Resistance exercise protects mice from protein-induced metabolic dysfunction

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Abstract

Lower intake of dietary protein is associated with improved metabolic health and reduced rates of age-associated diseases in humans, while low protein (LP) diets improve fitness and extend the lifespan of diverse animal species. Paradoxically, many athletes and bodybuilders consume high protein (HP) diets and protein or branched-chain amino acid supplements, yet remain fit and metabolically healthy. Here, we examine the effect of weight pulling, a validated progressive resistance exercise training regimen in mice fed either a LP diet or an isocaloric HP diet. We find that sedentary HP-fed male C57BL/6J mice gain significantly more fat mass than sedentary mice fed a LP diet, despite not consuming more calories. Resistance exercise was protective against this effect, blocking excess fat gain overall and in specific fat depots in HP-fed mice, but did not alter fat mass gain or distribution in LP-fed mice. In accordance with the widespread belief that high protein diets help promote muscle mass gain, the HP diet augmented the hypertrophy of the soleus, flexor digitorum longus (FDL) and the forearm flexor complex that occurred in response to exercise. While strength increased more rapidly in HP-fed mice, the maximum strength pulled by LP and HP-fed mice after 12 weeks was indistinguishable. Our results confirm the widespread belief that HP diets can augment muscle hypertrophy and strength gain induced by resistance exercise without negative metabolic consequences, while demonstrating that LP diets may be relatively advantageous in the sedentary. The results here highlight the need to consider both dietary composition and activity levels, not simply calories, when taking a precision nutrition approach to health.

Introduction

In contrast to the long-standing idea that “a calorie is a calorie,” research over the last two decades has shown that calories from different macronutrient sources have distinct impacts on health (Hall et al., 2015). Dietary protein in particular has been shown to have a critical role in the regulation of metabolic health and longevity; low protein diets are associated with reduced rates of diabetes and other age related diseases in human longitudinal studies, and promote leanness and insulin sensitivity in human randomized clinical trials (Ferraz-Bannitz et al., 2022; Fontana et al., 2016; Levine et al., 2014; Sluijs et al., 2010). In mice, low protein, high carbohydrate diets promote leanness, glucose tolerance, and extend lifespan (Hill et al., 2022b; Richardson et al., 2021; Solon-Biet et al., 2014; Solon-Biet et al., 2015); many of the benefits of a low protein diet are mediated by reduced consumption of the branched-chain amino acids (BCAAs), particularly isoleucine and valine, and we have recently shown dietary histidine is also associated with increased adiposity and body mass index (BMI) (Flores et al., 2022; Green et al., 2022b; Jang et al., 2016; Yu et al., 2021).

The idea that low protein diets are beneficial – and high protein diets are deleterious – challenges conventional wisdom in several ways. First, several high-protein “fad” diets have been popularized over the last two decades, and many researchers and medical professionals also support consuming more protein (Rodriguez, 2015). In addition to promoting satiety, some short-term studies have found that high protein intake improves glucose control in humans (Dong et al., 2013; Gannon et al., 2003; Seino et al., 1983). Critically, high protein diets are recommended for physically active individuals to support muscle growth and strength improvements (Andersen et al., 2005; Willoughby et al., 2007). Specifically, both training and amino acids can activate mechanistic target of (mTOR), which stimulates the protein synthesis machinery needed to stimulate skeletal muscle hypertrophy (Bodine et al., 2001). Therefore, The Academy of Nutrition and Dietetics recommends consuming 1.2-2.0 grams of protein per kg body weight (BW) per day

in physically active individuals (Thomas et al., 2016). These protein estimations are substantially higher than the Recommended Daily Allowance (RDA) for sedentary people of 0.8 g/kg body weight, but it remains controversial if protein intake above this level is detrimental in this population.

It is clear that high dietary protein intake is not always deleterious, or the millions of individuals consuming high protein diets or taking widely sold protein or BCAA supplements to maximize muscle growth following resistance training would have higher adiposity and an increased risk of diabetes. However, there is no data available to support such a surprising conclusion, and indeed the opposite is true: exercise reduces the risk of developing type 2 diabetes (Grontved et al., 2012). We are therefore left with a paradox: while the human and animal data suggesting that increased dietary protein intake is detrimental for metabolic health and increases the risk for numerous age-related diseases is robust, some of the people consuming the highest levels of protein – athletes – are metabolically very healthy.

Here, we examine the possibility that exercise can protect mice from the deleterious metabolic effects of a high protein diet normally observed in sedentary mice and the generally sedentary human population. As treadmill running – one of the most common ways of exercising mice – inevitably leads to leaner mice, we utilized a recently validated method of progressive resistance exercise for mice that does not cause weight loss (Zhu et al., 2021) to assess the interaction of dietary protein content and exercise in male C57BL/6J mice. We fed mice either a low protein (LP, 7% of calories from protein) or a high protein (HP, 36% of calories from protein) diet, and either pulled an increasing load of weight down a track 3x per week for 3 months, or pulled a sham unloaded cart. During the course of the experiment, we comprehensively assessed weight and body composition, metabolic parameters, and the fitness of the mice on these regimens. Finally, we euthanized the mice, determining the effect of both diet and exercise on tissue weight and muscle mass, muscle fiber type, and muscle mitochondrial function.

In agreement with our hypothesis that HP diets impair metabolic health in sedentary mice, we found that HP-fed sham-exercised mice gained excess fat mass and increased in adiposity relative to LP-fed mice. In sharp contrast, HP-fed mice engaged in resistance exercise showed hypertrophy of specific muscles and were protected from protein-induced fat accretion. Interestingly, while exercising HP-fed mice gained strength more rapidly than exercising LP-fed mice, the difference in maximum strength reached was negligible. Interestingly, neither diet nor exercise altered mitochondrial function. Our research shows that resistance exercise protects from HP-induced increases in adiposity, and suggests that metabolically unhealthy sedentary individuals consuming HP diet or protein supplements should either reduce their protein intake or begin a resistance exercise program.

Results

Resistance exercise protects against high-protein induced fat and body weight gain

Seven-week-old C57BL/6J mice were randomized to groups of equal weight and body composition, and placed on either a Low Protein (LP, 7% of calories from protein) or High Protein (HP, 36% of calories from protein) diet. The two diets were isocaloric, as calories from fat were kept at 19%, and carbohydrates were reduced in the HP diet. We have previously utilized this same LP diet, and the full composition of both diets can be found in **Table 1**. Throughout the course of the study, weight was collected weekly and body composition was analyzed every three weeks via EchoMRI (**Fig. 1A**). Starting after five weeks on the diets, we exercised half of the mice on each diet using a recently validated mouse model of resistance exercise training, weight pulling (WP), in which mice pull progressively heavier weights down a track; the other half of the mice were sham-exercised by pulling an unloaded cart.

All mice in each group ate approximately the same amount of food relative to their body weight; on a per animal basis, LP-diet fed mice ate more than HP-fed mice, consistent with the satiating effect of dietary protein (**Fig. 1B**). Despite this, mice on the HP diet gained more weight over the course of 18 weeks than LP-fed mice; and by the end of the experiment, HP-fed WP mice gained significantly less weight than HP-fed sham-exercised controls (**Fig. 1C**). As we expected based on previous studies investigating the effects of a LP diet as well as data demonstrating the beneficial effects of protein on muscle growth, HP-fed mice gained significantly more lean mass than LP-fed mice, although surprisingly exercise did not affect this gain (**Fig. 1D**). In accordance with our previous results, we found that sham-exercised HP-fed mice gained substantially more body fat than their LP-fed counterparts; however, we found that WP was protective against these effects (**Fig. 1E**).

At the conclusion of the experiment, we euthanized the animals and measured the weight of several fat depots and the liver. We found that the inguinal white adipose tissue (iWAT) and epididymal white adipose tissue (eWAT) was significantly heavier in sham-exercised HP-fed mice than in WP HP-fed mice (**Fig. 1F-G**). While there was no statistically significant effect on the mass of brown adipose tissue (BAT), the weight of this depot also trended higher in sham-exercised HP-fed mice than in WP HP-fed mice (**Fig. 1H**). Finally, there was a statistically significant effect of diet on liver weight, with HP-fed mice having heavier livers, and effect that was much greater in sham-exercised mice than in WP mice (**Fig. 1I**).

Mice fed a low protein diet have better glycemic control than high protein-fed mice

We and others have previously shown that consumption of a low protein diet improves glucose tolerance and insulin sensitivity, while the literature suggests that exercise improves insulin sensitivity in humans. We performed glucose and insulin tolerance tests; in agreement with

our previous findings, we found that LP-fed mice had improved glucose tolerance relative to HP-fed mice, and there was a trend (p=0.0865) towards a positive effect of WP on glucose tolerance (**Fig. 2A**). Similarly, while LP-fed mice were more insulin sensitive than HP-fed mice under sham conditions, we did not observe a difference between LP and HP-fed mice that performed WP (**Fig. 2B**).

In agreement with a model in which high dietary protein is deleterious to sedentary animals, we observed that HP-fed mice had higher fasting blood glucose than LP-fed mice under sedentary conditions (**Fig. 2C**). Interestingly, WP did not correct this difference – instead, WP further lowered the fasting blood glucose level of LP-fed mice, but did not alter the fasting blood glucose of HP-fed mice, resulting in a WP-induced exaggeration of the difference in fasting blood glucose between diet groups (**Fig. 2C**).

While ketogenesis is normally associated with fasting or the consumption of ketogenic, low carbohydrate diets (Goldberg et al., 2020; Keller et al., 2021), we recently showed that restriction of isoleucine or all three branched-chain amino acids induces ketogenesis in mice (Yu et al., 2021). In agreement with the idea that diets low in isoleucine promotes ketogenesis, we found that there was a significant effect of diet, with LP-fed mice having higher ketone levels than HP-fed mice (**Fig. 2D**). Intriguingly, the HP-fed WP mice had the lowest overall levels of ketones. Finally, we examined the lipid profile of the mice. Although we previously observed that a LP diet does not reverse hepatic steatosis in diet-induced obese mice (Cummings et al., 2018), some studies have shown altered lipid levels in mice and humans fed a LP diet (Trevino-Villarreal et al., 2018), and we considered it likely that WP would promote a healthy lipid profile. Surprisingly, while diet had no effect on triglyceride or lipid levels, there was an overall increase in triglyceride levels in WP mice (**Figs. 2E-F**).

In order to learn more about the effects of the HP diet on weight and adipose gain, we used metabolic chambers to evaluate food consumption, spontaneous activity and energy

expenditure. We observed no statistically significant effect of either diet or resistance exercise on either food intake or spontaneous activity, although in agreement with our expectations that dietary protein is satiating, there was an overall trend (p=0.1537) towards decreased food consumption by HP-fed mice during the dark cycle (**Figs. 3A-B**). We examined respiratory exchange ratio (RER) by calculating the ratio of O_2 consumed and CO_2 produced; an RER of close to 1.0 indicates that carbohydrates are being preferentially utilized for energy production, while a value near 0.7 indicates that lipids are the predominant energy source. As we anticipated, there was an overall effect of diet on RER, with mice consuming the LP diet having a higher RER during the dark cycle, reaching statistical significance in the WP groups (**Figs. 3C-D**). In agreement with previous works, we also observed a significant effect of the LP diet (Fontana et al., 2016; Hill et al., 2017; Rothwell et al., 1983), but not resistance exercise training, on energy expenditure, both on a per animal basis and when weight was considered as a covariate (**Figs. 3E-F**).

Training while consuming a LP diet does not compromise maximum strength or coordination but blunts WP-induced muscle hypertrophy

It is widely accepted in human weight training regimens that in order to maximize strength and muscle hypertrophy from resistance exercise, consumption of a large quantity of high quality protein is necessary (Phillips et al., 2005; Thomas et al., 2016). As we expected, there was an overall effect of diet (p=0.0322) on the maximum load mice were able to pull during their training sessions (**Fig. 4A**), which we interpret as HP-fed mice being able to pull more weight. However, the difference was much smaller than we anticipated; there was only a statistically significant difference in the maximum weight pulled by LP-fed and HP-fed mice during the third week, and the maximum weight pulled by LP-fed and HP-fed appeared to converge on virtually identical

values by week 12 (**Fig. 4A**). There was no overall effect of diet on the average number of sets per training bout (**Fig. 4B**) or the number of stimulatory touches by the investigator (**Fig. 4C**).

Despite an increase in max weight that the mice could pull, this effect did not translate into improved performance on other tasks assessing muscle function. Specifically, there was no difference in the performance of WP-trained LP-fed and HP-fed mice during an inverted cling test; indeed, there was a trend towards decreased performance in this task for HP-fed mice (p=0.1217) (**Fig. 4D**). We also evaluated the performance of mice on an accelerating Rotarod, a test of balance and coordination (Bellantuono et al., 2020). We observed no impact of either diet or training on the amount of time mice were able to remain on the Rotarod (**Fig. 4E**) or the maximum speed achieved (**Fig. 4F**).

At the conclusion of the weight training regimen, we euthanized the animals and collected numerous tissues. As noted above, WP in HP-fed mice reduced the mass of iWAT, eWAT, and liver (**Figs. 1F-I**). We also collected a diverse array of skeletal muscles from different regions of the body. We found that WP in HP-fed mice significantly increased the mass of several muscles, including the soleus, the flexor digitorum longus (FDL), plantaris, and forearm flexor complex, but did not increase the mass of the quadriceps (**Figs. 5A-E**). The mass of the FDL also increased in WP LP-fed mice relative to sham-exercised LP-fed mice. Similar positive effects on WP in HP-fed mice was seen when muscle mass was normalized to body weight (**Figs. 5F-J**).

Mitochondrial respiration is unaffected by diet and weight pulling

LP diets have been shown to alter mitochondrial respiration through upregulation of uncoupling protein 1 (UCP1) and browning of white adipose tissue (Hill et al., 2017; Pérez-Martí et al., 2017). In humans, mitochondrial respiration is impaired in the skeletal muscle of aged individuals (Cartee et al., 2016) and those with metabolic disorders (Mogensen et al., 2007; Wells

et al., 2008) and is increased after both aerobic and resistance training regimens (Konopka et al., 2015; Konopka et al., 2019; McKenna et al., 2022; Robinson et al., 2017). This could be due in part to the high energetic demands imposed by protein synthesis (Rolfe and Brown, 1997; Waterlow, 1984), an upregulated process in the 24 hours following a resistance training bout (Ogasawara et al., 2016). However, mitochondrial response to varying amounts of dietary protein with or without resistance training remains unclear. Here, we hypothesized the skeletal muscle mass gain driven by WP with HP compared to LP would also increase mitochondrial respiratory capacity in the FDL muscle.

To assess mitochondrial capacity following our training and diet interventions, we performed high-resolution respirometry in permeabilized muscle fibers from FDL samples that have been previously demonstrated to hypertrophy as a result of WP (Zhu et al., 2021) which increased in mass in the present study. Using complex-I linked substrates pyruvate, glutamate, and malate we found no change to leak respiration (**Fig. 6A**). We next evaluated sub saturating and saturating complex-I driven respiration by providing ADP at 0.25mM, 0.5mM and 5.5mM; none of these parameters was changed in any intervention group (**Figs. 6B-D**). Furthermore, we saw no change in any group following succinate addition for complex I+II linked respiration (**Fig 6E**).

Discussion

In model organisms and humans, dietary protein is a critical regulator of a wide variety of biological processes that determine metabolic health and lifespan (Trautman et al., 2022). Low protein diets promote healthspan and lifespan in flies and rodents (Fontana et al., 2016; Hill et al., 2022a; Solon-Biet et al., 2014), and emerging data suggests the same may be true for humans (Ferraz-Bannitz et al., 2022; Fontana et al., 2016; Laeger et al., 2014; Levine et al., 2014; Mair et

al., 2005; Yu et al., 2020). Paradoxically, while consumption of additional protein is recommended as a way of supporting muscle growth and strength gains during resistance exercise training, there is also evidence that high protein diets result in detrimental health effects (Lagiou et al., 2012; Mittendorfer et al., 2020; Zhang et al., 2020). Gaining skeletal muscle function and mass is associated with a host of health benefits including a reduced risk for diabetes and frailty (Liao et al., 2019; Phillips, 2007), but it is unclear if resistance training also protects against the negative metabolic effects of high protein diets.

Here, we investigated this paradox by feeding isocaloric diets with different levels of dietary protein to mice and subjecting them to a recently validated mouse model of progressive resistance exercise, weight pulling (WP) or sham control exercise. In agreement with previous studies from our lab and others, we found that in the absence of WP, LP-fed mice were metabolically healthier than HP-fed animals, remaining leaner and having better glycemic control; conversely, HP-fed animals were fatter and had worse glycemic control. However, when HP-fed mice were subjected to WP, the negative effects of the HP diet on fat mass and glycemic control were ablated.

Surprisingly, while HP-feeding did as expected support muscle mass gain and strength gain more than an LP diet, the superiority of the HP diet in the maximum weight pulled was transitory, and by the end of the training period, LP-fed mice could pull just as much weight as HP-fed mice. There was no effect of diet or WP in the effects on inverted cling time or Rotarod performance, which are typically more impacted by aerobic exercise (Graber et al., 2015). Mitochondrial respiration outcomes were also independent of diet and exercise.

Our study was subject to a number of limitations. We examined only young inbred C57BL/6J male mice. We have previously shown that sex, genetic background, and age impact the metabolic response to dietary protein (Green et al., 2022a), and we suspect that these factors may also influence the response to exercise. Further, as the metabolic response to dietary protein

is responsive to the exact level of protein in the diet, examining additional levels of dietary protein between the LP and HP diets we examined here might provide greater insight (Green et al., 2022a). Additionally, to keep our diets isocaloric, isonitrogenous and consistent in fat content, we had to reduce carbohydrates in the HP diet, which might play a role in the results found here. Many people who eat high protein diets or take protein supplements engage in aerobic exercise, which we did not examine in the present study. In the present study, we have not examined the effects of dietary protein and exercise on either muscle fiber type or molecular changes. Recent work has shown that WP results in significant fiber type changes in addition to muscle hypertrophy, but it is unknown how this might be affected by dietary protein content. In regards to molecular changes, we have not explained how WP inhibits HP-induced gains in adiposity. The hormone FGF21, which is induced by a LP diet, is responsive to exercise in mice and humans (Haghighi et al., 2022; Jin et al., 2022; Li et al., 2022) and we hypothesize that WP in HP-fed mice may activate beiging in iWAT or BAT activity. We have not explored the role of individual amino acids in the response; many athletes and body builders take supplements in the BCAAs, and future work should determine if there is a specific role for these or other dietary amino acids in the metabolic and physiological response to resistance exercise.

In summary, dietary protein and progressive resistance exercise interact to regulate metabolic health as well as strength and muscle mass. Specifically increasing protein consumption has – as we anticipated based on both animal and human data – negative effects on the metabolic health of mice that are not exercising. However, these negative effects are eliminated in mice performing progressive resistance exercise training. While mice consuming a HP diet gained strength during weight pulling more quickly than LP-fed mice, the ultimate max load the HP and LP-fed mice could pull by the end of twelve weeks was indistinguishable, despite clear differences in muscle mass. Our results suggest high protein diets as well as protein or amino acid supplements may be consumed without negative metabolic effects by individuals

engaged in resistance exercise training, while sedentary individuals would likely be metabolically healthier if consuming a low protein diet. Finally, our results suggest that precision nutrition approach to metabolic health must consider not only diet and genetic variation, but also take into account activity level.

Methods

Animal care, housing and diet

All procedures were performed in conformance with institutional guidelines and were approved by the Institutional Animal Care and Use Committee of the William S. Middleton Memorial Veterans Hospital. Male C57BL/6J mice were procured from the Jackson Laboratory (000664). All studies were performed on animals or on tissues collected from animals. All mice were acclimated to the animal research facility for at least one week before entering studies. All animals were housed 2 per cage in static microisolator cages in a specific pathogen-free mouse facility with a 12:12 h light–dark cycle, maintained at approximately 22 °C. At the start of the experiment, mice were randomized to receive either the 7% (Low Protein, TD.140712) or 36% (High Protein, TD.220097) plus or minus weight pulling; all diets were obtained from Envigo. In the low protein diet, calories from amino acids were replaced by calories from carbohydrates, while carbohydrates were reduced and amino acids increased in the high protein diet. Calories from fat were held fixed at 19%, making the diets isocaloric (3.9 kcal/g). Full diet descriptions, compositions and item numbers are provided in **Table 1**.

Weight training paradigm

The procedures of this weight pulling regimen (acclimation, weighted paradigm, and unweighted paradigm) were described previously (Zhu et al., 2021).

Metabolic Phenotyping

Glucose and insulin tolerance tests were performed following a 16 hour overnight or 4 hour fast, respectively and then injecting either glucose (1g/kg) or insulin (0.75U/kg) intraperitoneally (Bellantuono et al., 2020; Yu et al., 2019). Glucose measurements were taken using a Bayer Contour blood glucose meter and test strips. Blood ketone measurements were taken using a KetoBM ketone meter and test strips that detect beta-hydroxybutyrate, the most abundant ketone in the body and proxy for ketone level. Mouse body composition was determined using an EchoMRI Body Composition Analyzer. For assay of multiple metabolic parameters (O2, CO2, food consumption, and activity tracking), mice were acclimatized to housing in a Columbus Instruments Oxymax/CLAMS-HC metabolic chamber system for approximately 24 hours, and data from a continuous 24-hour period was then recorded and analyzed.

Tissue collection

Terminal collections were performed 60–96 h after the final training session and after a 3 hour fast. During this procedure, all potentially identifiable information (e.g., tail markings, etc.) was masked, and then the animals were weighed and subsequently anesthetized with isoflurane. The plantaris, flexor digitorum longus (FDL), forearm flexor complex (FF) (which consisted of the flexor carpi ulnaris, flexor carpi radialis, flexor digitorum superficialis, and all three heads of the flexor digitorum profundus), and soleus (SOL) muscles from both the left and right hindlimb were then weighed. The left and right plantaris and right FDL were submerged in optimal cutting temperature compound (OCT, Tissue-Tek; Sakura Finetek, The Netherlands) at resting length, and frozen in liquid nitrogen-chilled isopentane. Left FDL was prepared for mitochondrial analysis. At this point, the mice were euthanized by cervical dislocation, a photograph that included both a scale bar and the musculature of the left forelimb was obtained, and then quadriceps muscles from both the left and right side of the body were collected. In addition to skeletal muscles, the inguinal white adipose tissue (iWAT), brown adipose tissue (BAT), epididymal white adipose tissue (eWAT), liver, as well as the right tibia bone, were collected post-mortem and frozen in liquid nitrogen or

prepared for histology (OCT or formalin, to then be incubated in ethanol). All of the collection procedures were performed by investigators blinded to treatment and diet group.

High Resolution Respirometry

FDL muscle was excised, weighed, and cut in half. The distal half was snap frozen in liquid nitrogen while proximal half was placed in ice cold (4°C) Buffer X containing (in mM) 7.23 K2EGTA, 2.77 CaK2EGTA, 20 imidazole, 20 taurine, 5.7 ATP, 14.3 phosphocreatine, 6.56 MgCl2·6H2O, and 50 K-MES (pH 7.1). Muscle was mechanically permeabilized with fine tip forceps removing connective and adipose tissue. Fiber bundles were chemically permeabilized in Buffer X with saponin (50 µg/mL) for 30 min on ice. Permeabilized muscle fiber bundles were rinsed with MiR05 containing (in mM) 0.5 mM EGTA, 3 MgCl2, 60 K-lactobionate, 20 taurine, 10 KH2PO4, 20 HEPES, 110 sucrose, 1 g/L BSA essentially fatty acid free, (pH 7.1) and 25 µM blebbistatin. Fibers were then blotted on filter paper to remove excess buffer and weighed on a microscale. Fibers bundles (1.5-2.5 mg) were then placed into chambers of the Oxygraph-2k (O2K; OROBOROS INSTRUMENTS, Innsbruck, Austria) containing MiR05 plus 12.5 µM blebbistatin, a myosin ATPase inhibitor, at 37°C. Chambers were hyperoxygenated to ~425uM and mitochondrial respiration was supported by complex-I linked substrates 5mM pyruvate, 10mM glutamate, and 0.5mM malate. ADP was provided at 0.25mM, 0.5mM and 5.5mM to evaluate sub saturating and saturating complex-I driven mitochondrial respiration. Mitochondrial membrane integrity was evaluated using 10µM cytochrome c. A >15% change in oxygen consumption was considered a loss of mitochondrial membrane integrity and 3 samples were removed from final analysis. 10mM succinate was provided to evaluate complex I and II driven respiration. Data is normalized to muscle fiber bundle wet weight.

Statistics

Data are presented as the mean ± SEM unless otherwise specified. Statistical analyses were performed using one-way, two-way ANOVA, two-way RM ANOVA, or a mixed-effects model (Restricted Maximum Likelihood (REML)) followed by an appropriate posttest as specified in the figure legends. In all figures, n represents the number of biologically independent animals. Sample sizes were chosen based on our previously published experimental results with the effects of dietary interventions plus consideration of the training time required by personnel (Cummings et al., 2018; Fontana et al., 2016; Yu et al., 2021; Yu et al., 2019; Yu et al., 2018). Data distribution was assumed to be normal, but this was not formally tested.

CONFLICT OF INTEREST STATEMENT

D.W.L has received funding from, and is a scientific advisory board member of, Aeovian Pharmaceuticals, which seeks to develop novel, selective mTOR inhibitors for the treatment of various diseases.

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Figure 1

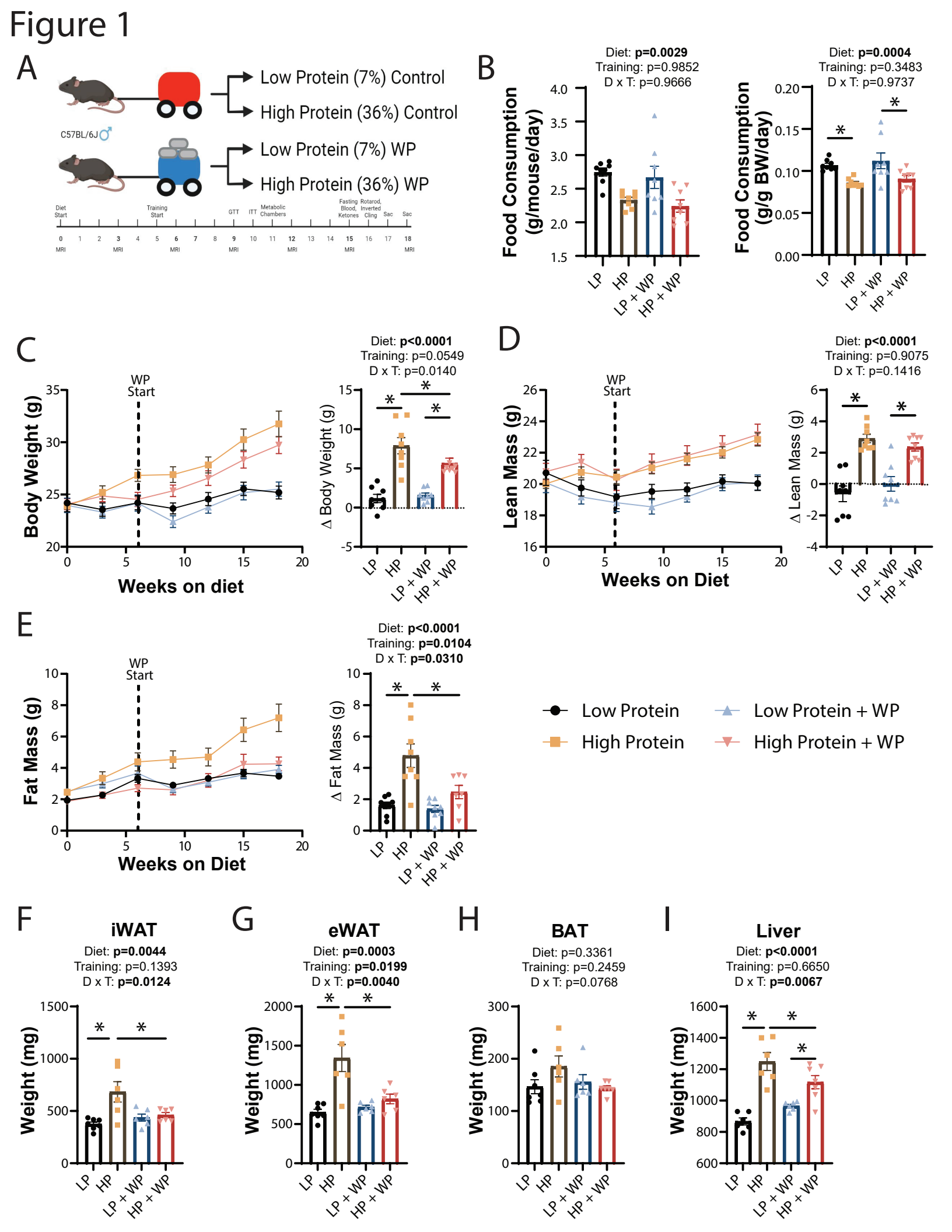
A
Low Protein (7%) Control
High Protein (36%) Control
C57BL/6J ♂
Low Protein (7%) WP
High Protein (36%) WP
Diet Start
Training Start
GTT
ITT
Metabolic Chambers
Fasting Blood, Ketones
Rotarod, Inverted Cling
Sac
MRI
B
Diet: p=0.0029
Training: p=0.9852
D x T: p=0.9666
Food Consumption (g/mouse/day)
Diet: p=0.0004
Training: p=0.3483
D x T: p=0.9737
Food Consumption (g/g BW/day)
LP
HP
LP + WP
HP + WP
C
WP Start
Body Weight (g)
Weeks on diet
Diet: p<0.0001
Training: p=0.0549
D x T: p=0.0140
Δ Body Weight (g)
D
WP Start
Lean Mass (g)
Weeks on Diet
Diet: p<0.0001
Training: p=0.9075
D x T: p=0.1416
Δ Lean Mass (g)
E
WP Start
Fat Mass (g)
Weeks on Diet
Diet: p<0.0001
Training: p=0.0104
D x T: p=0.0310
Δ Fat Mass (g)
Low Protein
High Protein
Low Protein + WP
High Protein + WP
F
iWAT
Diet: p=0.0044
Training: p=0.1393
D x T: p=0.0124
Weight (mg)
G
eWAT
Diet: p=0.0003
Training: p=0.0199
D x T: p=0.0040
Weight (mg)
H
BAT
Diet: p=0.3361
Training: p=0.2459
D x T: p=0.0768
Weight (mg)
I
Liver
Diet: p<0.0001
Training: p=0.6650
D x T: p=0.0067
Weight (mg)

Figure Legends

Figure 1. Weight pulling protects from high protein diet induced weight and fat gain.

(A) Experimental design. (B) Food consumption normalized to body weight after 5-6 weeks on the indicated diets. (C-E) Body weight (C), lean mass (D) and fat mass (E) over time, and change(Δ) from the beginning to end of study. (B-I) n=6-8 mice per group. Statistics for the overall effects of genotype, diet, and the interaction represent the p value from a two-way ANOVA; *$p<0.05$, Sidak's post-test examining the effect of parameters identified as significant in the 2-way ANOVA. Data represented as mean ± SEM.

Figure 2

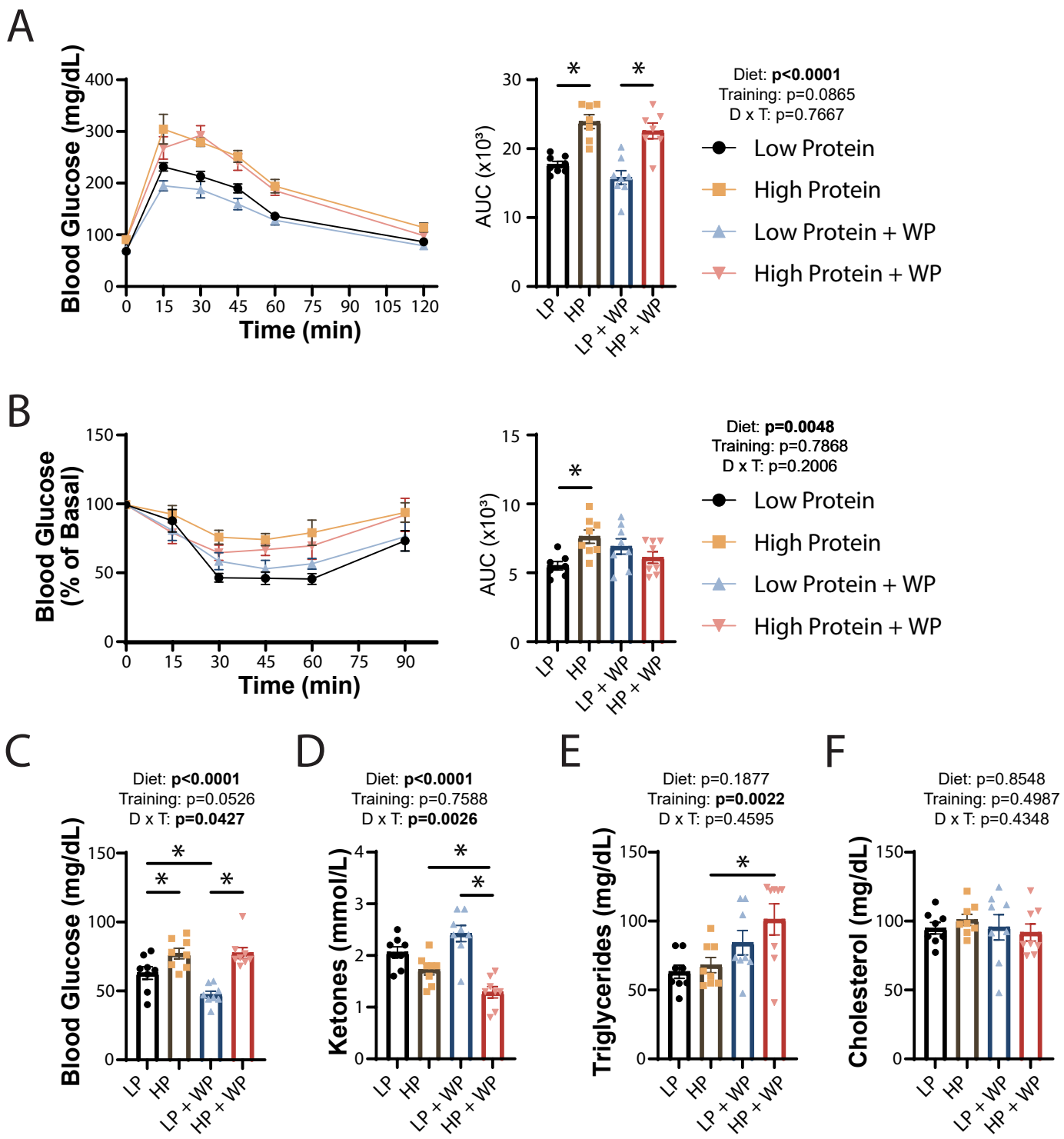
A
Blood Glucose (mg/dL)
Time (min)
AUC (x10³)
Diet: p<0.0001
Training: p=0.0865
D x T: p=0.7667
Low Protein
High Protein
Low Protein + WP
High Protein + WP
LP
HP
LP + WP
HP + WP
B
Blood Glucose (% of Basal)
Time (min)
AUC (x10³)
Diet: p=0.0048
Training: p=0.7868
D x T: p=0.2006
C
Blood Glucose (mg/dL)
Diet: p<0.0001
Training: p=0.0526
D x T: p=0.0427
D
Ketones (mmol/L)
Diet: p<0.0001
Training: p=0.7588
D x T: p=0.0026
E
Triglycerides (mg/dL)
Diet: p=0.1877
Training: p=0.0022
D x T: p=0.4595
F
Cholesterol (mg/dL)
Diet: p=0.8548
Training: p=0.4987
D x T: p=0.4348

Figure 2. Effect of diet and exercise on glycemic control and blood metabolites

(A-B) Glucose (A) and Insulin (B) tolerance tests were performed after 9-10 weeks on the diet, respectively, and area under the curve (AUC) was calculated. (C-F) Blood was collected from animals after a 16-hour overnight fast after 16 weeks on the diets. Fasting blood sugar (C), ketones (D), triglycerides (E) and cholesterol (F) was determined. (A-F) n=7-8 mice per group; statistics for the overall effects of diet, training and the interaction represent the p value from a two-way ANOVA, *$p<0.05$, Sidak's post-test examining the effect of parameters identified as significant in the 2-way ANOVA. Data represented as mean ± SEM.

Figure 3

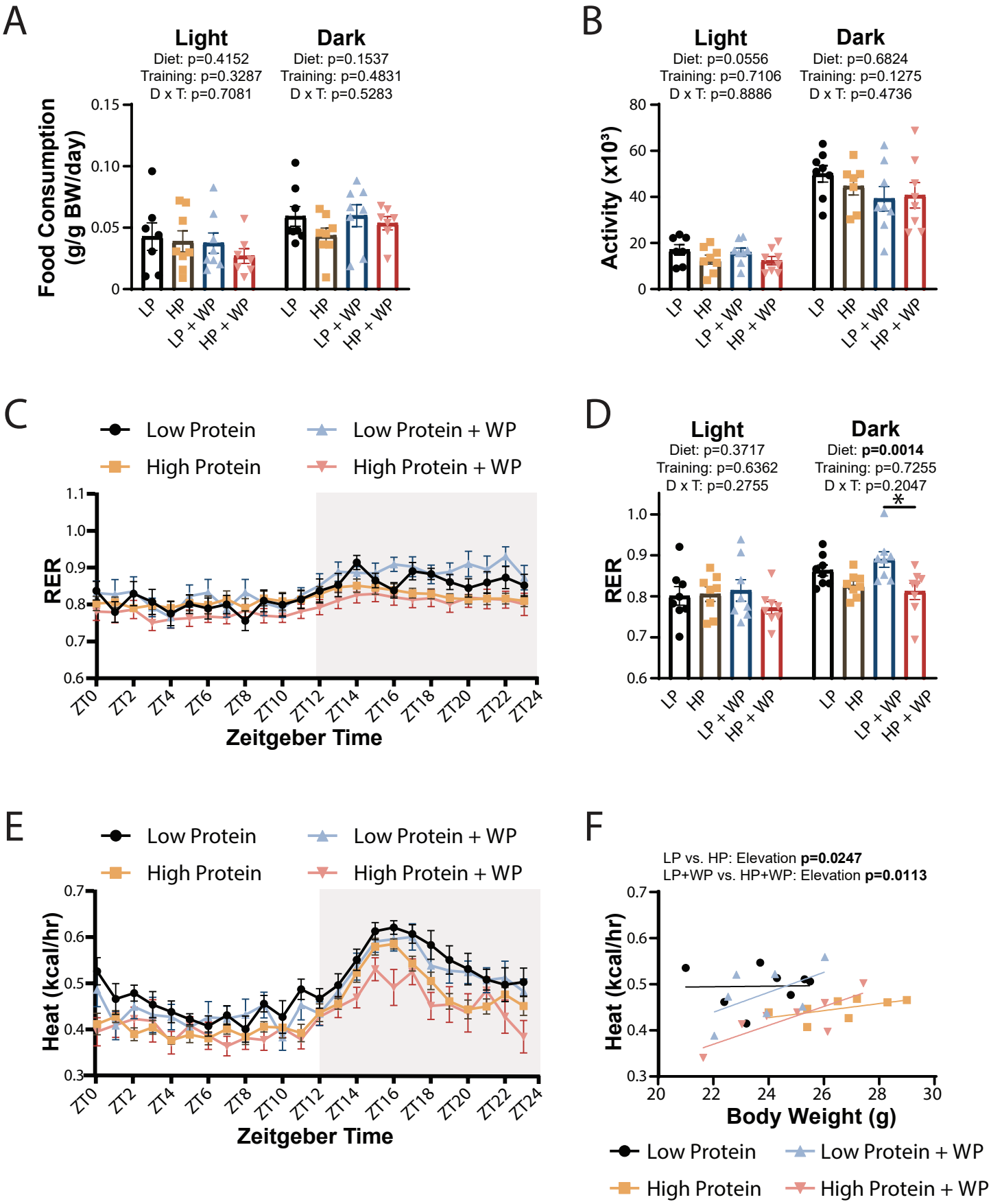
A
Light
Diet: p=0.4152
Training: p=0.3287
D x T: p=0.7081
Dark
Diet: p=0.1537
Training: p=0.4831
D x T: p=0.5283
Food Consumption (g/g BW/day)
0.15
0.10
0.05
0
LP
HP
LP + WP
HP + WP
B
Light
Diet: p=0.0556
Training: p=0.7106
D x T: p=0.8886
Dark
Diet: p=0.6824
Training: p=0.1275
D x T: p=0.4736
Activity (x10³)
80
60
40
20
0
C
Low Protein
Low Protein + WP
High Protein
High Protein + WP
RER
1.1
1.0
0.9
0.8
0.7
0.6
ZT0
ZT2
ZT4
ZT6
ZT8
ZT10
ZT12
ZT14
ZT16
ZT18
ZT20
ZT22
ZT24
Zeitgeber Time
D
Light
Diet: p=0.3717
Training: p=0.6362
D x T: p=0.2755
Dark
Diet: p=0.0014
Training: p=0.7255
D x T: p=0.2047
*
RER
1.0
0.9
0.8
0.7
0.6
E
Low Protein
Low Protein + WP
High Protein
High Protein + WP
Heat (kcal/hr)
0.7
0.6
0.5
0.4
0.3
Zeitgeber Time
F
LP vs. HP: Elevation p=0.0247
LP+WP vs. HP+WP: Elevation p=0.0113
Heat (kcal/hr)
20
22
24
26
28
30
Body Weight (g)
Low Protein
Low Protein + WP
High Protein
High Protein + WP

Figure 3. Low protein fed animals have increased energy expenditure regardless of training regimen

(A-F) Metabolic chambers were used to examine metabolic parameters after mice were fed the indicated diets for 8-9 weeks. These included food consumption (A), spontaneous activity (B), Respiratory Exchange Ratio (RER) (C-D), and energy expenditure (E-F) over a 24 hour period (n=7-8 mice per group). (A, B, D) Statistics for the overall effects of diet, training and the interaction represent the p value from a two-way ANOVA conducted separately for the light and dark cycles; *$p<0.05$ from a Sidak's post-test examining the effect of parameters identified as significant in the 2-way ANOVA. (F) Simple linear regression (ANCOVA) was calculated to determine if the elevation or intercepts are equal. Data represented as mean ± SEM.

Figure 4

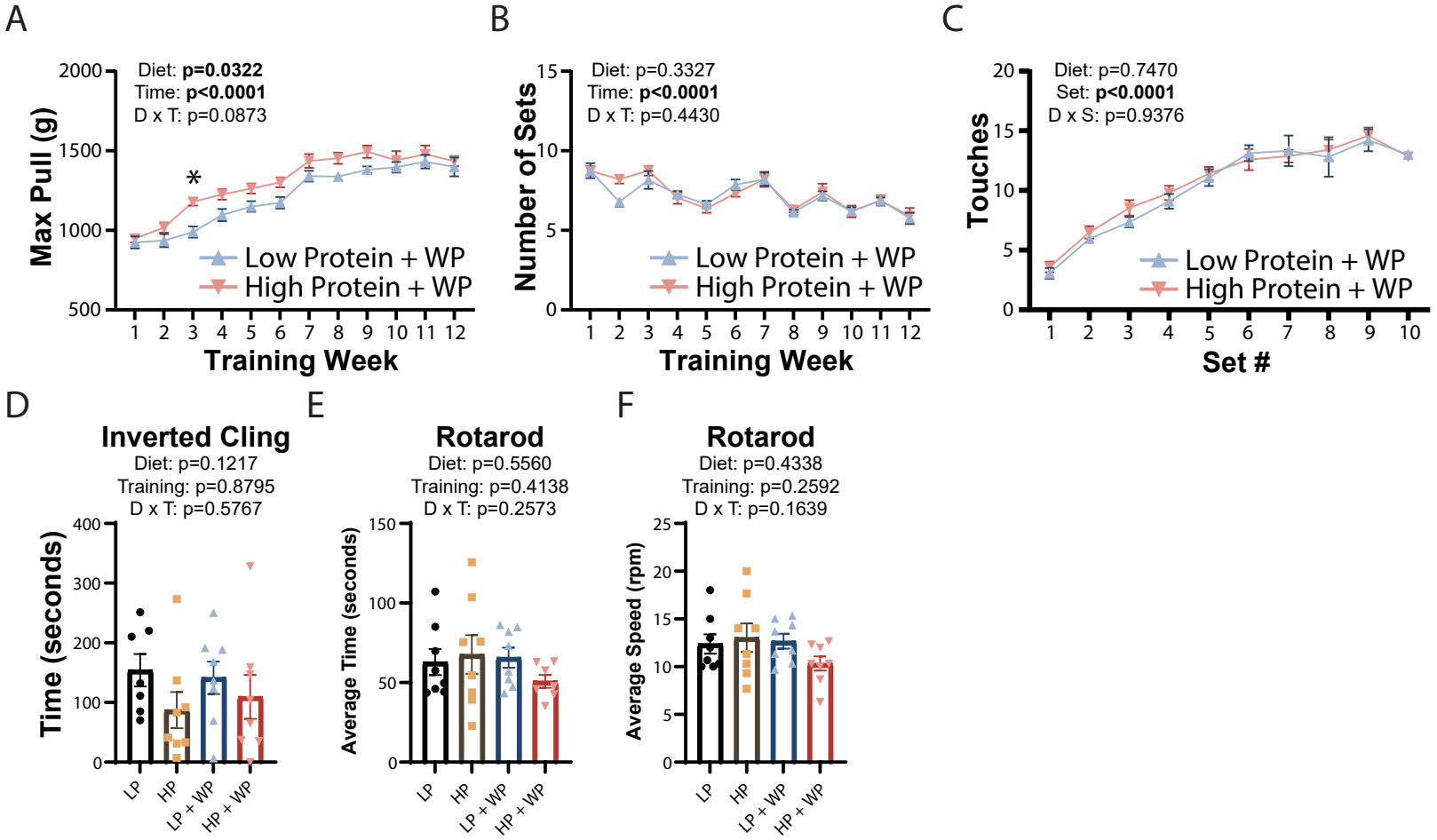
A
Diet: p=0.0322
Time: p<0.0001
D x T: p=0.0873
Max Pull (g)
Training Week
Low Protein + WP
High Protein + WP
B
Diet: p=0.3327
Time: p<0.0001
D x T: p=0.4430
Number of Sets
Training Week
Low Protein + WP
High Protein + WP
C
Diet: p=0.7470
Set: p<0.0001
D x S: p=0.9376
Touches
Set #
Low Protein + WP
High Protein + WP
D
Inverted Cling
Diet: p=0.1217
Training: p=0.8795
D x T: p=0.5767
Time (seconds)
LP
HP
LP + WP
HP + WP
E
Rotarod
Diet: p=0.5560
Training: p=0.4138
D x T: p=0.2573
Average Time (seconds)
LP
HP
LP + WP
HP + WP
F
Rotarod
Diet: p=0.4338
Training: p=0.2592
D x T: p=0.1639
Average Speed (rpm)
LP
HP
LP + WP
HP + WP

Figure 4. Strength and muscle growth is maximized by high protein and progressive resistance exercise.

(A-C) Weight pulling was performed three times per week for 12 weeks. The average maximum weight pulled each week (A), and the number of sets achieved (B) is shown. Similarly, the number of stimulatory touches (C) by the investigator was averaged through multiple weeks of training and plotted per set. (A-C) n=8 mice per group. Statistics for the overall effects of diet, time or set, and the interaction represent the p value from a two-way RM ANOVA or REML; *$p<0.05$ from a Sidak's post-test examining the effect of parameters identified as significant in the 2-way ANOVA. (D-F) Physical performance was assessed by an inverted cling test (D) or rotarod test (E-F). (D-F) n=7-8 mice per group. Statistics for the overall effects of diet, training and the interaction represent the p value from a two-way ANOVA, *$p<0.05$, Sidak's post-test examining the effect of parameters identified as significant in the 2-way ANOVA. Data represented as mean ± SEM.

Figure 5

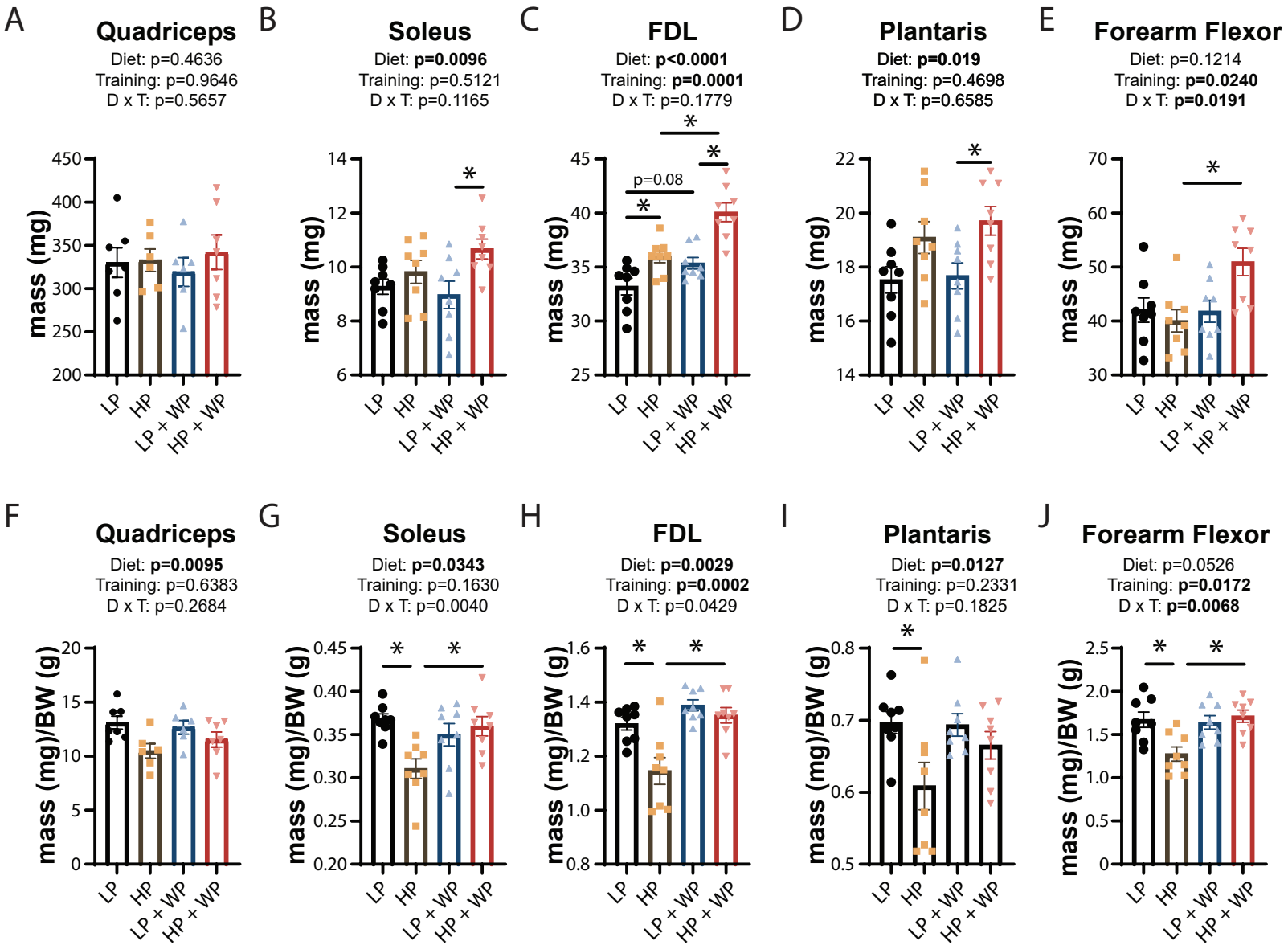
A
Quadriceps
Diet: p=0.4636
Training: p=0.9646
D x T: p=0.5657
mass (mg)
450
400
350
300
250
200
LP
HP
LP + WP
HP + WP
B
Soleus
Diet: p=0.0096
Training: p=0.5121
D x T: p=0.1165
mass (mg)
14
12
10
8
6
*
C
FDL
Diet: p<0.0001
Training: p=0.0001
D x T: p=0.1779
mass (mg)
45
40
35
30
25
p=0.08
*
D
Plantaris
Diet: p=0.019
Training: p=0.4698
D x T: p=0.6585
mass (mg)
22
20
18
16
14
*
E
Forearm Flexor
Diet: p=0.1214
Training: p=0.0240
D x T: p=0.0191
mass (mg)
70
60
50
40
30
*
F
Quadriceps
Diet: p=0.0095
Training: p=0.6383
D x T: p=0.2684
mass (mg)/BW (g)
20
15
10
5
0
G
Soleus
Diet: p=0.0343
Training: p=0.1630
D x T: p=0.0040
mass (mg)/BW (g)
0.45
0.40
0.35
0.30
0.25
0.20
*
H
FDL
Diet: p=0.0029
Training: p=0.0002
D x T: p=0.0429
mass (mg)/BW (g)
1.6
1.4
1.2
1.0
0.8
*
I
Plantaris
Diet: p=0.0127
Training: p=0.2331
D x T: p=0.1825
mass (mg)/BW (g)
0.8
0.7
0.6
0.5
*
J
Forearm Flexor
Diet: p=0.0526
Training: p=0.0172
D x T: p=0.0068
mass (mg)/BW (g)
2.5
2.0
1.5
1.0
0.5
0
*

Figure 5. Weight pulling increases muscle mass of HP-fed mice.

Absolute (A-E) and normalized to body weight (F-J) mass of a variety of muscles: the quadriceps (A, F), soleus (C, H), FDL (D, H), plantaris (D, I) and forearm flexor (E, J). n=8 mice per group. Statistics for the overall effects of diet, training and the interaction represent the p value from a two-way ANOVA, *$p<0.05$, Sidak's post-test examining the effect of parameters identified as significant in the 2-way ANOVA. Data represented as mean ± SEM.

Figure 6

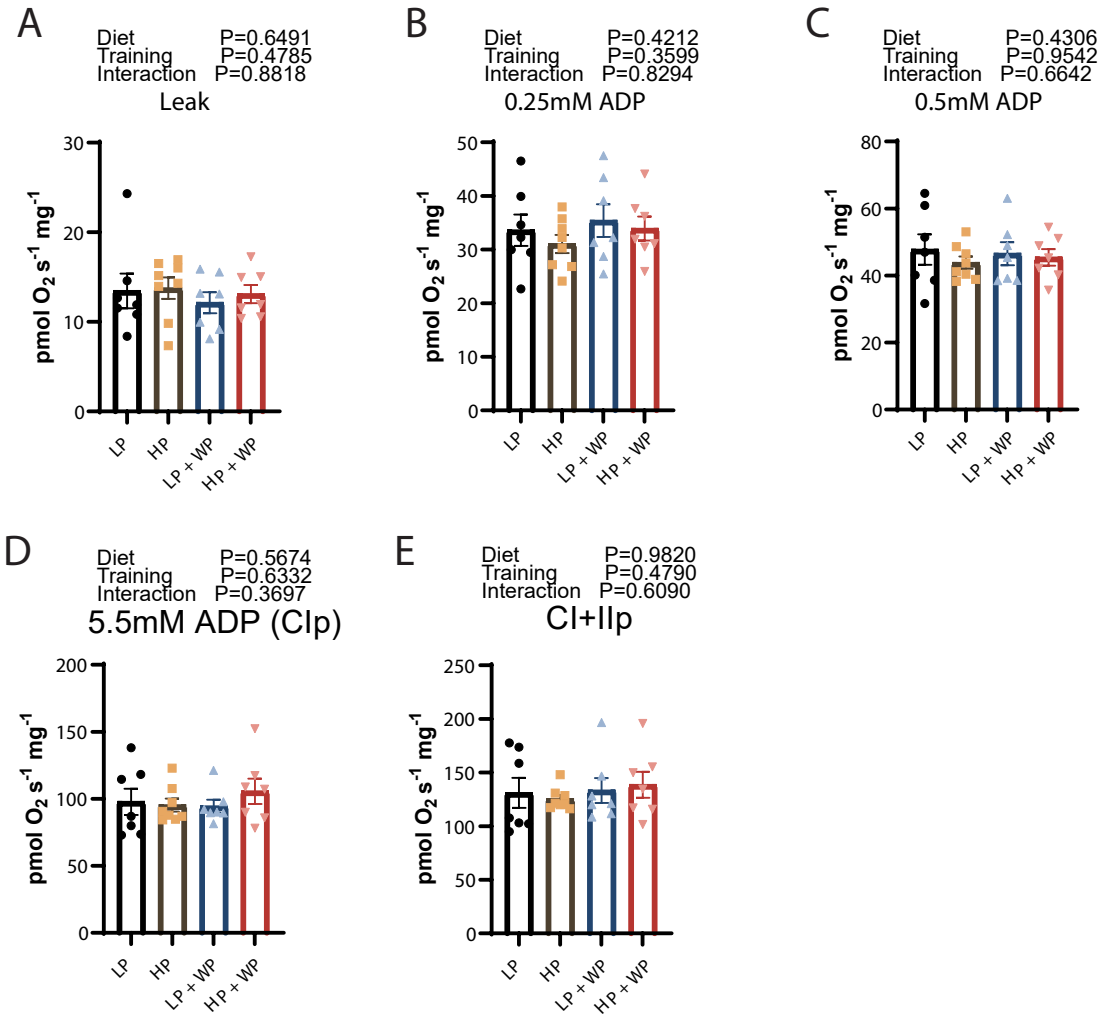
A
Diet P=0.6491
Training P=0.4785
Interaction P=0.8818
Leak
pmol O2 s-1 mg-1
0
10
20
30
LP
HP
LP + WP
HP + WP
B
Diet P=0.4212
Training P=0.3599
Interaction P=0.8294
0.25mM ADP
pmol O2 s-1 mg-1
0
10
20
30
40
50
LP
HP
LP + WP
HP + WP
C
Diet P=0.4306
Training P=0.9542
Interaction P=0.6642
0.5mM ADP
pmol O2 s-1 mg-1
0
20
40
60
80
LP
HP
LP + WP
HP + WP
D
Diet P=0.5674
Training P=0.6332
Interaction P=0.3697
5.5mM ADP (CIp)
pmol O2 s-1 mg-1
0
50
100
150
200
LP
HP
LP + WP
HP + WP
E
Diet P=0.9820
Training P=0.4790
Interaction P=0.6090
CI+IIp
pmol O2 s-1 mg-1
0
50
100
150
200
250
LP
HP
LP + WP
HP + WP

Figure 6. Mitochondrial respiration is not altered by protein or weight pulling.

(A) Mitochondrial leak. (B-D) Addition of 0.25 mM (B), 0.5 mM (C), and 5.5 mM ADP (D). (E) 10mM succinate was provided to evaluate complex I and II driven respiration. n=7-8 mice/group. (B-F) Statistics for the overall effects of diet, training and the interaction represent the p value from a two-way ANOVA, *$p<0.05$, Sidak's post-test examining the effect of parameters identified as significant in the 2-way ANOVA. Data represented as mean ± SEM.

Table 1. Diet composition

Amino Acid Defined Diets	**Low Protein**	**High Protein**
Teklad Diet Name	7% Protein Calories	36% Protein Calories
Teklad Diet Number	TD.140712	TD.220097
Colour	Blue	Green
Formula	**g/kg**	**g/kg**
Sucrose	291.248	214.867
Corn Starch	232.4	110.7
Maltodextrin	232.4	110.7
Corn Oil	52.0	52.0
Olive Oil	29.0	29.0
Cellulose	30.0	30.0
Mineral Mix, AIN-93G-MX (94046)	35.0	35.0
Calcium Phosphate, dibasic	8.2	8.2
Vitamin Mix, Teklad (40060)	10.0	10.0
% kcal from		
Protein	7.1	36.4
Carbohydrate	74.4	44.7
Fat	18.5	18.9
Kcal/g	3.9	3.9
Amino Acid Profile	**g/kg**	**g/kg**
L-Lysine HCl	6.64	33.308
L-Methionine	2.18	10.95
L-Cystine	2.34	11.767
L-Arginine	2.05	10.296
L-Phenylalanine	2.15	10.787
L-Tyrosine	2.25	11.277
L-Histidine HCl, monohydrate	1.15	7.518
L-Isoleucine	2.54	12.748
L-Leucine	8.27	41.512
L-Threonine	3.16	15.853
L-Tryptophan	1.1	5.557
L-Valine	2.735	13.729
L-Aspartic Acid	6.7	33.634
L-Glutamic Acid	9.43	47.347
L-Alanine	3.05	15.33
Glycine	0.96	4.838
L-Proline	2.41	12.111
L-Serine	2.41	12.111