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Paternal exercise protects against liver steatosis in the male offspring of mice submitted to high fat diet

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ABSTRACT

Parental lifestyle has been related to alterations in the phenotype of their offspring. Obese sires can induce offspring insulin resistance as well as increase susceptibility to obesity. On the other hand, obese sires submitted to voluntary exercise ameliorate the deleterious metabolic effects on their offspring. However, there are no studies reporting the effect of programmed exercise training of lean sires on offspring metabolism.

Aims: This study aimed to investigate the role of swimming training of sires for 6 weeks on the offspring metabolic phenotype.

Main methods: Male C57BL/6 mice fed a control diet were divided into sedentary and swimming groups. After the exercise, they were mated with sedentary females, and body weight and molecular parameters of the offspring were subsequently monitored.

Key findings: Swimming decreased the gene expression of *Fasn* and *Acaca* in the testes and increased the AMPK protein content in the testes and epididymis of the sires. The progeny presented a low weight at P1, which reached a normal level at P60 and at P90 the animals were challenged with HFD for 16 weeks. The male offspring of trained sires presented less body weight gain than the control group. The level of steatosis decreased in the male offspring from trained sires. The gene expression of *Prkaa2*, *Ppar-1α* and *Cpt-1* was also increased in the liver of male offspring from trained sires.

Significance: Taken together, these findings suggest that paternal exercise training can improve the metabolic profile in the liver of the progeny, thereby ameliorating the effects of obesity.

Abbreviations: Acaca, Acetyl-Coa Carboxylase-Alpha; ALT, Alanine Transaminase; AMPK, AMP-activatedproteinkinase; AST, Aspartate Transaminase; Cpt-1, Carnitinepalmitoyltransferase 1; Dnmt1, DNA (cytosine-5)-methyltransferase 1; Dnmt3a, DNA (cytosine-5)-methyltransferase 3A; Fasn, Fattyacidsynthase; GAPDH, Glyceraldehyde-3-Phosphate Dehydrogenase; GLUT4, Glucose Transporter 4; GTT, Glucose Tolerance Test; HFD, High Food Diet; ITT, InsulinTolerance Test; NAFLD, Non-alcoholicfattyliverdisease; PEPCK, Phosphoenolpyruvatecarboxykinase; PFA, Paraformaldehyde; PGC1α, Peroxisomeproliferator-activated receptor gamma-coactivator 1-alpha; PPARα, Peroxisomeproliferator-activated receptor alpha; Prkaa-2, ProteinKinase AMP-ActivatedCatalyticSubunit Alpha 2; TFAM, TranscriptionFactor A, Mitochondrial; SD, standard diet; TAG, Triacylglycerol

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1. Introduction

Physical training promotes molecular changes that culminate in the adaptation of metabolic tissues such as adipose tissue, skeletal muscle and liver that reduce the negative effects arising from obesity [1–3]. These changes occur due to the mobilization of intracellular proteins that activate biochemical pathway cascades and, in turn, recruit the substrates for use in energy production (ATP), with a consequent reduction in fat and increase in glycogen reservoirs. One of the major diseases related to obesity is NAFLD (non-alcoholic fatty liver disease), which consists of increased intrahepatic storage of triglycerides that reduces the metabolic activity in the liver and induces release of fat into the blood [4], increasing the risk of cardiovascular disease, acute myocardial infarction, hypertension, insulin resistance, glucose intolerance and type 2 diabetes [5–7]. Exercise alters the gene expression of CPT-1 (carnitine palmitoyl transferase 1), AMPK (AMP-activated protein kinase), ACC (acetyl-CoA carboxylase) and FAS (fatty acid synthase) in skeletal muscle and adipose tissue, which results in increased oxidative metabolic capacity and reduced lipogenesis; accordingly, exercise represents a relevant therapeutic tool to reduce body weight gain and the negative effects of obesity and type 2 diabetes [8–10]. In our previous study, we evaluated the role of swimming as exercise during pregnancy on the metabolic profile of F1 offspring fed a high-fat diet, and the results showed protection against HFD-induced obesity and a decrease in adipose depots in male offspring [11]. Recent studies confirm an important role for maternal lifestyle on the metabolic profile of progeny, suggesting involvement of key proteins of glycolytic and lipid metabolism, such as AMPK, PGC-1α, TFAM, and PEPCK [12–16].

Additionally, Wu and colleagues [17] revealed a strong correlation between altered hepatic gluconeogenesis in F1 offspring and paternal psychological stress and found alterations in DNA methylation and micro RNA concentrations in the sperm. Physical exercise reversed the adverse influence of paternal obesity on pancreatic islet morphology as well as reduced adiposity, incremented muscle mass and diminished free fatty acids in the male offspring [18]. This parental contribution was mediated by epigenetic changes, which mainly included DNA methylation, histone acetylation and synthesis of microRNA [19–21].

There are interesting articles addressing paternal exercise and metabolic effects on the offspring. The majority of them evaluate voluntary exercise in obese fathers and assess the amelioration of the metabolic profile of the offspring or show that long term voluntary exercise affect the offspring in a negative way [22–24]. Specifically, combined diet and exercise of obese fathers can protect the female offspring from metabolic syndrome [24] and when both obese breeders are submitted to voluntary wheels it leads to the protection of the offspring pancreas against deleterious effects [22]. However, to our knowledge, the role of lean sire programmed exercise training on diet-induced obesity and steatosis in the offspring has not yet been addressed; thus, our study aims to examine the influence of paternal physical training on the metabolic markers in the liver tissue of offspring fed a high fat diet (HFD).

2. Materials and methods

2.1. General protocol

Male C57BL/6 mice (n = 14), with 12 weeks of age and weighing between 23 and 26 g were obtained from the Animal Care Facility (Federal University of São Paulo). Animals were maintained in separate cages with water and standard diet ad libitum. The internal ethical institution committee of the Federal University of Sao Paulo approved all procedures employed in the study (project number 3767300414).

Seven mice were exposed to swim training throughout a period of 6 weeks, and 7 males did not exercise (sedentary group, Fig. 1). In the last week of training, males were mated with sedentary females of the same strain (one female was mated with each male and up to 8 pups were analysed from each mating). The offspring of both groups were evaluated in relation to body weight at 30, 60 and 90 days and, after 90 days, were fed a HFD for 16 weeks. After 16 weeks, the offspring fasted overnight was euthanized by the injection of a ketamine and xylazine as anesthetics followed by cervical dislocation. Tissues were collected and analysed (Fig. 1). The whole offspring was evaluated without averaging the litter in Fig. 4. For the remaining experiments, one pup per mother was randomly picked for analysis to avoid the maternal effect.

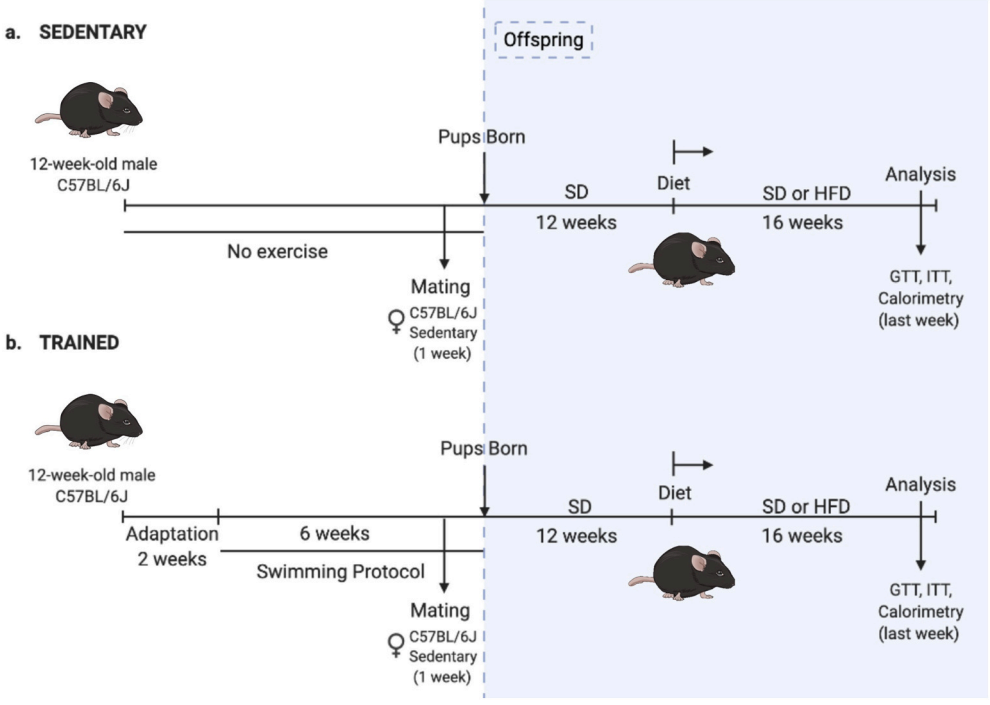

Fig. 1. Timeline and Design of the Experiment. Experimental Design: 12-week-old male C57Bl/6J mice were divided into sedentary (a) and trained (b) groups. The trained group (b) was submitted to an exercise adaptation period for 2 weeks, followed by the exercise protocol before and during the mating time (1–7 days). Male and female offspring of both groups were fed a standard diet (SD) until 12 weeks of age. Afterwards, pups received SD or high-fat diet (HFD) over a period of 16 weeks. During the last week of diet protocol, the glucose tolerance test (GTT), insulin tolerance test (ITT), and indirect calorimetry were performed. All animals were euthanized by cervical dislocation, and the tissues were used for a variety of analyses.

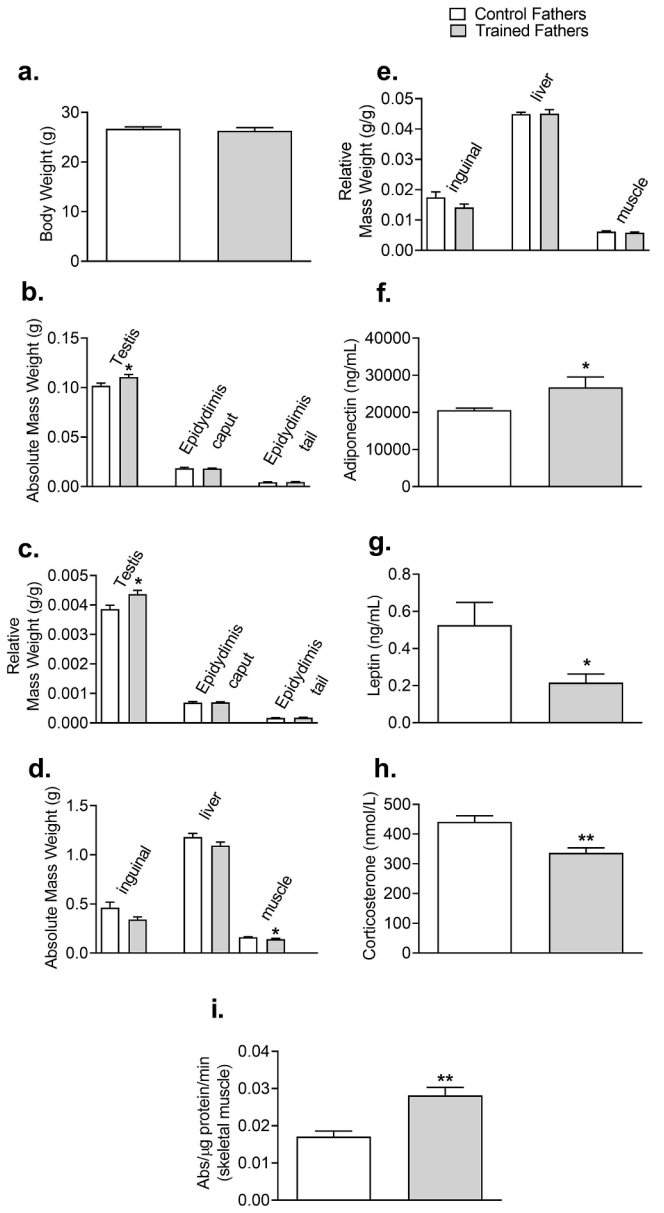

Fig. 2. Body weight (a), absolute (b,d) and relative (c,e) weight of dissected reproductive tissues, inguinal adipose tissue, liver and muscle from control sedentary (white bars) and trained sires (gray bars) at 48 h after the last bout of swimming exercise. Data are shown as mean ± SEM (n = 6 per group). Corticosterone (nmol/L), leptin (ng/mL) and adiponectin (ng/mL) levels in serum from trained and sedentary F0 mice (f, g and h, respectively). Citrate Synthase Activity in the skeletal muscle of trained and sedentary sires (i). Data are shown as mean ± SEM. Comparative analysis by Student's *t*-test. * $p < .05$ vs sedentary (n = 5–6 per group).

2.2. Exercise protocol

Male animals were exercised in a pool, with 300 L of 30 °C water that had been adapted for mice [25]. The tank had 10 lanes. Animals were adapted to the exercise for 2 weeks, gradually increasing the exercise time until reaching 60 min. After this adaptation period, animals were trained for 6 weeks. The training sessions were performed for 5 days/week. Importantly, a weight with 3% of body mass was attached to the tail. The training continued over 6 weeks under the same load. In the last week of exercise, trained males were mated with sedentary females (Fig. 1).

2.3. Obesity induction

The male and female progeny were kept alone in cages, and fed a standard diet until they were 3 months old. The standard diet contained 2.95 kcal/g and consisted of 6% calories from fat, 26.3% from protein, and 67.7% from carbohydrates (Nuvilab mod. CR-1). To assess food intake, the mice were housed singly. Then, mice of both sedentary and trained sires received a HFD (either a 'sedentary' or 'trained' HFD) consisting of 4.73 kcal/g − 1, 45% calories from fat, 20% from protein, and 35% from carbohydrates (Research Diets mod. D12451, New Brunswick, USA). Food and water was provided ad libitum and intake was equivalent between groups. The HFD was maintained for 16 weeks in order to evoke obesity. Animals were weighed weekly during this time, and the food intake was assessed by weighing pellets and multiplication by kcal from the diet.

2.4. Blood glucose assessment

A glucose tolerance test (GTT) and an insulin tolerance test (ITT) were performed after 15 weeks and a 12 h fasting period. GTT and ITT tests were performed allowing an interval of 7 days between the assessments. Glycaemia was probed using the Accu-Chek Advantage. For the GTT and ITT, 1 g glucose per kg body weight and 1 IU of human recombinant insulin per kg body weight, respectively, were injected intraperitoneally. Glucose levels were determined at different time points.

At the end of the experimental period, after euthanasia, the skeletal muscle (gastrocnemius), liver, white adipose tissue (gonadal) and brown adipose tissue were harvested and stored at −80 °C for analysis. The blood was obtained from cardiac puncture and allowed to clot for 30 min at room temperature, and serum was obtained by centrifuging at 10,000 rpm (10 min) in a refrigerated centrifuge.

2.5. Biochemical parameters

2.5.1. Triglyceride (TAG) quantification

The amount of TAG was measured by intrahepatic isolation of liver fragments that were homogenized in a chloroform, methanol and water (2:1:0.5) mixture. Then, the microtubes were centrifuged at 2000 rpm (10 min) at room temperature. Next, chloroform and methanol (2:1) were added, and the sample was subjected to centrifugation at 1500 rpm (5 min). The lower phase was recovered and dried at room temperature. A 1 mL aliquot of a 3% Triton-X solution was applied to each sample. The measurement was taken according to the manufacturer's instructions (Triglycerides Liquiform Ref. 87).

2.5.2. ALT and AST enzyme activity measurements

The serum was used for measuring enzyme activities. After serum isolation, the activity of both enzymes was measured following the manufacturer's instructions (LabTest Ref. 52 and LabTest Ref. 53).

2.6. Histological analysis procedure

Liver fragments were kept in a 4% PFA (paraformaldehyde) solution for 24 h for complete sample fixation. For dehydration, 70%, 96% and absolute ethanol baths were used for the samples. A 2 h xylol bath was used after the dehydration step, followed by paraffin exposure for 3 h. The 4 µm slices were stained with haematoxylin and eosin for 1 min. The histological analysis was performed in a single blind evaluation to determine the degree of steatosis. Counting of cells that presented intracellular lipid accumulation assessed the degree of steatosis.

2.7. Quantification of gene expression

Liver samples were frozen at −80 °C. Total RNA was isolated using TRIzol reagent according to the manufacturer's instructions (Invitrogen,

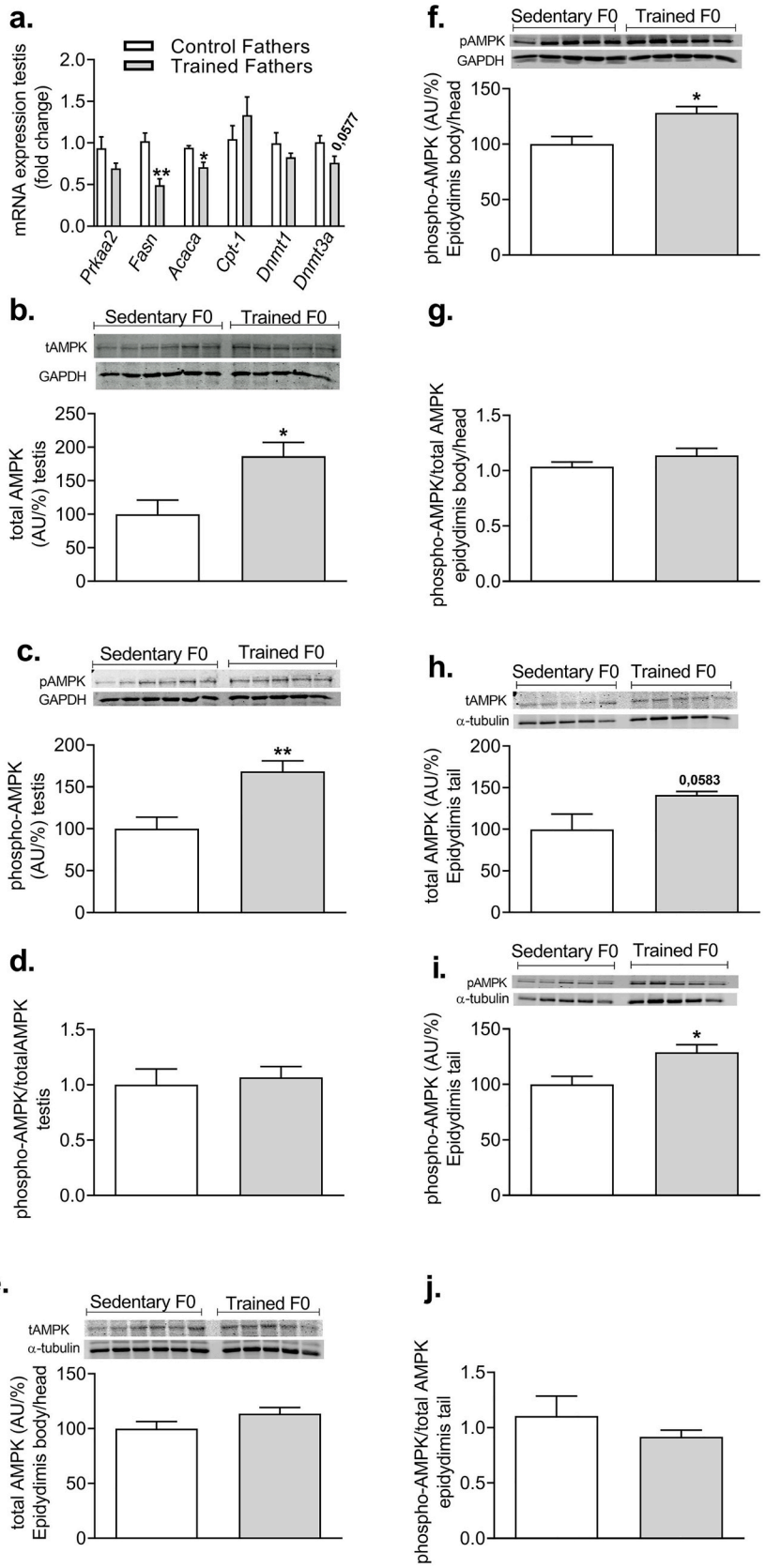

Fig. 3. Expression of metabolic and methylation related genes and protein abundance in reproductive organs from sedentary (white bars) or trained sires (gray bars) 48 h after the last bout of swimming exercise. Gene expression of *Ampk, Fasn, Acaca, Cpt-1, Dnmt1 and Dnmt3a* in testis (a). phospho-AMPK (Thr-172, pAMPK), total AMPK and pAMPK/AMPK total ratio protein abundance by Western blotting in testis (b,c,d), epididymis head/body (e,f,g) and epidydimis tail (h,i,j). Data are shown as mean ± SEM (n = 5–6 per group). Comparative analysis by Student's *t*-test. *p < .05. AMPK – 5′AMP-activated protein kinase; *Fasn* – Fatty Acid Synthase; *Acaca* – Acetyl CoA Carboxylase; *Cpt-1* – Carnitine Palmitoyltransferase 1; *Dnmt 1* and *Dnmt3a* – DNA Methyltransferase 1 and 3a. n = 5–6 per group.

Carlsbad, CA). First-strand cDNA was synthesized using a high-capacity cDNA reverse kit (Applied Biosystems). Real-time PCR was performed using SYBR Green system detection (Thermo Scientific, Waltham, MA) with the primers described in Table S1. The cycling conditions were as follows: 10 min at 95 °C, followed by 45 cycles of 30s at 95 °C, 30s at 60 °C, and 30s at 72 °C. Standard curves were created for each primer pair to check the amplification efficiency. Target mRNA expression was normalized to GAPDH (glyceraldehyde 3-phosphate dehydrogenase)

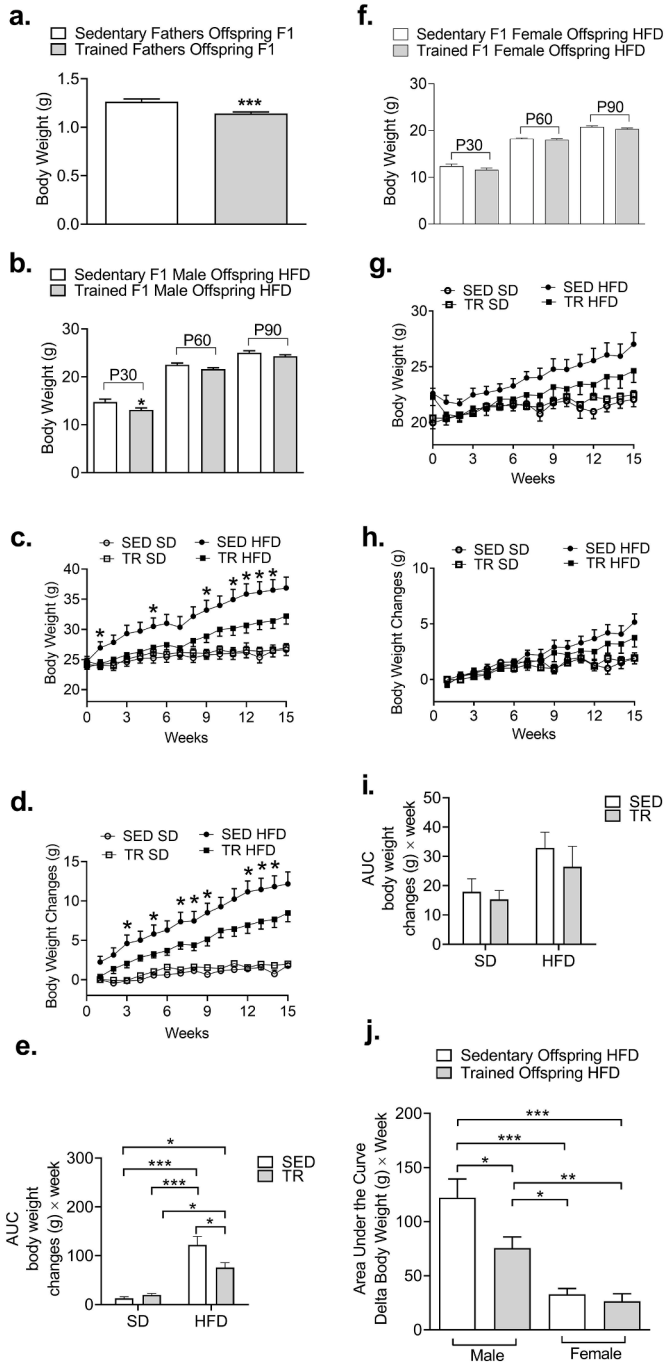

Fig. 4. Birth weight (P1) of offspring from sedentary and trained sires (n = 23 and 35 respectively, ***p < .001) (a). Body weight of the male offspring at P30, P60 and P90, *p < .05(b). Data in (a) and (b) were analysed by Student's *t*-test. Body weight in male offspring from sedentary and trained sires during SD and HFD started at P90 (c), delta body weight in male offspring from sedentary and trained sires during SD and HFD started at P90 (d), area under the curve of delta body weight of males during the time depicted in e (e). The graphics f-i are correspondent to the graphics (b-e), but represent females. Area Under The Curve of the body weight of the male and female offspring fed a HFD (j), from week 1 to week 15 was analysed. Data are shown as Mean ± S.E.M. and statistical analysis in c,d e, i and j was performed by Two-Way ANOVA and Tukey test. All groups were compared relative to each other in e,i and j. In c and d, SED HFD and TR HFD groups were compared. *p < .05, **p < .01, ***p < .001. SD: standard diet, HFD: high fat diet, SED: sedentary sire, TR: trained sire. (n = 17–27 per group).

and expressed as a relative value using the comparative threshold cycle (Ct) method ($2-\Delta\Delta Ct$) according to the manufacturer's instructions. The expression levels of the genes of interest were normalized to those of the control group.

2.8. Western blotting

Total protein was extracted from liver samples in RIPA buffer (25 mM Tris-HCl at pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS), and quantified via the BCA protein assay kit (Pierce, EUA). Approximately 50 μg of tissue protein was subjected to SDS-PAGE. The immunostaining was performed with the following primary antibodies: pAMPK α1\2 (sc-33524) obtained from Santa Cruz Biotechnology, Inc., and AMPK (#2532) which was purchased from Cell Signaling Technology. The loading controls were α-tubulin or GAPDH.

2.9. Indirect calorimetry

The metabolic activity in mice was assessed by indirect calorimetry using the CLAMS Oxymax monitoring system (Columbus Instruments). Briefly, O_2 consumption and CO_2 production was measured within isolated chambers containing single-housed mice for a period of 24 h with 12 h of light followed by 12 h of dark. Data were used to calculate the Respiratory Exchange Ratio (RER) and Energy Expenditure (EE). In addition, mouse activity was also recorded via infra-red (IR) measurements within the chambers.

2.10. Statistical analysis

To analyse the data, the Student's *t*-test, Two-Way ANOVA with Tukey test or ANCOVA test were employed. All values are represented as Mean ± S.E.M. Statistical significance was considered for p values of ≤.05.

3. Results

3.1. Training alters testis weight but not body weight, although corticosterone, leptin and adiponectin serum levels were altered in sires

The body weight of the sires was unchanged by the physical exercise protocol (Fig. 2a). The 6-week training led to increases in the weight of the testes (p < .05– Fig. 2b and c), but no changes were observed in different regions of the epididymis (head/body and tail). The groups did not display differences in inguinal adipose tissue and liver weight (Fig. 2d,e), while absolute muscle weight, but not relative muscle weight was different between groups (Fig. 2d,e). Swimming increased the adiponectin level (p = .0425, Fig. 2f) and decreased the leptin (p = .0463, Fig. 2g) and corticosterone (p = .0026, Fig. 2h) levels in serum. To evaluate whether exercise was effective in promoting beneficial effects, we measured the activity of citrate synthase in gastrocnemius, an important marker of aerobic metabolism efficiency. Our results showed that exercise was able to increase citrate synthase activity, meaning that our protocol can be considered a good physical exercise model (Fig. 2i).

3.2. Training regulates the expression of AMPK and its metabolic targets in the reproductive tract

In the testes, we observed downregulation of *Fasn* (fatty acid synthase - p < .0030) and *Acaca* (Acetyl CoA Carboxylase 1 – p < .0239) with a tendency towards downregulation in *Dnmt3a*(DNA cytosine-5 methyltransferase 3a) gene expression by physical exercise. There was no change regarding *Prkaa2* (AMPKα2- coding) and *Cpt-1* (carnitine palmitoyl transferase 1) or *Dnmt1* (Fig. 3a). The AMPKα2 protein level was increased in the testes of the sires subjected to exercise, in total

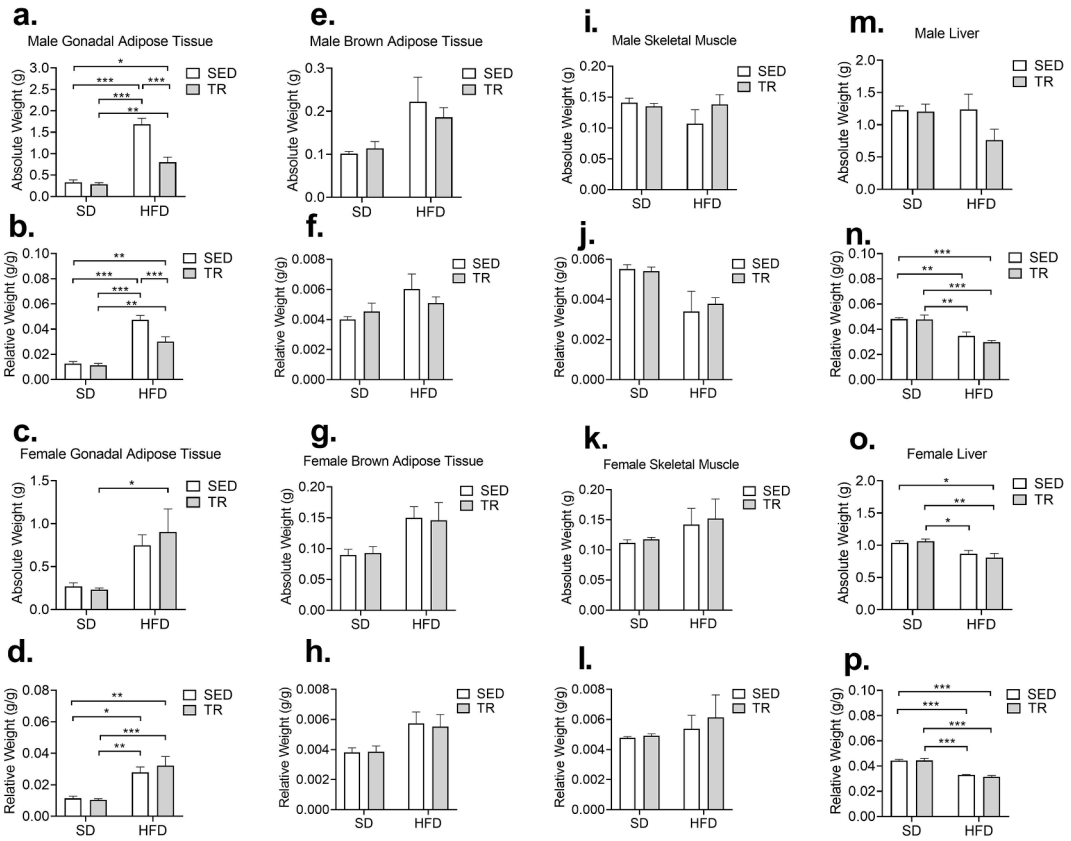

Fig. 5. Weight of desiccated tissues of male (a,b,e,f,i,j,m,n)and female (c,d,g,h,k,l,o,p) offspring from sedentary or trained sires. Absolute (a,c,e,g,i,k,m,o) and relative (b,d,f,h,j,l,n,p) values of gonadal adipose tissue (a–d), brown adipose tissue (e-h), skeletal muscle (i–l) and liver (m–p). Data are shown as mean ± SEM (n = 5 per group). Data was analysed with Two-Way ANOVA and Tukey test. *p < .05, **p < .01, ***p < .001. SD: standard diet, HFD: high fat diet, SED: sedentary sire, TR: trained sire. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(tAMPK - p = .0179– and in the phosphorylated form (pAMPK - p = .0059) (Fig. 3b and c, respectively). There was no difference in the ratio of pAMPK to tAMPK (Fig. 3d). In the epididymis (body/head region), the amount of tAMPK was unchanged (p = .1547), but the pAMPK level was increased by exercise (p = .0128). There was no change in the relative amounts of pAMPK to tAMPK (p = .2161) (Fig. 3e, f and g, respectively). In the tail of the epididymis, exercise increased pAMPK levels compared to the sedentary group (p = .0206) but did not significantly increase the protein abundance of tAMPK (p = .0583), and the pAMPK/tAMPK ratio (p = .3496) remained unchanged (Fig. 3h, i and j, respectively).

3.3. Paternal exercise regulates the birth weight of male but not female offspring and protects against high-fat-diet-induced body weight gain

Paternal exercise influenced the body weight in P1 (postnatal day 1) (p < .001, Fig. 4a) and P30 in the male offspring (p = .0215, Fig. 4b). Body weight was also measured at P60 and 90, with no alterations observed in the male offspring (Fig. 4b). When the male groups were fed a HFD (high fat diet) for 16 weeks after P90, we observed a significant difference in obesity progression, with a decrease in body weight gain in the offspring of the exercise group compared with the sedentary group (Fig. 4c–e). In the male groups fed a standard diet, no differences in body weight gain were observed (exercise versus sedentary groups, Fig. 4c–e). Regarding the female offspring, there was no difference in body weight at P30, P60, or P90 with a HFD (Fig. 4f), nor a difference in body weight gain in the offspring of the exercise group compared with the sedentary group on HFD (Fig. 4g–i).When we performed an Area Under the Curve (AUC) Analysis with the male and female offspring fed a HFD, we showed that exercise significantly affected weight in males but not in females (Fig. 4j).

3.4. Paternal exercise does not influence glucose tolerance in offspring fed a HFD

Glucose tolerance in mice fed a HFD was not significantly altered by paternal exercise in either the female (Fig. S1a, c) or male offspring (Fig. S1b and d). Additionally, in the insulin tolerance test (ITT), no difference was observed between female (Fig. S1e) and male offspring (Fig. S1f) from sedentary or trained sires.

3.5. Paternal exercise alters metabolism in offspring fed a HFD

To evaluate the metabolic parameters in vivo, we used indirect calorimetry. When we analysed our data with Student's *t*-tests, the male offspring from trained sires had better metabolic efficiency (increase of VO_2, Fig. S2); however, when we normalized the data for body weight using the ANCOVA test, we did not observe differences, which means that the reduced body weight in the trained group was the key factor in the metabolic adaptations measured by calorimetry (data not shown).

3.6. Paternal exercise reduces gonadal fat in HFD-fed male but not female offspring

The tissues related to metabolism (liver, gonadal adipose tissue, brown adipose tissue and skeletal muscle) were dissected and weighed. Paternal exercise reduced gonadal adipose tissue in HFD-fed male offspring, relative to the HFD-fed sedentary control, in terms of the total reduction and relative to body weight (Fig. 5a,b). For the weight of liver, skeletal muscle and brown adipose tissue, no differences were

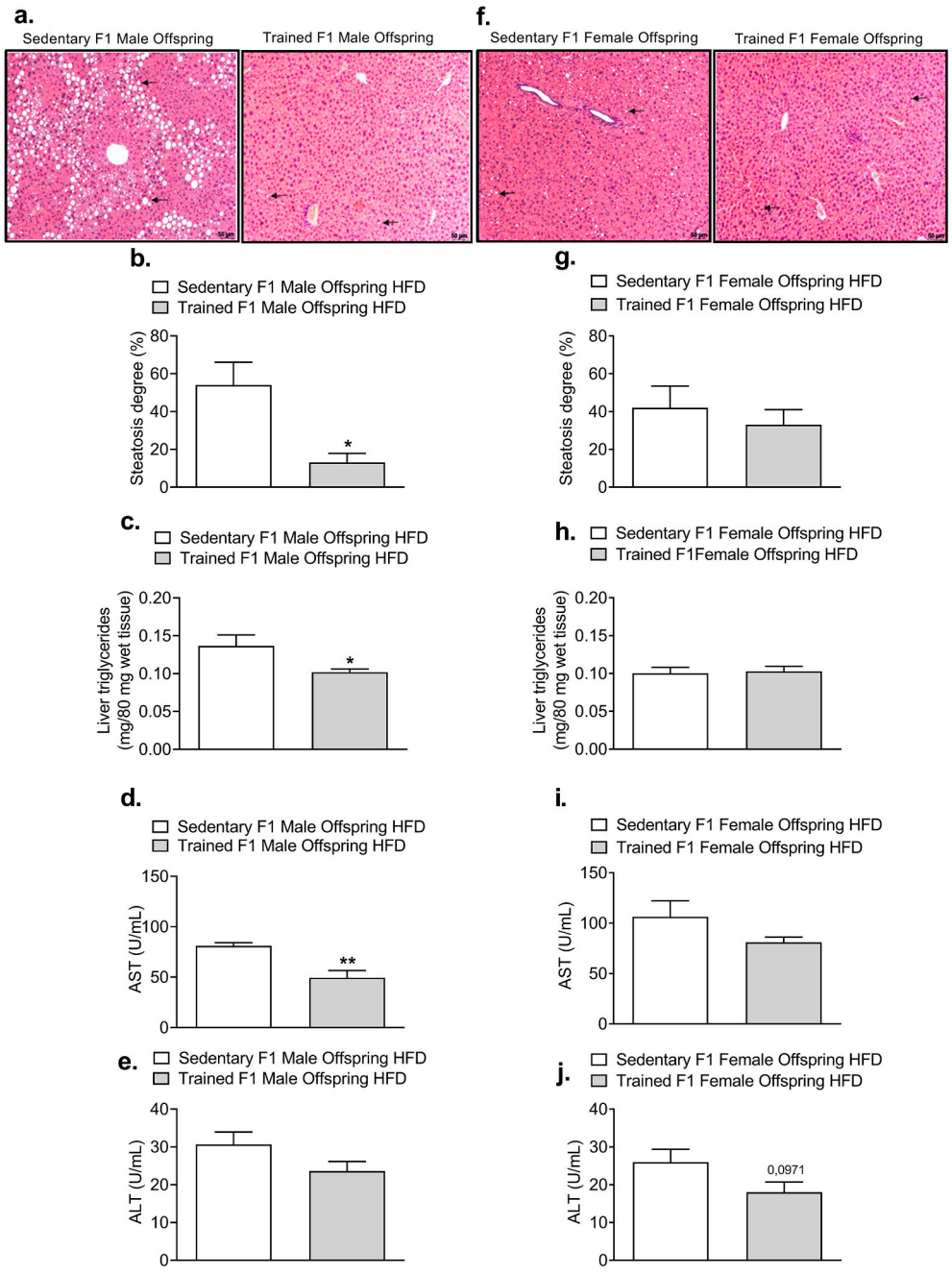

Fig. 6. HFD-induced steatosis in livers from male (a–e) and female (f–j) offspring of sedentary and exercised sires. Histological analysis (a,f). Degree of steatosis (%) (b,g). Liver triglycerides (c,h). Aspartate transaminase activity, AST (d,i), and alanine transaminase activity, ALT (e,j) in serum. Data are shown as mean ± SEM. *p < .05 (n = 5–6 per group).

observed relative to the HFD-fed sedentary control (Fig. 5e,f,i,j,m,n). For both sexes in the standard diet group (SD) and the HFD-fed females (Fig. 5c,d,g,h,k,l,o,p) from sedentary and trained sires, we did not observe any significant differences in tissue weight.

3.7. HFD-induced steatosis is ameliorated by paternal exercise in male offspring

Histological liver analysis (Fig. 6a) revealed a decrease in lipid droplets in HFD-fed male offspring from trained sires (p = .0153, Fig. 6b), but not after SD (data not shown). The levels of triglycerides confirmed these results (p = .0302) (Fig. 6c). An increase in AST activity, but not in ALT activity, was found in the serum of HFD-fed offspring from trained sires compared to offspring of sedentary sires (Fig. 6d and e). In the HFD-fed female offspring, no difference in lipid droplets, triglycerides, AST and ALT activities was observed (Fig. 6f–j).

3.8. Paternal exercise alters the expression of lipogenesis-related genes in the liver of male offspring, but not female offspring, fed a HFD

We observed the upregulation of lipogenesis-related genes induced by paternal exercise in the livers of male offspring. The expression levels of *Cpt1*, *Ppar-1α* and *Prkaa2* (AMPKα2-coding) were increased (p = .0408, p = .0499 and p = .0183, respectively) by paternal exercise in male offspring, while the *Fasn* gene was downregulated (p = .05). There was no significant difference in *Ppar-1γ* or *Acaca* gene expression in any group (Fig. 7a–f). Furthermore, in the female offspring, the gene expression in the liver was unchanged for all genes

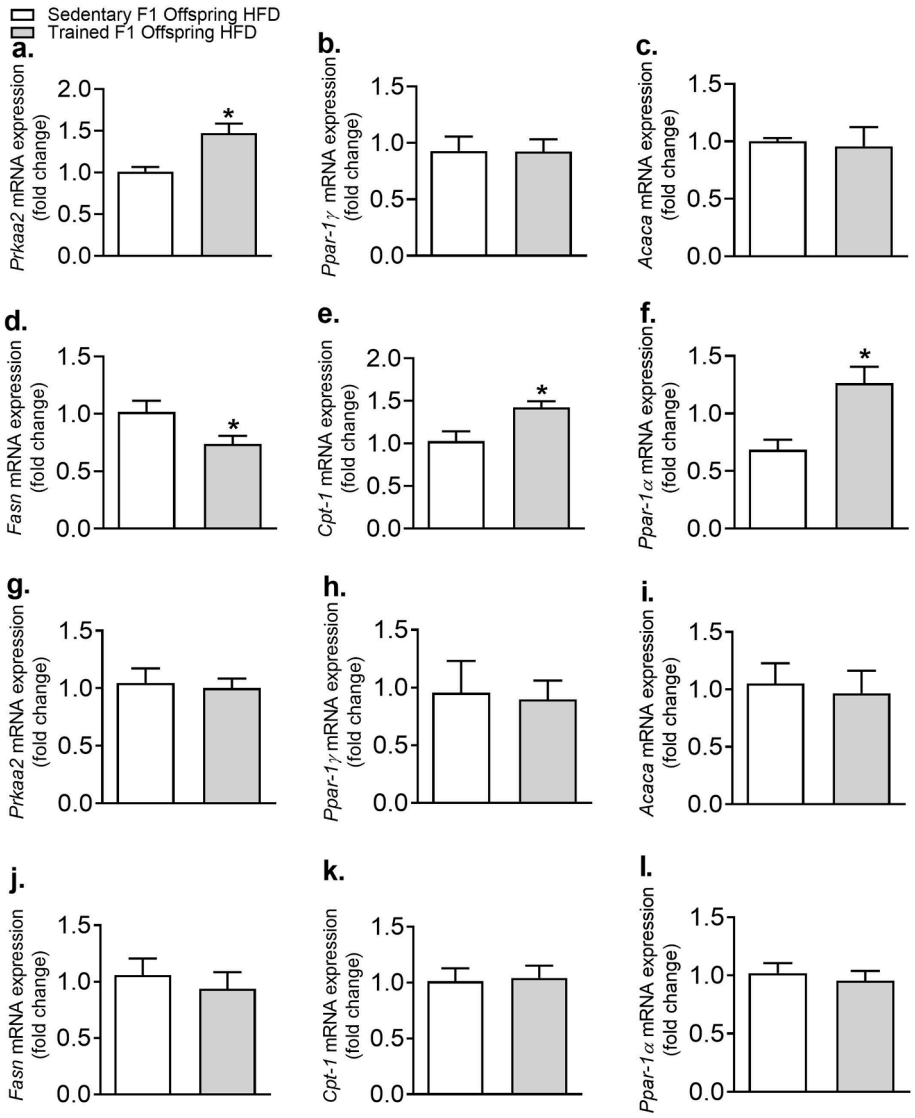

Fig. 7. HFD-induced gene expression in livers from HFD-fed male (a-f) and female (g-l) offspring of sedentary and exercised sires. Expression of *Prkaa2, Fasn, Ppar-1α, Ppar-1γ, Cpt-1* and *Acaca* genes. Data are shown as mean ± SEM. Comparison by Student's *t*-test. *p < .05 vs sedentary (n = 6 per group).

evaluated (Fig. 7g–l). The muscle and brown adipose tissue displayed an increase in *PGC1α* in the male offspring from trained sires, while *Prkaa2* increased in the muscle of the offspring from trained sires (Fig. S3).

Paternal exercise upregulates AMPK-protein in the liver of male offspring fed a HFD.

tAMPK and pAMPK (Thr-172) were upregulated in male HFD-fed offspring from trained sires (Fig. 8a and b) when compared to the offspring of HFD-fed sedentary sires (Fig. 8a) or SD-fed sedentary sires (Fig. 8b).

4. Discussion

Intergenerational effects of the sire on offspring obesity, memory, pancreas function, and kidney tubular changes have been described [19,22,26–31]. There are others showing the effect of exercise before and during pregnancy in dams affecting offspring metabolism [11,32].There are some studies evaluating whether physical exercise in HFD-fed sires would be able to ameliorate the metabolic profile in the offspring [18,24,33] and others with goals similar to ours, but using voluntary exercise [23,34]. To our knowledge, this is the first study to show paternal modulation of the offspring metabolism after programmed exercise protocol. Fathers were lean and fed with a regular chow.

In our model, only males were protected from metabolic alterations evoked by HFD. On the other hand, we did not observe an increase of body weight induced by HFD in females from trained or sedentary sires. A recent article clearly shows that females do not gain weight and are resistant to most metabolic alterations when fed a HFD around 3 months of age [35]. At a later age (32 weeks), females display the HFD-induced metabolic alterations and the possible mechanisms suggested involve the lower levels of G protein-coupled receptor kinase (GRK2) [35]. Thus, our HFD-induced weight gain protocol (3 months) is not suitable to analyse weight gain and metabolic modifications in females, only in males.

In our model, we found a positive effect of exercise on the regulation of pAMPK. Activation of this protein in the male reproductive tract is related to reproductive effects such as oxidative metabolism, spermatogenesis, sperm motility and viability, acrosome reaction, and sperm concentration and morphology [36–39]. We did not investigate sperm mobility and quality, and this is a limitation of our study. At the molecular level, we observed an increase in pAMPK in the testes and epididymis of trained sires. *Prkaa2* expression was not altered after training, possibly due to the long post-training time; however,

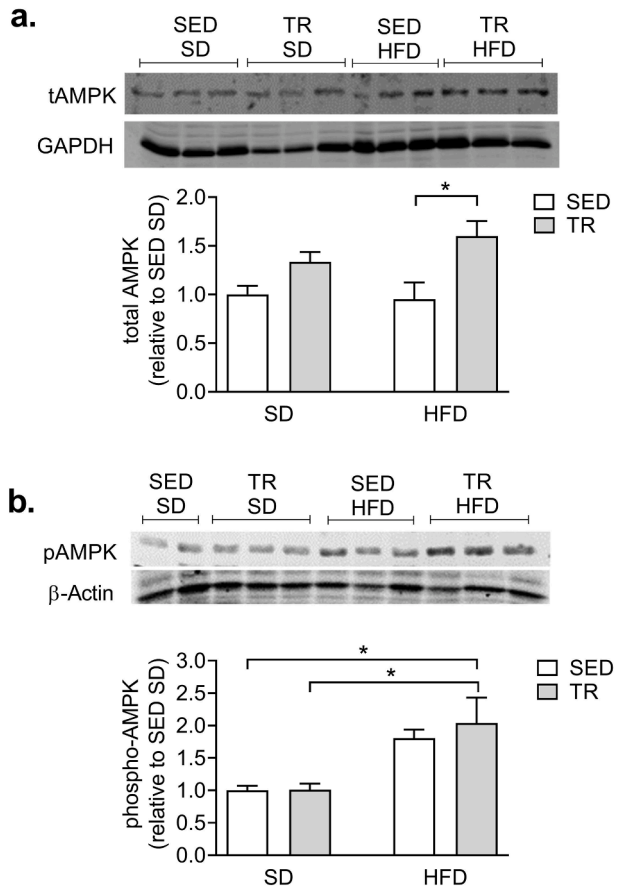

Fig. 8. AMPK protein abundance in liver from HFD-fed male offspring of sedentary and exercised sires. Total AMPK (tAMPK, a), AMPK-phosphyorylated at Thr172 (pAMPK, b) were detected in liver by Western blot. Data are shown as mean ± SEM. Comparison by Two-Way ANOVA and Tukey test. *p < .05 vs sedentary. (n = 3 per group (a) or 6 per group (b)).

expression of the AMPK-regulated genes *Fasn* and *Acaca* was reduced in the testes, suggesting low lipogenesis and possibly a reduction in oxidative stress.

An interesting result was the increase in *Prkaa2* (AMPK-coding), AMPK and pAMPK levels in the liver of male offspring from trained sires. This result suggests that *Prkaa2* can be regulated via epigenetic mechanisms and that sires have an important role in the expression of this gene. AMPK may be the main factor in the protection from HFD-induced obesity and hepatic steatosis, as observed in male offspring from trained sires. Lee et al. used mice in which E3 ubiquitin ligase was knocked out, and they observed lower AMPK degradation that resulted in chronic AMPK activation in liver and adipose tissue and protection against obesity and liver steatosis [40]. The suggested mechanisms for this protective effect against steatosis mediated by AMPK are reduction of de novo lipogenesis, increase in fatty acid oxidation in the liver and mitochondrial biogenesis in skeletal muscle [41]. Our findings in the livers of male offspring from trained sires suggest a decrease in lipogenesis mediated by low *Fasn* expression and increased fatty acid oxidation mediated by the high expression of *Cpt-1* and *Ppar-1α*. Despite this evidence, we cannot affirm whether the gene modulations observed in the liver are directly related to epigenetic mechanisms or are secondary to obesity resistance.

Low birth weight is related to complications later in life, including an increased prevalence of obesity [42], hypertension, and diabetes [43]. In our study, the birth weight of offspring from swimming sires was lower compared to offspring from sedentary sires. However, this birth weight did not predict metabolic dysfunctions later in life; on the contrary, male offspring of trained parents that were fed a high-caloric diet presented with protection against weight gain. Some authors described a positive effect of voluntary exercise in obese and lean fathers, ameliorating the metabolism of the offspring [33]. However, when we analyse the results of other studies [23,34], there are some conflicting findings regarding this aspect. In a study where the voluntary wheel was used as exercise model for 3 months, the offspring of trained sires had increased weight gain compared with the sedentary group [23]. The authors suggested that paternal exercise promoted a metabolic programming in offspring to "save" more energy, leading to increased weight gain when those were submitted to HFD [23]. On the other hand, a study with a similar exercise protocol (running wheel for 3 months) showed opposite results, as the physical exercise was able to protect the offspring from the deleterious effects of HFD [34].

Our group observed that the male and female offspring from mothers who participated in the swimming protocol during pregnancy were protected from obesity and that their male offspring presented with lower insulin resistance after being fed a HFD [11]. Similar results were obtained by Raipuria et al. [16] in a voluntary training model before and during gestation. The offspring were lighter at birth (both males and females), but the reduced visceral adiposity and plasma insulin was observed only in the male offspring. Moreover, the expression of the glucose uptake transporter GLUT4 was increased in the gastrocnemius muscle of the male offspring of trained mothers, while no significant changes were observed in the female offspring.

Interestingly, we did not observe GTT and ITT differences in the offspring after paternal swim training. Comparing with another protocol, where the authors observed better GTT results after 12 weeks of voluntary wheel training [34], we can suggest that an extended training period would be needed to observe skeletal muscle glucose uptake improvements.

In the calorimetric tests, our studies showed no differences, as determined by the ANCOVA test, which suggests that the oxygen consumption in the exercise group of sires was dependent on the reduced body weight of the mice in this group. It is noteworthy that the best strategy to evaluate this phenotype would be to use lean mass as a covariance factor [44]. Unfortunately, we do not have data on this parameter, which is a limitation of this study. However, Um et al. [45] found that the decrease in body weight induced by resveratrol in mice is dependent on AMPK activation in epididymal adipose tissue and skeletal muscle in conjunction with an increase in oxygen uptake.

5. Conclusion

This study showed that paternal programmed exercise can improve the selected metabolic outcomes in male offspring, ameliorating the effects of obesity in the liver. Other exercise modalities and at different intensities should be tested to evaluate the epigenetic role of the exercise regime of sires on obesity in their offspring.

Ethic statement

All procedures were previously reviewed and approved by the internal ethical institution committee of the Federal University of Sao Paulo (project number 3767300414).

CRediT authorship contribution statement

Designed the work: ROB and RCA. Performed experiments: ROB, AMA, MAFSG, TGRH, TS, MBS, FW and LMO. Analysed the data: ROB, MAFSG, LMO, AB, VL and RCA. Wrote the paper: ROB, AB, NOSC, MB, and RCA.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2020.118583.

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