coat condition
Musculoskeletal	Tumors
	Distended abdomen
	Kyphosis
	Tail stiffening
	Gait
	Tremor
	Forelimb grip strength
	Body condition
	Body weight
Vestibulocochlear	Head tilt
	Hearing loss
Ocular/Nasal	Cataracts
	Discharge
	Microphthalmia
	Corneal discoloration
	Vision loss
	Menace reflex
	Nasal discharge
Digestive/urogenital	Malocclusion
	Rectal prolapse
	Penile/uterine prolapse
	Diarrhea
Respiratory	Breathing rate
Discomfort	Mouse grimace
	Piloerection
	Surface body temperature

Figure 2.3.1: Instrument for measuring grip strength. Mice use their forelimbs to hold onto the metal mesh while being held upside down by their tail. Mice are gently pulled upwards, horizontal to the mesh. This process is done 4 times per mouse per timepoint. Grip strength is shown on the display in Newtons, as the highest force achieved for each pull.

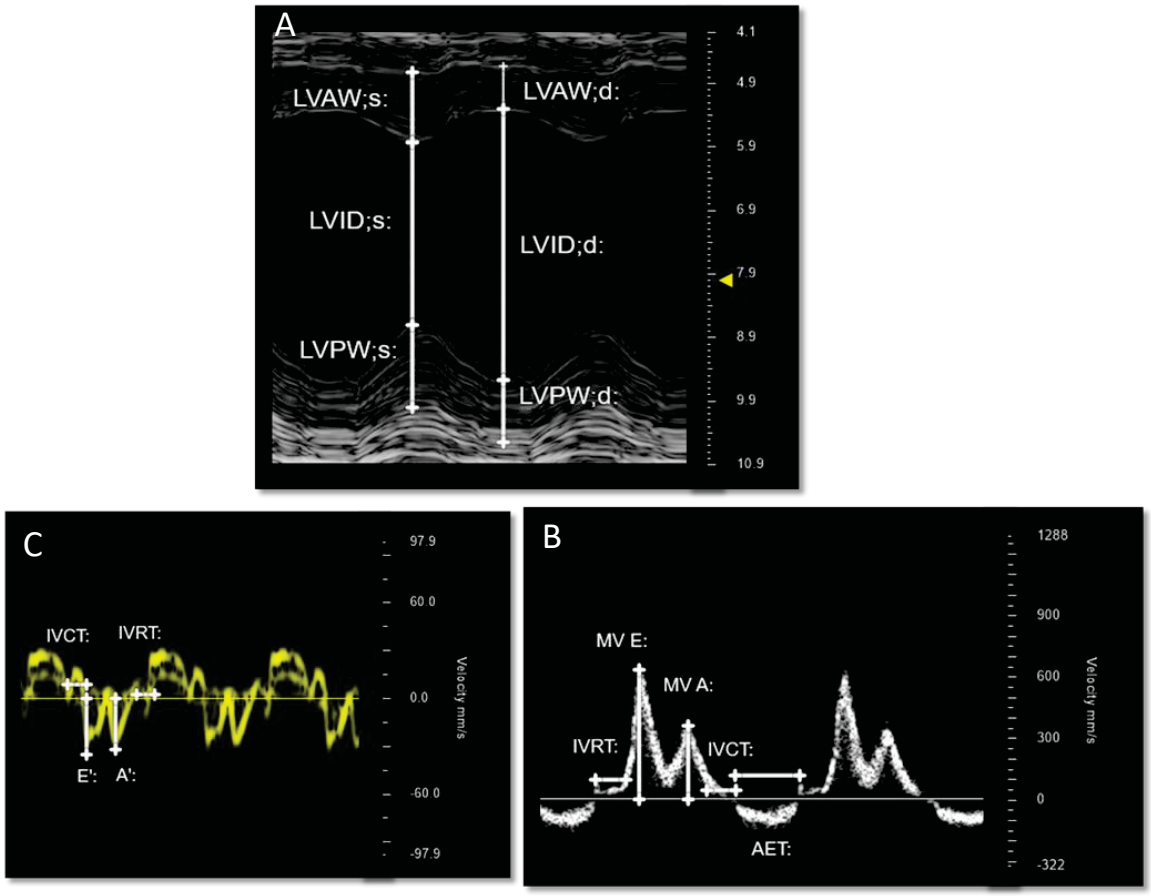

Figure 2.4.1: Examples of images used to calculate echocardiography parameters. **A** is an image of a short axis view of the left ventricle in M-mode. The light sections of the image are solid tissue while dark sections are blood or other fluids. Show in A are: LVAW is the left ventricular anterior wall thickness, LVPW is the ventricular posterior wall thickness, and LVID is the left ventricular internal diameter. These measurements were made in both diastole and systole. **B** is a doppler image of the blood through the mitral valve. Shown in B are: MV E is the mitral velocity of the early wave, and MV A is the mitral velocity of the active wave. **C** is an example of tissue doppler of a section of cardiac wall next to the mitral valve. Shown in C are: E' is the early wave and A' is the active wave.

Table 2.4.1: Echocardiography Parameters measured in this study

Abbreviation	Full name	Derivation	Calculations
HR	Heart rate	ECG	-
V;s and V;d	Volume at systole and diastole	M-mode short axis image	-
D;s and D;d	Diameter at diastole and systole		-
EF	Ejection fraction		100x((V;d-V;s)/V;d)
FS	Fractional shortening		100x((D;d-D;s)/D;d)
LV mass	Left ventricular mass		$(1.053x((D;d+LVPW;d+LVAW;d)^3 - D;d^3))x0.8$
LVAW;s and LVAW;d	Left ventricular anterior wall thickness at systole and diastole		-
LVPW;d and LVPW;d	Left ventricular posterior wall thickness at systole and diastole		-
MV E	Mitral valve E velocity	Mitral valve doppler	-
MV A	Mitral valve A velocity		-
AET	Aortic ejection time		-
IVRT	Isovolumetric relaxation time		-
IVCT	Isovolumetric contraction time		-
E’	Velocity at E’ wave	Mitral valve tissue doppler	-
A’	Velocity at A’ wave		-
MPI	Left ventricular myocardial performance index	Mitral valve doppler	(IVRT+IVCT-AET)/AET
MV E/A	Ratio E and A waves	Mitral valve doppler	(MV E)/(MV A)

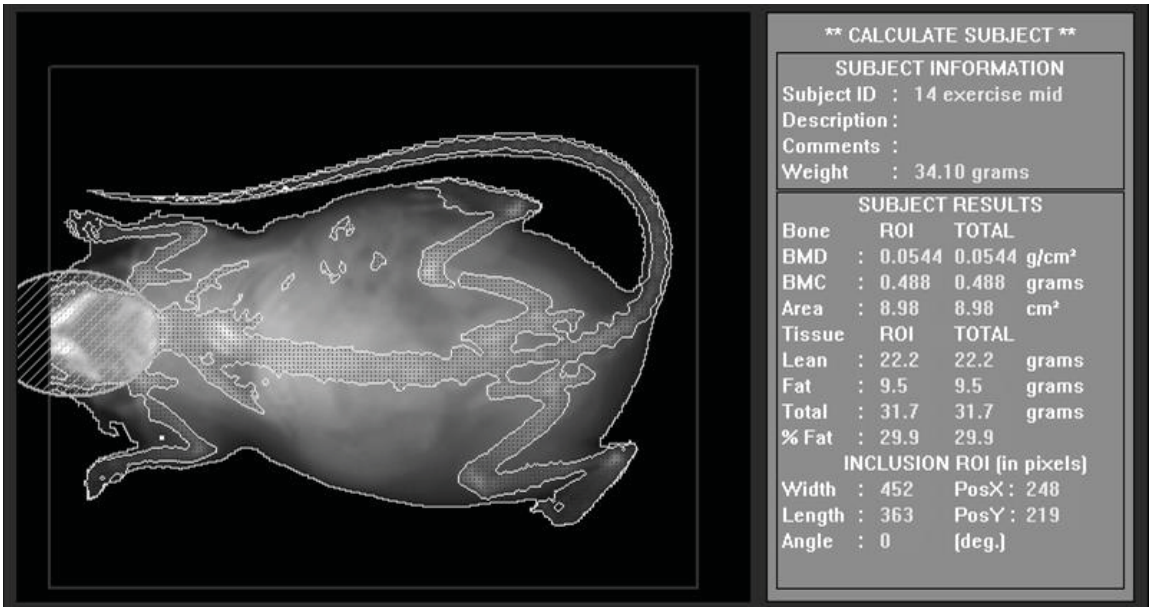

Figure 2.5.1: Example of a DEXA scan from exercised female #14 at midpoint. The head of the animal (circle at left) is excluded from the analysis. The animal was under anesthesia (1-2% isoflurane) during this scan.

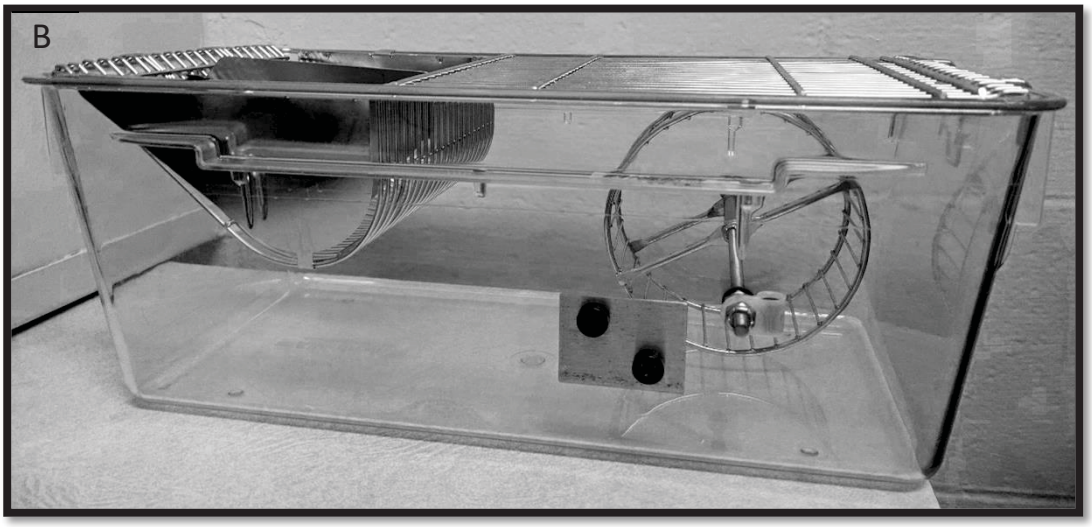

Figure 2.6.1: Examples of an exercise wheel cage. **A** shows a front view of the cage, without a lid. The cage is made from hard plastic while the wheel is metal. **B** shows a side view of the cage, with a metal grip lid. Also displayed in B is the side plug and magnet on the axis on the wheel, which is how wheel rotations are counted.

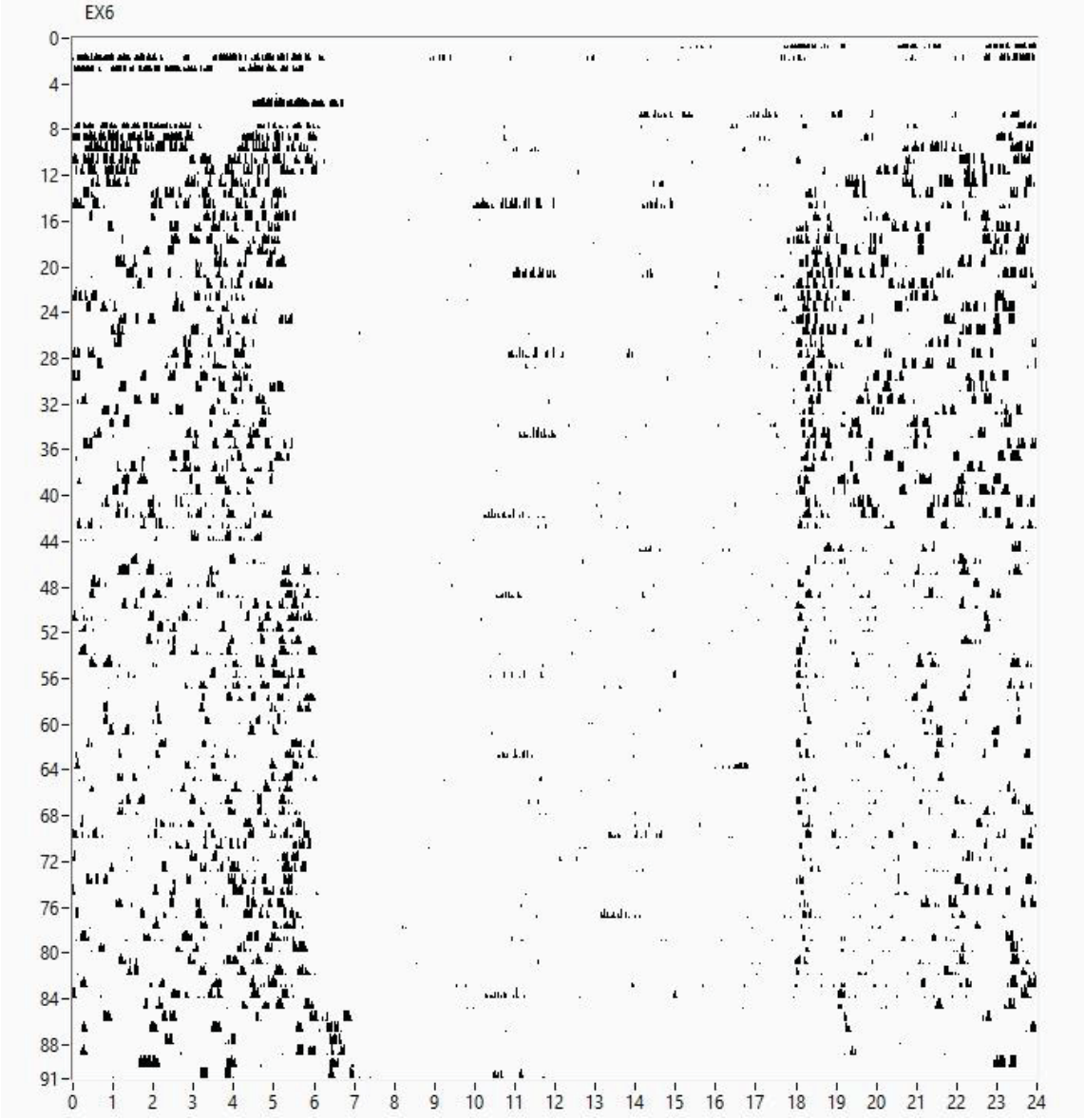

Figure 2.6.2: Example of an actogram recorded from exercised female #6. The days are shown on the Y-axis and time of day (24 hour clock) is shown on the x-axis. The black bars denote periods of activity. The periods of heavy activity during the daylight hours correspond to when mice were put in clean cages, a process done once a week.

Chapter 3: Results

3.1 Activity levels

3.1.1 There was high variability in running distance for both female and male mice

The first set of experiments compared running in aging male and female mice. Running levels were plotted as cumulative distance over the course of the study as seen in Figure 3.1.1. There was considerable variability in distance run for both sexes over the 13-week intervention. Female mice (Figure 3.1.1A) reached endpoint having run between 21 km to 111 km while male mice (Figure 3.1.1B) ran between 22 km to 122 km in cumulative distance. When mice were separated by activity, the highest performers (Figure 3.1.2A) in both males and females (111.1 km for female and 121.8 km for male) were very similar. The same can be seen in the lowest activity (Figure 3.1.2B: 21 km for female and 21.7 km for male), where male and female mice ran virtually the same distance. While both male and female mice had high inter-individual variability in activity, there were no apparent sex differences in the highest or lowest performers.

3.1.2 There were no sex difference in activity parameters

After investigating the cumulative distance that the mice ran, we next examined in more detail the running habits of the mice. This revealed that, on average, the male and female mice had very similar running parameters (Figure 3.1.3). There were no sex differences in the duration (Figure 3.1.3A), speed (Figure 3.1.3B), or frequency (Figure 3.1.3C) of running. While there was a slight trend for males to run more often per day than females (females: 12.0 ± 3.8 vs males: 14.9 ± 2.1 $p=0.15$) this was not statistically

significant. Similar to cumulative distance run (Figure 3.1.3D), there were no sex differences in activity levels.

3.1.3 Sex differences in time of day that mice ran

We next investigated sex differences in the time of day that the mice were active. A 24hr day was divided into four quartiles, with two during the light period (6:00-12:00 and 12:00-18:00) and two during the dark period (0:00-6:00 and 18:00-24:00). Activity in Figure 3.1.4 is shown as a percentage of total rotations, as explained in the methods. There was no sex difference for the night periods (0:00-6:00: $p=0.57$ and 18:00-24:00: $p=0.20$) but the females were more active during the day (6:00-12:00: $p=0.003$ and 6:00-12:00: $p=0.21$). All mice ran more often during the night cycle (Female: $82.4 \pm 3.8\%$ Males: $93.8 \pm 0.6\%$) than during the day cycle (Females: 17.6 ± 3.8 Males: $6.1 \pm 0.6\%$). While male and female mice ran more at night, females ran significantly more than males during the first quartile of the day cycle. Older female mice deviated from the natural circadian rhythm of the nocturnal C57Bl/6 strain (Valentinuzzi et al., 1997) while males did not.

In summary, both older male and female mice ran when given voluntary access to a running wheel. There was considerable variation in the distance run by individual mice for both sexes. There was no overall sex difference in cumulative distance run, or in activity levels. Most of this activity was done in the dark, however females did run more than males during the light cycle.

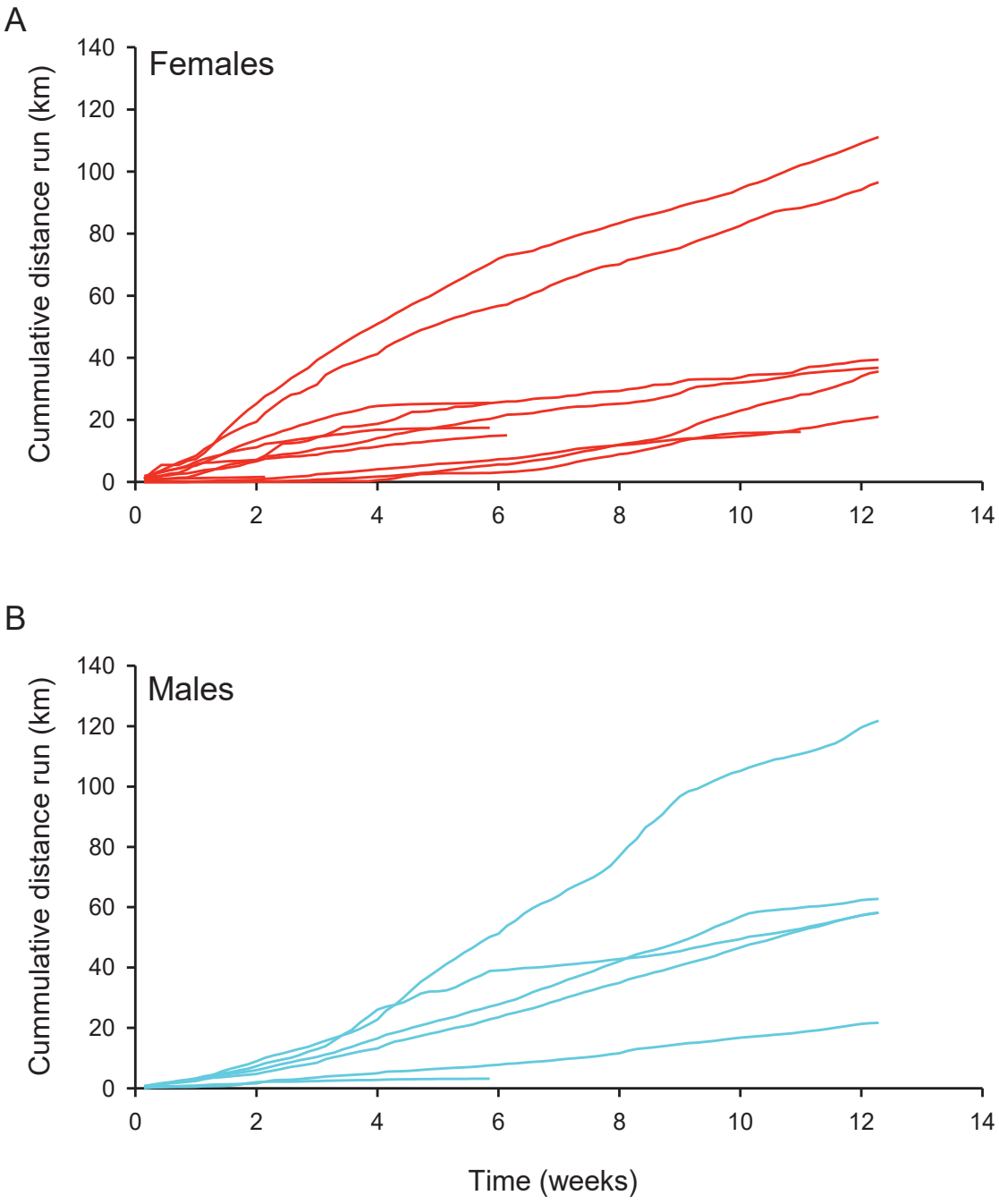

Figure 3.1.1: There was high inter-individual heterogeneity in running distances for older male and female mice. Both female (A: n=11) and male (B: n=6) running is shown as cumulative distance run. This was calculated by multiplying the number of wheel rotations per day by the circumference of the wheel (34.56 cm).

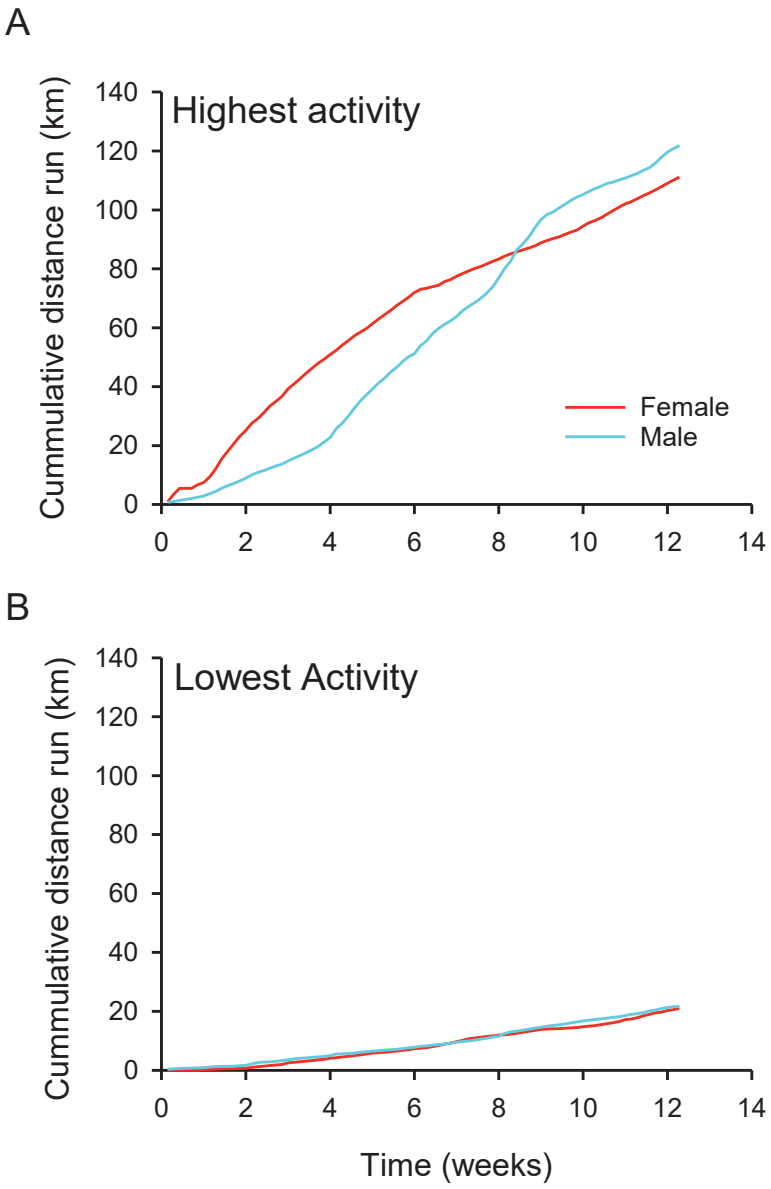

Figure 3.1.2: There were no sex differences between highest and lowest performing older mice. Highest performers (**A**: female Ex6 and male Ex30) and lowest performers (**B**: female Ex9 and male Ex40) were the mice who ran the furthest or shortest distances respectively throughout the study. Cumulative distance was calculated by multiplying the daily wheel rotations by the circumference of the wheel.

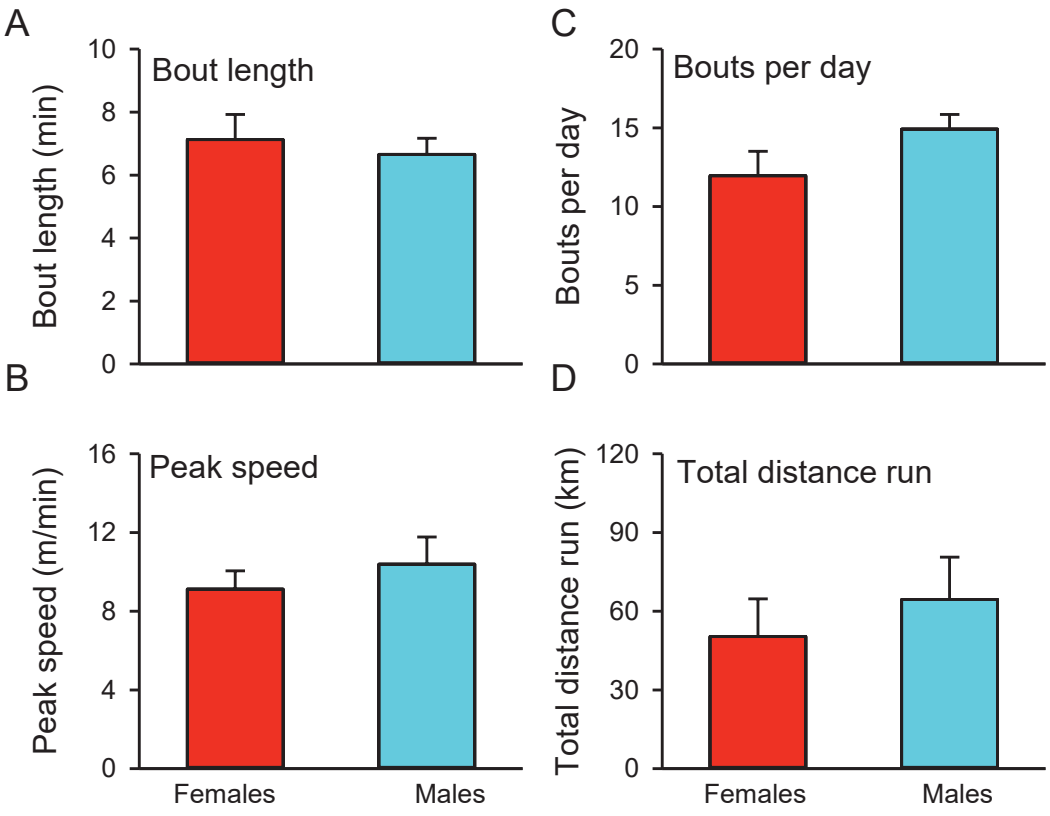

Figure 3.1.3: There were no sex differences in activity parameters. Activity parameters were measured using bout length (**A**), bouts per day (**B**), peak speed (**C**) and total distance run (**D**). For both female (A, B, C, D: n=6) and male mice (A, B, C, D: n=5) activity was averaged throughout the 13-week intervention. Total distance run (D) was the cumulative distance run by endpoint. Analysis was done using a Student's T-test, with error calculated as SEM.

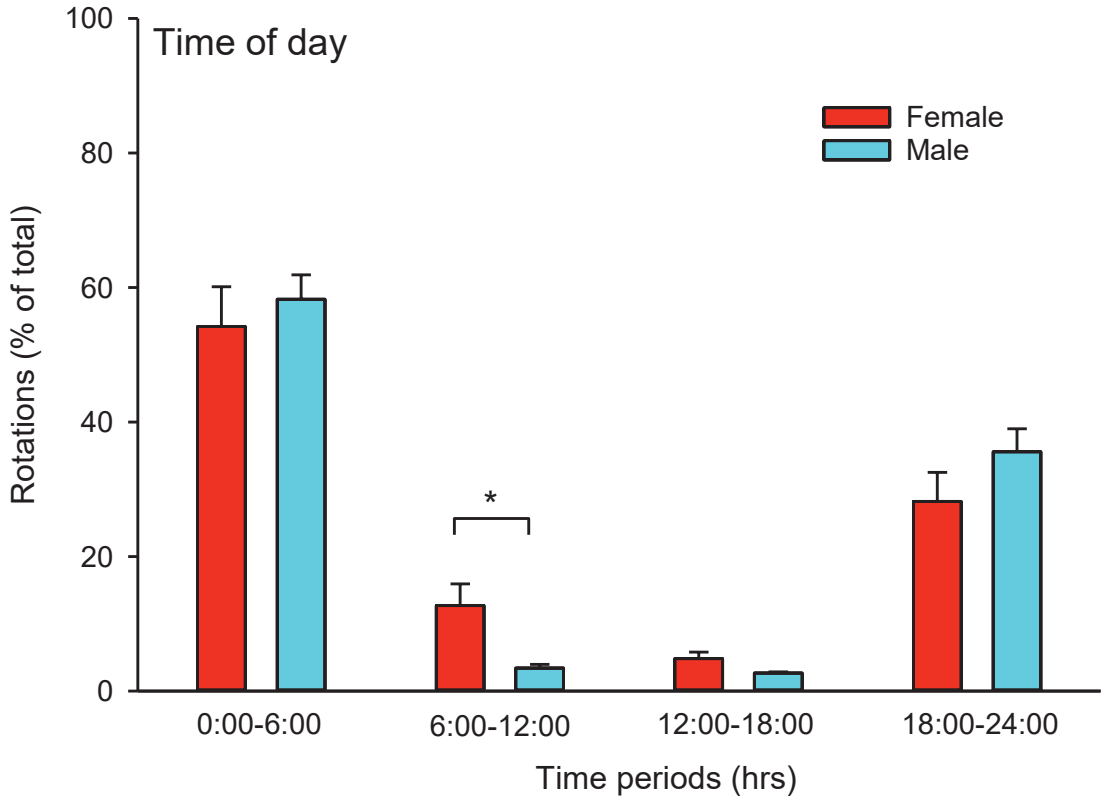

Figure 3.1.4: Aged females ran more during daylight hours than older males. Day was divided into quartiles using a 24hr clock where 6:00 - 18:00 is the daytime cycle. Activity is shown as percentage of rotations that occur during each quartile. Male (n=6) and female (n=11) activity was tested using a Student's T-test for each time period, and error is calculated as SEM. The * denotes significance for $p<0.05$.

3.2 Frailty and survival

3.2.1 Aerobic exercise attenuates the age-associated increase in frailty in both male and female mice

As all the mice in the study ran, it was possible to compare mice in the exercise group with their sedentary counterparts for both sexes. We next investigated whether aerobic exercise could attenuate the age-related deterioration of health, measured as frailty, in both male and female mice. Female mice (Figure 3.2.1A) in the sedentary group became more frail with time, becoming significantly higher than baseline at week 8 ($p=0.01$), week 12 ($p=0.026$), and endpoint ($p=0.045$). FI scores were significantly higher in sedentary females compared to the exercised group at 10 weeks ($p=0.003$) and 12 weeks of age ($p=0.019$) but not at endpoint ($p=0.054$). Conversely, the exercised group had lower levels of frailty compared to baseline at week 10 ($p=0.007$). This combined, resulted in a significant interaction between exercise and time ($p=0.001$) for the female mice.

The males had a similar overall effect of exercise (Figure 3.2.1B), with the sedentary group showing increased frailty over time. This was statistically significant at week 12 ($p=0.01$) and endpoint ($p=0.016$) when compared to baseline. This increase in frailty made the sedentary group significantly more frail than the exercised males at week 12 ($p=0.013$) and endpoint ($p=0.03$). At baseline (Figure 3.2.1C) females did have overall higher frailty scores (sedentary=0.25 ± 0.02, exercise= 0.24 ± 0.02) when compared to their male counterparts (sedentary= 0.21 ± 0.02, exercise= 0.19 ± 0.02), showing an overall effect of sex ($p=0.032$). This sex difference was abolished at endpoint (Figure 3.2.1D), where mice only showed a beneficial effect of activity.

When all groups were considered together in a 3-way linear mixed model, there was an overall effect of time ($p=0.0001$) and time*activity ($p=0.0003$). These observations demonstrate that aerobic exercise prevented the age-related increase in frailty in both male and female mice, and actually reduced frailty in older females at one timepoint.

We also determined if the reduction or attenuation in frailty was being driven by a single deficit or a small group of deficits. As shown in Figure 3.2.2, this was not the case. In the females, (Figure 3.2.2A, B) two deficits were higher in sedentary mice at endpoint (body composition and tremor) and there was also a baseline difference (kyphosis). In males, (Figure 3.2.2C, D) there were no group differences in proportion of deficits. In both female and male mice, the endpoint frailty differences are not a result of a few deficits but rather the cumulative effect across many systems.

3.2.2 There was no correlation between frailty and activity levels in either sex

We next investigated whether there was a relationship between the level of exercise done and frailty in the older mice of both sexes. Curiously, there was no correlation between the measures of activity and endpoint frailty in either sex. This lack of any relationship was similar in the duration (Figure 3.2.3A, D), speed (Figure 3.2.3B, E), and frequency (Figure 3.2.3C, F) of running. The attenuation of frailty by aerobic exercise was not graded by the amount of exercise, as it occurred regardless of activity levels.

3.2.3 Survival was not impacted by aerobic exercise.

As aerobic exercise improved overall health in older male and female mice, we next investigated if it affected survival. In both female (Figure 3.2.4A: p=0.77) and male (Figure 3.2.4B: p=0.95) mice there were no differences in survival regardless of activity. When all four groups were assessed together there were also no significant differences (p=0.38) between groups. While the female mice had higher mortality rates than their male counterparts, this difference was not statistically significant. Causes of the humane endpoints varied (Table 3.2.1), but the most common cause was dermatitis (Table 3.2.2). These observations indicate that, although aerobic exercise attenuated frailty in older male and female mice, it had no significant impact on survival.

3.2.4 Impact of aerobic exercise on FRIGHT and AFRAID scores

To analyze how exercise altered estimated lifespan and biological age, the FRIGHT and AFRAID frailty clocks were utilized. Both clocks use frailty deficit data along with chronological age to predict mouse health and life expectancy. FRIGHT scores (Figure 3.2.5A, C) are a predictor of biological age while AFRAID scores (Figure 3.2.5B, D) predict survival (Schultz et al. 2020). Although FRIGHT scores declined with time in the exercised female group (Figure 3.2.5A), there was no significant effect of time (p=0.33) or activity (p=0.10). In males, FRIGHT scores (Figure 3.2.5C) showed an effect of time (p=0.04), as they increased significantly from baseline in sedentary mice (759 ± 9 to 815 ± 27: p=0.049). FRIGHT scores increased moderately in exercised mice (782 ± 15 to 810 ± 9: p=0.05). Thus, biological age, or FRIGHT scores, increased with

time in males throughout the intervention regardless in both exercised and sedentary animals, an effect not seen in the females.

While the FRIGHT score predicts biological age, the AFRAID scores predict survival. In the female mice (Figure 3.2.5B), survival was affected by time ($p=0.011$) and this effect was driven by the sedentary females. AFRAID scores in sedentary females declined with time from baseline ($p=0.011$) while scores in the exercised mice did not ($p=0.13$). This resulted in the sedentary females having higher endpoint AFRAID scores than the exercised ones ($p=0.024$). Conversely, in the male mice (Figure 3.2.5D) there was an overall effect of time on AFRAID scores ($p=0.037$), this was due to a decrease in both the sedentary and exercised groups. Combining all groups together in a 3-way linear mixed effects model demonstrates an overall effect of time ($p=0.005$). Predicted survival decreased throughout the study in the sedentary females and males regardless of activity, while it remained constant in the exercised females.

As FRIGHT is a predicted age score, it was then correlated against the actual age of the mice at endpoint. Sedentary females (Figure 3.2.6A) showed a positive correlation ($R=0.98$, $p=0.001$) between the predicted and chronological age. By contrast, in exercised females there was no relationship between predicted and true age (Figure 3.2.6B: $R=0.40$, $p=0.43$). Interestingly, the males did not show any correlation regardless of activity level. Both sedentary males (Figure 3.2.6C: $R=0.32$, $p=0.60$) and exercised males (Figure 3.2.6D: $R=-0.69$, $p=0.19$) showed no significant relationship between biological and actual age. Combined, the FRIGHT score only showed a significant relationship with actual age in the sedentary female mice.

In summary, aerobic exercise attenuated the increase in frailty in both male and female mice, even resulting in a slight decrease in frailty in the females. Curiously, the endpoint frailty was unrelated to activity levels in either sex. In addition, activity did not significantly change survival, but in older female mice it did increase life expectancy, measured as the AFRAID score. With aerobic exercise, mice of both sexes did not live longer, but their healthspans increased.

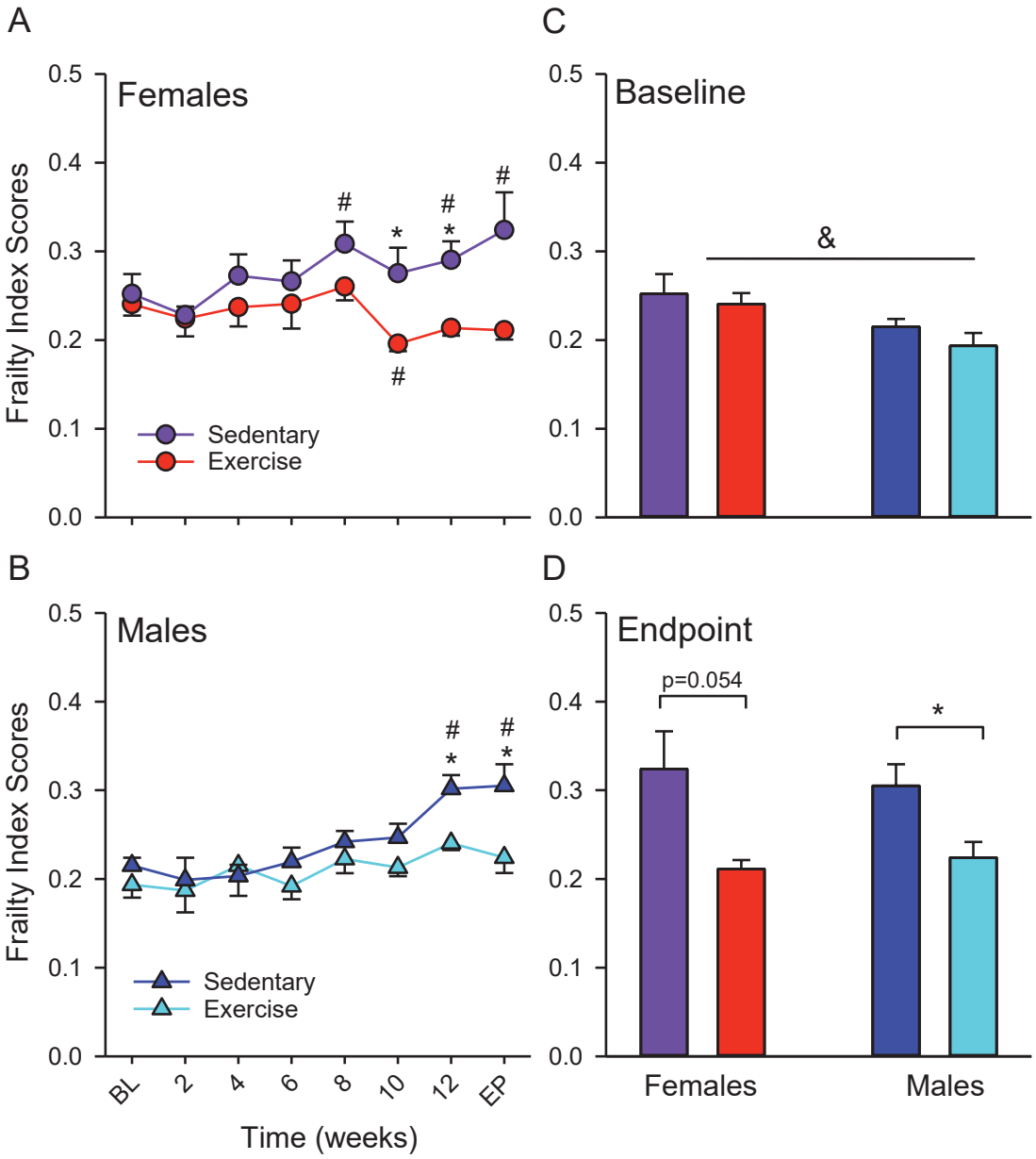

Figure 3.2.1: Exercise attenuated the age-associated increase in frailty in both male and female mice. Female (**A**: exercise n=11-6, sedentary n=11-5) and male (**B**: exercise n=6-5 and sedentary n=6-5) mice had frailty measured every two weeks using a frailty index tool. Data were analyzed using a linear mixed model. At baseline (**C**: sedentary female n=11, exercised female n=11, sedentary male n=6 and exercised male n=6) there was an overall sex difference with females having higher frailty scores than males. At endpoint (**D**: sedentary females n=5, exercised females n=6, sedentary males n=5, exercised males n=5) there was no longer an overall sex difference. Error calculated as SEM. Let BL denote baseline and EP denote endpoint. The * denotes differences between exercise and sedentary groups while # denotes a comparison with the group's own baseline, and & denotes a sex differences, $p<0.05$.

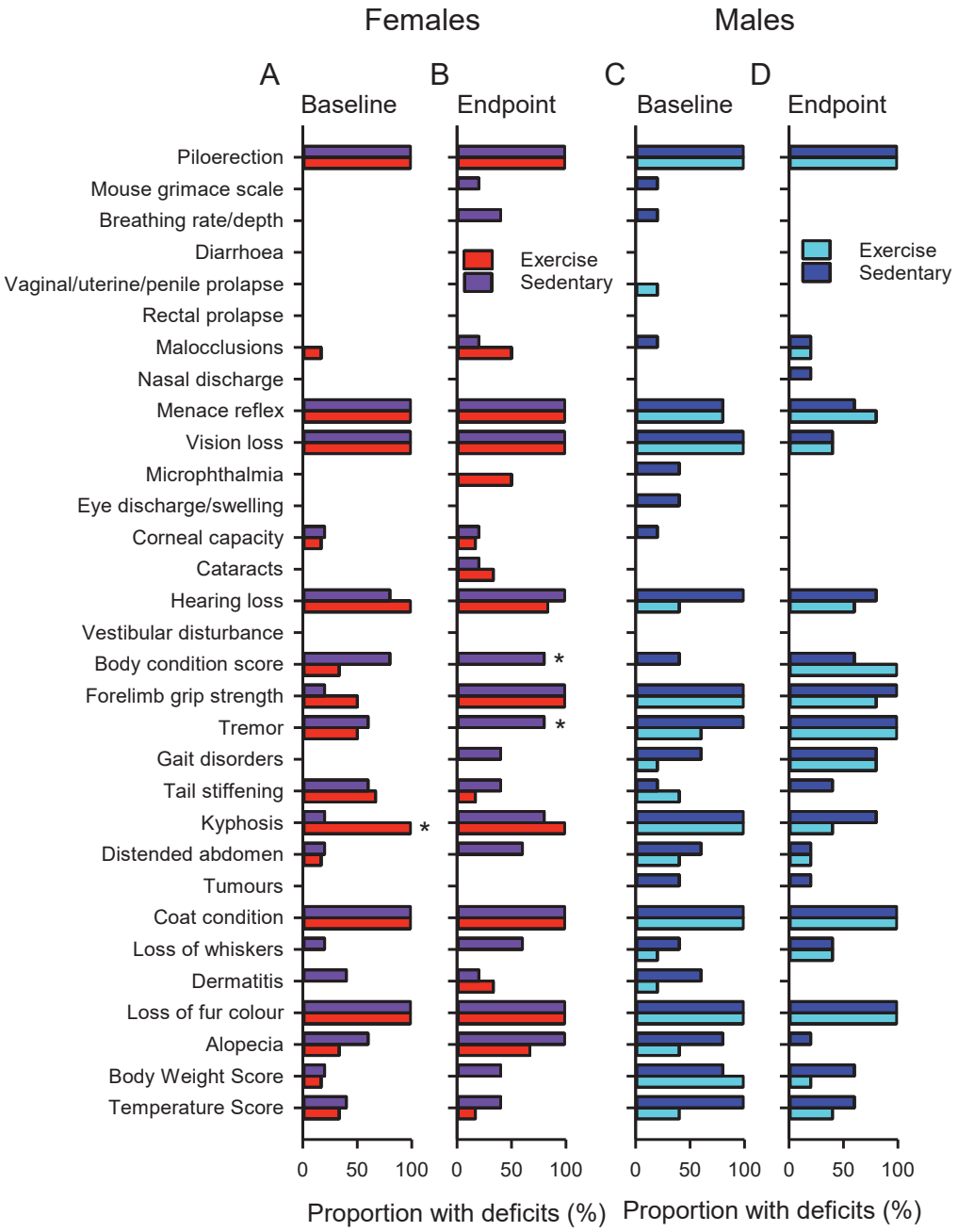

Figure 3.2.2: Increase in frailty index scores arises from many minor changes and is not driven by a single deficit. Females at baseline (**A**, sedentary n=5, exercise n=6) show that only kyphosis differs between the activity groups while at endpoint (**B**: sedentary n=5, exercise n=6) both body condition score and tremor differ between activity groups. Male mice (**C**, **D**, sedentary n=5, exercise n=5) show no differences between proportional frailty at baseline (**C**) or endpoint (**D**). Proportional frailty analyzed using Chi-squared analysis where every deficit was binarized into present (1) or absent (0) as described in the methods. The * denotes $p<0.05$.

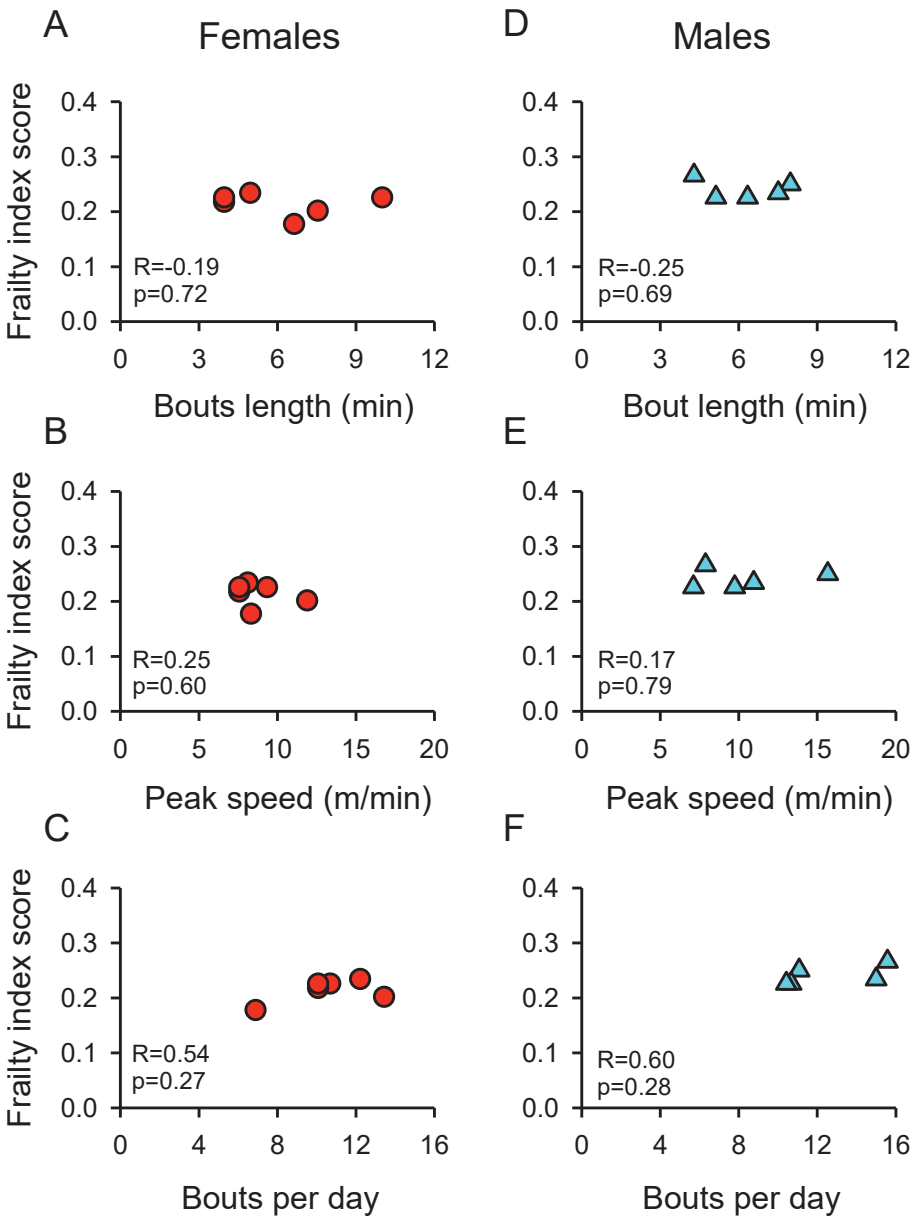

Figure 3.2.3: Neither sex had correlation between activity levels and frailty at endpoint. In aging females (**A**, **B**, **C**: n=6) and males (**D**, **E**, **F**: n=5) there were no significant relationships between exercise parameters and frailty index scores. Correlations were analyzed using a two-tailed Pearson's correlation with endpoint FI scores.

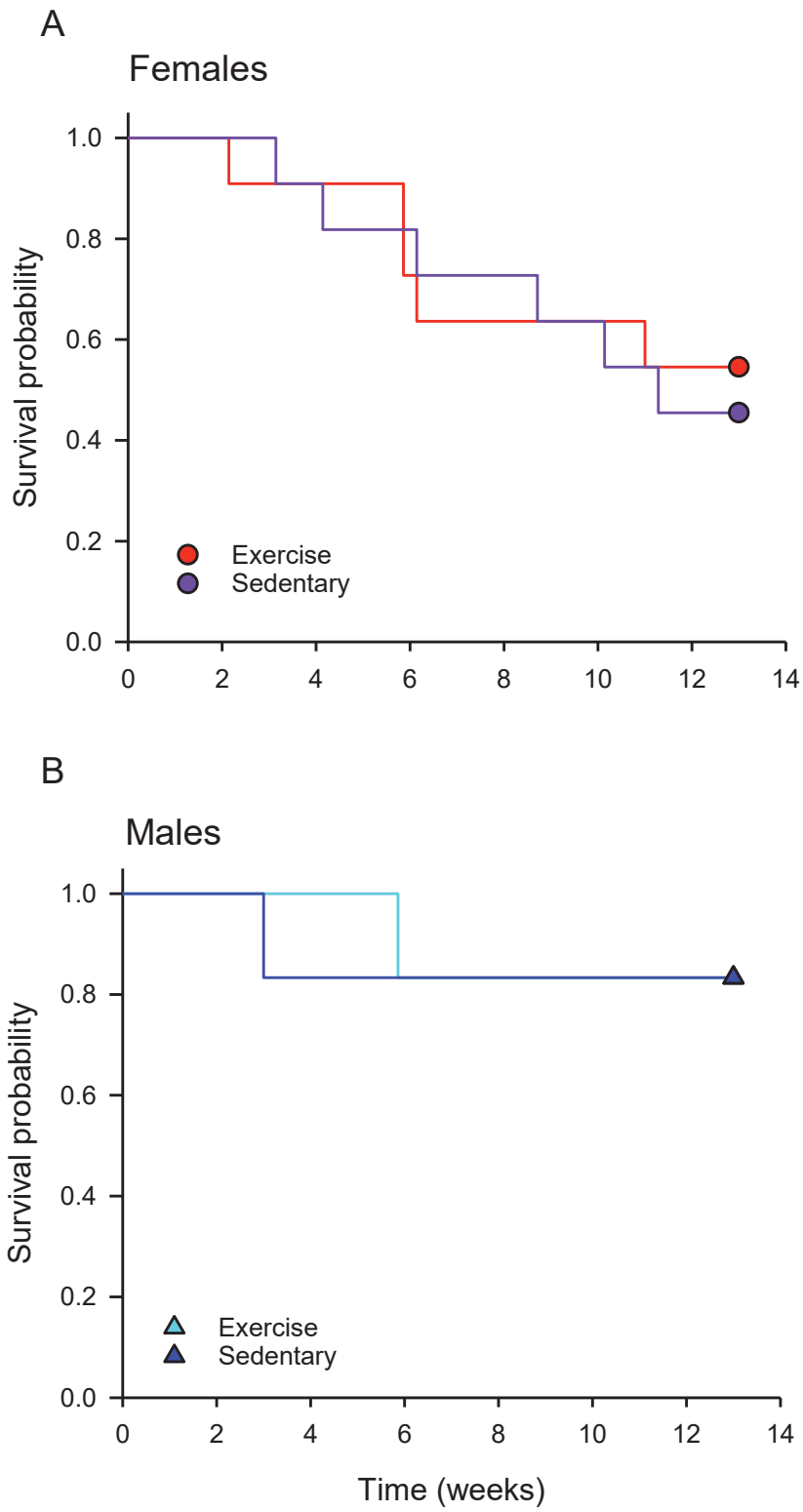

Figure 3.2.4: Exercise had no effect on survival in either sex. In females (**A**: sedentary n=11, exercise n=11) and males (**B**: sedentary n=6, exercised n=6) there was no effect of activity on survival. Survival was analyzed using a LogRank test.

Table 3.2.1: Cause for humane endpoint for all mice. If more than one cause exists, both are listed. Sudden weight loss was calculated as more than 10% loss since the previous week.

Mouse ID	Activity	Mouse Sex	Mouse age (days)	Cause of Humane endpoint
1	Exercise	F	749	Serous effusion
3	Exercise	F	750	Very thin
8	Sedentary	F	744	Serous effusion, enlarged liver
10	Sedentary	F	695	Dermatitis
13	Sedentary	F	682	Dermatitis
16	Sedentary	F	714	Dermatitis and swollen eye
17	Exercise	F	730	Sudden weight loss
19	Exercise	F	765	Liver cirrhosis, discolored intestines
20	Sedentary	F	801	Sudden weight loss
23	Sedentary	F	765	Very thin
24	Exercise	F	737	Dermatitis
35	Sedentary	M	673	Dermatitis
38	Exercise	M	693	Swollen eye

Table 3.2.2: Incidence for each humane endpoint. If a single mouse had more than one humane endpoint, both are reported.

Cause of Humane Endpoint	Number of mice	% Total
Dermatitis	5	33.3
Sudden weight loss/thin	4	26.7
Serous effusion	2	13.3
Swollen eye	2	13.3
Enlarged liver	2	13.3

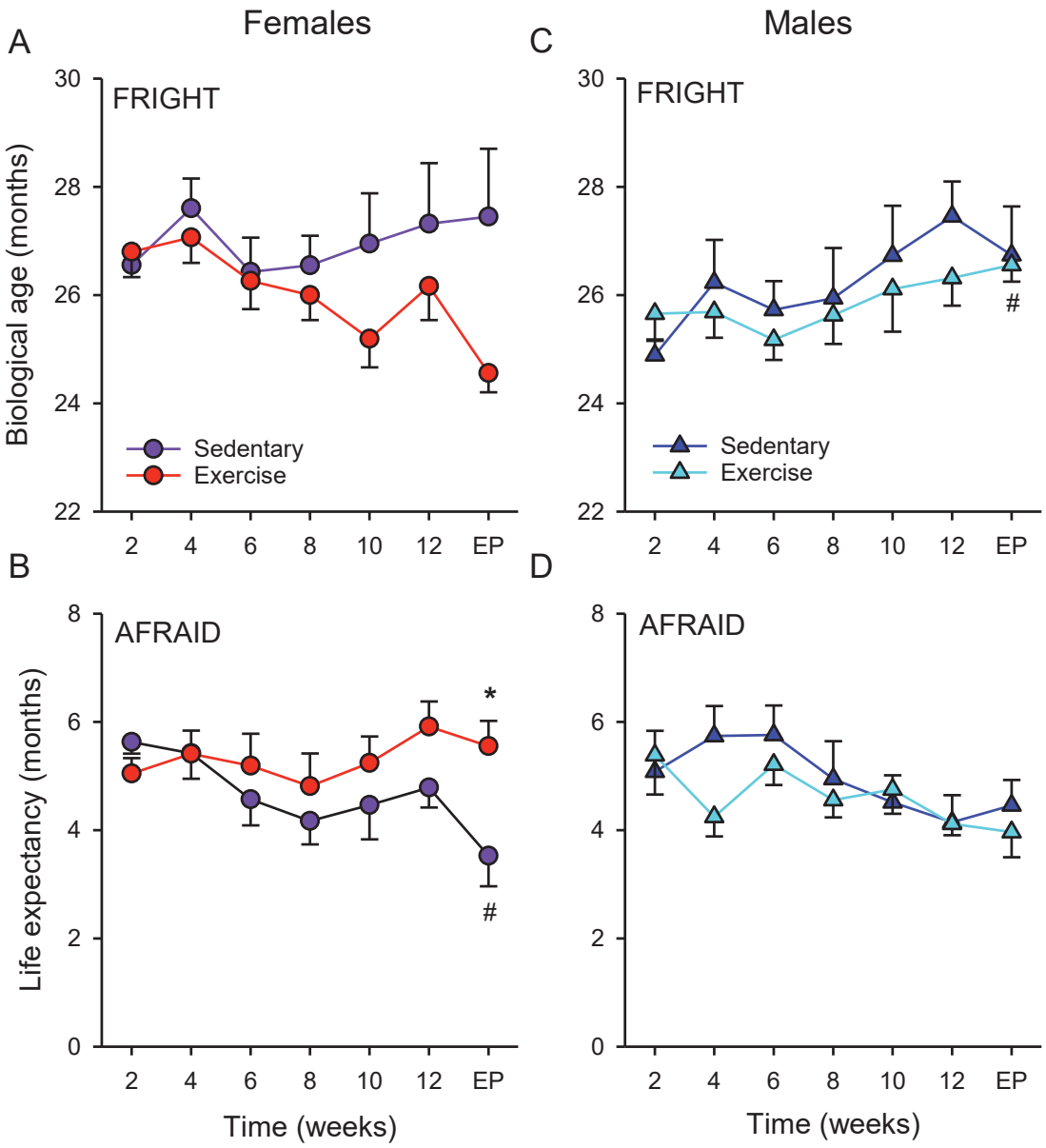

Figure 3.2.5: Exercise attenuated AFRAID scores in aging female mice but not in older males. In females, FRIGHT scores (**A**: Sedentary n=11-5, exercised n=11-6) were not altered by age or exercise. Conversely, AFRAID scores (**B**: sedentary n=11-5, exercised n=11-6) declined in sedentary but not exercised female mice. In males the FRIGHT scores (**C**: sedentary n=6-5, exercised n=6-5) and AFRAID scores (**D**: sedentary n=6-5, exercised n=6-5) showed no difference between exercised and sedentary mice. FRIGHT scores are a prediction of biological age while AFRAID scores predict time until death. Both AFRAID and FRIGHT scores were calculated using frailty data. All scores were analyzed using a linear mixed model and error is calculated as SEM. Let BL denote baseline and EP denotes endpoint. The * denotes difference between activity and sedentary while # denotes a difference within group from baseline, $p<0.05$.

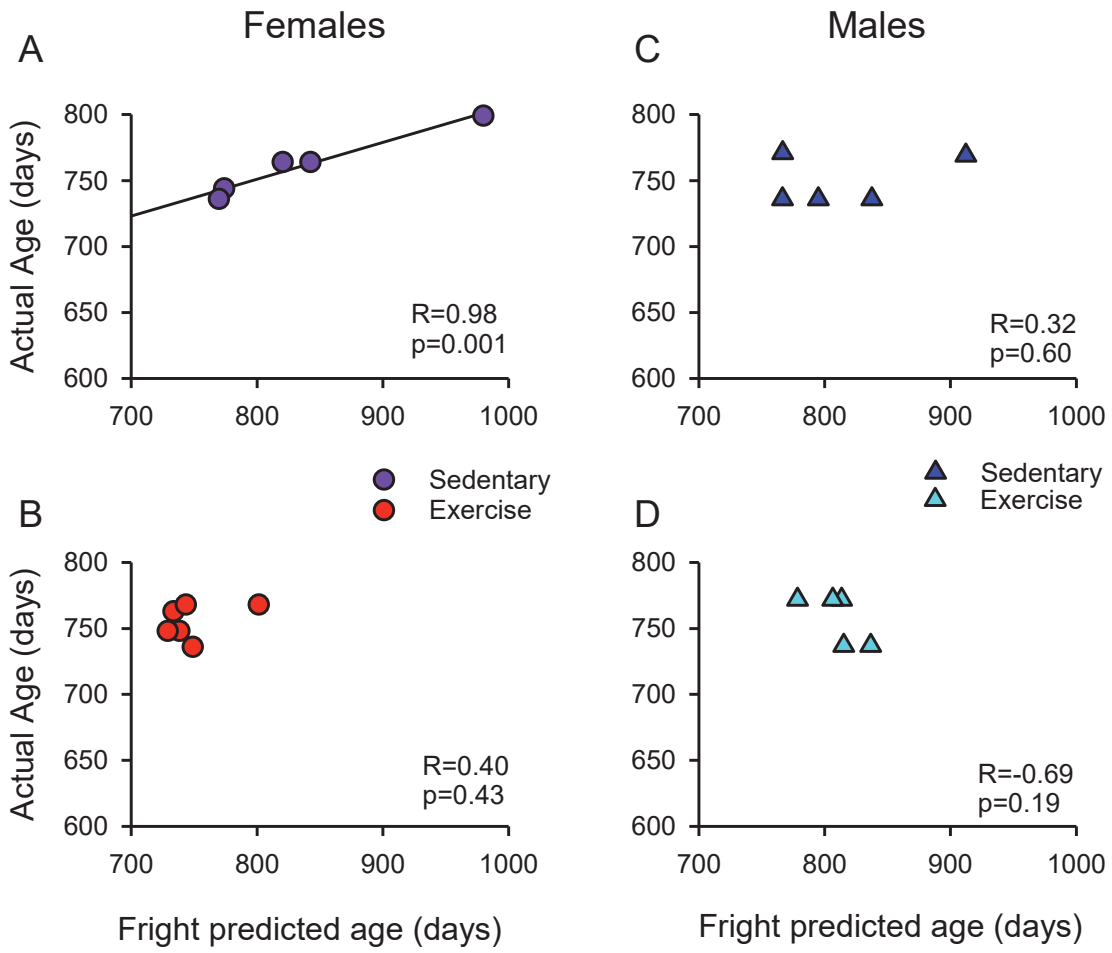

Figure 3.2.6: FRIGHT scores correlate with chronological age, but only in sedentary females. In exercised females (**A**: n=6) and sedentary females (**B**: n=5), the FRIGHT scores were positively correlated with the mouse's age, but only in sedentary animals. FRIGHT scores did not correlate with age in exercised males (**C**: n=5), or sedentary males (**D**: n=5). The analysis was done by correlating FRIGHT scores with frailty index at endpoint. Correlations were analyzed using a two-tailed Pearson's correlation. The regression line was only drawn if correlations were significant with a $p<0.05$.

3.3 Body composition

3.3.1 Exercise reduced signs of sarcopenia in female mice but had less impact in male mice.

As exercise had a positive impact in preventing the older mice from becoming more frail, we next investigated whether exercise attenuated age-related changes in body composition. In females, lean tissue (Figure 3.3.1A) increased significantly with time in the exercised group (64.2 ± 2.9% to 75.0 ± 2.6%: p=0.02) but only slightly in the sedentary mice (57.9 ± 4.7% to 69.4 ± 5.2%: p=0.06). Similarly, the exercised females exhibited a significant decline in fat tissue (Figure 3.3.1B: 35.6 ± 2.9% to 25.0 ± 2.6%: p=0.02), but this effect was attenuated in the sedentary mice (43.1 ± 4.9% to 30.6 ± 5.3%: p=0.06). In terms of total tissue (Figure 3.3.1C), while there was an overall effect of time (p=0.02), the decline was not significant in the sedentary (p=0.42) or exercised (p=0.06) female mice. In females, aerobic exercise increased lean tissue and reduced fat tissue without changing the overall tissue weight in the animals.

By contrast, the changes in body composition were similar regardless of activity in males. Lean tissue (Figure 3.3.1D) increased significantly in both sedentary (p=0.044) and exercised (p=0.003) mice. Likewise, the fat tissue (Figure 3.3.1E) decreased in both groups (sedentary p=0.045, exercise p=0.007). Conversely, for total tissue (Figure 3.3.1F), the exercised males showed a significant decline from baseline (50.9 ± 3.5 g to 36.2 ± 1.4 g: p=0.01) which was not present in the sedentary animals (p=0.075). In males, both sedentary and exercised animals gained lean and lost fat tissue, however only the exercised animals lost significant overall tissue weight.

Comparing all groups together (Table 3.3.1) with a 3-way linear mixed model reinforces the previously mentioned observations. The males showed similar results as the exercised, but not the sedentary females, giving overall interactions between time and sex. Interestingly, while the percent lean and fat tissue were similar across males and females, males had overall higher total tissue levels compared to females (Males: 41.1 ± 1.3 g vs Females: 34.3 ± 0.9 g: p=0.001).

3.3.2 Changes in body composition were not graded by frailty.

As the changes in body composition over time were different between the exercised and sedentary mice, particularly in the females, we next investigated whether these changes were graded by frailty. In the females (Figure 3.3.2) and males (Figure 3.3.3), neither the exercised nor sedentary mice exhibited any relationship between body composition at endpoint and frailty at endpoint. Interestingly, the only group with a strong, but still not significant relationship, is the total tissue compared to frailty in the sedentary males (Figure 3.3.3C: R=-0.86, p=0.06). This suggests that frailty and body composition may not have a linear relationship.

3.3.3 Changes in body composition were correlated with exercise in both male and female mice.

As the changes in body composition were unrelated to frailty at endpoint, we next investigated if they were instead related to activity levels. Both female and male mice (Figure 3.3.4) exhibited changes in body composition that were graded by the frequency of activity. In females, lean tissue increased with activity (Figure 3.3.4A: R=0.72, p=0.04) and fat tissue declined (Figure 3.3.4B: R=-0.71, p=0.05), while total tissue was

not correlated (Figure 3.3.4C: R=-0.52, p=0.19). In males, lean tissue and fat tissue showed similar trends, except that the relationships were not statistically significant. Conversely, in males, total tissue was negatively correlated with bouts per day (Figure 3.3.4F: R=-0.96, p=0.003). These correlations were only significant when comparing body composition to frequency of activity, and not the speed or duration (Table 3.3.2). The only exception is the males, where total tissue was also negatively correlated in peak speed (Table 3.3.2: R=-0.91, p=0.01). Thus, female mice who ran more frequently had higher lean tissue and lower fat tissue while male mice who ran more frequently had a decline in total tissue weight.

3.3.4 Exercised male mice had higher forelimb grip strength than sedentary males, a relationship not seen in female mice.

As aerobic exercise increased lean tissue in both sexes, and was even correlated with activity in females, we next investigated if changes in muscle strength also occurred. The sedentary female mice (Figure 3.3.5A), exhibited a decline in forelimb grip strength over time (baseline to endpoint: 0.71 ± 0.05 N to 0.41 ± 0.06 N: p=0.044) while exercised females did not decline (0.69 ± 0.04 N to 0.53 ± 0.04 N: p=0.22), but showed a slight decrease in grip strength at weeks 6 and 8 that was not seen by week 12. Sedentary male mice (Figure 3.3.5B) showed a decline in grip strength over time (0.53 ± 0.07 N to 0.40 ± 0.06 N: p=0.10) while exercised males gained strength over time (0.48 ± 0.05 N to 0.63 ± 0.05N: p= 0.09). The exercised males were stronger than the sedentary mice at week 10 (p=0.02) and week 12 (p=0.02). Interestingly, at baseline, females were stronger than the males (p=0.002). These findings indicate that, along with an increase in lean tissue over

time, exercised male mice had a corresponding increase in muscle strength while exercised females had slight protection against the decline seen in the sedentary females.

3.3.5 Exercise increased appetite in male mice.

As all mice lost fat tissue, we next investigated if the mice had consistent eating habits throughout the study. Females showed very little difference in food consumption with time ($p=0.48$) or activity ($p=0.26$) (Figure 3.3.6A). While sedentary females transiently ate more than exercised females at week 9 ($p=0.04$) and week 10 ($p=0.03$), this difference disappeared by week 11. However, in males (Figure 3.3.6C), exercise mice showed an increase in food consumption (baseline to endpoint: 3.9 ± 0.3g to 5.7 ± 0.5g: $p=0.002$) while sedentary males maintained their level of consumption throughout the study (baseline to endpoint: $p=0.19$). This increase was significantly higher than baseline at weeks 3, 4, and 8-12 in exercised males. Considering many of the mice, particularly the exercised males, lost weight throughout the study we next normalized food consumption to animal weight. In female mice, (Figure 3.3.6B) the daily food consumption was similar when normalized to the mouse's body weight, with no effect of time ($p=0.41$) or activity ($p=0.84$). The female mice ate the same amount throughout the study, regardless of activity. For the male mice (Figure 3.3.6D), the exercised group increased their food consumption from baseline. However, this increase was significant by week 2 ($p=0.05$) and continued until endpoint. The sedentary males showed a slight increase from baseline at weeks 3, 8, and 10. This suggests that the loss of tissue shown previously (Figure 3.3.1F) was not due to a reduction in calorie intake.

Both sedentary and exercised mice of both sexes exhibited changes in overall body composition. They lost fat tissue, gained lean tissue, and lost overall tissue over the course of the study. While these changes were not related to frailty, they were correlated with activity levels in both sexes. Exercise reduced the decline in forelimb grip strength in both sexes. The reduction in tissue weight could not be explained by a corresponding reduction in food consumption in the exercised animals.

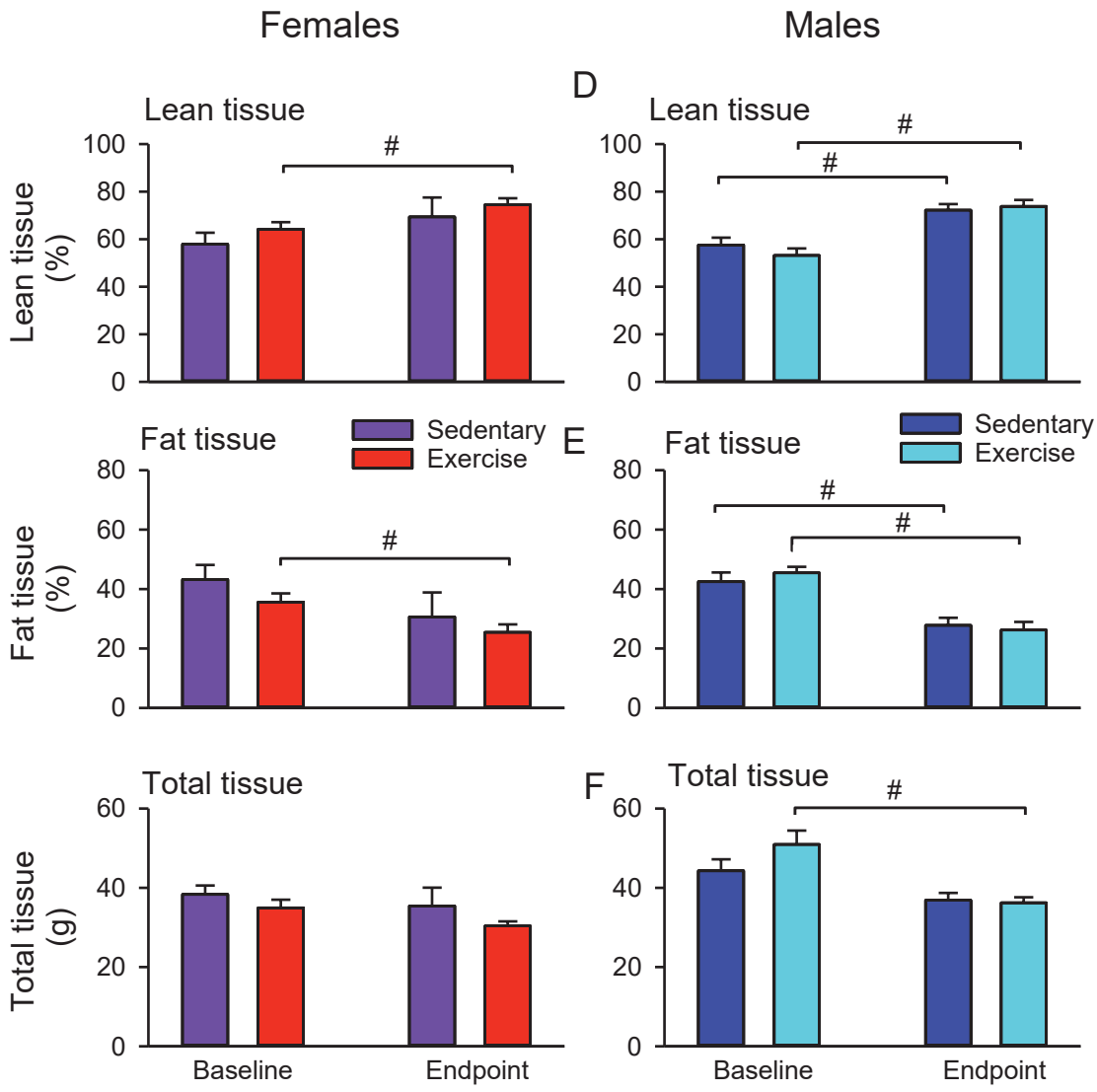

Figure 3.3.1: Exercise exacerbated the increase in lean tissue and decrease in fat tissue in female mice while having little impact on body composition in male mice. Female mice (**A**, **B**, **C**: sedentary n=11-5, exercise n=11-6) had a significant increase in lean tissue (**A**) and decrease in fat tissue (**B**) in exercised animals. Males (**D, E, F**: sedentary n=6-5, exercise n=6-5) showed similar trends in exercise and sedentary mice, where lean mass increased (**D**), fat mass declined (**E**) and total tissue declined (**F**). Analysis was done with a linear mixed model with time and activity as main variables, and error is calculated as SEM. Midpoint values were used in analysis but are not shown in the graph. Body composition was measured using dual x-ray absorptiometry. The # denotes a difference compared to baseline with $p<0.05$.

Table 3.3.1: The 3-way interactions of changes in body composition. Table shows the p values of a 3-way RM mixed model using body composition data from baseline, midpoint, and endpoint. The *denotes $p<0.05$ and a significant interaction.

Interaction	% Lean tissue	% Fat tissue	Total tissue
Time	**<0.001***	**<0.001***	**<0.001***
Sex	0.563	0.660	**0.001***
Activity	0.579	0.465	0.578
Time ^ Sex	**0.018***	**0.029***	**0.005***
Activity ^ Time	0.487	0.640	**0.033***
Sex ^ Activity	0.521	0.495	0.266
Sex ^ Activity ^ Time	0.394	0.448	0.074

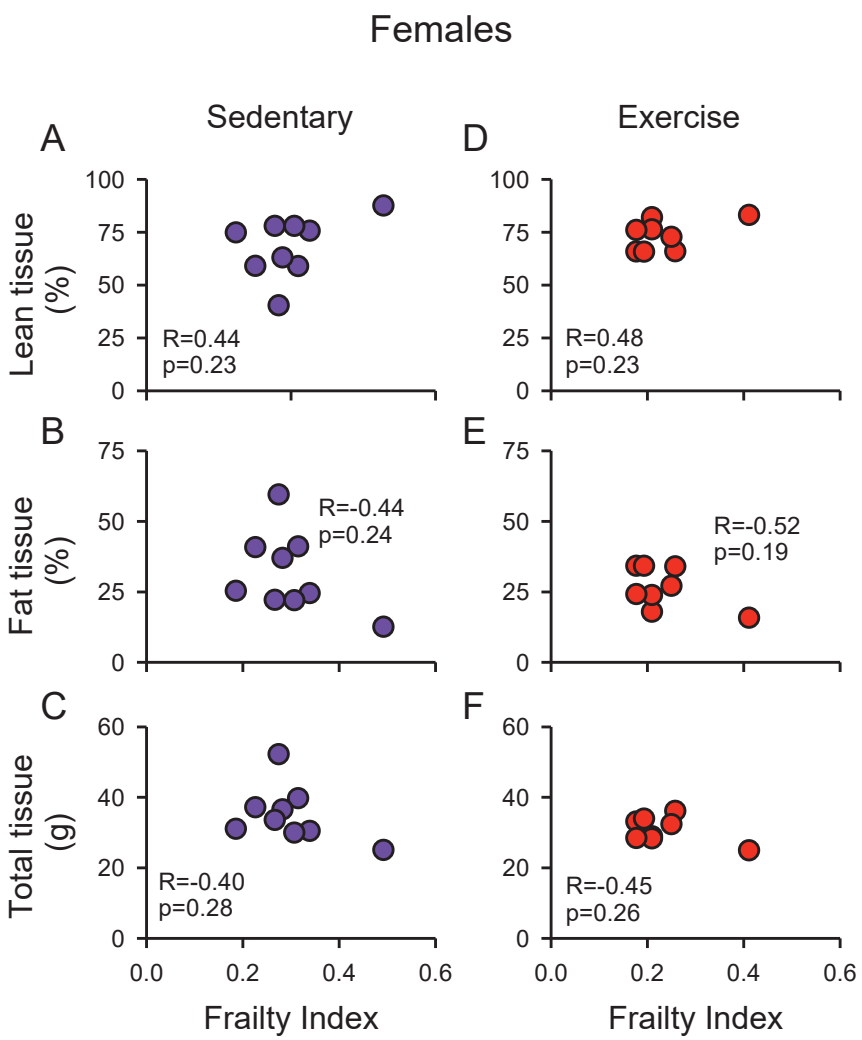

Figure 3.3.2: Changes in body composition were not graded by frailty in female mice. Body composition in sedentary females (**A**, **B**, **C**: n=8) and exercised females (**D, E, F**: n=9) showed no significant correlation with frailty. Correlations were done by plotting body composition at endpoint with the corresponding frailty index score and analyzed using a two-tailed Pearson's correlation test.

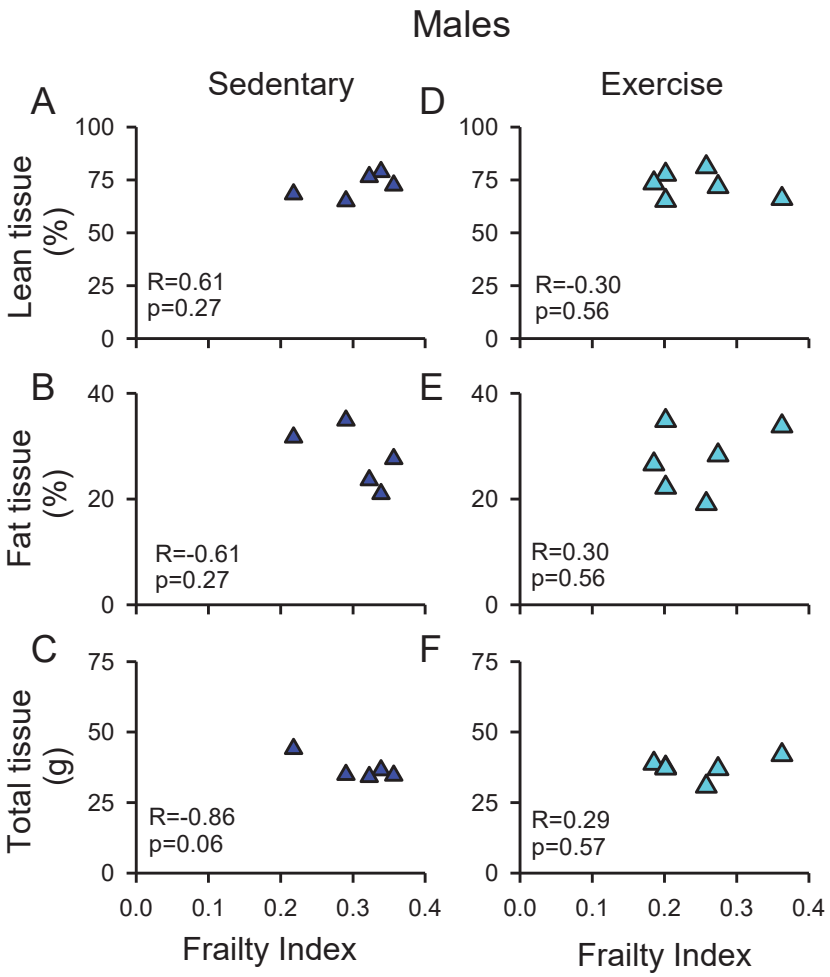

Figure 3.3.3: Changes in body composition were not graded by frailty in male mice. Body composition was not graded by frailty in either sedentary (**A**, **B**, **C**: n=5) or exercised males (**D, E, F**: n=6). Body composition was measured using dual x-ray absorptiometry and correlated with corresponding frailty scores from endpoint. Correlations were analyzed with two-tailed Pearson's correlation.

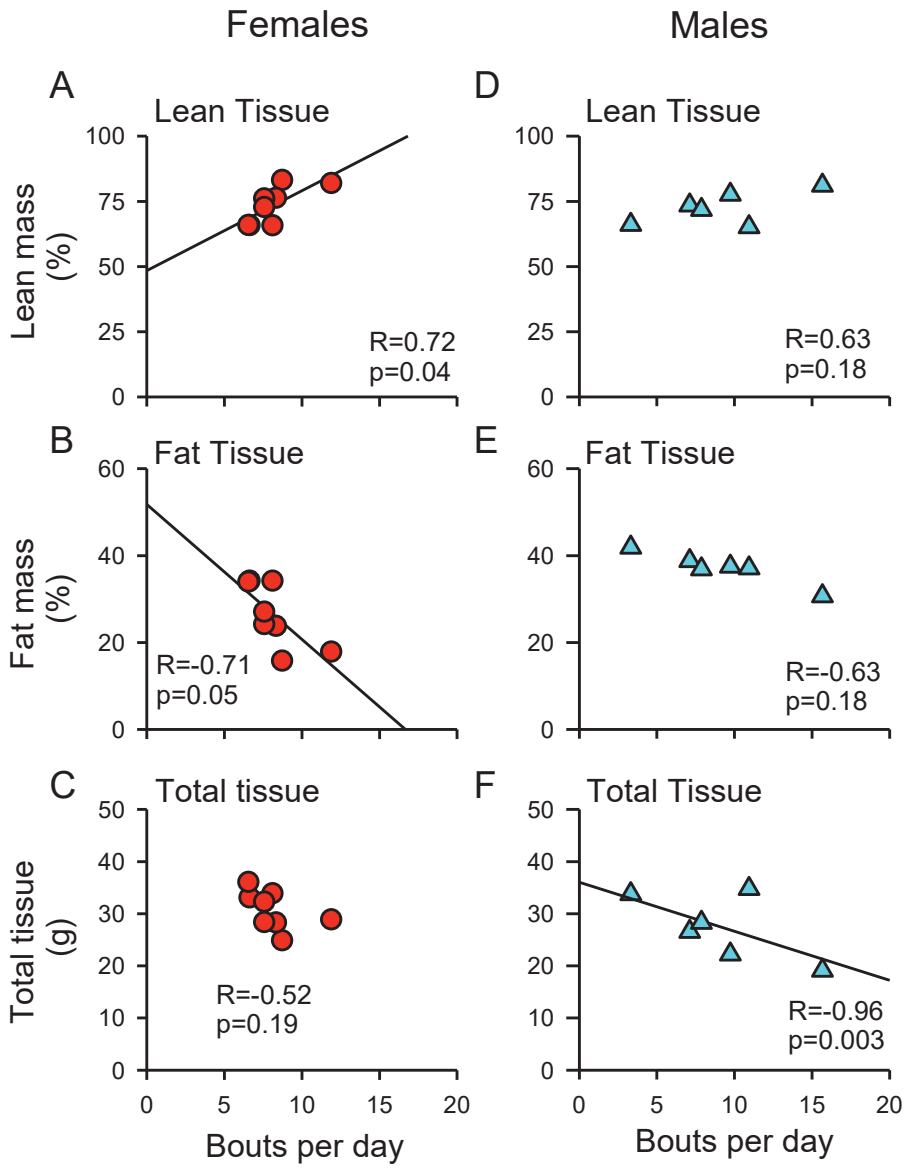

Figure 3.3.4: Changes in body composition were correlated with activity levels in both sexes. Female mice (**A, B**, **C**, n=8) showed a positive correlation between frequency of activity and lean mass (**A**) but a negative correlation with fat mass (**B**). Male mice (**D**, **E**, **F**, n=6) had a negative correlation between total tissue (**F**) and frequency of activity. Body composition was measured at endpoint using a DEXA scan while running frequency was calculated using bouts. Analysis was done with a Pearson's two-tailed correlation. Linear regression were only drawn if the correlation was significant.

Table 3.3.2: Activity parameters were correlated with changes in body composition. Table shows R values for body composition correlated with bouts. Analysis done with Pearson's correlations using endpoint DEXA values (Females n=8, males n=6). The * denotes $p<0.05$ and the correlation is considered significant.

	Body composition	**Bout length (min)**	**Peak speed (m/min)**	**Bouts per day**
Females	Lean tissue (%)	0.390	0.260	**0.724***
	Fat tissue (%)	-0.381	-0.229	**-0.715***
	Total tissue (g)	-0.336	-0.047	-0.516
	Average grip strength (N)	-0.044	0.124	-0.081
Males	Lean tissue (%)	0.434	0.677	0.634
	Fat tissue (%)	-0.430	-0.672	-0.627
	Total tissue (g)	-0.783*	**-0.911***	**-0.958***
	Average grip strength (N)	0.510	0.741	**0.822***

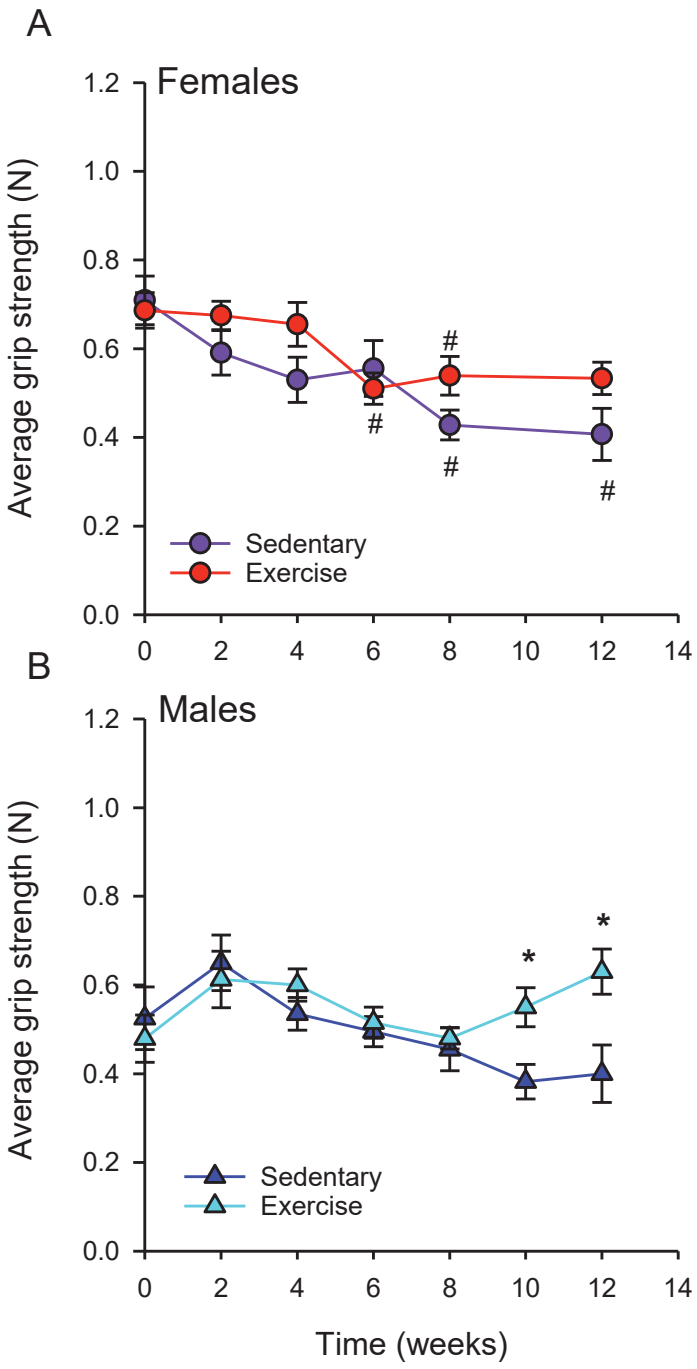

Figure 3.3.5: Exercise attenuated the age-related decline in grip strength in female mice but improved grip strength in male mice. Female mice (**A**: sedentary n=11-5, exercised n=11-6) and male mice (**B**: sedentary n=6-5, exercised n=6-5) had forelimb grip strength measured every two weeks. Analysis was done with a linear mixed, and error is calculated as SEM. The * denotes an effect of activity and # denotes an effect of time both with $p<0.05$.

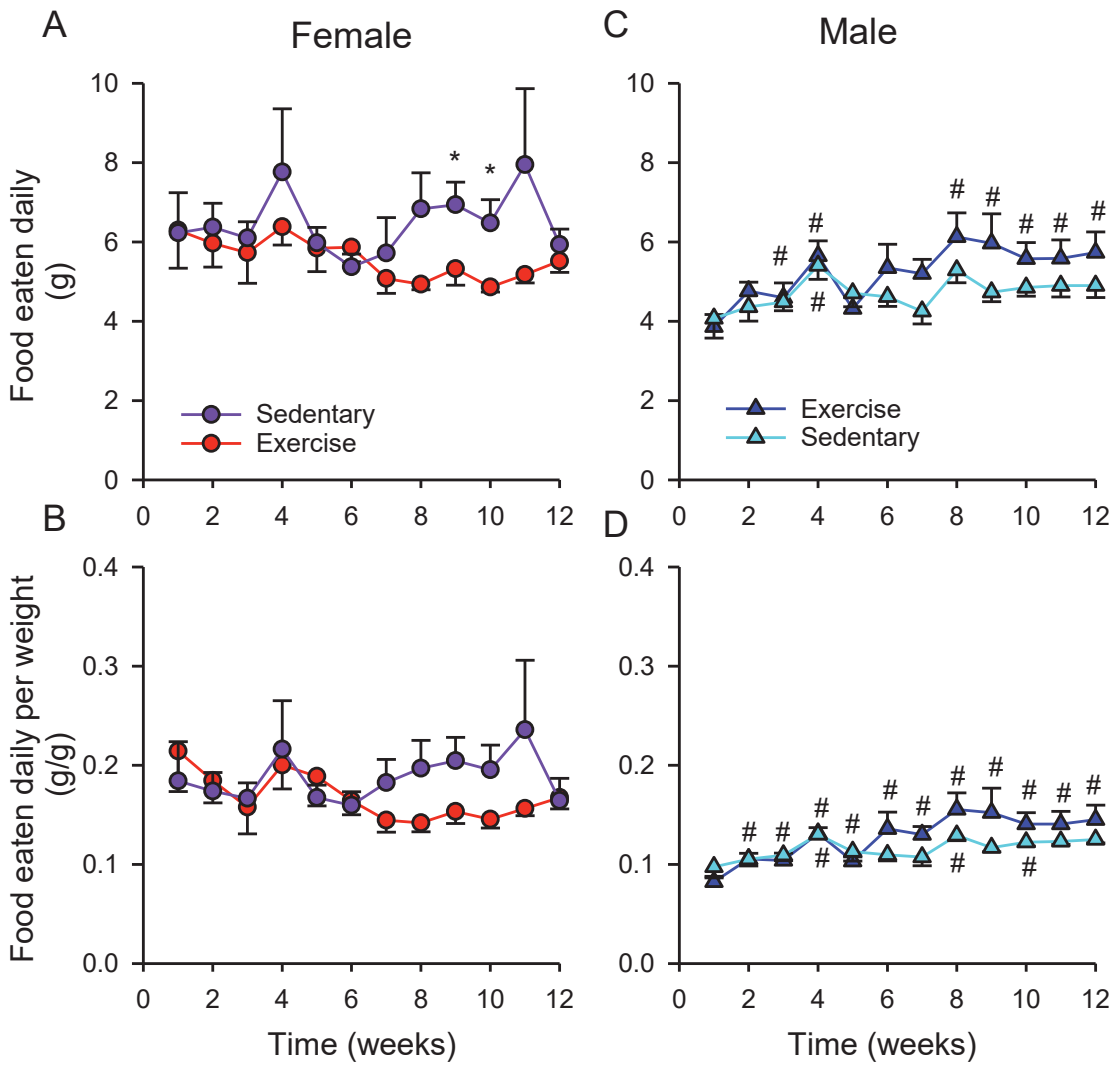

Figure 3.3.6: Male mice increased food consumption throughout the study, particularly in the exercised group. This was not seen in the female mice. Female mice (**A**, **B**: sedentary n=11-5, exercise n=11-6) and male mice (**C**, **D**: sedentary n=6-5, exercise n=6-5) had their food measured each week. This value per week was divided by 7 to get the amount eaten per day (**A**, **C**) and normalized to body weight (**B**, **C**) which was measured at the same time. Analysis was done with a linear mixed model, and error is calculated as SEM. The *denotes an overall effect of activity and # denotes a difference from baseline levels with $p<0.05$.

3.4 Cardiac structure and function

3.4.1 Changes in cardiac structure differ by sex and activity.

We next investigated whether late life aerobic exercise affected heart function. Interestingly, while most of the changes in cardiac structure over the course of the study were not significant, the adaptations were opposite in female and male mice. First, there were few changes in left ventricular size resulting from age or exercise (Figure 3.4.1). In females there were no significant changes in left ventricular (LV) diameter or volume over the course of the study regardless of exercise (Figure 3.4.1 A, B). By contrast, there were group differences between the exercise and control males (Figure 3.4.1 C, D), though these were due to random baseline differences not treatment effects. While only volume and diameter at systole are shown (Figure 3.4.1A-D), the same effects were seen in the heart at diastole.

When analyzing for three way interactions, all left ventricular size parameters did show interactions between sex and activity (Table 3.4.1). For the diameter and volume at diastole, these interactions were driven by endpoint values (D;d $p=0.04$, V;d $p=0.04$). For example, exercise reduced LV diameter in the females but increased LV diameter in the males. This resulted in sedentary females having similar levels to exercised males (D;d: female 4.46 ± 0.29 centimeters (cm) vs male 4.60 ± 0.17 cm), and exercised females having similar levels to sedentary males (D;d female 4.10 ± 0.12 cm vs male 4.17 ± 0.10 cm) at endpoint.

While left ventricular chamber size is a measure of hypertrophy, so is the mass of the left ventricle itself. Thus, we next investigated the mass of the left ventricle (Figure

3.4.2A, D) in both sexes, calculated from LV dimensions (Table 2.4.1). Female mice (Figure 3.4.2A) exhibited an overall effect of activity (p=0.017), driven by baseline differences (p=0.06). In all timepoints, sedentary females had heavier left ventricles than exercised females. Conversely, males (Figure 3.2.4D) both sedentary and exercised mice exhibited a non-significant increase in left ventricular mass.

There were also sex-specific differences in LV wall thickness. The LV anterior wall (Figure 3.4.2B) significantly declined with age in the sedentary female mice (p=0.03) but not exercised mice (p=0.39). For males, LV anterior wall thickness declined with age in both the sedentary and exercised mice (Figure 3.4.2E: p=0.005). Interestingly, the posterior wall thickness was unaffected by time in mice of both sexes for both activity levels (Figure 3.4.2 C, F). There was a decline in LV anterior wall thickness with time in all mice, except the exercised females.

3.4.2 Exercise affected cardiac function in older males but not females.

We next investigated the impact of exercise on measures of systolic function. Females showed no changes with activity or age in either ejection fraction (Figure 3.4.3A) or fractional shortening (Figure 3.4.3B). In males, there were baseline differences in EF and FS. There was also an interaction between activity and time for both ejection fraction (Figure 3.4.3C: p=0.006) and fractional shortening (Figure 3.4.3D: p=0.005). This was driven by the age-related decrease in sedentary males (EF: p=0.032, FS: p=0.038) which did not occur in the exercised males.

Systolic function also showed the same interaction as seen for LV volume and diameter mentioned above. There was a significant interaction between sex and activity

(Table 3.4.1) for EF and FS, which was driven by endpoint values. In summary, at endpoint the sedentary female and exercised male mice had similar contractile parameters (EF: females 52.5 ± 9.6% vs males 52.2 ± 4.5%) while exercised females resembled sedentary males (EF: female 66.1 ± 3.4% vs males 64.5 ± 3.5%). Like cardiac structure, systolic function showed that hearts in male and female mice adapted differently to age and exercise.

We next investigated the effect of aerobic exercise on another measure of systolic function, the IVCT. Female mice (Figure 3.4.4A) exhibited no effect of age (p=0.31) or activity (p=0.77) as seen for previous systolic parameters. Curiously, the IVCT in males (Figure 3.4.4C) was also not impacted by age or activity. We then examined the MPI, which is a parameter calculated from both systolic and diastolic parameters (IVRT, IVCT, and AET). Female mice (Figure 3.4.4B) exhibited an overall age-related decline in MPI (p=0.024), while male mice (Figure 3.4.4D) did not. This suggests that while systolic function was preserved in exercised male mice, diastolic function may have also been affected.

3.4.3 Exercise prevented the age-related decline in diastolic function in male but not female mice.

Having found systolic function was differentially impacted by sex, age, and exercise, we next investigated diastolic function using both the E/A ratio and IVRT. There was no effect of time (p=0.32) or exercise (p=0.77) in E/A ratios for female mice (Figure 3.4.5A). There was no effect of time or activity on IVCT measured in sedentary and exercised females (Figure 3.4.5B). As for systolic function, diastolic function did not change during the intervention in either the exercise or sedentary female mice.

Conversely, the male mice did show signs of diastolic dysfunction which was affected by exercise. Exercise protected male mice against the age-related decline in the E/A ratio (Figure 3.4.5C) seen in the sedentary males (1.40 ± 0.13 to 0.98 ± 0.10: p=0.022). Likewise, exercise protected the male mice against the age-related shortening of the IVRT (Figure 3.4.5D). While there was a baseline group difference in male IVRT (p=0.04), only the sedentary males exhibited a decrease over the course of the study (Sedentary p=<0.001, exercise p=0.32). In male mice, exercise protected hearts against age-related diastolic dysfunction, and this was not seen in the females.

Considering that exercise modified diastolic function, at least in male hearts, we next investigated changes in cardiac fibrosis were involved. Cardiac fibrosis was analyzed by measuring hydroxyproline concentrations in ventricular tissue (Figure 3.4.5E). Interestingly, though exercise had no impact on cardiac fibrosis, there were clear sex-specific effects. Levels of cardiac fibrosis were higher in female hearts than male hearts regardless of activity level. This suggests that changes in fibrosis are not involved in the effects of aerobic exercise on diastolic function.

3.4.4 Measures of cardiac structure and function only graded by frailty in the sedentary male mice.

As several cardiac parameters were affected both by age and exercise, we investigated the relationship between frailty and these parameters. Table 3.4.2 shows correlations between endpoint cardiac parameters and the corresponding frailty index score. There were no significant correlations in the female groups and the only significant correlations were found in the sedentary male mice. They showed a negative correlation between frailty and left ventricular size (D;d: R=-0.93 p=0.02, V;d: R=-0.93 p=0.02) and

a positive correlation with posterior wall thickness (LVPW;s: R=0.91, p=0.03). Thus, sedentary males with higher FI scores had larger and thicker hearts.

3.4.5 Measures of cardiac structure and function were only graded by activity in the female mice

As the cardiac parameters showed no correlation with frailty in the exercised animals of either sex, we next investigated if the endpoint values were instead graded by activity. In female mice (Table 3.4.3), many of the measures of activity were correlated with measures of endpoint cardiac structure and function. Exercise duration was positively correlated with left ventricular mass (LV mass: R=0.79, p=0.02) and anterior wall thickness (LVAW;d: R=0.90, p=0.002). The peak speed was also positively correlated with both anterior wall thickness (LVAW;d: R=0.78, p=0.02) and posterior wall thickness (LVPW;s: R=0.82, p=0.01). The frequency of running was positively correlated with isovolumetric contraction time (IVCT: R=0.90, p=0.002). In contrast, there were very few correlations between activity and cardiac parameters in males. The only exception was a positive correlation for isovolumetric relaxation time (IVRT: R=0.86, p=0.03). This shows that females with higher activity had hearts with thinner walls and faster contraction times but males with higher activity had longer relaxation times.

To conclude, when exercise was introduced to older male and female mice the resulting changes were subtle but had sex-specific results. Systolic function and ventricular size showed general opposite adaptions in male and female mice. Exercise protected male hearts from signs of diastolic dysfunction. These adaptations were

correlated with frailty only in sedentary male mice and graded by exercise only in female mice.

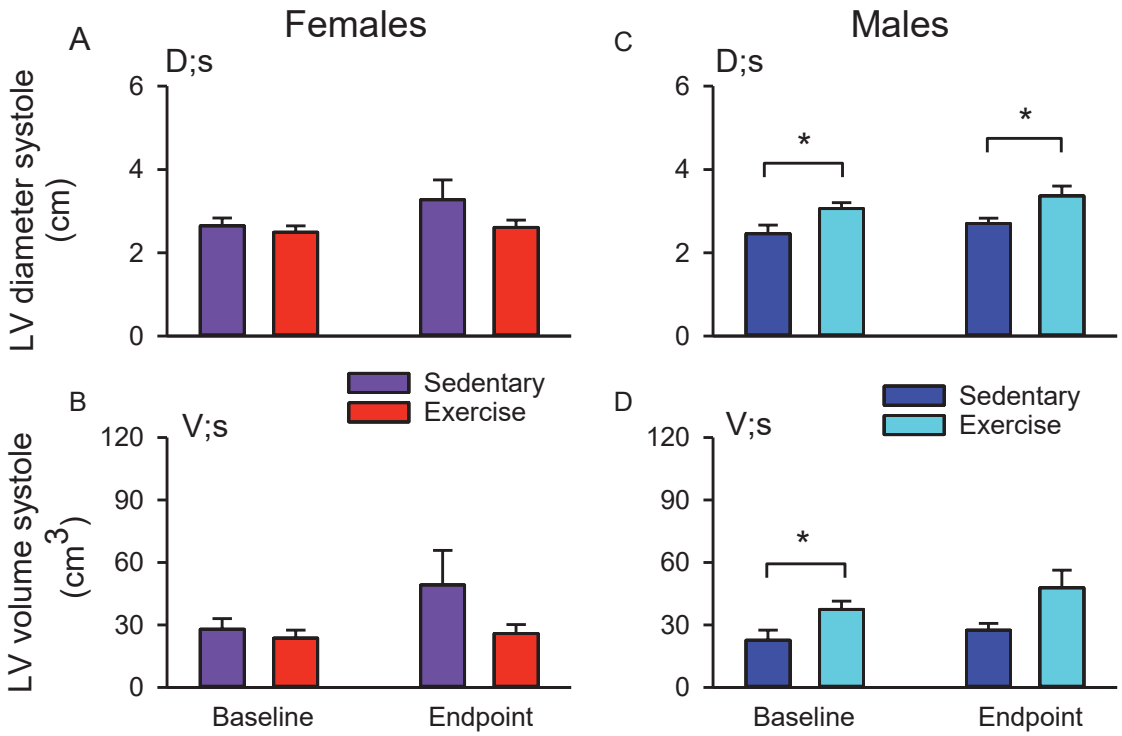

Figure 3.4.1: Neither age nor activity affected left ventricular size. Female mice (**A, B**: baseline sedentary n=11, baseline exercise n=11, endpoint sedentary n=5, endpoint exercise n=6) and male mice (**C, D**: baseline sedentary n=6, baseline exercise n=6, endpoint sedentary n=5, endpoint exercise n=5) had their left ventricular size derived from M-mode short axis images at baseline, midpoint (not shown) and endpoint. Analysis was done using a linear mixed model with activity and time as main factors, and error is calculated as SEM. The * denotes $p<0.05$.

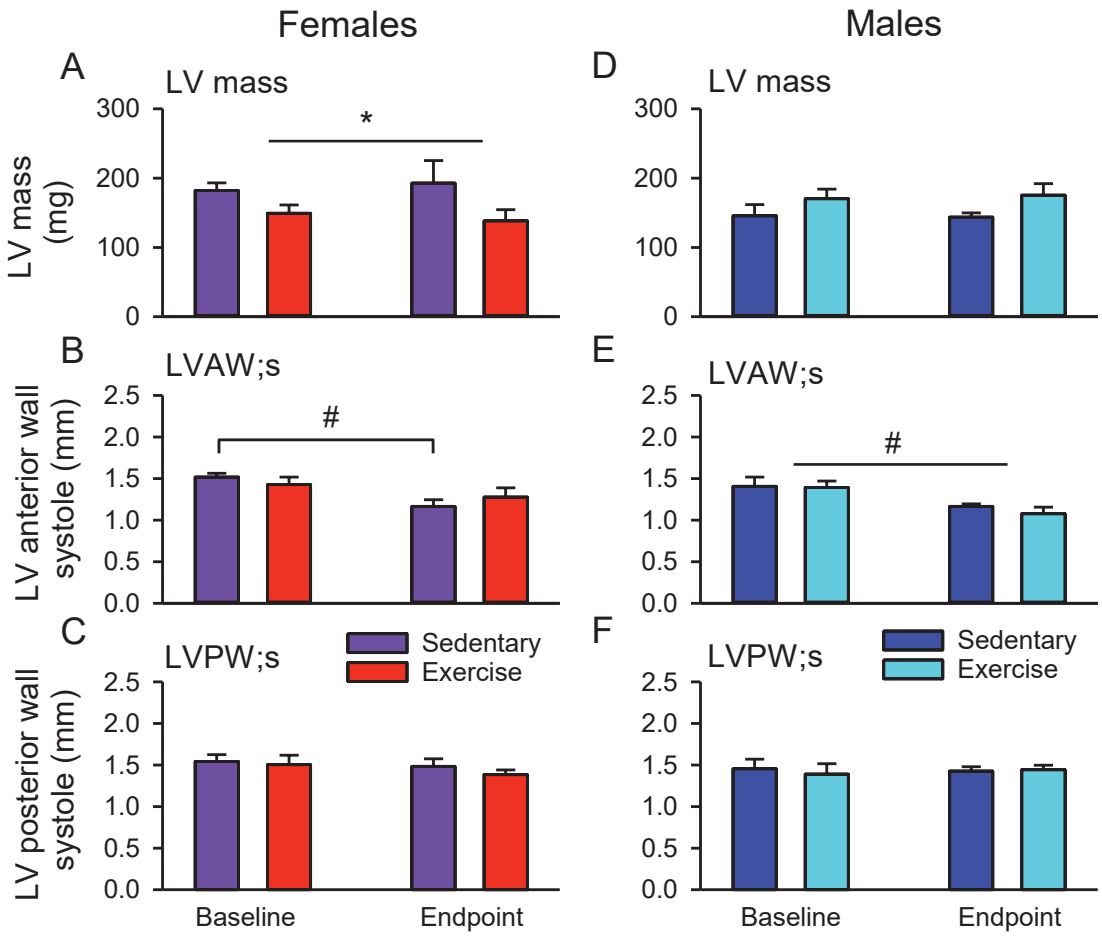

Figure 3.4.2: In females, the reduction in cardiac wall thickness occurred only in the sedentary mice while in males both wall thickness and left ventricular weight declined regardless of activity. Female mice (**A-C**: baseline sedentary n=11, baseline exercise n=11, endpoint sedentary n=5, endpoint exercise n=6) and male mice (**D-F**: baseline sedentary n=6, baseline exercise n=6, endpoint sedentary n=5, endpoint exercise n=5) had their left ventricular mass and wall thickness derived from M-mode short axis images at baseline, midpoint (not shown), and endpoint. Analysis was done using a linear mixed model with activity and time as main factors, and error is calculated as SEM. The * denotes a significance difference with activity while # denotes a difference with time with $p<0.05$.

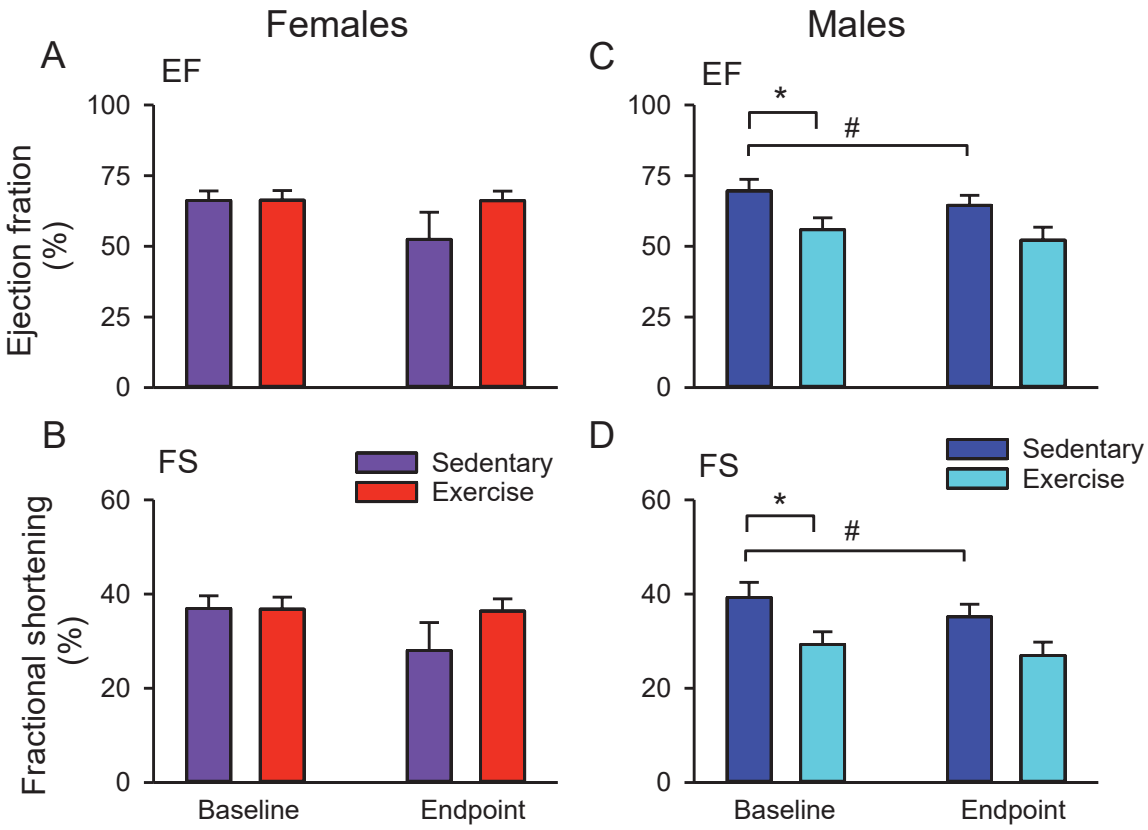

Figure 3.4.3: Exercise prevented the age-related decline in EF and FS in male hearts but had little effect in female hearts. Female mice (**A, B**: baseline sedentary n=11, baseline exercise n=11, endpoint sedentary n=5, endpoint exercise n=6) and male mice (**C, D**: baseline sedentary n=6, baseline exercise n=6, endpoint sedentary n=5, endpoint exercise n=5) had their fractional shortening and ejection fraction calculated from M-mode short axis images at baseline, midpoint (not shown) and endpoint. Analysis was done using a linear mixed model with activity and time as main factors, and error is calculated as SEM. The * denotes a significance difference between exercise and sedentary while # denotes difference from baseline with $p<0.05$.

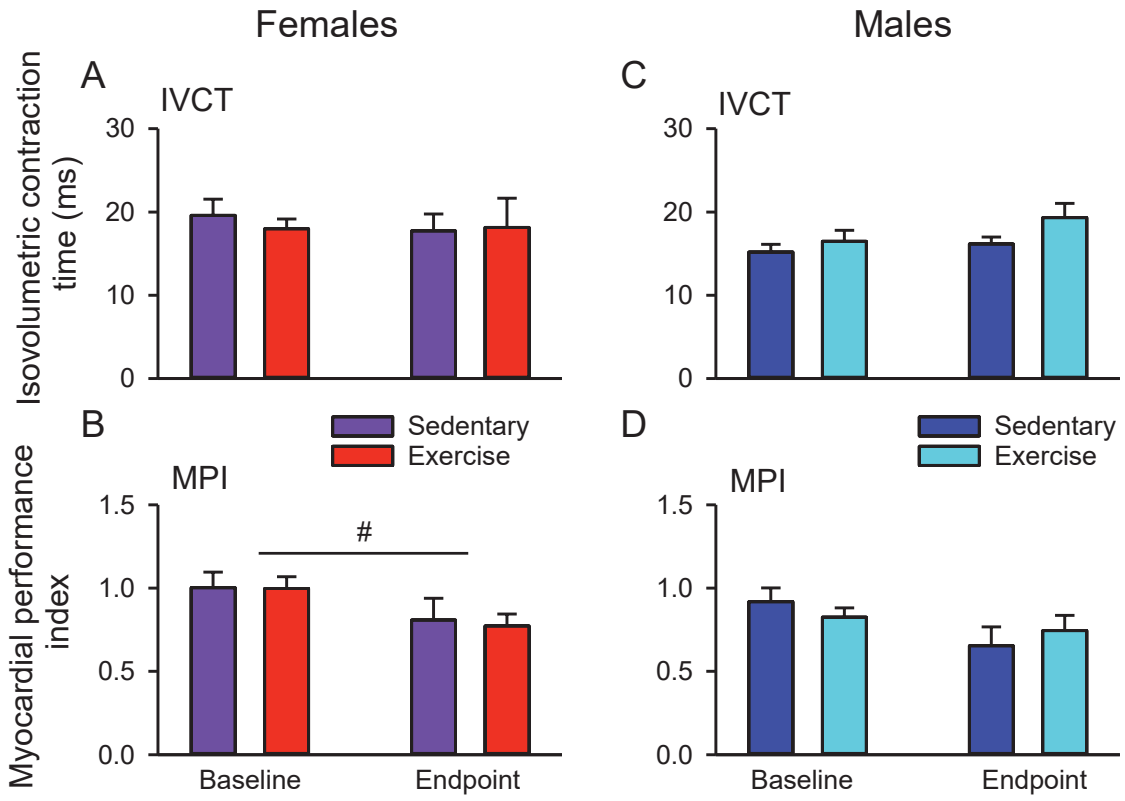

Figure 3.4.4: The combined measures of systolic and diastolic function were not impacted by exercise in the older female or male mice. Female mice (**A, B**: baseline sedentary n=11, baseline exercise n=11, endpoint sedentary n=5, endpoint exercise n=6) and male mice (**C, D)**: baseline sedentary n=6, baseline exercise n=6, endpoint sedentary n=5, endpoint exercise n=5) had their mitral valve function measured at baseline, midpoint (not shown), and endpoint using tissue and pulse wave doppler of the apical 4 chamber view of the mitral valve. Analysis was done with a linear mixed model where error was calculated as SEM. The # denotes a significant effect of time with $p<0.05$.

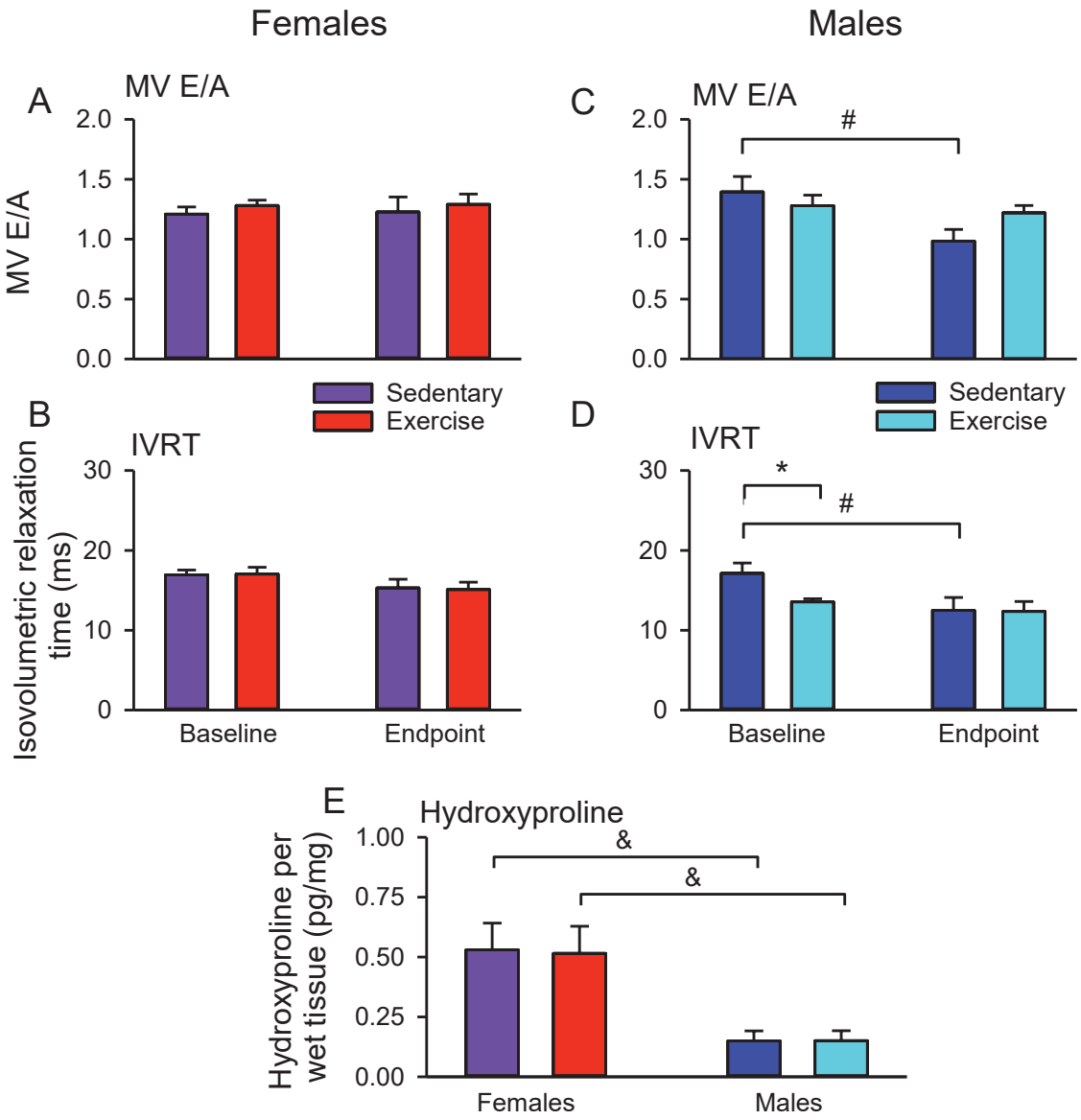

Figure 3.4.5: Exercise prevented the decline in diastolic function in male mice but not female mice, even though female mice had more cardiac fibrosis. Female mice (**A, B**: baseline sedentary n=11, baseline exercise n=11, endpoint sedentary n=5, endpoint exercise n=6) and male mice (**C, D)**: baseline sedentary n=6, baseline exercise n=6, endpoint sedentary n=5, endpoint exercise n=5) had their mitral valve function measured using tissue and pulse wave doppler in the apical 4 chamber view. Hydroxyproline (D: sedentary female n=9, exercise females n=11, sedentary males n=6, sedentary males n=6) levels were measured using tissue harvested at endpoint (terminal or censored) as detailed in methods. Analysis was done with a linear mixed model and error was calculated as SEM. The * denotes difference in activity, # denotes a significant difference from baseline and & denotes a sex difference with $p<0.05$.

Table 3.4.1: 3-way interactions for echocardiography parameters. The table shows p-values. Mice had cardiac parameters measured using echocardiography at baseline, midpoint, and endpoint. Details on calculations and abbreviations are found in the methods. The * denotes a $p<0.05$.

Group	Time	Sex	Activity	Time ^ Sex	Time ^ Activity	Sex ^ Activity	Sex^ activity^ timepoint
D;s	**0.023***	0.369	0.531	0.917	0.445	**0.014***	0.155
D;d	**0.047***	0.171	0.592	0.993	0.953	**0.006***	0.423
V;s	**0.037***	0.553	0.688	0.853	0.579	**0.011***	0.071
V;d	0.055	0.212	0.599	0.986	0.986	**0.006***	0.392
EF	0.066	0.590	0.566	0.678	0.051	**0.037***	0.086
FS	0.067	0.537	0.537	0.766	**0.046***	**0.045***	0.125
LV mass	0.053	0.414	0.212	**0.024***	**0.440**	**0.045***	0.217
LVAW;s	**0.001***	0.767	0.572	0.169	0.875	0.657	0.576
LVAW;d	**0.031***	0.188	0.197	**0.019***	0.397	0.560	0.586
LVPW;s	0.700	0.816	0.613	0.410	0.846	0.429	0.667
LVPW;d	0.756	0.217	0.254	0.478	0.990	0.198	0.755
A'/E'	0.868	0.164	**0.026***	0.200	0.382	0.122	0.467
E'/A'	0.407	**0.046***	0.070	0.406	0.127	0.575	0.808
MPI IV	**0.002***	0.050	0.884	0.929	0.722	0.958	0.704
MV E/A	0.178	0.534	0.901	0.427	0.516	0.551	0.380
IVCT	0.632	0.515	0.611	0.203	0.720	0.364	0.952
IVRT	**0.001***	**0.003***	0.271	0.569	0.325	0.644	0.085

Table 3.4.2: Correlation between frailty and cardiac parameters. The table shows R-values calculated by the two-tailed Pearson's correlation. Relationships is between echocardiography parameters and frailty index at endpoint. The * denotes $p<0.05$.

Group	Female sedentary	Female Exercise	Male Sedentary	Male Exercise
D;s	-0.225	0.349	-0.492	-0.280
D;d	-0.115	0.323	**-0.935***	-0.242
V;s	-0.236	0.304	-0.498	-0.294
V;d	-0.132	0.313	**-0.934***	-0.249
EF	0.294	-0.303	0.174	0.254
FS	0.294	-0.318	0.195	0.254
LV mass	0.096	0.048	-0.234	0.271
LVAW;s	-0.101	0.040	-0.278	0.680
LVAW;d	-0.183	0.054	-0.366	0.531
LVPW;s	0.499	-0.202	0.525	0.339
LVPW;d	0.244	-0.259	**0.907***	0.199
A'/E'	-0.084	0.295	-0.244	-0.539
E'/A'	0.070	-0.333	0.222	0.598
MPI IV	-0.154	0.181	-0.789	-0.483
MV E/A	-0.006	-0.259	-0.638	0.523
IVCT	0.107	0.045	0.715	-0.609
IVRT	0.325	0.053	-0.819	-0.582

Table 3.4.3: Correlations between activity parameters and cardiac parameters in female mice. Table showing R values calculated using a two-tailed Pearson's correlation between bouts and echocardiography parameters at endpoint (n=8-6). Bout measurements come from an average of the 5 previous days before the echocardiography was performed. The * denotes $p<0.05$.

Group	Bout length (min)	Peak rate (m/min)	Bouts per day
D;s	0.167	-0.177	-0.131
D;d	-0.200	-0.514	-0.610
V;s	0.148	-0.174	-0.122
V;d	-0.199	-0.502	-0.591
EF	-0.395	-0.088	-0.219
FS	-0.426	-0.122	-0.263
LV mass	**0.794***	0.625	0.415
LVAW;s	0.697	0.675	0.694
LVAW;d	**0.901***	**0.777***	0.686
LVPW;s	0.104	0.472	0.585
LVPW;d	0.650	**0.822***	0.671
A'/E'	-0.316	-0.391	-0.623
E'/A'	0.140	0.273	0.382
MPI IV	0.535	0.379	0.491
MV E/A	-0.028	0.235	-0.240
IVCT	0.623	0.645	**0.903***
IVRT	-0.271	-0.275	-0.024

Table 3.4.4: Correlations between activity and cardiac structure and function in male mice. Table showing R values calculated using a two-tailed Pearson's correlation between bouts and echocardiograph parameters from endpoint. Bout measurements come from an average of the 5 previous days before the echocardiography was performed. The * denotes a significant effect with $p<0.05$.

Group	Bout length (min)	Peak rate (m/min)	Bouts per day
D;s	0.123	0.242	0.200
D;d	-0.116	-0.109	-0.098
V;s	0.136	0.234	0.187
V;d	-0.097	-0.088	-0.082
EF	-0.336	-0.543	-0.463
FS	-0.325	-0.542	-0.463
LV mass	-0.339	-0.132	-0.240
LVAW;s	-0.195	0.172	0.082
LVAW;d	-0.176	0.169	0.067
LVPW;s	-0.555	-0.733	-0.753
LVPW;d	-0.349	-0.410	-0.497
A'/E'	0.061	0.017	-0.032
E'/A'	-0.164	-0.116	-0.041
MPI IV	0.639	0.340	0.351
MV E/A	-0.454	-0.428	-0.334
IVCT	-0.067	-0.245	-0.183
IVRT	**0.863***	0.633	0.620

3.5 Chronic inflammation

3.5.1 Exercise prevented the age-related decline in female serum cytokines

The aging of the immune system is a key mechanism of frailty. As such, we next investigated the levels of inflammation in the serum of older male and female mice and to determine how this was impacted by aerobic exercise. Serum cytokine levels at each endpoint were normalized tothe baseline for each sex. For example, serum cytokine levels in females (Figure 3.5.1A) were divided by baseline levels then compared between the exercised and sedentary groups. There were significant differences in the endpoint cytokine levels between the sedentary and exercised female mice for IL-3 ($p=0.02$), IL-12(p70) ($p=0.03$), C-GSF ($p=0.05$), TNFα ($p=0.03$), IL-4 ($p=0.04$), and macrophage inflammatory protein (MIP-1α) ($p=0.03$) where levels were lowest in the sedentary females. This was driven by a decline in cytokines in sedentary females and an attenuation of cytokines in exercised mice. By contrast, there was no difference in cytokine levels between exercised and sedentary males (Figure 3.5.1B). Of note, the difference in the females was driven by the decline in cytokine levels in the sedentary group. When comparing cytokine levels in female and male sedentary mice with a T-test, the female sedentary mice had significantly lower levels than the males for IL-2 ($p=0.02$), IL-12(p70) ($p=0.03$), IL-17A ($p=0.008$), IL-13 ($p=0.03$), keratinocyte-derived chemokine (KC) ($p=0.008$) and MIP-1β ($p=0.03$). When comparing the two exercised groups, there was no difference between the sexes in serum cytokine levels. The age-related decline in cytokines was only seen in the sedentary females and was prevented by exercise.

3.5.2 The females exhibited a positive correlation between serum cytokines and exercise, which was not seen in the males

As exercise protected female hearts from the age-related decline in cytokines, we next examined whether this effect was graded by activity levels**.** In female mice (Figure 3.5.2), serum cytokine markers were positively correlated with exercise. This positive relationship was seen with the pro-inflammatory cytokines IL-2 (R=0.87, p=0.01), IL-3 (R=0.82, p=0.01), IL-5 (R=0.78, p=0.02), IL-6 (R=0.85, p=0.01), IL-9 (R=0.79, p=0.02) and IFNγ (R=0.83, p=0.01). The females also exhibited a positive correlation between the pro-inflammatory chemokine KC (R=0.75, p=0.03) and frequency of activity. This relationship between activity and cytokines levels was not seen in the males (Figure 3.5.3). In females, higher levels of activity corresponded to higher levels of pro-inflammatory cytokines, an effect that was not seen in the males.

In older male and female mice, markers of inflammation were only affected by age and activity in the females. Serum cytokine levels were correlated positively with the frequency of exercise in the females. Exercise protected the older females against the systemic decline in serum cytokines observed with age in the sedentary females.

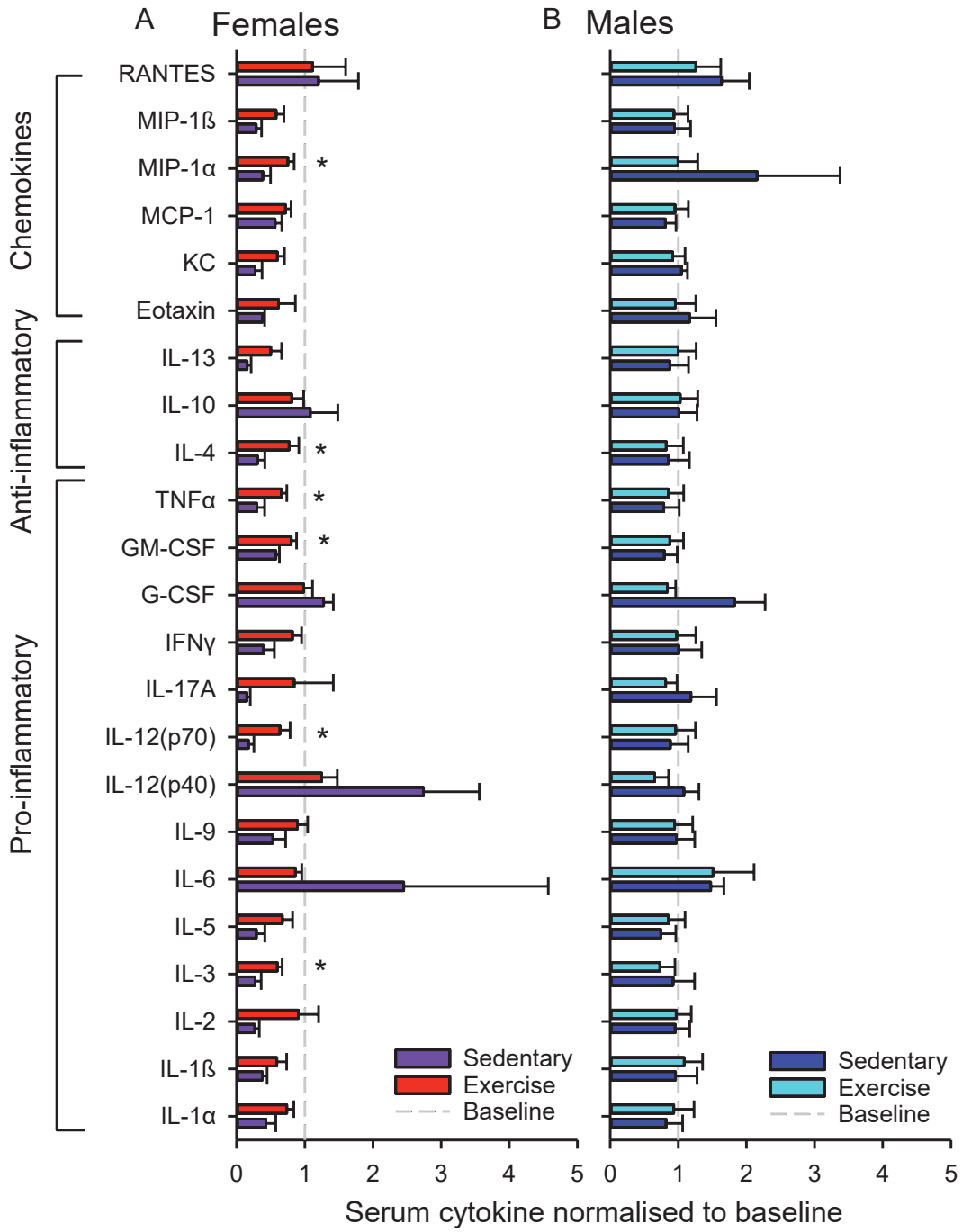

Figure 3.5.1: Exercise prevented the decline in serum cytokines seen in sedentary females but neither age nor exercise had an effect in male mice. Female (**A**, sedentary n=5, exercise n=6) serum cytokines were normalized to female baseline values. Male mice (**B**, sedentary n=5, exercise n=5) had serum cytokines normalized to male baseline values. A reference line was drawn at x=1 for both female and male mice. The difference between sedentary and male mice was calculated using a two-tailed Student's T-test, and error was calculated as SEM. The * denotes $p<0.05$

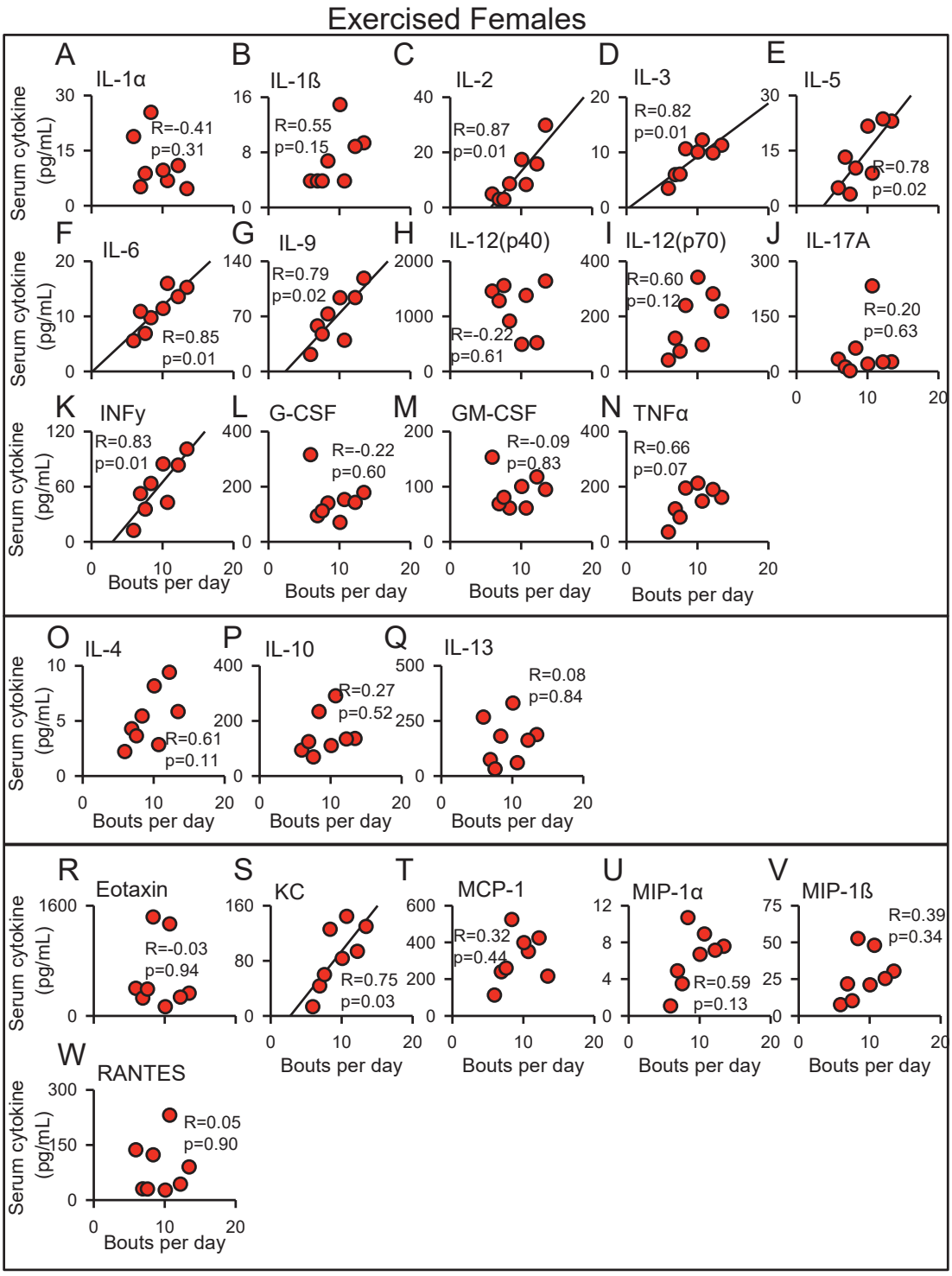

Figure 3.5.2: Exercise levels were correlated with serum cytokines levels in female mice. Female mice (**A-W**: n=8) had exercise levels compared to inflammatory markers at endpoint. Exercise levels were calculated as bouts per day. Correlations were calculated as two-tailed Pearson's correlations. Linear regressions were only drawn if the relationship was significant with $p<0.05$.

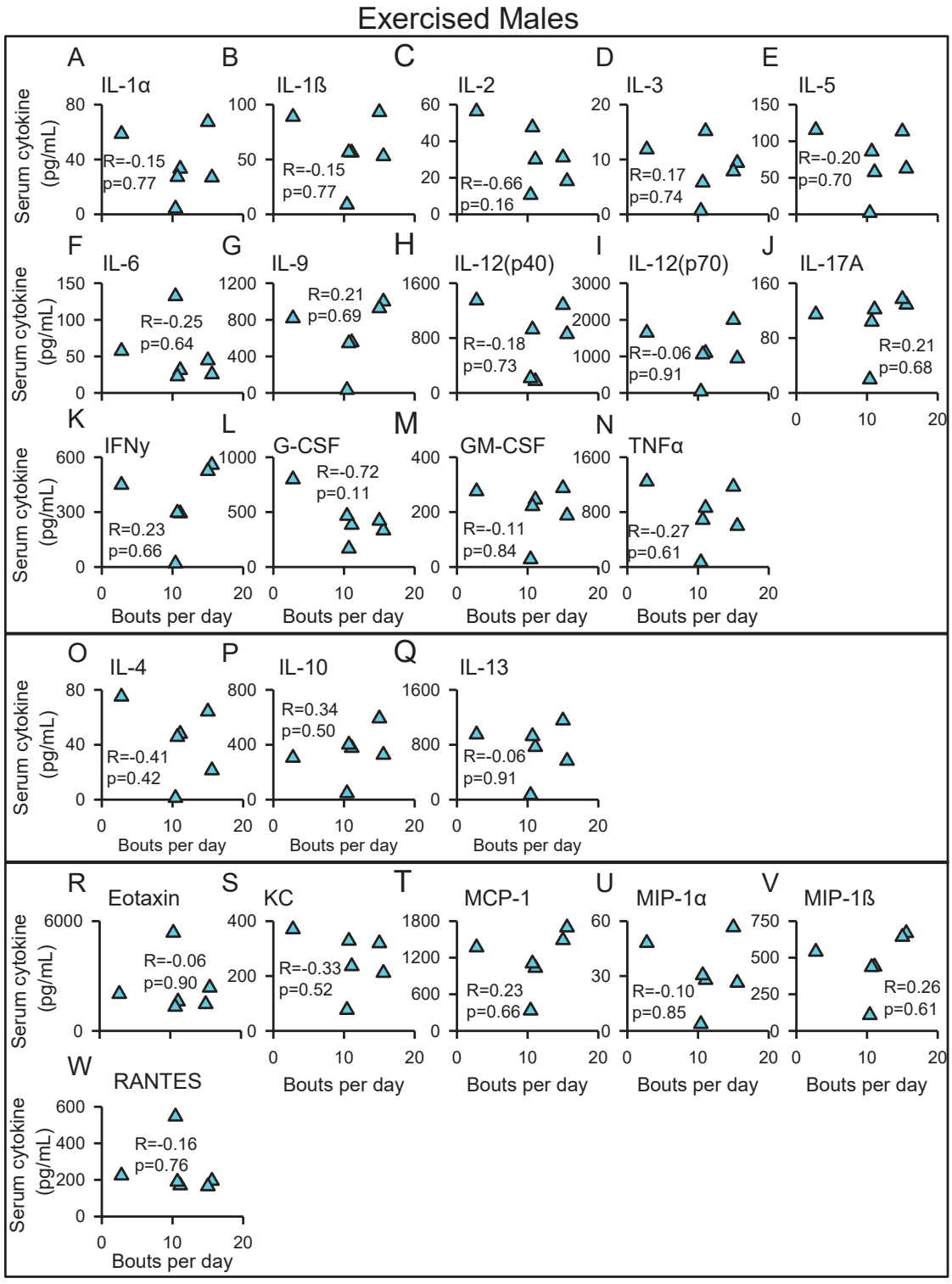

Figure 3.5.3: Exercise levels were not correlated with serum cytokines in male mice. Male mice (**A-W**: n=6) had their exercise levels compared to inflammatory markers at endpoint. Exercise levels were calculated as bouts per day. Correlations were calculated as two-tailed Pearson's correlation

Chapter 4: Discussion

4.1 Summary of principal results

Voluntary aerobic exercise when introduced late into life, was able to attenuate the development of frailty in mice of both sexes and actually reduce frailty in female mice without altering survival in either sex. The changes in frailty scores were a result of cumulative small effects and were not driven by any one deficit, or group of deficits. Changes in body composition, a mechanism of frailty, showed that exercise promoted an increase in lean tissue and loss of fat tissue in female mice, and both these changes were correlated with the frequency of activity. In males, while both sedentary and exercised mice gained lean and lost fat tissue, only the exercised group lost total tissue weight. This weight loss was also graded by frequency of activity. The gain in lean tissue in both male and female exercise groups corresponded with a either a protection in females grip strength or increased grip strength in males. A muscle of particular interest, the heart, showed sex-dependent adaptations to age and exercise. Exercise protected male hearts against an age-related decline in both diastolic and systolic function which did not occur in females. Lastly, exercise preserved cytokines in female mice, an effect that was graded by activity. By contrast, there was no relationship between cytokines and activity levels in males. To summarize, aerobic activity lengthened the healthspan of older male and female C57Bl/6 mice, though the health benefits on frailty, body composition, inflammation and the hearts were sex-specific.

Figure 4.1.1: Table of principal results. Table shows directional change of the main significant results from the present study.

Outcome	Females		Males	
	Sedentary	Exercise	Sedentary	Exercise
FI scores	↑	↓,↔	↑	↔
FRIGHT score	↑	↔	↑	↑
AFRAID score	↓	↔	↓	↓
Lean tissue	↔	↑	↑	↑
Fat tissue	↔	↓	↓	↓
Total tissue	↔	↔	↔	↓
EF (%)	↔	↔	↓	↔
E/A ratio	↔	↔	↓	↔
LV mass	↔	↔	↔	↔
LV wall thickness	↓	↔	↓	↓
Pro-inflammatory cytokines	↓	↔	↔	↔
Anti-inflammatory cytokines	↓	↔	↔	↔
Chemokines	↓	↔	↔	↔

4.2 Aerobic exercise

4.2.1 Running distance variability

While all the mice in the exercise group did run, there was no sex difference in activity levels. This was surprising, but perhaps not unexpected. While young mice do have distinct sex differences in running levels, with female mice running considerably more than their male counterparts (Manzanares et al., 2019; Rosenfeld., 2017), this difference tends to decline with age (McMullan et al., 2016). There has been previous work that suggests that this reducing sex difference is a result of the age-related decline in estradiol levels, which are highest in young females (Bartling et al., 2017; Konhilas et al., 2004; De Bono et al., 2006; Lightfoot et al., 2004) and then decline with age (Ghimire et al., 2019). This theory was later expanded upon using ovariectomized female mice. The absence of ovaries significantly reduced running levels which were re-established with estrogen supplementation (Cabelka et al., 2019). Hence, female mice with lower circulating estrogen run significantly less, and mice naturally have lower estrogen levels with age, so older females run less. This decline in older female activity levels helps explain why there is no difference in running distances between older male and female mice in the present work. Interestingly, the biggest difference in running distance was not between the sexes, but between individual mice. As exemplified by the highest and lowest activity levels, the difference between the sexes was negligible, while the longest runners ran approximately three times more than the slowest runners.

There are several possible explanations for this high degree of variation in running and one of them is genetic. Previous work has managed to selectively breed mice

for greater running distances (Swallow et al., 1998). For each generation, mice would be tested for wheel running rotations and the higher performers would be used to breed the next generation. After 10 generations, selectively bred mice ran more than 70% further than their wild type counterparts (Swallow et al., 1998). This suggests that activity, running speed in particular, is a heritable trait. Curiously, at generation 0 the study also noted a large range of activity levels in individual mice of both sexes (Swallow et al., 1998). While these were very young mice, the marked range in activity very similar to what was reported here. Looking more closely at the mice selectively bred for increased running, these mice oddly do not have higher aerobic capacity, or adaptations to either the heart or skeletal muscle (Rhodes et al., 2005). These adaptations would be the expected changes to account for increased running capacity, but they were not observed. Instead, the current hypothesis is not that the selectively bred runners have a higher capacity to run, rather that they have a greater desire to run (Rhodes et al., 2005). This is potentially a result of changes in the neural reward network (Rhodes et al., 2005). Therefore, it is possible that the mice showing the highest activity in the present study have the same increased motivation to run. While the ability to test this theory is beyond the scope of this thesis, it serves as an interesting explanation for inter-individual running variation. It also helps reinforce that, although the mice used in this study have the same background genetics, they still show marked variation.

4.2.2 Circadian rhythm

While there were no sex-specific differences in activity levels, the female mice did show a difference in temporal running patterns compared to the males. During the 13-week intervention, females ran more often during daylight hours than the males.

Importantly, while mice were often woken for a visual health inspection during the morning, this occurred at approximately the same time for male and female mice, so would not account for sex differences in running times. The observation that females run more during daylight is a similar result to previous work done by Bruns and colleagues (Bruns et al., 2020). They used voluntary wheel running to investigate the shift in circadian rhythm with age from young (4 months) to old (18 month) mice of both sexes (Bruns et al., 2020). While young females, and males of both ages returned to basal running levels when the lights were turned on, older females continued running 2 hrs into the daytime (Bruns et al., 2020). While the mice used in the current study are older than those used by Bruns et al (2020), they show the same pattern, where older female mice ran more during daylight hours than older male mice. Another study that used older male mice (22 months of age) also showed a shift in the circadian rhythm with age (Valentinuzzi et al., 1997). While these male mice did not shift to running during the day cycle, they initiated running later into the dark cycle (Valentinuzzi et al., 1997). Both these previous studies, along with the present one show how voluntary wheel running can help identify age-related shifts in circadian rhythm and how these shifts can change with sex. This is vital as only 20% of research regarding circadian rhythm in mice involved female cohorts (2008-2013) (Kuljis et al., 2013) and recent clinical work have also shown sex-differences in the aging circadian clock (Anderson & FitzGerald, 2020).

4.2.3 Aerobic exercise as a drug?

This study uses aerobic exercise as an intervention, with the goal of improving the health status of mice. As a beneficial intervention, it shares many similarities to more traditional drug treatments (Vina et al., 2012). For example, a metadata study compared

drug and exercise trials with the primary outcome of reducing mortality, with most trials focusing on cardiovascular diseases (e.g. coronary heart diseases and stroke) (Naci & Ionannidis, 2015). Interestingly, exercise was more effective than medications (e.g. anticoagulants) at reducing mortality in patients with stroke, and had a similar reduction in mortality to medication (e.g. statins) in patients with coronary heart disease (Naci & Ionannidis, 2015). This suggests that exercise can have equal efficacy to drugs in the treatment of cardiovascular diseases. In another serious condition, cancer-related fatigue, exercise was more effective than traditional pharmaceuticals (e.g. dexymethylphenidate) at reducing fatigue (Mustian et al., 2017). Like drugs, exercise also has dose-dependent effects. For example, sedentary adult women were trained using aerobic exercise for 6 months in three groups and a control (Martin et al., 2009). The three exercise groups would train at different levels and were then tested for quality of life using a survey (Martin et al., 2009). At the endpoint, quality of life improved in a dose-dependent fashion with exercise training (Martin et al., 2009). This effect is also found in pre-clinical studies where an 8-month voluntary wheel program for mice reveal dose-dependent effects on working memory performance (Robison et al., 2018). Therefore, not only can exercise have the same efficacy as drugs but also shares one of their principal features. Although exercise can serve as an invaluable treatment for multiple diseases and chronic conditions (Warburton et al. 2007), few studies have focused on exercise as an intervention in older pre-clinical models of both sexes. It will be interesting to investigate whether exercise can be combined with drug therapy to produce additive benefits in the treatment of aging and frailty in preclinical models. The previously proposed drug for frailty (examples: metformin, rapamycin, and enalapril) would be

interesting combination therapies with aerobic exercise with the goal of a synergistic effect. This could point the way to clinical trials of beneficial combinations for frailty interventions in vulnerable older adults.

4.3 Frailty and survival

4.3.1 Frailty scores

The first set of studies were performed to analyze whether aerobic exercise had a beneficial impact on health status, measured as frailty. While voluntary wheel running did improve frailty scores, this occurred in a sex-specific manner. The knowledge that frailty differs in severity between sexes is well established, where females tend to have higher FI scores than males (Baumann et al., 2019; Kane et al., 2019). This was demonstrated at baseline in the present study, where the females were frailer than the males. Even at endpoint, although not statistically significant, the sedentary females had higher FI scores than the sedentary males.

As expected, the present study did show beneficial health effects of aerobic exercise in aged animals as seen in earlier mostly male studies (Garcia-Valles et al., 2013; Gomez-Cabrera et al., 2017; Graber et al., 2015; Schafer et al., 2016). However, this is the first study to measure FI scores during a late-life intervention of voluntary wheel running in mice of both sexes. In male mice, we demonstrated that, while FI scores increased over the 13 weeks in sedentary animals, there was no increase in exercised males. In females, not only did exercise attenuate the development of frailty seen in the sedentary group, but exercise reduced frailty from baseline levels, at least at one timepoint. The group difference between exercise and sedentary mice also occurred

sooner in females than in males. This difference cannot simply be explained by running levels because there were no sex differences in activity levels. The FI scores were also not directly graded by exercise. This suggests that exercise is directly impacting a shared mechanism of frailty. This study examined both sarcopenia and chronic inflammation as physiological targets of exercise and mechanisms of frailty. Other examples of mechanisms not studied here include psychological effects (Morgan et al., 2018) or oxidative stress (Lee et al., 2019). It is therefore possible that aerobic exercise improves one, or many of these targets which in turn reduce frailty, and this may explain how exercise impacts frailty.

4.3.2 Survival rates

Interestingly, aerobic exercise did not affect survival rates in either sex. Whether aerobic exercise can alter lifespan is controversial. A lifelong treadmill training intervention did increase median mice lifespan (males by 19% and females by 9%) (Navarro et al., 2004). Conversely, in a lifelong voluntary wheel program, survival either did not change (Garcia-Valles et al., 2013), or only moderately improved (6% higher compared to control) (Nilsson et al., 2019). This latter study is comparable to human studies where aerobic exercise is associated with a modest average increase in lifespan (Reimers et al., 2012). If longer interventions can only produce modest effects at best, it is perhaps not surprising that a shorter intervention yielded no change in survival.

While not statistically significant, there was a sex difference in survival rates where females died more often than males, regardless of their intervention status. This may be in part due to the analytical technique used. LogRank curves test only the

significance between survival curves and not the magnitude of the difference (Bland & Altman, 2004). Significance is also most likely when the risk of mortality is consistently greater for one group (Bland & Altman, 2004). The sedentary females showed a consistent decline in survival throughout the 13 weeks, while the exercised females tended to have periods of higher mortality. This lack of consistency may help drive the lack of significance between the male and female survival curves. To calculate significance with the reported mean and variance, with a power of 0.80, the male sample size would have to be increased to 20. Significance aside, there is still an 80% male survival rate compared to a 50% female survival rate. This is consistent with some previous work (Turturro et al., 1999) where female C57Bl/6 mice live shorter lives, although not all studies agree (Austad & Fischer, 2016). This has interesting implications for the "morbidity-mortality paradox" seen in humans, where women tend to be frailer but live longer (Gordon et al, 2016). The present study shows that female mice are also frailer but tend to die earlier. Additional studies to explore this in a larger sample of male and female mice are now warranted.

4.2.3 Frailty clocks

To better understand the survival and frailty data, we used Frailty Clocks to examine predicted age and lifespan. Both clocks use multivariate regression models of frailty deficit data to predict both age and mortality (Schultz et al., 2020). The FRIGHT clock uses only the deficits with a strong correlation to frailty (21 of the 31 items). This gives a good predictor of chronological age, with a mean error of 1.6 months (Schultz et al., 2020). On the other hand, the AFRAID clock uses all 31 deficit items along with

chronological age to predict survival with a mean error of 2.3 months (Schultz et al., 2020). These are both very recent development and no studies have previously used these clocks to test exercise as an intervention.

In the males, there were no group differences between the exercised and sedentary mice in clock scores. Over time, the FRIGHT scores increased, and AFRAID scores decreased. As the male mice aged, they got biologically older (FRIGHT), and their life expectancy declined (AFRAID). This is the expected relationship over time for sedentary animals, however the present study shows the exercised males did not significantly deviate from this relationship. Conversely exercised females showed a non-significant decline in FRIGHT scores and an attenuation in AFRAID scores. As mentioned previously, both clocks use the FI to calculate clocks scores. Therefore, the difference between the sedentary and exercised female's FRIGHT or AFRAID scores can potentially be explained by differences in FI scores. However, this does not explain the sex-difference in the exercised mice. In the AFRAID score, the FI and the mouse's chronological age are used in the analysis. As the exercised males and females had the same increase in chronological age during the study, so the difference in AFRAID scores is primarily due to frailty. As mentioned previously, aerobic exercise had a more significant effect on frailty in female mice, and the AFRAID score shows this same relationship. Thus, voluntary aerobic exercise increased predicted lifespan and healthspan in aging females.

Voluntary aerobic exercise was able to protect mice against the age-related increase in frailty, with females showing a greater health benefit than males. However, exercise did not affect survival in either sex. Thus, the male and female mice were not

living longer but dying healthier, a goal that has been highlights in recent clinical studies (Annas & Galea, 2018).

4.3 Body composition

4.3.1 Signs of sarcopenia

As aerobic exercise beneficially impacted frailty in both sexes, we next wanted to investigate its effects on one underlying mechanism of frailty, sarcopenia. Sarcopenia is integrally linked to both aging and frailty (Rosenberg, 1997; Bisset & Howlett, 2019; Cesari et al., 2014) and can be altered through voluntary wheel running (McMullan et al., 2016). As such, we theorized that the improved frailty scores may result at least in part, from body composition improving with aerobic exercise.

The traditional signs of sarcopenia are a decrease in lean tissue, increase in fat tissue and overall weight loss (Rosenberg, 1997). As such, the exercised female's increase in lean tissue and loss of fat tissue can be viewed as beneficial changes. Importantly, both these changes were graded by the frequency of activity done by the female mice. These changes can therefore be viewed as a direct consequence of exercise. This is similar to previous work done by McMullan and colleagues who showed that, over a year long voluntary wheel training program, exercised females had lower fat and higher lean tissue than their sedentary counterparts (McMullan et al., 2016). However, this study also found that exercised females maintained baseline physical parameters while the sedentary females gained fat and lost lean tissue (McMullan et al., 2016). As their baseline levels were calculated when the mice were 1 year old, the analysis is not comparable to the present study. Curiously, McMullan and colleagues also noted that,

between 1.6-2 years, sedentary males gained lean tissue and lost fat tissue, giving them similar levels to the exercised males (McMullan et al., 2016). This mirrors the present study where both the sedentary and exercised males lost fat and gained lean tissue. The only change distinct to exercised males was the loss of overall tissue, and this was the only parameter graded by activity. This suggests that the loss of fat and gain of lean in the male mice is more a result of age than exercise. Of note, the reduction in overall tissue in the exercised males cannot be explained by a decrease in caloric intake, as male exercised mice actually increased their food consumption throughout the study. Therefore, the weight loss can be viewed as a direct consequence of exercise.

Importantly, both the lean and fat tissues in this study were normalized to overall tissue. This was done to better compare between the sexes, as it would negate baseline body weight differences (males had higher baseline weights than females) and be more comparable to clinical data (where fat is generally expressed as a percentage). Another important limitation related to DEXA as a tool is that lean tissue is a sum of all non-fat, non-bone tissue. As previously shown in our own humane endpoint data (e.g. endpoint caused by swollen liver) and by Marino and colleagues, organ weights tend to increase with age (Marino, 2012). Lean tissue in this study is not solely composed of muscle but may also reflect age-related organ hypertrophy. Organ hypertrophy with aging would help explain why previous studies saw an increase in soleus and extensor digitorum longus muscles in older trained male mice but a decrease in lean tissue weight (Seldeen et al., 2018).

4.3.2 Mouse grip strength

Forelimb grip strength is another common measurement of age-related deterioration and frailty. In terms of a biomarker, it is correlated with all-cause mortality, cardiovascular disease, and decline in physical function in a clinical setting (Bohannon, 2019). In preclinical work, grip strength has been used as a component of physical frailty (Liu et al., 2014; Gomez-Cabrera et al., 2017), and as a component of the FI (Whitehead et al., 2014) to indicate mouse health. Here we used a version of the modified grip strength test (Takeshita et al., 2017). Traditionally rodent grip strength is measured using a horizontal test, but Takeshita and colleagues tested vertically instead to obtain more accurate results (Takeshita et al., 2017). Our own version of this test gently pulled the mouse up from the bar while Takeshita and colleagues gently pulled the mouse downwards (Takeshita et al., 2017). Our version better mimics the FI forelimb grip deficit test and avoids potential mouse falls. Takeshita and colleagues also previously demonstrated that normalizing grip strength to body weight resulted in inconsistent results (Takeshita et al., 2017). The theory of normalizing grip to body weight assumes body weight as a proxy for muscle weight. However, as shown in the present study, much of that weight is due to fat mass and not muscle. Hence, our study did not normalize forelimb grip strength.

Consistent with previous studies, forelimb grip strength declined with age in both female and male sedentary mice (Takeshita et al., 2017; Gomez-Cabrera et al., 2017; Ge et al., 2016). Interestingly, exercised male mice actually exhibited an increase in forelimb grip strength whereas exercised females were protected against the age-related decline in

strength. This is similar to results from an exercise program in younger male mice, where the increase in forelimb grip strength occurred in a voluntary wheel training program (Kim et al., 2020). Another study found that treadmill running could increase forelimb grip strength in male mice (Zelikovich et al., 2019). This is important because treadmill running, unlike wheel running, does not require mice to use their forelimbs to grab metal bars. This suggests that there may be an underlying effect of running itself that improves forelimb grip strength in mice.

To summarize, the increase in lean tissue in exercised male and female mice occurred along with an increase in grip strength, with many changes correlated with activity levels. This suggests that exercise can improve signs of sarcopenia in older mice. However, male mice showed similar relationships in both sedentary and exercised mice for both lean and fat tissue, which suggests that other mechanisms may be involved in the effects on frailty.

4.4 Cardiac structure and function

4.4.1 Diastolic and systolic function

Having found that aerobic exercise improved health (e.g. attenuated frailty) and body condition in both sexes, we then investigated whether cardiac health was affected by this intervention. Cardiac function was measured in both sexes using echocardiography. Results showed that sedentary males exhibited signs of age-related diastolic and systolic dysfunction, which were not observed in the exercised males. By contrast, female mice did not exhibit cardiac dysfunction regardless of age or activity.

This suggests that aerobic exercise may have particular benefit on hearts from older males.

Interestingly sedentary male mice displayed more significant signs of diastolic dysfunction with age than females. This is similar to results from a mouse model of heart failure with preserved ejection fraction, where the increase in hypertrophy and signs of diastolic dysfunction were more prominent in younger males than females (Tong et al., 2019). Much of the difference in young animals is likely due to the cardioprotective effect of estrogen (Blenck et al., 2016). However, this is unlikely to be the mechanism in older female mice due to age-related declines in circulating estradiol. Reasons for the lack of age-related diastolic dysfunction in the sedentary females presents an interesting future area for research.

While the sedentary male mice displayed signs of diastolic dysfunction, the exercised males were protected for this adverse effect of aging. The ability of aerobic exercise to improve diastolic function in older individuals has shown complex and often contradictory results. In one clinical study, older male athletes had similar diastolic parameters to those seen in older sedentary controls (Fleg et al., 1995). They concluded that age, and not aerobic capacity, was a predictor of diastolic dysfunction (Fleg et al., 1995). Conversely, another clinical study discovered that signs of age-related diastolic dysfunction were less pronounced in older adults who exercised (Takemoto et al., 1992). Studies in male rats show that a 12-week treadmill program can reduce signs of diastolic dysfunction measured using E and A waves and the E/A ratio (Brenner et al., 2001). Brenner and colleagues concluded that aerobic exercise reduced signs of diastolic dysfunction (increased E/A ratio) and improved early-diastolic filling (shorter E waves)

(Brenner et al., 2001). Other work has demonstrated that treadmill training programs in mice can improve signs of diastolic dysfunction without the ventricular stiffness due to cardiac fibrosis (Chang, 2020). These previous studies agree with many of the observations of the present study, in that aerobic exercise can protect against signs of diastolic dysfunction. However, all previous work used only male mice. The present study is unique in that it revealed that the effects of aerobic exercise and age on diastolic dysfunction are sex-specific and seen more in males than females. Further work in this area could be illuminating.

Cardiac fibrosis could be involved in diastolic dysfunction, as stiffer ventricles are known to impair relaxation (Du et al., 2003). However, our study showed no difference in hydroxyproline concentrations between hearts from exercised and control mice. This lack of an exercise-induced fibrotic change has been previously noted in young males (Soares et al., 2019), and in older males (Chang, 2020). It is possible that we saw no impact of exercise on fibrosis due to the voluntary nature of exercise. As mentioned previously, older mice voluntarily run significantly less than younger ones. Therefore, this level of exercise may not result in enough activity to facilitate changes in fibrosis in aging hearts. As hydroxyproline measures all fibrosis, this may mask shifts in the type of collagen as a function of exercise. For example, the shift from type 1 collagen to type 3 collagen in clinical models has been linked to increased LV stiffness in older adults (Meschiari et al., 2017). Future work should investigate not just the amount of cardiac collagen, but also the type.

Interestingly in our study, the female mice had substantially higher level of fibrosis than the males. Previous work has shown that older female hearts have higher

interstitial reactive and epicardial fibrosis while older males have higher levels of replacement fibrosis (Achkar et al., 2020). As replacement fibrosis occurs in areas of necrosis (Hinderer & Schenke-Layland, 2019), our sampling method may have simply missed areas of higher male fibrosis. Histological investigations may also be helpful in exploring the impact of exercise on cardiac fibrosis in male and female hearts.

Similar to diastolic dysfunction, systolic function showed sex-dependent effects of age and exercise. Sedentary male mice saw an age-related decline in EF and FS and non-significant increase in IVCT, all signs of systolic dysfunction (Medrano et al., 2016). Exercised males were protected against this maladaptive change. Of note, IVCT also increases with diastolic heart failure, so is not purely a measure of systolic dysfunction (Kono et al., 2010). The idea that male mice exhibit more significant signs of systolic dysfunction with age than females is well established (Keller & Howlett, 2016). As both EF and FS are calculated from left ventricular chamber size, the exercise-induced protection is likely a result of cardiac remodeling, which is discussed in detail below. Interestingly, many of the changes in systolic function were subtle, although significant, in the male animals. To better investigate changes, future studies should also analyze peak systolic strain which is more sensitive to age-related systolic dysfunction (Derumeaux et al., 2008). Importantly, our study reported both FS and EF though they are both derived from LV chamber dimension and are both measures of cardiac contraction. While FS requires fewer assumptions in the calculation (Table 2.4.1), EF is more often reported in a clinical setting (McDonald, 1975).

4.4.2 Left ventricular structure

We found no difference in cardiac size between exercise and sedentary groups, as seen in previous work (Shanmugam et al., 2017; Walton et al., 2016). Interestingly, we did see an interaction between sex and activity in many measurements of cardiac size, driven by differences in the endpoint values. This showed that sedentary females trended towards pathological hypertrophy (LV chamber dilation, thinner LV walls, and decreased systolic function) while this was not seen in exercised females. The variation in values measured at the endpoint in sedentary females was large, and this may account for a lack of significance. As such, having a larger cohort may reduce variation to better show changes in cardiac structure with age and exercise. Calculating required sample size using the reported means and variance and a power of 0.80 reveals that for endpoint LV volume, a sample size of 40 for each sedentary and exercised females would be required to show significance. Exercised males, on the other hand, had larger hearts with no change in systolic function or wall thickness, similar to physiological eccentric hypertrophy. This too was non-significant, so additional studies with a larger sample size could be informative.

In terms of activity, females did show correlations between wall thickness, and LV mass to the volume of exercise. This suggests that signs of cardiac hypertrophy may be linked to activity. If females had been trained at greater intensity, more pronounced changes may have been seen. In addition, as previously found by Walton and colleagues (2016), the changes in cardiac structure may be found on the cellular level. After training mice on a treadmill, the study reported no difference in heart weight, but cardiomyocyte width and area were different between exercise and sedentary older males (Walton et al.

2016). Future studies should investigate cellular signs of hypertrophy in response to age and exercise, and if they differ between male and female hearts.

To summarize, while the effects were modest, aerobic exercise did protect male hearts against signs of both systolic and diastolic dysfunction, but it did not protect female hearts. This change was not explained by stiffer ventricular tissue due to cardiac fibrosis. It is therefore possible that other cellular or molecular changes contributed to these differences, although this was not investigated here. While aerobic exercise improved the health of both male and female mice, this was done with only moderate improvements to cardiac health.

4.5 Chronic inflammation

4.5.1 Serum cytokines

Previous work has shown that chronic inflammation plays a role in the development of frailty (Bisset & Howlett, 2018; Kane et al., 2019). Here we assessed the relationship between exercise and frailty by comparing serum levels of pro- and anti-inflammatory cytokines in exercised and sedentary mice of both sexes. Curiously, the effect of aerobic exercise on chronic inflammation was sex-specific. In male mice, there was no difference in the 23 serum cytokines between exercised and sedentary groups. Levels of both pro- and anti-inflammatory cytokines were similar at baseline and endpoint, with no significant differences between the sedentary and exercised mice over the course of the study. Furthermore, serum cytokine levels in male mice were not correlated with the volume of activity. These findings agree with a previous study of treadmill exercise in 20-month-old male rats, where activity had no effect on levels of the

pro-inflammatory cytokines IL-6 and TNFα (Moon et al., 2012). No links between serum markers of inflammation and activity were seen in a study of older men and women, even after periods of acute exercise (Windsor et al., 2018). However, this latter study included very few women (*e.g.* only 2 women per group) so it was not powered or designed to test sex differences and the data reflect observations primarily in men (Windsor et al., 2018).

While activity had no impact on markers of inflammation in male mice, there were profound differences in cytokines between exercised and sedentary female mice. While many studies have focused on the idea that aging is accompanied by an increase in pro-inflammatory cytokines and decrease in anti-inflammatory cytokines (known as "inflammaging" (Franceschi et al., 2000)), this is not universally observed. Indeed, when blood cells from older people were challenged with lipopolysaccharide to induce inflammation, individuals who produced the lowest levels of pro- and anti-inflammatory cytokines had the lowest chance of survival over a five year follow up (van den Biggelaar et al., 2004). Another clinical study found that serum collected from older people showed that the pro-inflammatory cytokines/chemokines granulocyte colony stimulating factor (G-CSF), IL-17 and MIP-1α were higher in younger people (Elisia et al., 2017). Interestingly, these higher levels of pro-inflammatory cytokines were graded by the frequency of activity (Elisia et al., 2017). These findings are similar to the present study, where the pro-inflammatory cytokines in female mice were graded by exercise. In another clinical trial, intense exercise has been shown to increase levels of pro-inflammatory cytokines, often for extended periods of time (Suzuki, 2019). Our data suggests that immune system dysregulation may occur in aging female mice, and that this

may be attenuated by voluntary exercise. This could contribute to the beneficial effects of exercise on frailty in older females, although additional studies will be required to further investigate underlying mechanisms.

4.5.2 Inflammation and age

There are a few potential mechanisms for the detrimental dysregulation of the immune system seen in the sedentary female mice. One that also links to aerobic exercise and frailty is aging adipose tissue. While only briefly mentioned earlier, the shift in body composition with age not only encompasses the amount of fat but also its function (Stout et al., 2017). Age-related dysfunction in adipocytes can result in the release of pro-inflammatory cytokines (Mau & Yung, 2018). This may contribute to the rise of chronic inflammation, known as "inflammaging" (Franceschi et al., 2000). While sedentary female mice were the only group not to lose a significant amount of fat during the study, and the only ones to have depressed cytokine levels, this is unlikely to be the reason for the dysfunctional immune system. Age-related dysfunctional adipocytes increase inflammation, not reduce it. In addition, previous work on aerobic exercise in older male mice showed that, while exercise reduced adipocyte size and the accumulation of alternatively activated M2 macrophages, it did not change IL-6, IL-10, or TNFα expression (Ziegler et al., 2019). Thus, immune system deregulation in the sedentary females is likely not related to body composition and therefore the exercise-induced protection is unrelated to improved signs of sarcopenia.

A more promising mechanism is age-related immunosenescence, a state of both B and T-cell exhaustion (Aiello et al., 2019). Immunosenescence occurs when the immune

system becomes less efficient, making the body more susceptible to infections, reducing the response to vaccines and increasing the susceptibility to inflammatory diseases (Aiello et al., 2019). In mice, older T-cells produce significantly less IL-2 and IFNγ upon stimulation (Maue, et al., 2009). Interestingly, previous research has suggested that exercise may play a role in mitigating immunosenescence in humans (Turner & Brum, 2017), and that immunosenecence develops differently between the sexes (Aiello et al., 2019). This is potentially an interesting avenue for future work on the aging immune system in mice of both sexes and how exercise may help prevent deterioration.

4.6 Limitations

There are a few important limitations to the work presented, which should be taken into consideration when interpreting the results. Firstly, frailty was measured in an unblinded fashion, which could have introduced bias. Secondly, a larger cohort would improve analysis of certain parameters, particularly mortality and correlations with frailty. Next, only a few tools were used to measure cardiac function. For example, pressure-volume loops could better elucidate cardiac function and measuring cardiomyocyte size could better examine cardiac hypertrophy. Lastly, this study uses only voluntary wheel running which allows mice to run a variable amount. A treadmill running program would eliminate this variation by putting all mice in a similar training program and better examine specific doses of exercise.

4.7 Conclusion and future work

Future studies should use this work to focus on the mechanisms of frailty involved. For example, examining cellular changes in body composition would give a

better understanding the role of sarcopenia. As mentioned previously, muscle fiber types also shift with age, so it would be useful to measure how myosin isoforms shift both with age and exercise. Another direction would be to focus on the changes in the immune system with age and exercise, perhaps to determine why the cytokine levels declined in the sedentary females.

In conclusion, voluntary aerobic exercise attenuated frailty in older male and female mice, even when introduced late in life, but had no effect on survival. In both sexes exercise improved body composition, a result graded by the frequency of activity. While results were subtle, exercise did help protect male hearts against signs of systolic and diastolic dysfunction but had little effect on female hearts. Finally, aerobic exercise prevented the age-related decline in cytokines but only in female mice. Voluntary aerobic exercise had health benefits on both older male and female mice, but in a sex-specific manner.

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Appendix A

Works Published and in Preparation for Publication

Introduction

Sections 1.2.3, 1.3.2 and 1.3.3 are versions of a paper currently being writing as a review of senolytics drugs and aerobic exercise as an intervention for frailty in older mouse models (2021). This work is being done in collaboration with Will Johnston, Dr Susan Howlett and Dr Scott Grandy. I am helping with both the research and writing of this work.

Figure 1.2.1 comes from a published review from myself and Dr Susan Howlett (Bisset & Howlett, 2019) about the mechanisms of frailty. I assisted in the research and writing of this review, along with the creation of this figure.

Results

Results in sections 3.1.1, 3.1.2, 3.2.1, 3.2.4 and 3.5.2 have been written and submitted to the Journal of Gerontology series A for publication under the title “Impact of aerobic exercise on frailty, mortality, and inflammation in aging male and female C57Bl/6 mice”. This work is currently under review (2021). This work was done in collaboration with Stefan Heizne Milne, Dr Scott Grandy and Dr Susan Howlett. I performed most of the experiments, analysis of data and collaborated in writing this research paper.

Discussion

Section 4.2.1 contains a citation to the work by Ghimire, Dr Howlett, and myself (Ghimire et al., 2019). Here we measured serum estradiol levels in young and old mice of both sexes. I did the measurement and analysis of the serum estradiol levels in the mice and wrote relevant sections of the manuscript, which was included in the published paper

Appendix B

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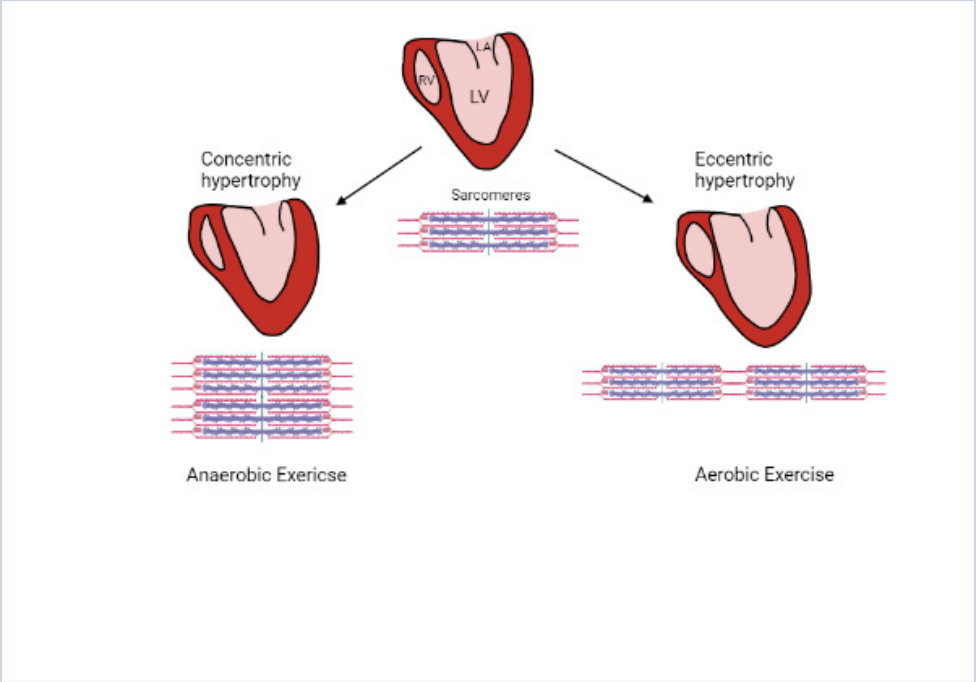

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