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Four weeks of exercise early in life reprograms adult skeletal muscle insulin resistance caused by paternal high fat diet

Filippe Falcão-Tebas[1,2*], Jujiao Kuang[1], Chelsea Arceri[1], Jarrod P. Kerris[1], Sofianos Andrikopoulos[3], Evelyn C. Marin[1,3], Glenn K. McConell[1,4]

[1] Institute for Health and Sport (IHES), Victoria University, Melbourne, Australia.
[2] The Ritchie Centre, Hudson Institute of Medical Research, and Department of Obstetrics and Gynaecology, Monash University, Melbourne, Australia
[3] Department of Medicine, Austin Hospital, The University of Melbourne, Melbourne, Australia
[4] College of Health and Biomedicine, Victoria University, Melbourne, Australia

Running title: Offspring exercise after a high fat fed father

*Corresponding author: Current affiliation - The Ritchie Centre, Hudson Institute of Medical Research, and Department of Obstetrics and Gynaecology, Monash University, Clayton, VIC, Australia, filippe.oliveira@monash.edu

Key points

- Paternal high fat diet/obesity before mating can negatively influence the metabolism of offspring.

- Exercise only early in life has a remarkable effect to reprogram adult rat offspring exposed to detrimental insults before conception.
- Exercise only early in life normalized adult whole body and muscle insulin resistance due to having a high fat fed/obese father.
- Unlike the effects on the muscle, early exercise did not normalise the reduced adult pancreatic beta cell mass due to having a high fat fed/obese father.
- Early life exercise training may be able to reprogram the individual whose father was obese, inducing long-lasting beneficial effects on health.

Abstract

Paternal high fat diet (HFD) impairs female rat offspring glucose tolerance, pancreatic morphology and insulin secretion. We examined whether only 4 weeks of exercise early in life could reprogram these negative effects. Male Sprague-Dawley rats consumed a HFD for 10 weeks before mating with chow-fed dams. Female offspring remained sedentary or performed moderate intensity treadmill exercise (5 days/week, 60 min/day, 20 m/min) from 5 to 9 weeks of age. Paternal HFD impaired ($P<0.05$) adult offspring whole body insulin sensitivity (intraperitoneal insulin sensitivity test) and skeletal muscle *ex vivo* insulin sensitivity and TBC1D4 phosphorylation. It also lowered β-cell mass and reduced *in vivo* insulin secretion in response to an intraperitoneal glucose tolerance test. Early life exercise in offspring reprogrammed the negative effects of paternal HFD on whole body insulin sensitivity, skeletal muscle *ex vivo* insulin-stimulated glucose uptake and TBC1D4 phosphorylation and increased GLUT4 protein. However, early exercise did not normalise the reduced pancreatic β-cell mass or insulin secretion. In conclusion, only 4 weeks of exercise early in life in female rat offspring reprograms reductions in insulin sensitivity in adulthood caused by a paternal high fat diet without affecting pancreatic β-cell mass or insulin secretion.

The dramatic rise in type 2 diabetes (T2D) can partially be understood by phenotypic plasticity (Hanson & Gluckman, 2014), when diverse environmental conditions modify the expression of a phenotype characteristic from a single genotype (West-Eberhard, 1989). The developmental origins of health and disease (DOHaD) paradigm uses this concept to interpret how environmental cues early in life (gestation, lactation and early childhood) impact on long-term disease susceptibility, such as T2D (Bateson *et al.*, 2004; McMullen & Mostyn, 2009; Wells, 2014). In humans, obese mothers are more likely to have obese offspring (Gale *et al.*, 2007). Similarly, maternal high fat diet in rodents causes offspring insulin resistance (Nivoit *et al.*, 2009), hyperphagia and hypertension (Samuelsson *et al.*, 2008) in adulthood, as well as reduced skeletal muscle mitochondrial transcription factors and oxidative phosphorylation (Latouche *et al.*, 2014; Pileggi *et al.*, 2016).

In addition, paternal high fat diet/obesity before mating can negatively influence the phenotype and metabolism of offspring (Soubry, 2015). In fact, a father's body fat (percentage or total) is predictive of long-term changes in body fat in premenarcheal girls (Figueroa-Colon *et al.*, 2000) and a prospective epidemiological study observed that obesity in fathers before conception is linked with obesity in his offspring (Loomba *et al.*, 2008). In rodents, studies are controversial in regards to effects on offspring body weight (Ng *et al.*, 2010; Fullston *et al.*, 2013; Cropley *et al.*, 2016). Nevertheless, paternal high fat diets seem to reduce glucose tolerance, islet size and function as well as insulin secretion in female offspring (Ng *et al.*, 2010). Similarly, obesity induced by paternal high fat diets results in offspring with impaired glucose tolerance and insulin sensitivity (Fullston *et al.*, 2013), suggestive of reduced insulin sensitivity.

In regards to possible interventions to overcome the effects of paternal high fat diets, it is known that there is an increased window of opportunity of adaptation from gestation up to infancy (Hanson & Gluckman, 2014). Indeed, higher physical activity in youth was associated with lower rates of T2D (and hypertension) in adulthood, independent of current physical activity (Fernandes & Zanesco, 2010).

We and others have proposed early life exercise training as a positive stimulus to reprogram the offspring exposed to detrimental insults *in utero*, such as placental restriction (Laker *et al.*, 2011; Laker *et al.*, 2012; Gatford *et al.*, 2014; Street *et al.*, 2015). We found that exercise training early in life in rats (from 5-9 weeks of age only, treadmill running) normalised the 50% lower relative islet surface area and β-cell mass in adulthood in rats born small for gestational age (Laker *et al.*, 2011). This was remarkable especially as early

exercise did not have a sustained effect on skeletal muscle mitochondrial biogenesis in adult offspring using this design (Laker *et al.*, 2012). No study has investigated whether exercise early in life can overcome the negative metabolic consequences of a paternal high fat diet before conception. The 'classical' insulin-stimulated glucose uptake pathway involves a series of signals to recruit serine/threonine protein kinases. Once protein kinase B (PKB, also known as AKT) is activated, the signal then propagates to activate an AKT substrate of 160 kDa (AS160, more recently being called as TBC1 domain family member 4 – TBC1D4; and TBC1D1) (Middelbeek et al. 2013, Sakamoto and Holman 2008). Next, TBC1D4 activates a Rab GTPase protein (Tan et al. 2012a), which will release glucose transporter (GLUT) 4 and allows it to be translocated to the plasma membrane. Some phosphorylation sites were found to be stimulus-specific. For instance, phospho-AKT Ser308 is a specific site for insulin only, whereas phospho-AKT Ser473 and phospho-TBC1D4 Thr642 are responsive to both insulin and contraction (exercise) (Treebak et al. 2014). The long-term effects of paternal high fat diet and exercise early in life have not been investigated in terms of insulin signaling in the skeletal muscle.

Therefore, we aimed to determine if paternal high fat diet impairs insulin sensitivity and mitochondrial function in skeletal muscle and pancreatic morphology in adult rat offspring and whether exercise early in life can attenuate these negative effects. We hypothesised based on our previous studies in rats born small for gestational age that early life exercise would normalise reduced pancreatic β-cell mass without having effects on skeletal muscle.

Research Design and Methods

Ethical approval and animals

All procedures were performed according with the Australian Code for the Care and Use of Animals for Scientific Purposes (2013), after approval by Victoria University Animal Ethics Committee (#13/008). The authors understand the ethical principles under which this journal operates and verify that this work meets the standards of the journal's animal ethics checklist.

Sprague Dawley rats were obtained from the Animal Research Centre (Perth, Australia). Male rats were fed control chow diet (Rat and Mouse Cubes, 12.0% energy as fat; Specialty Feeds, Western Australia) or high fat diet (combined SF01-025 and SF03-020, with

40.7% and 43.0% energy as fat; Specialty Feeds, Western Australia) from 4 to 14 weeks of age (Ng et al., 2010). All female breeders were chow fed and sedentary (Figure 1). The rats were exposed to a 12 hour light-dark cycle (lights on at 0700 h) and standard environmental conditions of 18-22°C and ~50% relative humidity. All animals had access to food and water *ad libitum*, as well as nesting materials and enrichment items. Mating was performed between 0800 and 1700 (during the light cycle) when rats were 12 weeks old (1 male to 1 female) and on control chow diet. Fourteen male breeders were obtained at 3 weeks of age and equally allocated into control diet or HFD groups randomly. All male rats were housed with female rats, and out of the seven rats in each group, five control diet and five high fat diet rats mated successfully.

Offspring

This study only included litter sizes between 9-15 pups. Birth was considered the postnatal day (PND) 1. At PND21, offspring were weaned and only female pups remained in the study as per Ng et al. (Ng *et al.*, 2010). Five dams were used to generate the female offspring, hence, two pups from each breeding pair were used in the study. There were no interventions in the offspring from birth until 4 weeks of age. Female offspring were randomized into sedentary or exercised group. All offspring performed acclimatization to the treadmill. Trained rats were subjected to a protocol of moderate intensity treadmill exercise training (5 d/wk, 60 min/d, at ~65-75% $\dot{V}O_{2max}$) from 5-9 weeks of life (Figure 1), while sedentary rats were kept in their cages. This protocol was based on previous studies from our group (Laker *et al.*, 2011) and others (Bedford *et al.*, 1979). All offspring had body weight and food intake measured from 3 weeks up to 25 weeks of life. We have defined adolescent offspring as 11-12 weeks of age and adult offspring as 23-24 weeks of age. All analysis was conducted under blinded conditions using coded samples.

Insulin sensitivity tests and glucose tolerance tests

Intraperitoneal insulin sensitivity tests (IPIST; 1 U/kg body weight) were performed after 2 hours of fasting at 12 weeks and 23 weeks of age while intraperitoneal glucose tolerance tests (IPGTT; 1.0 g/kg body weight) (Figure 1) were performed after an overnight fast one week later, as described previously (Ng *et al.*, 2010; Laker *et al.*, 2011). Tail vein blood glucose was measured by glucometer (Accu-Chek Performa Nano, Roche Diagnostics,

Mannheim, Germany). Insulin was analyzed by commercial radioimmunoassay (SRI-13K RI-13K, Linco Research, St Charles, Missouri, USA).

From the IPGTT at 24 weeks of age, the homeostatic model assessment of insulin resistance (HOMA-IR) and the insulinogenic indexes were obtained. The insulinogenic index provided an estimate of early insulin response to glucose (a measure of β-cell function *in vivo*) (Singh & Saxena, 2010), and has been used previously in female Sprague-Dawley rats (Ng *et al.*, 2010).

Insulin-stimulated glucose uptake in skeletal muscle

At 25 weeks of age, rats were deeply anaesthetized (60 mg/kg intraperitoneally; pentobarbitone, Virbac, New South Wales, Australia) and anesthesia checked every 10 min by performing a tail pinch and observing no change in the respiratory rate. Epitrochlearis (EPI) and soleus (SOL) muscles were dissected, longitudinally split in half (Sharma *et al.*, 2015) and incubated in chambers with Krebs Henseleit solution (in mM): 118.5 NaCl, 24.7 $NaHCO_3$, 4.74 KCl, 1.18 $MgSO_4$, 1.18 KH_2PO_4, 147.02 $CaCl_2$, 32 mannitol, 7.5% BSA and MilliQ H_2O, with pH 7.4, maintained at 30°C and continuously oxygenated with 95% O_2 and 5% CO_2. Muscles were incubated for 20min in the Krebs Henseleit solution with 8mM glucose. Muscles were then transferred to another Krebs Henseleit solution for 30min, with 36mM mannitol, and 4mM pyruvate. This second buffer also contained either 0nM or 1.2nM of insulin, which is considered a physiological concentration of insulin (Sharma *et al.*, 2011). The third incubation was in Krebs Henseleit solution with the addition of 8 mM 2-deoxyglucose, 0.75µCi ml^{-1} 2-[1,2-^{3}H] deoxy-D-glucose and 0.225µCi ml^{-1} [1-14C] mannitol, and 0 nM or 1.2nM insulin for 10min.

Approximately 30mg of EPI and 35mg of SOL were digested in 300µl of 1M NaOH at 95ºC for 10min, neutralized with 300µl of 1M HCl, vortexed and centrifuged for 5min at 13,000g in room temperature. Samples sat for at least 1h at room temperature prior to reading on a β-scintillation counter (Liquid Scintillation Analyser, Tri-Carb 2810TR, PerkinElmer, Boston, MA) using dual counts for ^{3}H and ^{14}C with each sample read for 10min (Stephens *et al.*, 2004).

Skeletal muscle mitochondrial respiration and reactive oxygen species production

Approximately 2 mg of plantaris (PLA) muscle was placed in cold Biopsy Preservation Solution (BIOPS) as described previously (Pesta & Gnaiger, 2012; Granata *et al.*, 2016). Muscle fibers were mechanically separated under a microscope and 50µg/ml saponin was used for chemical permeabilization of plasma membrane (30min incubation) in BIOPS. Mitochondrial respiration was performed on permeabilized muscle fiber at 37ºC using high-resolution Oxygraph-2k (OROBOROS, Innsbruck, Austria) (Pesta & Gnaiger, 2012) combined with the Fluorescence-Sensor Green of the O2k-Fluo LED2-Module for H_2O_2 measurement. The substrate–uncoupler–inhibitor titration (SUIT) protocol was performed as described elsewhere (Granata *et al.*, 2016). Briefly, we measured leak respiration (*L*) through complex I (CI) (CI_L), Maximum oxidative phosphorylation (oxphos) capacity (*P*) through CI (CI_P), *P* through CI+II combined ($CI+II_P$), electron transport system (ETS) capacity (*E*) through CI+II ($CI+II_E$), *E* through CII (CII_E) and finally residual oxygen consumption (ROX) and emission of H_2O_2 (Pesta & Gnaiger, 2012).

Citrate synthase (CS) activity assay was adapted to be performed on a 96-wells plate (Srere, 1969; McConell *et al.*, 2015), by examining the increase of 5,5-dithiobis-2-nitrobenzoate (DTNB) at a wavelength of 412 nm.

Western Blots

EPI muscle with and without insulin, and PLA muscle were homogenized in ice-cold buffer, and lysates were prepared as described previously (Betteridge *et al.*, 2016). Briefly, western blots were carried out using 7.5-12% hand-cast TGX Stain-Free gels (Bio-Rad, Hercules, CA, USA) loaded with 4 µg of skeletal muscle lysate. The polyvinylidene fluoride membranes (Bio-Rad) were imaged to quantify total protein using Imagelab 4.1 (Bio-Rad) (Murphy, 2011). After overnight incubation with primary antibodies, the membranes were incubated for 1h with secondary antibodies (anti-mouse or rabbit IgG, HRP-linked Antibody; 1:10000), and exposed to SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific). Raw signal was normalized by total protein content and bands were analyzed with ImageLab software (Version 5.2.1, Bio-Rad).

Antibodies used were anti-GLUT4 (abcam, #ab37445), anti-GLUT1 (abcam, #ab652), AKT2 (Cell Signaling, #5239), anti-TBC1D4 (Cell Signaling, #2670), anti-Tfam (abcam, #ab131607), anti-PGC1α (Merck Millipore, #ST1202), and anti-PHF20 (Cell Signaling, #3934). The primary phosphorylated (p)-specific antibodies were anti-p-AS160 Thr642 (Cell

Signaling, #4288), anti-p-AKT Thr308 (Cell Signaling, #9275), anti-p-AKT Ser473 (Cell Signaling, #9271).

Pancreas morphology

After dissection of skeletal muscles, rats were euthanized by cardiac puncture, and pancreases were weighed and stored in 10% Neutral Buffered Formalin at room temperature for up to one week, transferred to 70% ethanol and kept at 4ºC until processed. Five sections per pancreas were immunostained to identify and localise insulin-positive β-cells (n=8-10 per group). Fixed tissue was sent to Anatomical Pathology, Department of Medicine, University of Melbourne (Parkville, Victoria, Australia) to be paraffin embedded, sectioned at 100 µm and stained for insulin using a guinea pig polyclonal anti-porcine insulin antibody (DAKO Corporation, Denmark) diluted 1:100 and counterstained with haematoxylin. Digital images of microscopic sections were obtained through the Austin Health, Victorian Cancer Biobank Slide Scanning service (Heidelberg, Victoria, Australia). Following standard protocols, whole slide sections were line scanned using an Aperio ScanScope XT (Aperio Technologies, Vista, CA, USA) at 40x magnification at a resolution of 0.5 µm/pixel. Digital images were analysed using the Aperio image software (ImageScope version 12.2.2). Measurements were performed as described previously (Laker *et al.*, 2011).

Five random cross-sections from different parts of the pancreas (head, body and tail) were analyzed. Sampling from various parts of the pancreas is important as islet localisation, composition and architecture may vary depending on physiological and pathological states (Kharouta *et al.*, 2009). Relative islet surface area, β-cell area, number of islets, β-cell mass, islet number were obtained as described previously (Tikellis *et al.*, 2004; Laker *et al.*, 2011).

Statistical analyses

Data are presented as means ± SEM. Data were checked for normality using the Shapiro-Wilk test. If the test was significant, data were log transformed and reanalyzed. Statistical analyses were performed with SPSS (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.) and GraphPad Prism (GraphPad Prism version 5.00 for Windows, GraphPad Software, La Jolla California USA). The Student t test was used to investigate differences in paternal HFD in body weight and fat mass as well as newborn offspring in regards to organ masses. Two-way ANOVA was used having 'Paternal diet' and

'Exercise early in life' as main factors for offspring phenotype, protein expressions, enzyme activities, mitochondrial function, and pancreas morphology. Three-way ANOVA with repeated measures was used for experiments that included the two factors previously mentioned as well as different time points or treatment, for instance, IPIST, IPGTT, insulin incubation during 2DG uptake. If an interaction was found, a two-way ANOVA was applied in cases of three main factors for each time-point. For two-way ANOVA, if an interaction was found, a post-hoc analysis using the least significant difference test was used. The α-level of statistical significance was set *a priori* at $P<0.05$.

Results

High fat diet, fat mass and insulin resistance in fathers

The high fat diet intervention from 4 to 14 weeks of age prior to mating in male rats was successful in achieving breeder rats with higher body and fat mass and insulin resistance ($P<0.05$, data not shown).

Paternal high fat diet and offspring phenotype from birth until weaning

Paternal HFD did not change the number of male or female rats born, birth weight, or body weight from birth until PND 21 ($P>0.05$). At PND 21, there was no significant difference between pups from HFD or chow fed fathers for soleus or EDL muscle mass ($P=0.07$ and $P=0.09$, respectively) or the mass of the liver, spleen, heart, kidneys and pancreas ($P>0.05$).

Effect of paternal high fat diet and early life exercise on body weight, food intake and glucose tolerance in adolescent and adult offspring

Offspring sired by HFD fathers had lower body weight and food intake after 12 and 16 weeks of age, respectively ($P<0.05$, data not shown). In adolescent offspring, paternal HFD did not affect insulin sensitivity but it impaired glucose tolerance, increasing the glucose AUC. Exercise early in life normalized body weight after week 16 of age and normalized food intake from week 18 until week 24 of age. In adolescent rats, exercise early in life did not alter glucose tolerance in offspring of normal diet fathers. But when offspring sired by high-fat eating fathers performed early exercise, the negative metabolic effects on glucose tolerance were attenuated ($P<0.05$, data not shown). In adult offspring (24 weeks of age), insulin sensitivity was impaired by paternal HFD, whereas glucose tolerance was

unaffected. Insulin levels during the IPGTT increased less in offspring sired by HFD fathers (Figure 2). During our experiments, one animal from the NE group suddenly died. An autopsy was conducted by the university animal welfare officer and the cause of death was not identified in the samples examined.

Effect of paternal high fat diet and early life exercise on offspring phenotype at 25 weeks of age (post mortem)

Body weight, soleus and gastrocnemius skeletal muscle weights were lower in offspring of paternal HFD with no changes in body length ($P<0.05$, data not shown). When offspring sired by HFD father exercised early in life, their body weight, soleus and plantaris muscle weights were normalized but there were no effects on tibialis anterior, liver and heart.

Effect of paternal high fat diet and early life exercise on offspring skeletal muscle insulin-stimulated glucose uptake

There were no differences in basal and insulin-stimulated glucose uptake ex vivo in soleus muscle between groups (Figure 3). However, offspring sired by HFD fathers had reduced basal and insulin-stimulated glucose uptake *ex vivo* in the EPI muscle with no difference in the delta glucose uptake between basal and insulin-stimulated glucose uptake. In the EPI muscle, exercise early in life had no effect in offspring from chow fed fathers but normalized both basal and insulin-stimulated 2DG uptake in rats sired by HFD fathers (Figure 3).

Effect of paternal high fat diet and early life exercise on skeletal muscle insulin signaling in offspring of HFD fathers

Since glucose uptake was impaired in EPI muscle but not the SOL muscle, immunoblotting for insulin signaling were performed only in the EPI muscle (Figure 3 and 4). Basal (0nM), p-Akt^{Thr308}, p-AKT^{Ser473} and p-TBC1D4^{Thr642} at 25 weeks were not affected by paternal HFD and exercise early in life (Figure 4). Insulin-stimulated (1.2nM) p-Akt^{Thr308} was unaffected but surprisingly p-Akt^{Ser473} was increased in offspring of paternal HFD irrespective of early exercise (Figure 4). Importantly, insulin-stimulated p-TBC1D4^{Thr642} was lower in offspring of paternal HFD and early life exercise normalized this (Figure 4). Paternal HFD did not reduce skeletal muscle GLUT1 and GLUT4 protein content in adult offspring. Early life exercise increased both GLUT1 and GLUT4 in adult offspring above the control

group irrespective of paternal diet (Figure 3). Because there was no change in the total proteins we measured, the phosphorylated and the ratio of phosphorylated to total were similar.

Effect of paternal high fat diet and early life exercise on mitochondrial respiration and flux control ratios, mitochondrial ROS production and mitochondrial protein expression

Paternal HFD reduced most aspects of mitochondrial respiration by 20-30% in adult offspring (Figure 5). Exercise early in life in offspring born from control diet fathers increased CI_L. Early life exercise increased several aspects of mitochondrial respiration in adult offspring of HFD fathers, such that the levels were higher than sedentary offspring of HFD fathers (Figure 5). CS activity (Figure 5) and respiratory control ratios ($P>0.05$, data not shown) in adulthood were not affected by paternal HFD or exercise early in life. Given that adult offspring CS activity was unaffected by paternal diet or early life exercise in offspring, no normalization of respiration to CS activity was undertaken to avoid introduction of variation during a normalization process. Mitochondrial H_2O_2 production was not altered by either paternal HFD or exercise early in life during any stages of respiration ($P>0.05$, data not shown). Offspring sired from HFD fathers had lower PHF20 but there was no difference in Tfam protein expression ($P=0.07$). PGC1α was also unaffected. Exercise early in life did not affect the expression of these proteins (Figure 6).

Effect of paternal high fat diet and early life exercise on pancreas morphology in adult offspring

Relative islet surface area, number of islets, β-cell area and proportion did not change due to paternal HFD (Figure 7). However, in offspring of HFD fathers β-cell mass and the insulinogenic index was lower and islet size distribution was altered with more small size islets and less large and very large islets (Figure 7). Exercise early in life increased the number of islets in both normal and high fat father offspring while β-cell mass and the insulinogenic index remained lower in exercised offspring sired by HFD fathers (Figure 7). Exercise early in life normalized the amount of 20,000-50,000μm^2 islets, but did not change the number of <5,000μm^2 and 10,000-20,000μm^2 islets (Figure 7).

Discussion

The main findings of this study were that adult offspring sired by HFD fed fathers had reduced whole body and *ex vivo* skeletal muscle insulin sensitivity and reduced glucose-stimulated insulin secretion (with lower pancreatic β-cell mass). Only 4 weeks of exercise early in life normalized whole body and *ex vivo* insulin sensitivity in these adult offspring of HFD fed fathers. The benefits of the early exercise appeared to be mainly at the level of skeletal muscle since lower glucose-stimulated insulin secretion and lower pancreatic β-cell mass were not normalized by early exercise training.

Similar to previous studies (Fullston *et al.*, 2013; Masuyama *et al.*, 2016), paternal HFD before mating did not affect the number of pups born, the ratio between males and females or the birth weight of the litter. In humans, paternal body mass index (BMI) is positively associated with offspring total and central fatness in youth (Labayen *et al.*, 2010), but in rodents, body weights tend to be higher or not different after HFD in fathers (Ng *et al.*, 2010). However, we found that body weight was lower in female adult offspring fathered by rats fed with high fat diet, which was also reported in male offspring (Lecomte *et al.*, 2017). Ng *et al* found no difference in body weight of their female offspring sired by HFD father at 12 weeks of age (Ng *et al.*, 2010), but it is possible that the differences in older animals (e.g. 25 weeks of age) may have been missed in that study, as we examined in our investigation. It is also possible that their offspring were not lighter as the fathers in that study were fed some standard chow in addition to the high fat diet while our fathers were fed only the high fat diet (Ng, 2011). Although not mentioned in the Ng et al. (Ng *et al.*, 2010) paper, it was stated in Ng's PhD thesis that in addition to high fat pellets "a small amount of standard laboratory chow was also available in the cage" (Ng, 2011).

Exercise early in life increased food intake and normalized the reduced body weight in offspring of HFD fed fathers by increases non-fat mass including skeletal muscles, liver and heart weights. It is very interesting that only 4 weeks of exercise early in life is able to result in these changes 4 months later when the rats were 25 weeks of age.

In vivo insulin sensitivity, observed from the IPIST, was lower in offspring at 23 weeks of age after paternal HFD which fits with findings by others at 16, 26, and 39 weeks of age (Fullston *et al.*, 2013). The lower insulin sensitivity was likely due to reduced skeletal muscle insulin sensitivity since *ex vivo* insulin-stimulated glucose uptake and TBC1D4 Thr642 phosphorylation were also lower in the EPI muscle of offspring of HFD fed fathers. Only 4 weeks of exercise early in life in offspring sired by fathers fed HFD normalized insulin

sensitivity, *ex vivo* insulin-stimulated glucose uptake and TBC1D4 Thr642 phosphorylation in adulthood. The early life exercise also resulted in greater GLUT4 protein levels in adulthood which may have contributed to the normalizing of insulin sensitivity. The effects of HFD fathers and early life exercise on skeletal muscle insulin sensitivity appeared to be at the level of TBC1D4 since insulin-stimulated was not reduced by having a HFD fed father and also there was no effect of early life exercise training on pAkt. It is not clear why offspring sired by fathers fed HFD actually had higher insulin-stimulated $pAkt^{Ser473}$. Although our results suggested that the effects of HFD fed fathers and early life exercise on insulin sensitivity were at the level of the skeletal muscle, further studies are needed using tracer and clamp techniques to investigate if hepatic insulin resistance might also be playing a role.

We also observed lower basal *ex vivo* EPI glucose uptake in adult offspring of HFD fed father. Basal glucose uptake in skeletal muscle is thought to be related to GLUT1 protein, an insulin-independent glucose transporter found in the sarcolemmal membrane (Ebeling *et al.*, 1998). We found GLUT1 protein content was 23% lower in HFD offspring but this was not significant (P=0.11). Early exercise training normalized the reduced basal *ex vivo* EPI glucose uptake and increased GLUT1 protein content in adulthood. It is without precedent that these changes in basal and insulin-stimulated glucose uptake are observed 4 months after exercise is ceased and suggest epigenetic changes may have occurred. We normally say "use it or lose it" when it comes to exercise because adaptations to exercise are lost within days to weeks after exercise training is ceased. Yet, as was the case after short term exercise early in life in our previous study in rats born small for gestational age, the effects are observed 4 months later (Laker *et al.*, 2011). It will be important for future studies to examine possible epigenetic changes in regards to this.

Some studies have showed indications that skeletal muscle mitochondrial function can influence insulin sensitivity (Szendroedi *et al.*, 2012; Holloszy, 2013; Martin & McGee, 2014). Skeletal muscle mitochondrial function of all measured complexes were lower (20-30%) in offspring sired by HFD fed fathers but there was no indication of an effect on respiratory ratios, reactive oxygen species production or citrate synthase activity. Exercise early in life in rats from chow fed fathers did not affect mitochondrial respiration, but when offspring sired by HFD fed fathers performed exercise early in life, their mitochondrial respiration was improved in adulthood. This remarkable effect of early life exercise on mitochondrial function months later in offspring of HFD/obese fathers may have contributed

to the normalization of insulin-stimulated glucose uptake in skeletal muscle. Similarly, the positive effects of maternal exercise are only observed in offspring of mothers on a high fat diet (Laker *et al*, 2014) or a low-protein diet (Falcao-Tebas *et al.*, 2012), but maternal exercise in chow fed mums has no effects on the offspring. Similar effects might apply to exercise early in life, where positive effects are only observed when there was a prior negative event on the offspring.

Given the reprogramming effects of early life exercise on skeletal muscle, we were surprised that early life exercise had no effect on the reduced β-cell mass and reduced insulin secretion in adult offspring from fathers fed a HFD. However, we found changes in islet numbers and distribution in offspring of HFD fed fathers, these alterations were just partially normalized by early life exercise. We had previously demonstrated that exercise early in life fully restored the large decrease in β-cell mass (~65%) and pancreatic islet surface area in rats born small for gestational age (Laker *et al.*, 2011). This suggests that the mechanisms causing decreases in β-cell mass with intrauterine growth restriction are different to those that involve the father's sperm. It will be important to determine which genes are associated with the lower β-cell mass in the two models. In addition, the disparate effects of exercise early in life in the two models may have related to sex differences, as Laker *et al*. used male and only females were used in this study, or may be due to rat strain differences between Wistar-Kyoto used in the small for gestational age study (Laker *et al.*, 2011) and Sprague-Dawley rats used in this investigation.

The underlying mechanism of how offspring exercise may modulate offspring's phenotype later in life might be related to epigenetic adaptations. Considering exercise early in life as an environmental factor, it would be able to modify cellular and physiological phenotypic traits in the offspring, by switching genes on and off (Handy et al. 2011). Different epigenetic markers, such as microRNAs, acetylation and methylation of histones might be involved to the long-term responses observed in the offspring. Although other studies have provided some evidence to support that TBC1D4^{Thr642} phosphorylation increases during clamp, 5h into exercise recovery (healthy men, 25-28 years) (Pehmoller et al. 2012) or 2 days after the last bout of exercise training (Frosig et al. 2007), we have demonstrated for the first time its long-term effects (many weeks after exercise training had ceased). Sixteen weeks after the last exercise session, the positive effects of exercise early in life were still observed in adult offspring sired by obese fathers fed with HFD before conception. Further

experiments investigating epigenetic markers in these offspring should provide useful insights to explain these findings.

In conclusion, exercise early in life attenuated the negative effects of paternal HFD whole body and *ex vivo* insulin sensitivity in adulthood. Exercise early in life increased GLUT4 protein and normalised the reduced skeletal muscle insulin-stimulated TBC1D4 phosphorylation and also increased mitochondrial respiration. Surprisingly, early life exercise did not have any positive effects on the reduced pancreatic β-cell mass in offspring from HFD fed fathers, which likely explains why exercise had no effects on insulin secretion in adulthood. Taken with our previous findings in small for gestational age offspring, these results provide strong support for the importance of exercise early in life especially in children who suffered some kind of developmental insult.

Additional information section

Competing interests
None declared.

Author contributions
F.F.T. and G.K.M. conceived and designed the work; F.F.T., J.K., C.A., J.P.K., S.A. and E.C.M. conducted acquisition of data; F.F.T., E.C.M. and G.K.M. performed analysis and interpretation of data for the work; F.F.T., E.C.M. and G.K.M. drafted the manuscript; F.F.T., J.K., C.A., J.P.K., S.A., E.C.M. and G.K.M. revised it critically for important intellectual content.

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Table 1. Effects of exercise early in life on adult (25 weeks of age, post mortem) female offspring sired by high fat fathers.

Parameters	NS n = 10	HS n = 10	NE n = 9	HE n = 10
Body weight (g)	333.5 ± 13.9	299.1 ± 6.9 *	352.8 ± 11.8#	322.0 ± 9.3 +
Length (cm)	24.3 ± 0.2	24.0 ± 0.1	25.1 ± 0.1 #	24.6 ± 0.2 #
Abdominal circumference (cm)	18.33 ± 0.45	18.18 ± 0.20	19.12 ± 0.69	18.73 ± 0.24
EDL (g)	0.16 ± 0.07	0.14 ± 0.05	0.17 ± 0.03 #	0.16 ± 0.03 #
Soleus (g)	0.15 ± 0.05	0.13 ± 0.06 *	0.16 ± 0.05 #	0.15 ± 0.04 +
Gastrocnemius (g)	1.75 ± 0.05	1.62 ± 0.02	1.70 ± 0.4	1.72 ± 0.04
Tibialis anterior (g)	0.67 ± 0.02	0.63 ± 0.01	0.69 ± 0.01 #	0.68 ± 0.12 #
Plantaris (g)	0.33 ± 0.01	0.31 ± 0.07 *	0.35 ± 0.01 #	0.33 ± 0.03 +
Liver (g)	8.52 ± 0.31	8.15 ± 0.28	9.28 ± 0.31 #	9.22 ± 0.46 #
Pancreas (g)	1.36 ± 0.07	1.20 ± 0.09	1.46 ± 0.13	1.27 ± 0.11
Retroperitoneal fat (g)	6.17 ± 0.35	6.98 ± 0.59	7.46 ± 1.05	6.95 ± 0.91
Kidneys (g)^	0.9 ± 0.0	0.9 ± 0.03	0.9 ± 0.03	1.0 ± 0.04

	8		3	0		*	8			0		+
Heart (g)	1.0		0.0	0.9			1.0		0.03	1.0		0.04
	3	±	3	5	±	0.02	8	±	#	4	±	#
Fasting glucose	5.6		0.0	5.6			5.6			5.5		
(mmol/l)	6	±	9	3	±	0.28	4	±	0.12	9	±	0.23
Fasting insulin (ng ml^{-1})	0.1		0.0	0.1			0.1		0.01	0.1		0.01
	5	±	1	3	±	0.02	1	±	#	2	±	#
HOMA-IR	0.0		0.0	0.0		0.00	0.0		0.005	0.0		0.003
	35	±	06	34	±	8	26	±	#	29	±	#

EDL, extensor digitorum longus. HOMA-IR, homeostatic model assessment of insulin resistance. Equation was as follows: HOMA-IR = [fasting plasma glucose (mg/dl) x fasting plasma insulin (μU/ml)]/2,430 (Cacho, Sevillano et al. 2008). NS, offspring sired by control diet fathers; HS, offspring sired by high fat diet fathers; NE, early life exercised offspring sired by control diet fathers; HE, early life exercised offspring sired by high fat diet fathers. ^, kidneys left and right were combined as there was no statistical difference between them. Values are presented as mean ± SEM. * Paternal HFD effect $P < 0.05$, 0.01. #, Exercise early in life effect $P < 0.05$. +, Paternal HFD vs. Exercise early in life interaction $P < 0.05$.

Figure legends

Figure 1. Experimental groups and timelines. A: Experimental groups based on paternal diet and offspring exercise interventions. Male breeders were mated with only one female (1:1). B. Experimental design with timelines for main experiments. NS, pups sired by control diet fathers that remained sedentary in life. HS, pups sired by high fat diet fathers which remained sedentary in life. NE, pups sired by normal diet fathers and exercised early in life. HE, pups sired by high fat diet fathers and exercised early in life.

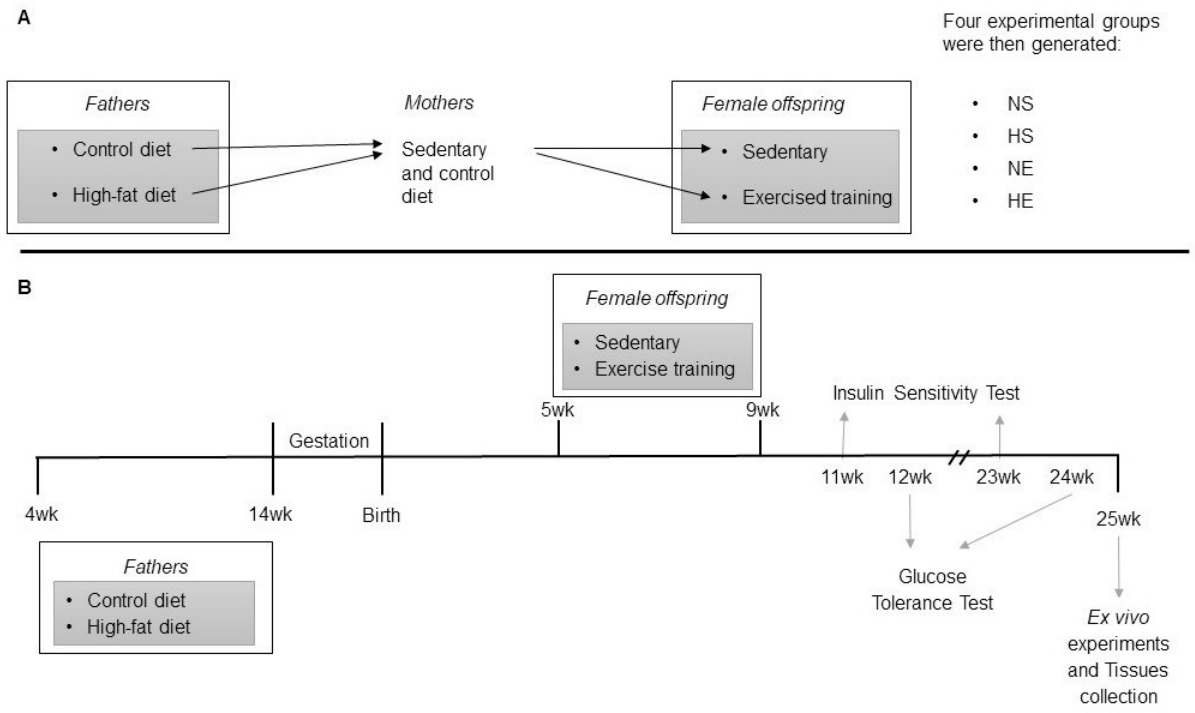


Figure 2. Exercise early in life protects adult female offspring sired by high fat fathers to develop impaired glucose tolerance. Glucose levels (A) and glucose area under the curve (AUC, B) during the intraperitoneal insulin sensitivity tests (IPIST, 23 weeks of age). Glucose (C) and insulin (E) levels during a glucose tolerance test (GTT, 24 week of age), and their respective areas under the curves (D, F). NS, offspring sired by control diet fathers (n=10); HS, offspring sired by high fat diet fathers (n=10); NE, early life exercised offspring sired by control diet fathers (n=9); HE, early life exercised offspring sired by high fat diet fathers (n=10). Values are presented as mean ± SEM. * Paternal HFD effect $P < 0.05$. +, Paternal HFD vs. Offspring exercise interaction $P < 0.05$, followed by LSD test (HS vs. HE).

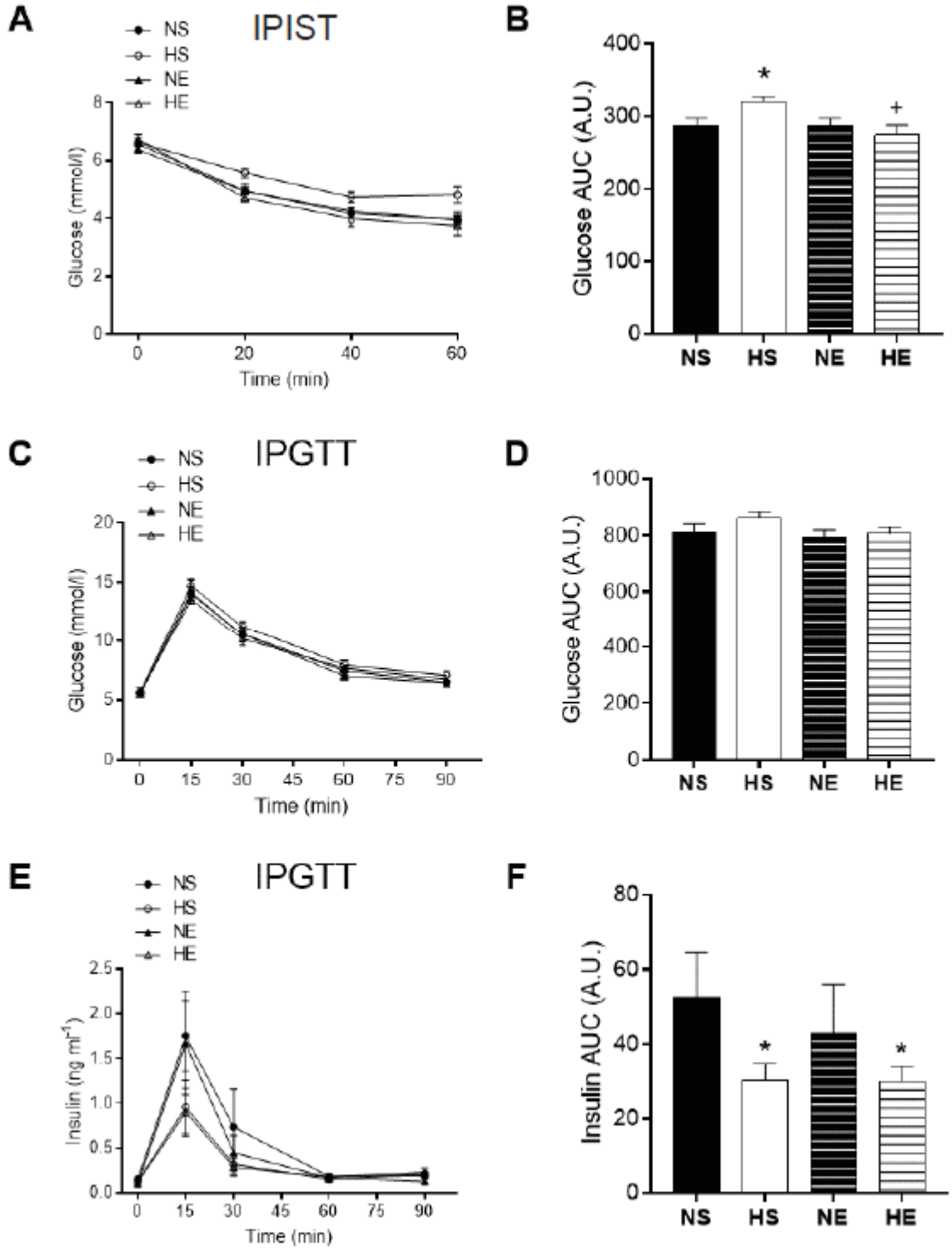


Figure 3. Paternal high fat diet before conception impairs basal and insulin-stimulated glucose uptake in epitrochlearis (B) but not soleus (A) muscle, while exercise early in life protects adult female offspring. Early life exercise increases GLUT1 and GLUT4 in adult offspring epitrochlearis muscle. For GLUT1 two bands were identified and used

to measure protein expression. Representative western blots show the quality and signal obtained with the respective antibodies; because they represent one animal, they do not necessarily represent an exact mean of their experimental group. NS, offspring sired by control diet fathers (n=6); HS, offspring sired by high fat diet fathers (n=6); NE, early life exercised offspring sired by control diet fathers (n=5); HE, early life exercised offspring sired by high fat diet fathers (n=5). Values are presented as mean ± SEM. & Insulin effect P < 0.05. *, ** Paternal HFD effect P < 0.05, 0.01. #, Exercise early in life effect P < 0.05. +, Paternal HFD vs. Offspring exercise interaction P < 0.05, followed by LSD test (HS vs. HE).

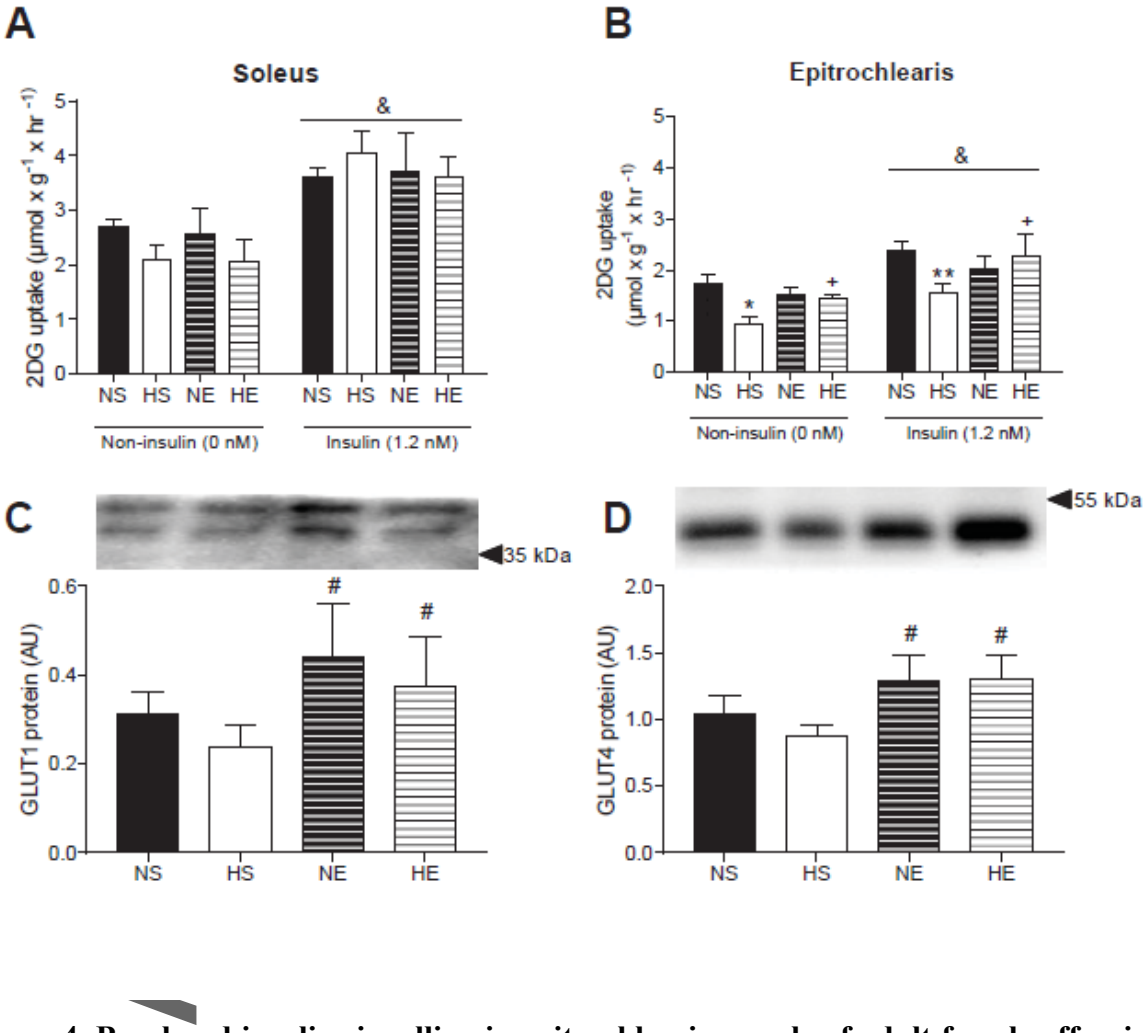


Figure 4. Basal and insulin signalling in epitrochlearis muscle of adult female offspring which exercised early in life or not, sired by high fat eating fathers or control diet eating fathers. Akt Thr308 (A) and Ser473 (B) as well as TBC1D4 Thr642 (C)

phosphorylation. For p-TBC1D4^{Thr672} in insulin-stimulated condition (1.2 nM) two bands were identified and used to measure protein expression. NS, offspring sired by control diet fathers (n=6); HS, offspring sired by high fat diet fathers (n=6); NE, early life exercised offspring sired by control diet fathers (n=5); HE, early life exercised offspring sired by high fat diet fathers (n=5). Values are presented as mean ± SEM. &, && Insulin effect P < 0.05, 0.01. * Paternal HFD effect P < 0.05. +, Paternal HFD vs. Offspring exercise interaction P < 0.05, followed by LSD test (HS vs. HE).

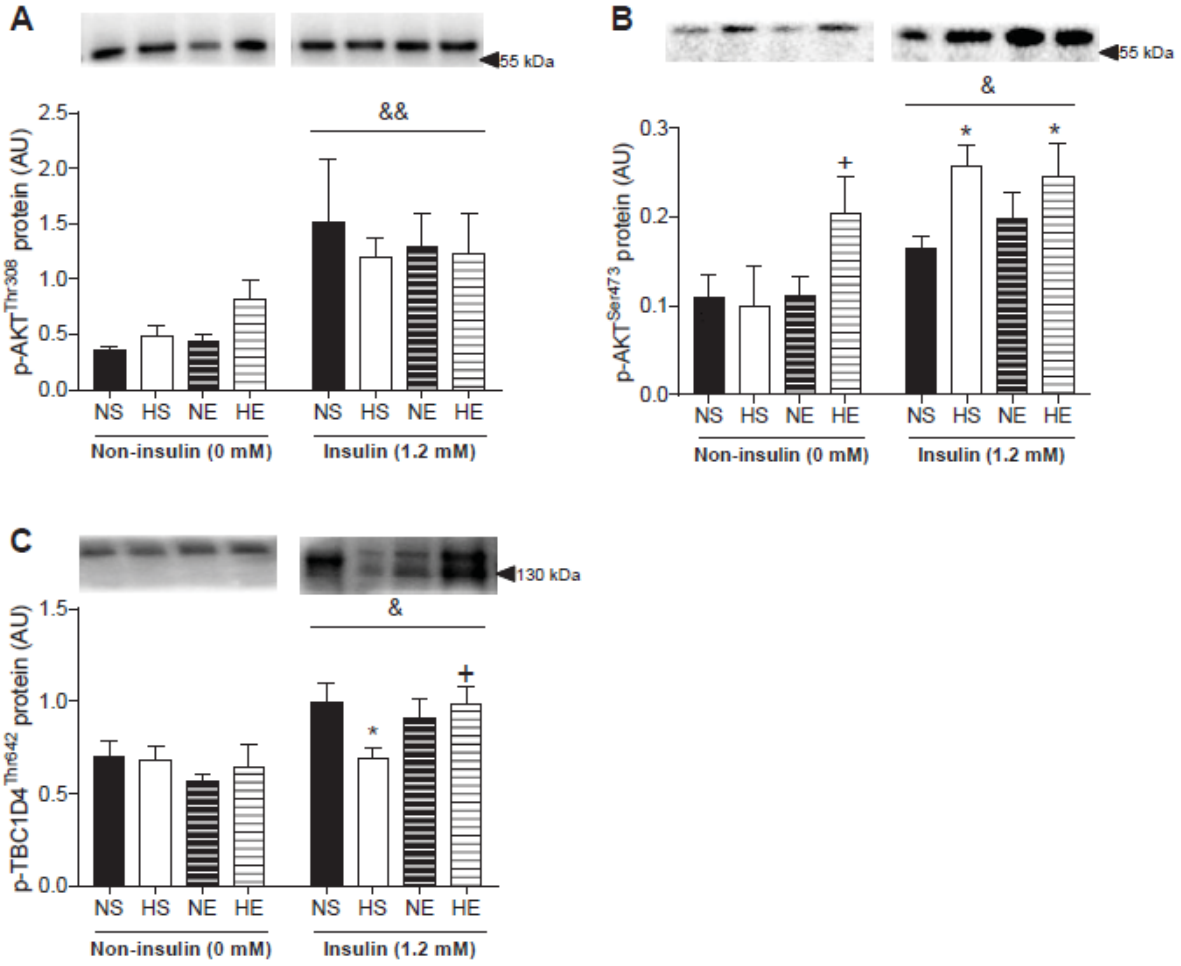


Figure 5. Mass-specific mitochondrial respiration in adult offspring which performed exercise early in life or not, sired by control or high fat eating fathers before conception. The following parameters were measured: leak respiration through complex I (CI_L; A), Maximum oxidative phosphorylation capacity (*P*) through CI (CI_P; B), *P* through CI+II combined ($CI+II_P$; C), electron transport system capacity (*E*) through CI+II ($CI+II_E$; D), *E* through CII

(CII_E; E). Citrate synthase activity was also measured in the plantaris muscle (F). NS, offspring sired by control diet fathers (n=9); HS, offspring sired by high fat diet fathers (n=8); NE, early life exercised offspring sired by control diet fathers (n=7); HE, early life exercised offspring sired by high fat diet fathers (n=8). F. Paternal high fat diet and early in life does not affect citrate synthase activity. NS, offspring sired by control diet fathers (n=10); HS, offspring sired by high fat diet fathers (n=10); NE, early life exercised offspring sired by control diet fathers (n=9); HE, early life exercised offspring sired by high fat diet fathers (n=10). Values are presented as mean ± SEM. #, Exercise early in life effect $P < 0.05$. +, Paternal HFD vs. Offspring exercise interaction $P < 0.05$, followed by LSD test (HS vs. HE).

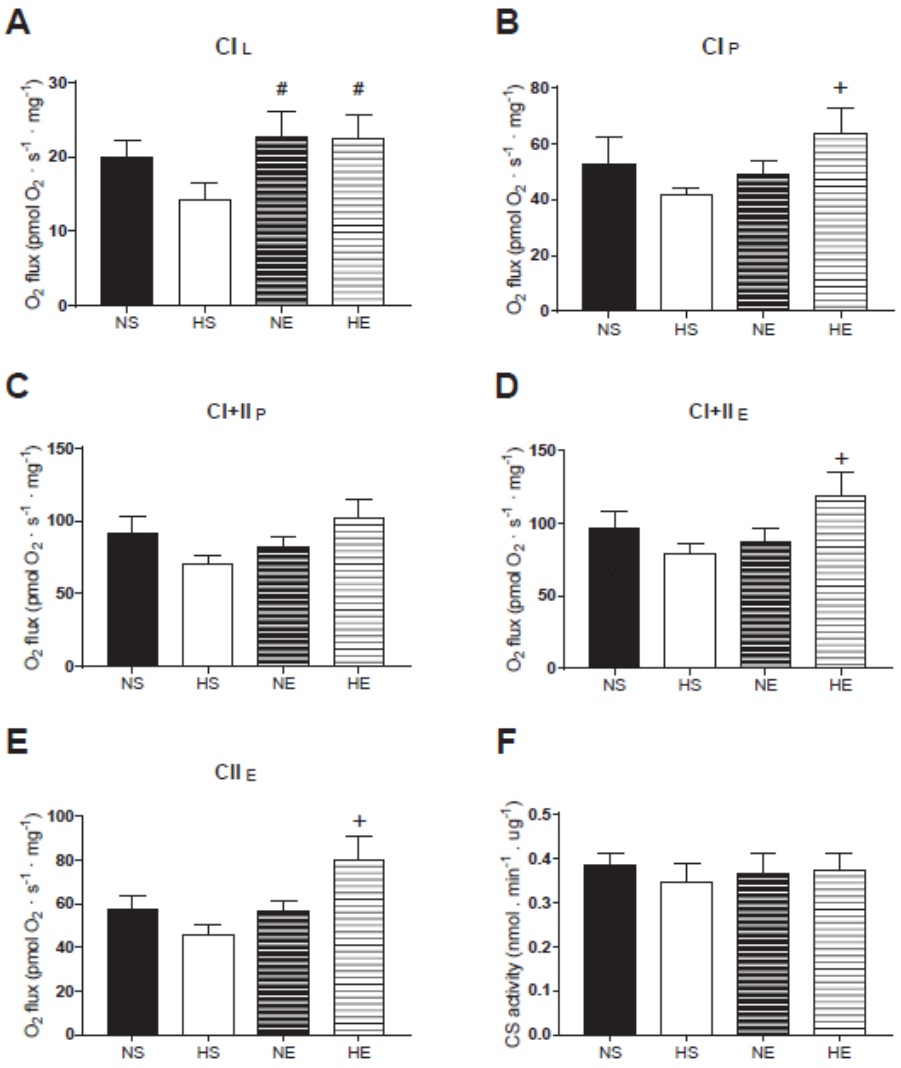


Figure 6. Protein expression in plantaris muscle of adult offspring which performed exercise early in life or not, sired by control or high fat eating fathers before conception. For TFAM and PHF20, two band were identified and used to measure protein expression. NS, offspring sired by control diet fathers (n=10); HS, offspring sired by high fat diet fathers (n=10); NE, early life exercised offspring sired by control diet fathers (n=9); HE, early life exercised offspring sired by high fat diet fathers (n=10). Representative western blots show the quality and signal obtained with the respective antibodies; because they represent one animal, they do not necessarily represent an exact mean of their experimental group. Values are presented as mean ± SEM. * Paternal HFD effect P < 0.05.

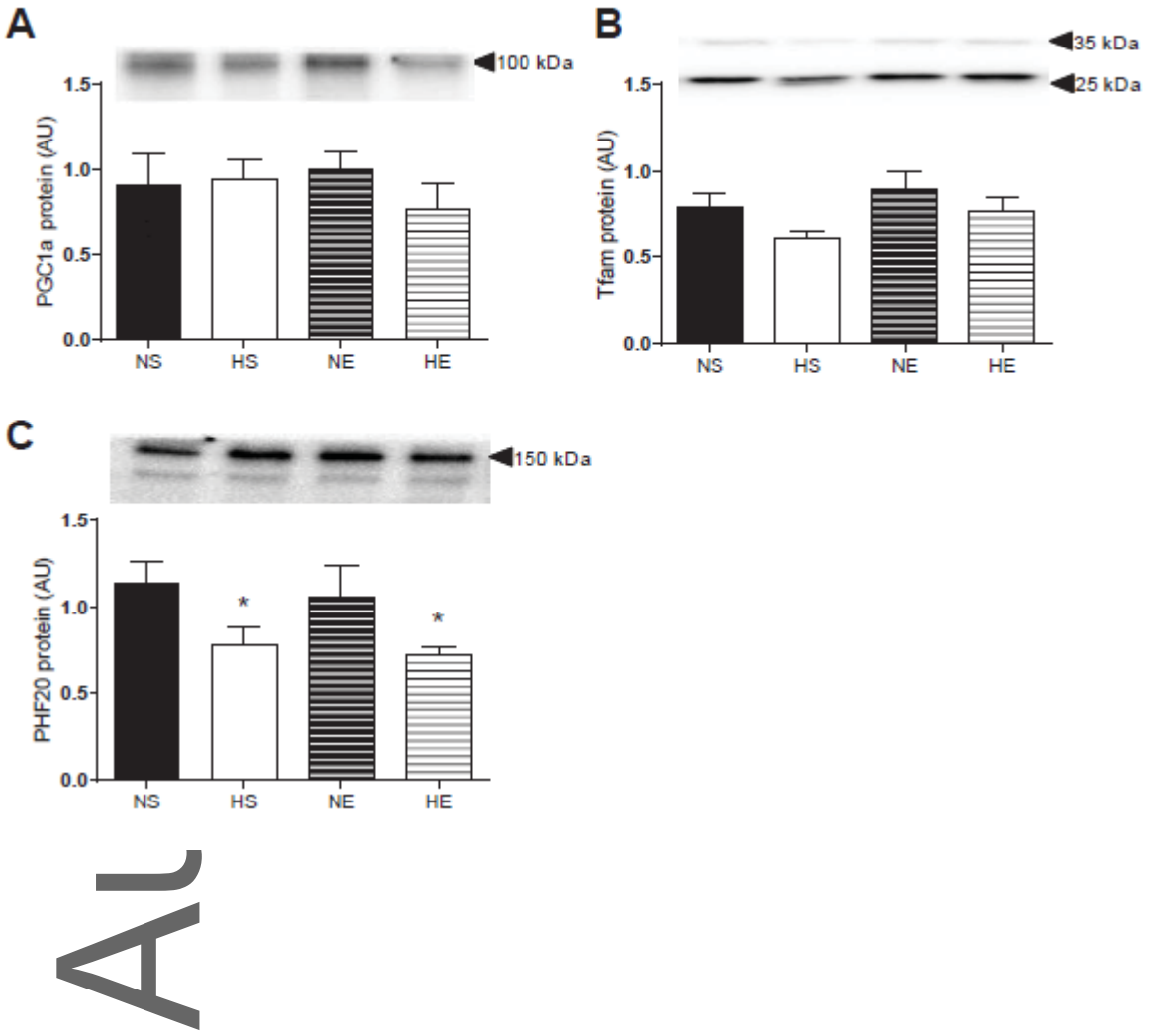
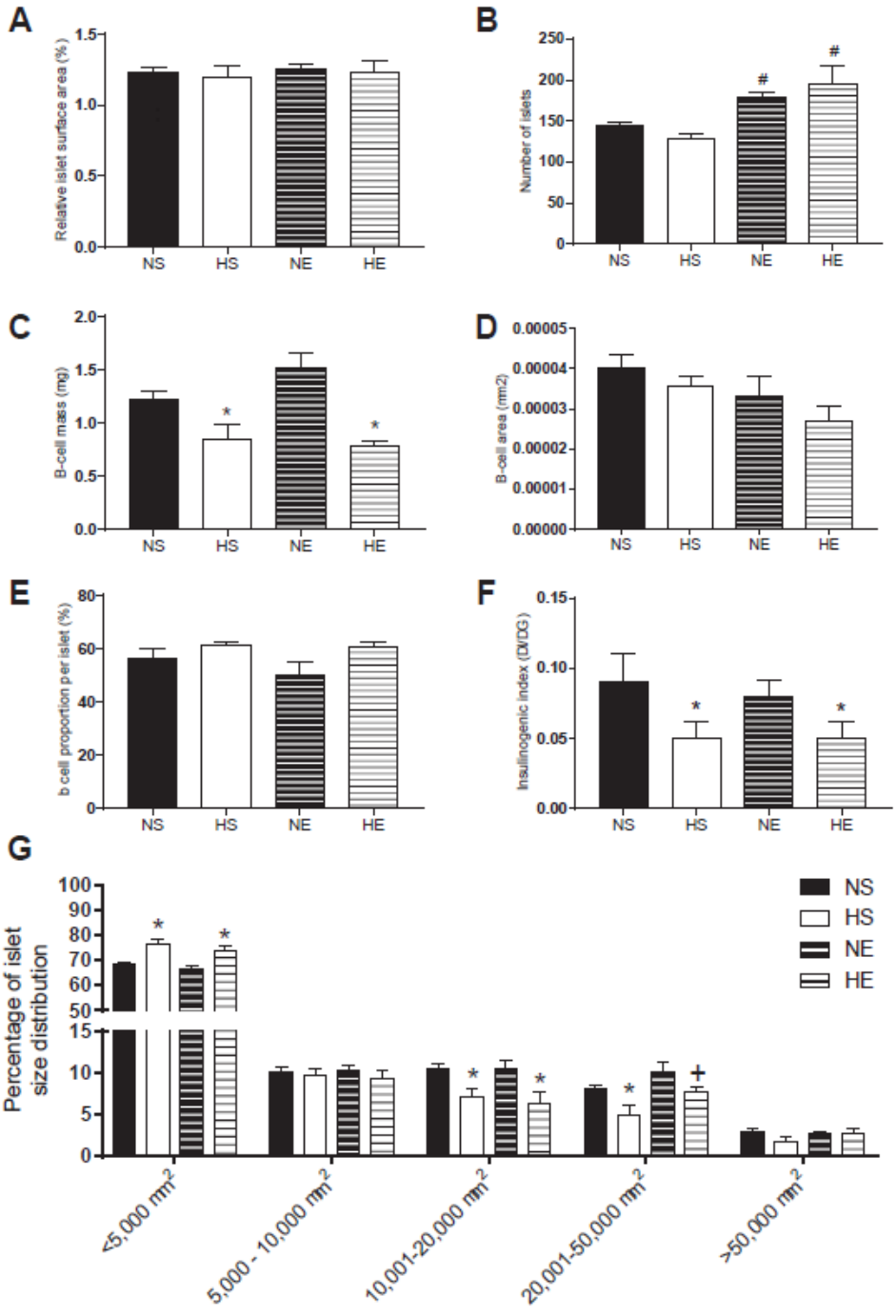


Figure 7. Pancreas morphology in adult offspring sired by fathers fed high fat or control diets which performed or not exercised early in life. The following parameters were measured: Relative islet surface area expressed as a percentage of total pancreas surface area (A), number of islets (B), β-cell mass (C) was calculated as the product of whole pancreas weight before fixation and the ratio of insulin positive/total pancreas cross-sectional area, β-cell area (D) and β-cell proportion per islet (E). The insulinogenic index (F), derived from intraperitoneal glucose tolerance test (IPGTT) at 24 weeks of age with the following formula: Insulinogenic index = $AUC_{insulin(0–30\ min)}$ / $AUC_{glucose(0–30\ min)}$ (Ng et al., 2010). Islet distribution (G) was arbitrarily classified according to their size: <5,000 μm^2, 5,000 – 10,000 μm^2, 10,001 – 20,000 μm^2, 20,001 – 50,000 μm^2 and >50,000 μm^2. NS, offspring sired by control diet fathers (n=10); HS, offspring sired by high fat diet fathers (n=10); NE, early life exercised offspring sired by control diet fathers (n=9); HE, early life exercised offspring sired by high fat diet fathers (n=10). Values are presented as mean ± SEM. * Paternal HFD effect P < 0.05. #, Exercise early in life effect P < 0.05. +, Paternal HFD vs. Offspring exercise interaction P < 0.05, followed by LSD test (HS vs. HE).



Author profile

Filippe Falcão-Tebas obtained his Master's degree in Nutrition at the Federal University of Pernambuco (Brazil) and completed his PhD at the Institute for Health and Sport, Victoria University (Australia). Filippe is interested in the role of exercise and diet during developmental stages and their potential to reprogram metabolic diseases later in life.

Author/s:
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