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Sex-specific Effects of Exercise Ancestry on Metabolic, Morphological, and Gene Expression Phenotypes in Multiple Generations of Mouse Offspring

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Abstract

Early life and pre-conception environmental stimuli can affect adult health-related phenotypes. Exercise training is an environmental stimulus affecting many systems throughout the body and appears to alter offspring phenotypes. The aim of this study was to examine the influence of parental exercise training, or "exercise ancestry," on morphological and metabolic phenotypes in two generations of mouse offspring. F0 C57BL/6 mice were exposed to voluntary exercise or sedentary lifestyle and bred with like-exposed mates to produce an F1 generation. F1 mice of both ancestries were sedentary and sacrificed at 8 wk or bred with littermates to produce an F2 generation, which was also sedentary and sacrificed at 8 wk. Small, but broad generation- and sex-specific effects of exercise ancestry were observed for body mass, fat and muscle mass, serum insulin, glucose tolerance, and muscle gene expression. F1 EX females were lighter than F1 SED females, and had lower absolute tibialis anterior and omental fat masses. Serum insulin was higher in F1 EX females compared to F1 SED females. F2 EX females had impaired glucose tolerance compared to F2 SED females. Analysis of skeletal muscle mRNA levels revealed several generation- and sex-specific differences in mRNA levels for multiple genes, especially those related to metabolic genes (e.g., F1 EX males had lower mRNA levels of *Hk2*, *Ppard*, *Ppargc1α*, *Adipoq*, and *Scd1* than F1 SED males). These results provide preliminary evidence that parental exercise training can influence health-related phenotypes in mouse offspring.

Keywords

Exercise; Pregnancy; Metabolism

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Disclosures

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Introduction

The beneficial effects of exercise training on metabolic health are well characterized. Among other outcomes, exercise training leads to increased mitochondrial oxidative capacity, which may be protective against a variety of chronic diseases (Tseng *et al.*, 1995). In addition, exercise training improves glucose homeostasis and fat oxidation (Hoppeler & Flück, 2003; Fluck, 2006). In contrast, lack of exercise (or sedentary lifestyle) is associated with chronic disease development and all-cause mortality (Mokdad *et al.*, 2004).

Mounting evidence suggests early life (even pre-conception) events can affect adult health-related phenotypes, such as disease risk; this is referred to as the developmental origins of health and disease hypothesis (Gluckman & Hanson, 2006). Maternal protein restriction results in offspring with lower birth weights and adult metabolic dysfunction (Peixoto-Silva *et al.*, 2011). Excess maternal caloric intake also affects offspring health, leading to excess adiposity (Bayol *et al.*, 2008) as well as reduced muscle force production (Bayol *et al.*, 2009) in exposed offspring compared to mothers consuming a normal diet. The existing literature is primarily focused on maternal factors, however there is emerging evidence that dietary and other environmental factors can also influence offspring health through the paternal line (Ng *et al.*, 2010).

Exercise training is an environmental stimulus affecting many systems throughout the body, and it may be capable of inducing transgenerational modifications similar to these more commonly studied nutritional interventions. The clinical literature concerning the effect of maternal exercise on maternal, fetal, and child health has been reviewed elsewhere (Kalisiak & Spitznagle, 2009). In summary, exercise during pregnancy appears to have beneficial effects in the mother and fetus. Child health outcomes are less clear, with studies reporting greater, lower, or no difference in body weight between neonates whose mothers did or did not exercise during pregnancy (Kalisiak & Spitznagle, 2009). In those studies that observed decreased body weight, the difference appeared to be due to a lower percent body fat (Clapp & Capeless, 1990). The impact of maternal exercise on adult health outcomes or the impact on multiple generations has not been studied in humans. Likewise in rodents, varying effects of maternal exercise have been observed. Maternal treadmill running has been linked to smaller litters in some (Treadway *et al.*, 1986) though not all (Monteiro *et al.*, 2010) studies, but has not been associated with fetal or offspring body mass or length (Treadway *et al.*, 1986; Monteiro *et al.*, 2010). Similarly, maternal swimming has been associated with lower birth weight offspring in one (Pinto & Shetty, 1995) but not all studies (Treadway & Lederman, 1986). Two recent studies documented beneficial adaptations in body composition and glucose and insulin dynamics in the mature offspring of dams who had access to a voluntary running wheel during the perinatal period (Carter *et al.*, 2012*a*, 2012*b*). To the best of our knowledge, only one study has examined the impact of maternal exercise over multiple generations of offspring; there, maternal swimming led to low-birth-weight pups in the first generation. This growth retardation was also observed in a second generation of rat pups born to the offspring of exercised dams, regardless of whether the first generation offspring were also exposed to exercise (Pinto & Shetty, 1995). Whether these observed changes in body size and/or composition are associated with metabolic health or function has not been elucidated. Additionally, the potential impact of paternal exercise has not been examined.

Thus, we sought to determine how body morphology, metabolic phenotypes and skeletal muscle gene expression are affected by exercise ancestry in multiple generations of mouse offspring. Our aims were 1: determine body, fat, organ and skeletal muscle mass differences, 2: examine metabolic phenotypes such as glucose tolerance and circulating insulin and lipids, and 3: determine gene expression differences by skeletal muscle microarray and

targeted gene expression analyses. We hypothesized that multiple generations of offspring from exercise ancestry would exhibit advantageous morphological, metabolic and gene expression phenotypes compared to offspring from a sedentary ancestry.

Methods

Ethical Approval

All animal procedures were performed in accordance with the National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee at the University of Maryland.

Animal Procedures

We performed a breeding experiment including three generations of animals that were either exposed to exercise or kept sedentary in the F0 generation. An overview of our experimental design and timeline is provided in Figure 1. A standard diet (Purina Prolab RMH 3000, 60% carbohydrate, 14% fat, 26% protein) and water were provided *ad libitum* for all generations in all conditions. 20 male and 20 virgin female 5-wk old C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME); these mice made up the F0 generation. C57BL/6 is an inbred strain of mouse that was chosen based on their propensity to perform voluntary wheel running, their common use in research studies of exercise, nutrition, metabolism, and gene expression, and their fecundity. Until 8 weeks of age, these animals were kept sedentary (i.e., unexposed to voluntary running wheel; standard cage conditions) and group-housed with same-sex mice.

At 8 weeks of age, each sex was randomly split into two condition groups: exercise (EX) and sedentary (SED) (n=10 per group). F0 animals were placed into standard cages (F0 SED) or in cages with computer-monitored voluntary running wheels (F0 EX). F0 mice were housed individually in their respective cages for 10 weeks. Following this intervention males and females from like conditions (EX or SED) were randomly paired for mating. The F0 EX breeding pairs had continued access to the running wheel throughout the breeding period, however running activity could not be monitored during the breeding period as there were two mice in each cage. Males were removed after pregnancy was visually confirmed by abdominal distention, vaginal plug and/or and body weight changes. After removal, F0 males had continued access to the running wheel (EX) or SED condition until sacrifice at 20 weeks (a total of 12 weeks of wheel access). F0 females had continued access to the running wheel (EX) or SED condition during pregnancy and lactation and were sacrificed at 25 weeks after the F1 mice were weaned (a total of 17 weeks of wheel access). Eight of ten breeding pairs in each condition produced viable litters. All of the F0 animals that underwent the intervention period (EX or SED condition) were included in the F0 analyses. The pups resulting from this original mating were designated as F1. A random group of 10 male and 10 female F1 pups from each condition (EX and SED) were designated as F1 breeders and were mated at 8 weeks with F1 littermates to produce the F2 generation. Of the 10 breeding pairs per condition, 7 F1 EX and 9 F1 SED pairs produced viable litters. The remaining F1 pups were glucose tolerance tested and sacrificed at 8 weeks. F2 pups from each condition were glucose tolerance tested and sacrificed at 8 weeks. Body weight was monitored weekly for all animals. F1 and F2 offspring remained sedentary throughout the experiment. Following weaning at 21–25 days, all F1 and F2 offspring were group-housed with same-sex littermates. We used 1–7 offspring per litter for the 8-week analyses.

Intraperitoneal Glucose Tolerance Test (IPGTT)

Glucose tolerance tests were performed on all F0, F1, and F2 mice at 8 (F1 and F2 generations), 20 (F0 males), or 25 (F0 females) weeks. For F0 EX mice, the voluntary

running wheels were locked 36 hours prior to the glucose tolerance test to limit the effect of acute exercise. All animals were fasted overnight (12 hr) prior to glucose tolerance testing. Baseline blood glucose measurements were made and then each mouse was injected intraperitoneally with 2.0 mg of D-glucose (Sigma-Aldrich, St. Louis, MO) per gram of body mass. Blood glucose was measured 30, 60, 90, and 120 minutes after injection in all animals; blood glucose was also measured 15 min after injection in F2 males and females. Area under the curve for concentration vs. time was calculated using the linear trapezoidal rule. Blood glucose measurements were made using a rodent-specific glucometer (AlphaTRAK; Abbott Laboratories, Abbott Park, IL) on blood removed from a tail snip. Following the glucose tolerance test, wheels were unlocked and animals were returned to *ad libitum* food and water.

Tissue & Serum Collection

Animals were euthanized 2–5 days following the glucose tolerance test. To limit the effects of acute exercise and feeding, running wheels were locked 24 hours and animals were fasted for 4 hours prior to euthanasia. Euthanasia was performed under isoflurance anesthesia; the method of euthanasia was exsanguination by cardiac puncture followed by removal of the heart. Heart, liver, omental fat, cerebellum, tibialis anterior (TA), extensor digitorum longus (EDL), soleus, plantaris, gastrocnemius, and quadriceps muscles were dissected, weighed, and flash frozen in liquid nitrogen and then stored at −80°C until analysis. Approximately 1 mL of blood was obtained from a cardiac puncture and allowed to coagulate. The coagulated blood was centrifuged at 1750 × g for 15 minutes to obtain serum. Serum was removed to a fresh tube and stored at −80°C until analysis.

Serum Measures

Serum triglyceride and glycerol were measured using the Serum Triglyceride Determination Kit (TR0100; Sigma-Aldrich, St. Louis, MO). Serum insulin was measured following a 4-hour fast using a mouse-specific ELISA kit (80-INSMS-E01, ALPCO Diagnostics, Salem, NH).

Tissue Preparation

The gastrocnemius muscle was chosen for analysis because it is a mixed-fiber type muscle (Augusto *et al.*, 2004) and therefore may be more representative of the average mouse skeletal muscle than a predominantly fast or slow muscle. Whole gastrocnemius muscle was powdered in liquid nitrogen. Total RNA was isolated with Trizol reagent (15596-026, Life Technologies, Grand Island, NY), DNase-treated, and quantified with the NanoDrop (Bio-Rad, Hercules, CA) spectrophotometer. Reverse transcription was performed with 1μg of total RNA with the High-Capacity cDNA RT kit (4368813, Life Technologies, Grand Island, NY).

Microarray

Genome-wide analyses of gene expression were performed on subsets of F1 and F2 males. Total RNA was extracted from powdered gastrocnemius muscle using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Germantown, MD). All samples were diluted to 98 ng/μl and pooled by generation and condition (5 pooled samples per array). A total of 8 arrays were performed (2 arrays each for F1 EX males, F1 SED males, F2 EX males, and F2 SED males). Microarray experiments were performed as two-color experiments using GeneChip Mouse Exon 1.0 ST Array chips (Affymetrix, Santa Clara, CA). The microarray gene expression data were imported from the probe cell intensity CEL files and the Affymetrix Expression Console software was used to calculate the summary measure of the probe level data. These microarray data have been deposited in the NCBI Gene Expression Omnibus

(GEO, http://www.ncbi.nlm.nih.gov/geo/) website and can be accessed through GEO Series accession number GSE40469.

Gene Expression

Real-time quantitative PCR was used to assess the gene expression level of adiponectin (*Adipoq)*, cell death-inducing DFFA-like effector c (*Cidec)*, and stearoyl-Coenzyme A desaturase 1 (*Scd1)* (n=10–20 per group). Primer and probe sequences were designed for each gene's mRNA sequence using PrimeTime qPCR Assay designer (IDT). *18S* rRNA was used as an expression control for both real-time and gel-based PCR and did not differ between treatment groups. RT-PCR was used to measure the expression of metabolic genes (peroxisome proliferator-activated receptor gamma, coactivator 1 alpha, *Ppargc1a*; pyruvate dehydrogenase kinase, isozyme 4, *Pdk4*; peroxisome proliferator activated receptor alpha, delta, and gamma, *Ppara, Ppard*, and *Pparg*, aminolevulinate, delta-, synthase 1, *Alas1*; hexokinase 2, *Hk2*; cytochrome c, somatic, *Cycs*; citrate synthase, *Cs*; and cytochrome c oxidase subunit I, *Cox1*) (n=7–10 per group). Primer and probe sequences and PCR conditions are available upon request. Products were visualized on 1.5% agarose gels using ethidium bromide. qPCR data were normalized to *18S* using the −ΔCt method (Schmittgen & Livak, 2008) and expressed as fold induction ($2^{-\Delta Ct}$) of mRNA expression compared to the corresponding EX group (1.0-fold induction). Relative band intensities from PCR gels were be analyzed with NIH ImageJ software and normalized to *18s*. Values are shown as means ± standard error.

Statistics

Two-tailed t-tests were used to compare body and tissue masses, serum measures, and IPGTT AUC between EX and SED groups within each sex and generation. A repeated-measures ANOVA was used to compare the IPGTT response between EX and SED groups within each sex and generation. One- (F1 male RT-qPCR analysis) and two- (all other analyses) tailed t-tests were used to compare gene expression between EX and SED groups within each sex and generation using SPSS version 18. Statistical significance was accepted at $p < 0.05$. A 2-stage ANOVA procedure was carried out on the 4 arrays of the F1 generation and F2 generations, respectively. Details about this procedure can be found in Lee (Lee, 2004). Briefly, in the first-stage ANOVA, a one-way ANOVA model for EX vs. SED ancestry was fitted on the entire dataset, resulting in normalized estimates of gene expression centered by the ancestry factor. These normalized values were used in a second-stage ANOVA to identify which probe sets were significantly differentially expressed between EX and SED ancestries.

Results

During the pre-breeding intervention period, F0 EX males ran an average of 6558 ± 503 m × 24 hr^{-1} while F0 EX females ran a significantly greater ($p < 0.05$) average of 8378 ± 533 m × 24 hr^{-1}. Running behavior was only measured prior to mating, though the F0 EX mice had continued access to the voluntary running wheel throughout breeding, pregnancy and lactation. To examine the effects of parental exercise, we characterized the body mass, various tissue masses, glucose tolerance, and serum insulin, glycerol, and triglyceride levels in the original parent generation (F0) and F1 and F2 offspring from EX and SED ancestries.

F0 generation

Males—After the intervention period, F0 EX males weighed significantly less than F0 SED males (p=0.024; Table 1). F0 EX males also had significantly less omental fat mass compared to SED males (p=0.003; Table 1), a difference that was maintained after normalizing to body mass (p=0.009; Table 2). Additionally, when normalized to body mass,

F0 EX males had greater EDL mass and lower omental fat mass compared to F0 SED males (p=0.021 and p=0.001, respectively; Table 2). There were no other differences in organ or tissue mass in F0 males. Baseline blood glucose and glucose tolerance was not affected by the EX intervention in F0 males (Figure 2A).

Females—F0 female body mass was not affected by the EX intervention, but EX females had significantly greater soleus mass and normalized soleus mass than SED females (p=0.001 for both; Tables 1 & 2). Baseline blood glucose concentration was not affected by the EX intervention in females. However, glucose tolerance tended to be better (p=0.051, 16.4%) in EX compared to SED females as indicated by AUC and EX females had significantly lower (p=0.001) blood glucose 60 minutes following the glucose injection (Figure 2B).

F1 generation

The F1 offspring sacrificed at 8 wk of age for analysis was comprised of 20 EX males, 17 SED males, 12 EX females, and 18 SED females. F1 litter size ranged from 3–9 (EX) and 4–9 (SED). The average litter size was not affected by EX (6.5 offspring/litter for F1 EX and 6.9 offspring/litter for F1 SED, p>0.05).

Males—There were no differences in body mass in F1 male offspring at 8 weeks. F1 males from EX parents had lower soleus, EDL, and quadriceps masses (all $p < 0.05$; Table 1) than F1 males from SED parents, though only EDL mass remained significantly lower in F1 EX males after normalizing to body mass (p=0.015, Table 2). When normalized to body mass, F1 EX males had greater heart mass compared to F1 SED males (p=0.01, Table 2). No differences were observed in baseline blood glucose concentration, glucose tolerance (Figure 2C) or serum insulin, free glycerol, and triglyceride (Table 3) between EX and SED F1 males.

Females—F1 female offspring from EX parents were significantly lighter (5%) at 8 weeks (p=0.001; Table 1) compared to offspring from SED parents. F1 females from EX parents also had lower TA and omental fat masses compared to F1 females from SED parents ($p < 0.05$; Table 1). After normalizing to body mass, the difference in TA mass was no longer significant, but omental fat mass remained lower in F1 EX females (p=0.017; Table 2). No differences in baseline blood glucose concentration or glucose tolerance were observed between EX and SED in F1 females (Figure 2D), but serum insulin was significantly higher (55%) in F1 EX females compared to SED females (p=0.008; Table 3). Serum free glycerol and total triglyceride concentration were not affected by EX ancestry in F1 females (Table 3).

F2 generation

The F2 generation was comprised of 18 EX males, 18 SED males, 14 EX females, and 23 SED females. F2 litter size ranged from 3–7 (EX) and from 1–7 (SED). The average litter size was not affected by EX ancestry (4.6 F2 offspring for both F2 EX and F2 SED, p>0.05).

Males—At 8 weeks, no differences in absolute or normalized body, organ, or muscle weights were observed between F2 male offspring with EX and SED ancestries (Tables 1 & 2). No differences in baseline blood glucose concentration or glucose tolerance were observed in F2 male offspring between EX and SED ancestries (Figure 2E). No differences in serum insulin, free glycerol or triglycerides were observed between EX and SED F2 male offspring (Table 3)

Females—There were no differences in body, organ, or muscle weights between F2 EX and SED female offspring (Table 1), however after normalizing to body mass, F2 EX females had lower soleus, EDL, and omental fat mass (p=0.003, 0.015, and 0.048, respectively; Table 2). Baseline blood glucose concentration did not vary between EX and SED F2 female offspring, but EX offspring exhibited slightly impaired glucose tolerance compared to SED offspring as indicated by a larger (14%) AUC (P=0.015; Figure 2F). Additionally, EX female offspring had significantly higher blood glucose concentration at both 60 and 120 minutes after the glucose injection (p=0.049 and <0.001, respectively). There were no differences in serum insulin, free glycerol, or triglyceride between EX and SED F2 offspring (Table 3).

Gene Expression Analyses

In order to broadly assess the impact of EX ancestry on muscle gene expression, we performed a genome-wide gene expression microarray on gastrocnemius muscle of F1 and F2 males. In F1 males, 86 targets were upregulated and 23 targets were downregulated with EX ancestry (see Supplementary Figure 1, Supplementary Table 1). In F2 males, 142 targets were upregulated and 35 targets were downregulated with EX ancestry (see Supplementary Figure 2, Supplementary Table 2). Examination of the differentially regulated genes revealed three genes that have been linked to lipid metabolism: *Adipoq*, *Cidec*, and *Scd1* were all downregulated in F1 EX males. We used real-time qPCR analysis of these genes to validate the microarray findings and further examine the expression of these genes in our remaining groups.

Real-time qPCR analysis confirmed the lower gastrocnemius expression of *Adipoq* and *Scd1* in F1 EX males ($p < 0.05$, Figure 4C) and demonstrated a tendency for lower expression of *Cidec* ($p = 0.06$, Figure 4C). We also examined the expression level of these genes in F0 and F2 males and found lower expression of *Scd1* in F2 males with EX ancestry compared to F2 males of SED ancestry (p=0.07; Figure 4E), but no other differences. We also measured the gastrocnemius expression levels of these genes in all three generations of females. Interestingly, many of the patterns observed in the males were reversed in females. In F0 females, there was a tendency for higher expression of *Adipoq* and *Cidec* in the EX group (p=0.06–0.09; Figure 4B). *Adipoq* expression was significantly higher in F1 EX females and *Scd1* expression was significantly higher in F2 EX females (Figure 4F).

To further investigate processes that might contribute to some of the observed body and tissue mass and glucose metabolism differences, we also performed targeted gene expression analyses in gastrocnemius muscle. We examined a number of genes that have been previously associated with acute exercise, exercise training responses or other metabolic health outcomes (McClelland, 2004; Leick *et al.*, 2007). These included *Alas1*, *Cox1*, *Cycs*, *Cs*, *Hk2*, *Pdk4*, *Ppara*, *Ppard*, *Pparg*, and *Ppargc1a*. No significant differences in expression of any of these genes were observed in F0 males. In F0 females, *Pdk4* expression was significantly lower in exercised animals (Figure 3A). There was a tendency for *Pparg* expression to be higher in exercised females, but this difference did not reach statistical significance (p=0.07; Figure 3A). No expression differences were observed for any of these genes in F1 females. F1 males with EX ancestry had significantly lower expression levels of *Hk2*, *Ppard*, and *Ppargc1a* (all $p<0.05$; Figure 3B). Additionally, F1 males with EX ancestry tended to have lower expression levels of *Cs*, *Ppara*, and *Alas1*, though these differences did not reach statistical significance (p=0.06–0.09; Figure 3B). F2 males with EX ancestry had significantly higher expression levels of *Cycs* and significantly lower expression levels of *Cox1* and *Pparg* (all $p<0.05$; Figure 2C). F2 EX females had significantly lower expression levels of *Pparγ* and significantly higher expression levels of *Ppargc1a* (all $p<0.05$; Figure 2D). In addition, F2 females with EX ancestry tended to have

higher expression levels of *Alas1*, *Hk2*, and *Ppard*, though these differences did not reach statistical significance (p=0.06–0.09; Figure 3D).

Discussion

In this study, we sought to determine the effect of EX ancestry on the morphological and metabolic phenotypes of two generations of offspring. This study investigated the influence of maternal and paternal EX ancestry on anatomical characteristics and metabolic phenotypes, including skeletal muscle gene expression. Our results indicate broad effects of EX ancestry on various offspring phenotypes, including body mass, fat and muscle mass, fasting serum insulin, and glucose tolerance. Further, we observed effects of EX ancestry on the expression of several gastrocnemius muscle mRNAs through two generations of offspring.

Following the EX training period, F0 EX males were lighter with less omental fat mass and greater normalized muscle mass than SED males. F0 EX females had greater soleus mass than SED females but did not differ in fat or body mass. Other researchers have also observed lower body mass in male, but not female mice after a period of voluntary exercise (De Bono *et al.*, 2006), though this is not universal as some have observed decreased body mass in both sexes (Swallow *et al.*, 2005) or no differences in either sex (Allen *et al.*, 2001). There were no differences in glucose tolerance in males following the exercise intervention and a tendency for improved glucose tolerance in exercised females. Though forced exercise can induce improvements in glucose tolerance in mice (James *et al.*, 1983), no other studies that we are aware of have observed an effect of voluntary exercise on glucose tolerance in non-obese mice on standard chow. Our voluntary wheel running approach alleviates the induction of systemic handling stress seen with forced running or swimming, however it does not allow us to control exercise volume or intensity. We anticipated that voluntary running wheel would be sufficient to produce significant adaptations as measurable changes in muscle fiber type content have been observed in comparable studies in the gastrocnemius muscle in the same mouse strain as used here (Allen *et al.*, 2001).

F1 females with EX ancestry were lighter than those with SED ancestry at 8 weeks of age; no other differences in offspring body mass were observed. Maternal exercise training has previously led to offspring with lower (Pinto & Shetty, 1995) or not different (Carter *et al.*, 2012*a*) body mass. Our study does not allow us to identify a specific mechanism leading to lower body mass in F1 EX females because F0 dams were exposed to exercise from pre-conception through gestation and lactation (e.g. epigenetic, in utereo, or lactational mechanisms). There does not appear to be a transgenerational effect (e.g., F2 offspring were unaffected) of parental exercise on body mass at 8 weeks of age in our study. Only one other study we are aware of has examined the effect of maternal exercise on offspring mass over multiple generations. Pinto and Shetty (1995) found EX ancestry led to decreased body mass in both F1 and F2 offspring; however, the exercise intervention in that study was forced swimming, which is often questioned as a form of exercise in a rodent (Baldwin, 1985) and may have induced an additional maternal stress independent of the exercise stimulus. Maternal stress alone has induced both higher (Schulz *et al.*, 2011) and lower (Patin *et al.*, 2002) offspring body mass in other studies.

Although we did not measure body composition, we examined individual tissue masses to identify potential differences in organ mass in the offspring. F1 males with EX parents had lower muscle masses than F1 males from SED parents, and F1 females from EX parents had lower TA and omental fat mass compared to F1 females from SED parents. Recent evidence suggests that the maternal environment can have significant effects on developmental partitioning of muscle, adipose tissue, and connective tissue precursors (Du *et al.*, 2011);

however, we are unable to confirm if this would explain our differences. Interestingly, F1 EX males had lower muscle masses compared to F1 SED males, however, there were no corresponding differences in omental fat pad mass. Carter *et al.* (2012*a*) observed lower fat mass and higher lean mass percentages in mature (39 wk) male, but not female, offspring of exercised dams, while in a similar study, no body composition differences were observed (Carter *et al.*, 2012*b*). Though these results are not directly comparable due to the difference in offspring age and method of body composition assessment, it is interesting that we observed a contrasting effect, with lower fat mass in only female offspring. In the F2 offspring, EX females had lower relative muscle and omental fat pad mass compared to F2 SED females. The differential effects of EX ancestry on offspring body and tissue mass between sexes and generations suggest the observed differences may be induced through sex- and/or generation-specific mechanisms.

Glucose tolerance was affected only in F2 female offspring where those with EX ancestry had slightly reduced glucose tolerance than those with SED ancestry. It is possible that the greater fasting insulin concentration observed in F1 EX females could have led to impaired glucose disposal and thus hyperglycemia during pregnancy if insulin resistance developed in the F1 EX females. Mild maternal hyperglycemia during pregnancy has been linked with reduced glucose tolerance in rat offspring (Gauguier *et al. 1991*). Two recent studies have examined the influence of maternal exercise on glucose tolerance in healthy animals. In both, offspring from exercised dams had improved glucose tolerance as well as insulin sensitivity (Carter *et al.*, 2012*a*, 2012*b*). It is critical to note, however, that these improvements were not observed until offspring were 31–32 weeks (Carter *et al.*, 2012*a*) or 10 months of age (Carter *et al.*, 2012*b*). Together with our results this indicates that while the positive effects of exercise training on glucose tolerance may be transmittable to offspring, they are not yet apparent at a young age.

The effects of EX and EX ancestry on gene expression were examined by a combination of global mRNA profiling and targeted gene expression analysis of gastrocnemius muscle. Our study is the first to examine the effect of maternal exercise or exercise ancestry on skeletal muscle gene expression. Our microarray approach revealed a number of mRNA transcripts were differentially expressed as a function of EX ancestry in both generations, with a greater number of transcripts affected in the F2 compared to the F1 generation. Surprisingly, there was no overlap in differentially expressed transcripts between F1 and F2 offspring, again highlighting the difference between *in utero* versus transgenerational effects. Three of the differentially regulated transcripts (*Adipoq*, *Cidec*, and *Scd1*) have previously been associated with lipid metabolism and specifically intramuscular lipogenesis. *Adipoq* codes for the adipokine adiponectin, that when secreted from adipocytes promotes fatty acid oxidation and enhances insulin sensitivity (Tomas *et al.*, 2002) in muscle. *Adipoq* mRNA is expressed in muscle (Delaigle *et al.*, 2004; Liu *et al.*, 2009), but it is unclear whether the resultant protein has similar effects to circulating adiponectin. *Adipoq* mRNA in muscle is associated with greater intramuscular fat (Wang *et al.*, 2008) and lipotoxicity (Delaigle *et al.*, 2006), but also enhanced insulin sensitivity (Liu *et al.*, 2009). *Cidec* codes for cell death-inducing DFFA-like effector c, which promotes apoptosis (Liang *et al.*, 2003) and is upregulated in adipose tissue during adipogenesis (Puri *et al.*, 2007). In muscle, *Cidec* mRNA expression is associated with de novo lipogenesis (Wang *et al.*, 2010). *Scd1* codes for steroyl-CoA desaturase-1, which enzymatically regulates the formation of monounsaturated fatty acids within the cell (Kim *et al.*, 2011). *Scd1* mRNA expression in muscle is associated with the increased intramuscular triglyceride concentrations observed with both obesity (Hulver *et al.*, 2005) and exercise training (Dobrzyn *et al.*, 2010). These lipogenic transcripts were downregulated in F1 EX male offspring, with a similar pattern observed in the F0 and F2 generations. In females, we found the opposite result, an overall tendency towards increased expression of *Adipoq*, *Cidec*, and *Scd1* in EX/EX ancestry

females. Recalling that F1 EX females had less omental fat than F1 SED ancestry females while there were no differences in fat mass in male offspring, we speculate that EX ancestry females preferentially decreased adipose tissue lipid storage compared to SED ancestry females while EX ancestry males preferentially decreased ectopic (including muscle) lipid storage.

In addition to the targets identified by the microarray, we examined a number of *a priori* metabolic gene expression targets. Direct exposure to EX did not lead to differences in basal expression of the *a priori* metabolic genes in F0 males and only *Pdk4* expression was significantly lower in EX females. Although altered expression levels of the selected genes have been observed following exercise in previous studies (Puntschart *et al.*, 1995; Schmutz *et al.*, 2006), the lack of expression differences in our study is not surprising because sacrifice occurred 24 hours after the last bout of wheel running and in humans, the exercise-induced increase in transcription of some metabolic genes is transient and expression returns to baseline by 24 hours post-exercise (Pilegaard *et al.*, 2000). In F1 offpring, EX males tended to have lower expression levels of *Cs*, *Ppara*, and *Alas1*. If these gene expression differences reflect a more extensive effect of EX ancestry on skeletal muscle gene expression then perhaps the morphological differences seen in the F1 EX males (smaller soleus, EDL, and quadriceps muscle mass) can be partially explained by an impact of EX ancestry on skeletal muscle gene expression and tissue development. Interestingly, there were more significant effects of EX ancestry on metabolic gene expression in F2 offspring than F1 offspring. The magnitude of the difference between EX and SED ancestries, however, was smaller, which may explain why the differences in gene expression were not associated with gross morphological changes in the F2 generation. Alternatively, EX may uniquely affect skeletal muscle gene expression in the F2 offspring through the germ line whereas the gross morphological effects observed in the F1 generation may be related to a direct influence of exercise during the F1 *in utero* period.

Altered maternal nutrition and other stressors have been associated with epigenetic changes in a number of genes and tissues, however in many of these studies the associated physiological effects of these gene changes do not present until later in life or following a metabolic challenge such as a high fat diet (Vickers, 2011). Similarly, the lack of overt physiological phenotype observed in the present study may be related to the young age (8 wk) of our animals and/or their exposure to only typical cage conditions and diet. The body composition differences between offspring of sedentary and exercised dams observed by Carter *et al.* (2012*a*) were observed at 39–40 weeks of age. Further, the authors note that the observed differences in glucose tolerance were not detectable until 7 months of age (Carter *et al.*, 2012*a*). Thus we believe future studies should investigate the effects of EX ancestry on metabolic phenotypes such as glucose tolerance in animals following aging or a metabolic challenge.

We observed differences in gene expression patterns between males and females in multiple offspring groups. For several genes, the expression pattern was inverted between F1 males and F2 females, with *Alas1*, *Hk2*, *Ppard*, and *Ppargc1a* all lower in EX F1 male compared to SED while they were higher in F2 EX females compared to SED. Overall, EX ancestry led to primarily greater mRNA expression in female offspring and lower mRNA expression in male offspring. These patterns along with the body and tissue weight results demonstrate an apparent sex-specific effect of EX ancestry on offspring outcomes. Sex-specific environmental influences have been observed previously for offspring phenotypes in both humans (Stein *et al.*, 2007; Lumey *et al.*, 2009) and rodents (Dunn & Bale, 2009; van Straten *et al.*, 2012; Carter *et al.*, 2012*a*). In humans, prenatal exposure to famine was associated with increased BMI (Stein *et al.*, 2007) and blood lipids (Lumey *et al.*, 2009) in women, but not men. In mice, *in utero* protein restriction influenced the development of metabolic

dysfunction in female, but not male offspring (van Straten *et al.*, 2012). Additionally, maternal high fat diet induced greater body length in both sexes of second-generation offspring, but higher Igf1 levels in females only (Dunn & Bale, 2009). With regard to exercise, voluntary maternal wheel running in the perinatal period led to higher lean and lower fat mass percentages in mature male, but not female offspring (Carter *et al.*, 2012*a*). One proposed mechanism for these sex-dependent observations is a difference in placental function between male and female fetuses. Chronic maternal stress in mice led to sex-specific differences in placenta gene expression where male placentas had greater expression levels of genes important to growth while female placentas were not affected (Mueller & Bale, 2008). Several possible mechanisms could lead to this sex-specificity, such as an effect of the environmental stimulus on early development (gametogenesis or embryonic sexual differentiation). Alternately, the environment could interact with the offspring's own sex chromosomes or hormones later in development.

A critical difference between our study and the other studies of maternal exercise and offspring phenotypes is the inbred status of the mice used in our study. The rats used in the Pinto and Shetty (1995) study and the mice and rats used in the Carter *et al.* (2012*a*; 2012*b*) studies were both from outbred lines, while we studied C57Bl/6 mice, an inbred strain. Though there are additional confounding factors, the effect of maternal exercise in outbred lines appears to be more dramatic than the effect of exercise ancestry observed in the present study. Interestingly, other environmentally-induced transgenerational effects on offspring health have also been shown to be dependent on the inbred vs. outbred status of the rodent line used for the experiment (Skinner *et al.*, 2010; Guerrero-Bosagna *et al.*, 2012). Guerrero-Bosagna et al. (2012) found maternal vinclozolin treatment led to increased adult onset disease in an outbred, but not an inbred mouse model. This is the only study we are aware of to directly compare transgenerational phenotypes in an inbred and outbred mouse strain within the same study. The mechanisms contributing to this differential response between inbred and outbred lines of rodents have not yet been identified. However, we speculate that the stress of continued inbreeding may modify the epigenetic mechanisms responsible for manifestation of environmentally induced phenotypic changes, thus making the inbred organism less susceptible to epigenetic reprogramming of the germ line in response to environmental factors. Together these studies suggest a critical role of rodent strain when examining the role of the environment in modifying transgenerational phenotypes.

We would like to acknowledge some limitations to our study related to the breeding and pre-weaning methodology. The litter sizes reported reflect those offspring that were weaned from each breeding pair. We did not collect information on litter size, survival or body mass prior to weaning. Additionally, we recognize that the variability in litter size and weaning time could have affected early offspring nutrition. Overnutrition induced by culling litters during lactation leads to neonatal overgrowth and can induce the development of obesity and glucose intolerance (Pentinat *et al.*, 2010) as well as a number of other cardiometabolic risk factors (Habbout *et al.*, 2013). As noted previously, 1–7 offspring per litter were used for analysis in this study. The use of multiple offspring per litter was unavoidable due to sample size limitations. However, it is important to note that the use of multiple offspring per litter could confound the findings due to the "litter effect", where pups within litters are more similar to one another than pups between litters (Holson *et al.*, 1976).

In summary, EX ancestry affects various offspring phenotypes across two generations, but in a generation- and sex-dependent manner. We have observed effects at both the whole-body (body and tissue mass and glucose tolerance) and skeletal muscle gene expression levels, which together reflect a broad impact of EX ancestry. This study is the first to examine the effect of exercise ancestry on a broad range of metabolic phenotypes, however this study is descriptive in nature and the specific mechanisms responsible for the observed

phenotypes were not elucidated. Future studies should examine the potential influences of maternal vs. paternal exercise, and their cooperative effects. Similarly, additional work should isolate the EX exposure to discrete phases (i.e. pre-conception, gestation, lactation) to provide further insight into potential mechanisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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New Findings

a. What is the central question of this study? How does parental exercise training directly and/or transgenerationally affect offspring phenotypes over two generations of mice?

b. What is the main finding and what is its importance? We observed preliminary evidence that parental exercise training can influence health-related phenotypes (e.g., body and fat mass, muscle gene expression) in mouse offspring. These findings indicate parental exercise may be another environmental factor (like altered nutrition) capable of influencing offspring phenotypes in adulthood.

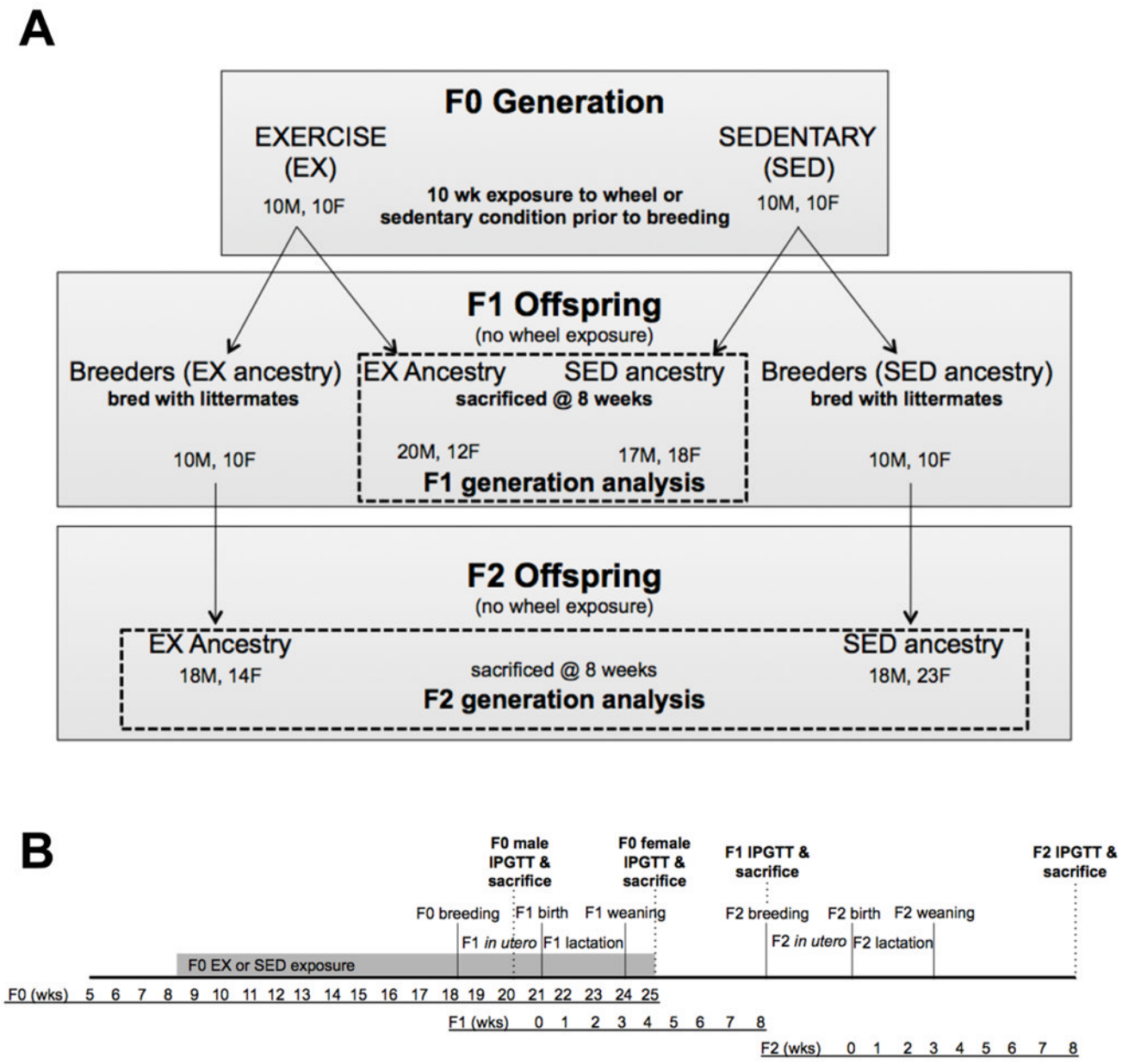

Figure 1.
Experimental design (A) overview and (B) timeline.

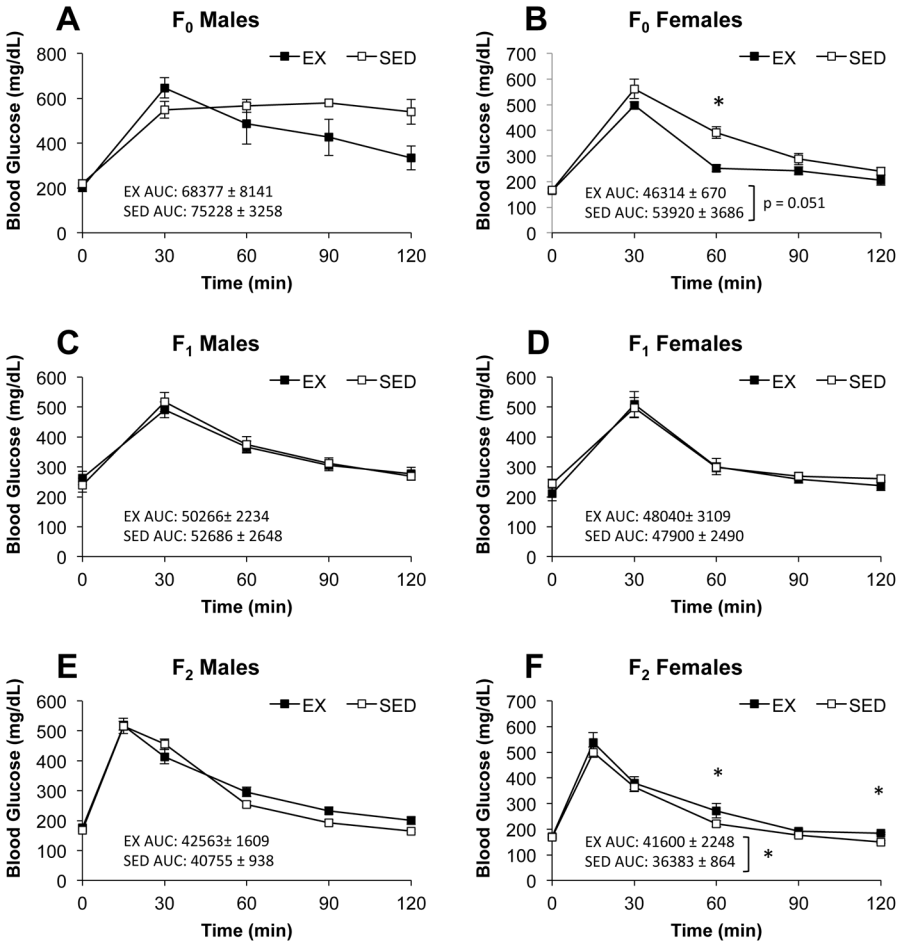

Figure 2.

Blood glucose concentration during an intraperitoneal glucose tolerance test in: (A) F0 males, (B) F0 females, (C) F1 males, (D) F1 females, (E) F2 males, and (F) F2 females. Mice were fasted 12 hours and given a 2 g/kg body mass glucose load at time 0. Glucose levels were determined before and 30, 60, 90, and 120 min after injection. Blood glucose was also measured 15 min after injection in F2 males and females. Area under the curve for concentration vs. time was calculated using the linear trapezoidal rule. Values are means ± SE. Sample sizes were n=4–5 for F0, 9–13 for F1, and 14–19 for F2. *significant difference in AUC between EX and SED within sex and generation ($p < 0.05$)

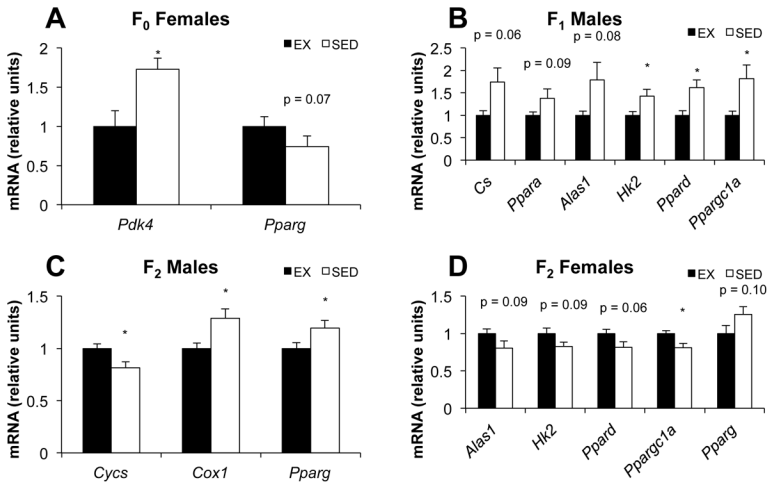

Figure 3.

Relative gastrocnemius muscle mRNA levels of *Alas1*, *Cox1*, *Cycs*, *Cs*, *Hk2*, *Pdk4*, *Ppara*, *Ppard*, *Pparg*, and *Ppargc1a* determined by RT-PCR in: (A) F0 females, (B) F1 males, (C) F2 males, and (D) F2 females. For each offspring group, only mRNA targets with $p \leq 0.10$ between EX and SED ancestry are shown in the figure; no mRNA targets met this threshold for F0 males or F1 females. Average expression level in EX was set to 1.0. Sample sizes were n=7–10. *significantly different from EX within sex and generation ($p < 0.05$)

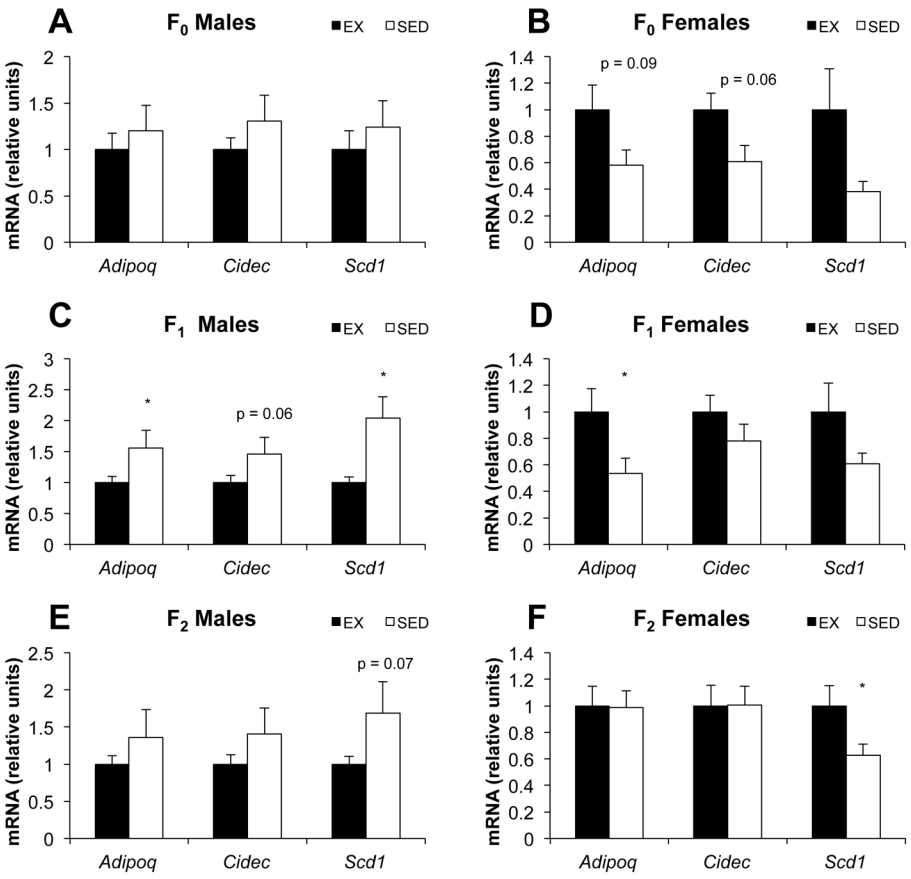

Figure 4.

Relative gastrocnemius muscle mRNA levels of *Adipoq*, *Cidec*, and *Scd1* determined by RT-qPCR in: (A) F0 males, (B) F0 females, (C) F1 males, (D) F1 females, (E) F2 males, and (F) F2 females. Average expression level in EX was set to 1.0. Sample sizes were n=10–20. *significantly different from EX within sex and generation ($p < 0.05$)

Table 1

Mean body, organ, and muscle mass in exercise and sedentary ancestry groups for males and females of each generation (F0, F1, and F2).

	Males		Females	
F_0 generation	**EX (N=10)**	**SED (N=10)**	**EX (N=10)**	**SED (N=10)**
Body Mass (g)	29.1 ± 0.1	31.1 ± 0.7*	27.4 ± 0.7	26.7 ± 0.5
Heart (mg)	143.6 ± 4.0	145.7± 4.3	157.7 ± 9.2	155.6 ± 6.3
Cerebellum (mg)	51.9 ± 3.8	53.2 ± 2.7	64.5 ± 3.7	63.2 ± 3.3
Gastrocnemius (mg)	144.7 ± 3.6	140.6 ± 2.9	117.1 ± 3.4	111.1 ± 3.9
Soleus (mg)	11.6 ± 0.4	10.9 ±0.7	9.5 ± 0.3	7.1 ± 0.4*
EDL (mg)	16.6 ± 0.7	14.3 ± 0.9	7.5 ± 0.8	8.5 ± 0.4
Plantaris (mg)	21.5 ± 0.6	21.2 ± 1.1	16.2 ± 1.0	17.2 ± 1.8
TA (mg)	55.8 ± 1.8	55.3 ± 1.2	39.3 ± 1.2	39.2 ± 1.8
Quadriceps (mg)	216.8 ± 7.6	212.2 ± 3.5	124.6 ± 6.3	116.9 ± 36.2
Omental Fat (mg)	608.9 ± 34.7	929.8 ± 78.0*	387.1 ± 49.9	346.6 ± 44.1
Liver (mg)	1042.9 ± 34.8	1274.9 ± 52.5	1238.8 ± 33.1	1241.9 ± 76.1
F_1 generation	**EX (N=20)**	**SED (N=17)**	**EX (N=12)**	**SED (N=16)**
Body Mass (g)	22.3 ± 0.2	22.5 ± 0.3	17.5 ± 0.2	19.0 ± 0.3*
Heart (mg)	107.6 ± 1.8	103.3 ± 1.7	92.2 ± 1.4	96.0 ± 2.6
Cerebellum (mg)	51.4 ± 2.0	51.5 ± 2.9	51.0 ± 2.8	53.4 ± 1.7
Gastrocnemius (mg)	108.2 ± 1.8	107.8 ± 1.4	77.7 ± 2.4	82.5 ± 2.3
Soleus (mg)	6.6 ± 0.2	7.3 ± 0.3*	5.9 ± 0.5	6.0 ± 0.2
EDL (mg)	8.9 ± 0.4	12.4 ± 1.4*	10.7 ± 2.5	9.5 ± 1.0
Plantaris (mg)	14.3 ± 0.4	14.2 ± 0.05	10.3 ± 0.5	11.4 ± 0.5
TA (mg)	39.0 ± 0.8	41.2 ± 0.9	29.8 ± 0.5	33.0 ± 0.8*
Quadriceps (mg)	112.1 ± 6.8	135.4 ± 8.8*	98.3 ± 6.3	98.5 ± 6.2
Omental Fat (mg)	257.6 ± 8.3	274.9 ± 15.6	111.3 ± 7.6	154.7 ± 11.2*
Liver (mg)	965.5 ± 17.8	973.3 ± 32.3	770.0 ± 22.0	799.3 ± 24.6
F_2 generation	**EX (N=18)**	**SED (N=18)**	**EX (N=14)**	**SED (N=23)**
Body Mass (g)	22.1 ± 0.2	22.5 ± 0.2	18.6 ± 0.3	18.2 ± 0.2
Heart (mg)	119.2 ± 4.3	113.1 ± 2.4	103.6 ± 3.0	96.0 ± 2.4
Cerebellum (mg)	47.3 ± 2.4	54.0 ± 3.3	43.8 ± 2.8	49.4 ± 3.0
Gastrocnemius (mg)	92.1 ± 2.7	97.0 ± 3.3	71.8 ± 1.5	74.3 ± 1.3
Soleus (mg)	6.9 ± 0.3	7.1 ± 0.4	5.3 ± 0.2	5.7 ± 0.1
EDL (mg)	7.7 ± 0.3	7.9 ± 0.6	5.8 ± 0.2	5.5 ± 0.3
Plantaris (mg)	11.6 ± 0.6	12.4 ± 0.5	8.8 ± 0.3	9.3 ± 0.3
TA (mg)	36.9 ± 0.7	36.8 ± 15.7	28.7 ± 0.6	29.4 ± 0.8
Quadriceps (mg)	97.5 ± 27.2	101.4 ± 25.6	81.2 ± 2.7	75 ± 2.1

	Males		Females	
F_0 generation	**EX (N=10)**	**SED (N=10)**	**EX (N=10)**	**SED (N=10)**
Omental Fat (mg)	253.3 ± 15.7	279.0 ± 8.9	129.7 ± 35.4	147.0 ± 61.7
Liver (mg)	866.6 ± 29.2	973.3 ± 45.8	736.3 ± 35.4	727.7 ± 21.2

Values are means ± SEM.

* significantly different from EX within sex and generation ($p < 0.05$)

Table 2

Organ and muscle masses relative to body mass in exercise and sedentary ancestry groups for males and females of each generation (F0, F1, and F2).

	Males		Females	
F_0 generation	**EX (N=10)**	**SED (N=10)**	**EX (N=10)**	**SED (N=10)**
Heart	4.94 ± 0.10	4.70 ± 0.15	5.76 ± 0.28	5.84 ± 0.25
Cerebellum	1.78 ± 0.13	1.71 ± 0.09	2.36 ± 0.13	2.48 ± 0.14
Gastrocnemius	4.98 ± 0.08	4.54 ± 0.12*	4.29 ± 0.11	4.16 ± 0.13
Soleus	0.40 ± 0.01	0.35 ± 0.03	0.35 ± 0.01	0.27 ± 0.01*
EDL	0.57 ± 0.03	0.47 ± 0.03*	0.27 ± 0.03	0.32 ± 0.02
Plantaris	0.74 ± 0.02	0.68 ± 0.03	0.59 ± 0.04	0.64 ± 0.05
TA	1.92 ± 0.07	1.78 ± 0.04	1.44 ± 0.04	1.46 ± 0.06
Quadriceps	7.46 ± 0.26	6.87 ± 0.24	4.58 ± 0.30	4.39 ± 0.16
Omental Fat	20.89 ± 1.06	29.60 ± 2.01*	14.04 ± 1.76	12.84 ± 1.50
Liver	45.50 ± 0.79	41.06 ± 1.58	45.64 ± 1.80	46.50 ± 2.75
F_1 generation	**EX (N=20)**	**SED (N=17)**	**EX (N=12)**	**SED (N=16)**
Heart	4.83 ± 0.07	4.58 ± 0.06*	5.28 ± 0.10	5.07 ± 0.10
Cerebellum	2.20 ± 0.15	2.28 ± 0.12	2.92 ± 0.16	2.95 ± 0.09
Gastrocnemius	4.86 ± 0.07	4.52 ± 0.29	4.44 ± 0.13	4.46 ± 0.05
Soleus	0.30 ± 0.01	0.33 ± 0.01	0.34 ± 0.03	0.32 ± 0.01
EDL	0.40 ± 0.02	0.55 ± 0.06*	0.61 ± 0.14	0.53 ± 0.06
Plantaris	0.64 ± 0.01	0.63 ± 0.02	0.59 ± 0.03	0.61 ± 0.02
TA	1.75 ± 0.03	1.83 ± 0.03	1.71 ± 0.03	1.76 ± 0.03
Quadriceps	5.05 ± 0.32	6.00 ± 0.37	5.66 ± 0.40	5.42 ± 0.32
Omental Fat	11.57 ± 0.35	12.12 ± 0.55	6.36 ± 0.42	8.29 ± 0.60*
Liver	43.44 ± 0.84	43.13 ± 1.16	43.97 ± 1.00	43.16 ± 0.95
F_2 generation	**EX (N=18)**	**SED (N=18)**	**EX (N=14)**	**SED (N=23)**
Heart	5.40 ± 0.19	5.04 ± 0.12	5.58 ± 0.14	5.28 ± 0.95
Cerebellum	2.15 ± 0.12	2.42 ± 0.16	2.35 ± 1.50	2.73 ± 1.74
Gastrocnemius	4.17 ± 0.11	4.10 ± 0.28	3.86 ± 0.06	4.09 ± 0.44*
Soleus	0.31 ± 0.01	0.32 ± 0.02	0.28 ± 0.01	0.32 ± 0.01*
EDL	0.35 ± 0.01	0.35 ± 0.03	0.32 ± 0.01	0.30 ± 0.02
Plantaris	0.53 ± 0.02	0.55 ± 0.02	0.47 ± 0.01	0.51 ± 0.02
TA	1.67 ± 0.03	1.65 ± 0.07	1.54 ± 0.03	1.62 ± 0.04
Quadriceps	4.42 ± 0.12	4.53 ± 0.13	4.38 ± 0.15	4.13 ± 0.10
Omental Fat	11.49 ± 0.68	12.42 ± 0.37	6.95 ± 0.47	8.10 ± 0.33*
Liver	39.33 ± 1.38	43.45 ± 1.99	39.60 ± 1.64	40.17 ± 1.23

Values are means ± SEM, all units are mg g^{-1} body mass.

* significantly different from EX within sex and generation ($p < 0.05$)

Table 3

Serum insulin, glycerol, and triglyceride levels in exercise and sedentary ancestry groups for male and female offspring (F1 and F2).

	Males		Females	
F_1 generation	**EX (N=9)**	**SED (N=8)**	**EX (N=12)**	**SED (N=16)**
Insulin (ng mL^{-1})	0.30 ± 0.04	0.26 ± 0.06	0.33 ± 0.05	0.15 ± 0.02 *
Free Glycerol (mg dL^{-1})	43.0 ± 4.0	58.2 ± 6.6	51.3 ± 8.0	54.0 ± 5.6
Total Triglyceride (mg dL^{-1})	94.9 ± 8.8	109.8 ± 10.8	80.8 ± 6.9	80.8 ± 5.6
F_2 generation	**EX (N=18)**	**SED (N=18)**	**EX (N=14)**	**SED (N=23)**
Insulin (ng mL^{-1})	0.28 ± 0.04	0.25 ± 0.03	0.23 ± 0.04	0.22 ± 0.04
Free Glycerol (mg dL^{-1})	31.9 ± 4.3	36.5 ± 7.6	34.0 ± 7.7	46.7 ± 7.7
Total Triglyceride (mg dL^{-1})	100.4 ± 3.3	94.0 ± 11.8	67.6 ± 6.1	77.8 ± 7.4

* significantly different from EX within sex and generation ($p < 0.05$)