TITLE:

Diet during early life defines testicular lipid content and sperm quality in adulthood

RUNNING HEAD: Diet in childhood defines testes' lipid content in adulthood

KEYWORDS: High-fat diet; early-life obesity, diet intervention; lipidomics; male fertility

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Supplemental Material available at

URL: https://figshare.com/s/785af8effa28e8bc2935

DOI: https://doi.org/10.6084/m9.figshare.12302171

ABSTRACT

Childhood obesity is a serious concern associated with ill health later in life. Emerging data suggest that obesity has long-term adverse effects upon male sexual and reproductive health but few studies addressed this issue. We hypothesized that exposure to high-fat diet during early life alters testicular lipid content and metabolism leading to permanent damage to sperm parameters. After weaning (day 21 after birth), 36 male mice were randomly divided into 3 groups and fed with different diet regimen for 200 days: CTRL–standard chow; HFD–high-fat diet (Carbohydrate: 35.7%, Protein: 20.5%, Fat: 36.0%); HFDt–high-fat diet for 60 days then replaced by standard chow. Biometric and metabolic data were monitored. Animals were then sacrificed, and tissues collected. Epididymal sperm parameters and endocrine parameters were evaluated. Testicular metabolites were extracted and characterized by 1H-NMR and GC-MS. Testicular mitochondrial and antioxidant activity were evaluated. Our results show that mice fed with high-fat diet, even if only until early adulthood, had lower sperm viability and motility, and higher incidence of head and tail defects. Although diet reversion with weight loss during adulthood prevents the progression of metabolic syndrome, testicular content in fatty acids is irreversibly affected. Excessive fat intake promoted an over-accumulation of pro-inflammatory n-6 polyunsaturated fatty acids in testis, which are strongly correlated with negative effects upon sperm quality. Therefore, the adoption of high-fat diets during early life correlates to irreversible changes in testicular lipid content and metabolism, which are related to permanent damage to sperm quality later in life.

New & Noteworthy

The adoption of high-fat diets from early-age promotes a pro-inflammatory environment in testis, due to the accumulation of n-6 polyunsaturated fatty acids. Diet reversion to standard chow in early adulthood does not revert testicular lipid content completely. This testicular lipid remodeling is correlated to poorer sperm parameters later in life, notably sperm motility, viability and morphological defects. Hence, high-fat diets cause irreversible damage to male reproductive function.

INTRODUCTION

The fast and global increase of overweight/obesity in recent decades led the World Health Organization to declare it the “Epidemics of the XXI Century” (72). Excessive fat deposition has been associated with the onset of non-communicable diseases, particularly type 2 diabetes (T2DM) (22). Consumption of energy-dense, high-fat diet (HFD), and low physical activity are two major drivers of the obesity epidemic (22). In developed countries, consumption of energy-dense foods occurs at an ever-younger age (41). Childhood obesity rates continue to soar worldwide, with obesity-associated comorbidities occurring at a younger age (33, 71). Concurrently, concerns on the impact of excessive adiposity and T2DM on sperm quality and fertility outcomes have been raised (14, 42, 51). Although there are not many studies demonstrating the impact of fat-rich diets in sperm quality, men attending fertility centers are often advised to lose weight and adopt a balanced diet. Unlike the other lifestyle interventions such as physical activity (62), the effectiveness of dietary intervention in recovering normal sperm quality or its positive effects on testicular metabolism has never been demonstrated (64).

Normal male reproductive function requires a well-orchestrated balance of endocrine and metabolic factors to secure proper energy flow towards meiosis and cellular remodeling of differentiating germ cells (55). In this regard, testicular lipid dynamics are crucial, not only as energy substrates, but also as structural elements of the future sperm cells. Moreover, mitochondrial function and antioxidant defenses are crucial for providing energy for spermatogenesis and sperm motility, while avoiding oxidative damage which can critically compromise cell functions. Previously we have shown that HFD during early life

causes irreversible changes in testicular metabolism, even after reversion, and those changes are correlated to defects in sperm motility, viability and morphology (12). The relevance of fatty acids in testicular physiology has been reported, specially the repercussions for spermatogenesis (20, 28, 37, 57). We hypothesize that the adoption of HFDs affects testicular lipid dynamics resulting in poorer sperm quality. We further address whether an HFD early in life can irreversibly affect sperm quality later in life, due to testicular lipid dysmetabolism, even after diet intervention in early adulthood. We have performed our study in a rodent model. Biometry, glucose homeostasis, endocrine function, testicular antioxidant system, testicular bioenergetics and sperm parameters were assessed. We have further performed a multivariate analysis supported by targeted metabolomics and lipidomics, based on semi-quantitative GC-MS and ^{1}H-NMR, to determine changes in testicular lipid metabolism.

MATERIALS & METHODS

Animal Model

Mus musculus C57BL6/J male mice (n=36) were randomly divided into three groups after weaning (21-23 days): control (CTRL) (n=12), HFD (n=12) and transient HFD (HFD_t) (n=12). All mice were generated from normoponderal males and females and were subjected to the same random *in utero* stimuli, although generated from different litters. Briefly, CTRL group mice were fed with a standard chow (#F4031, BioServ, USA – Carbohydrate: 61.6%, Protein: 20.5%, Fat: 7.2% - 16.3% Kcals). HFD group mice received a HFD (#F3282,

BioServ USA – Carbohydrate: 35.7%, Protein: 20.5%, Fat: 36.0% - 59.0% Kcals) for 200 days after weaning. HFD_t group mice were fed with a HFD (#F3282, BioServ, New Jersey, USA) for 60 days and then switched to standard chow (#F4031, BioServ, New Jersey, USA). At 120 days post-weaning, mice were randomly assigned to a normoponderal female, in mating pairs, 6 hours per day, for seven consecutive days, for breeding. During this period, female breeders were kept in the same standard diet. Mating pairs had no access to water or food during mating (6h). Reproductive success rate, litter size, and litter male to female ratio were assessed. Animals were killed by cervical dislocation 200 days after weaning, and tissues collected for further analysis. Total body weight, water and food intake were monitored weekly until sacrifice. The animal model is compliant with the ARRIVE guidelines and was licensed by the Portuguese Veterinarian and Food Department (0421/000/000/2016).

Endocrine and metabolic function

Fasting glycemia was measured before sacrifice (200 days post-weaning), after overnight fast (8h), using a glucometer (One Touch Ultra Lifescan-Johnson, Milpitas, CA, USA) by collecting a drop of blood from the tail vein. Blood was then collected by cardiac puncture and centrifuged at 1500 *g*, 4 ºC, for 10 minutes to collect the serum. Serum insulin was quantified using a Rat/Mouse Insulin ELISA assay (EZRMI-13K, Millipore). Glucose homeostasis was evaluated according to the HOMA2 scores (30), using the HOMA2 calculator (University of Oxford, United Kingdom). Disposition index (DI) was calculated as suggested by Caumo et al. (9). Similarly, FSH, LH, 17β-estradiol and

testosterone were quantified in the purified serum using ELISA kits (respectively, ENZ-KIT108-0001, ENZ-KIT107-0001, ADI-900-174 and ADI-900-176, Enzo Life Sciences, USA).

Evaluation of epididymal sperm parameters

Epididymides were isolated and placed in pre-warmed (37°C) Hank's Balanced Salt Solution (HBSS) pH 7.4, minced with a scalpel blade and the suspension was incubated for 5 min (37°C). Sperm parameters were evaluated as previously described (12, 53). Sperm motility was calculated as the average proportion of motile sperm in 10 random microscope fields, observing a drop of sperm suspension on a warmed slide (37 °C) using an optical microscope (×100 magnification). Epididymal sperm concentration was determined using a Neubauer counting chamber and an optical microscope (×400 magnification), in a diluted the sperm suspension (1:50 in HBSS). Sperm viability and morphology were assessed in differently stained epididymal sperm smears, counting 333 spermatozoa in random fields using an optical microscope (×400 magnification). Sperm viability smears were stained with eosin-nigrosin (29), as membrane-compromised spermatozoa stain pink. Sperm morphology smears were stained with Diff-Quick (Baxter Dale Diagnostics AG, Dubinger, Switzerland). Sperm morphology categories were mutually exclusive, *i.e.,* spermatozoa displaying more than one defect were assigned according to the most serious defect category (decapitated > pinhead > flattened head > bent neck > coiled tail) (29).

NMR spectroscopy

A combined extraction of polar and nonpolar metabolites from testicular tissue was performed as previously described (2, 12). Testes were washed with saline and decapsulated prior to metabolite extraction to minimize contamination by blood metabolites. The aqueous phase containing polar water-soluble metabolites was lyophilized and analyzed by ^{1}H-NMR spectroscopy as described (25). Lipid metabolites were identified by comparing recorded spectra with reference spectra and the Human Metabolome Database (HMDB) (69) and according to Metabolomics Standards Initiative (MSI) guidelines for metabolite identification (65). Identification levels are indicated in Table S1 (Supplementary data). ^{1}H spectra were processed using previously described methods (25). Obtained peak areas were normalized by the total spectral area and analyzed by univariate analysis. Areas were obtained using AMIX software (Bruker BioSpin GmbH, Rheinstetten, Germany) and expressed as arbitrary units defined by the software.

GC-MS analysis

Fatty acid methyl esters of total lipids were obtained by base-catalyzed transmethylation (2 M KOH in methanol) in the presence of nonadecanoic fatty acid (C19:0), used as the internal standard. The obtained hexane fatty acid methyl esters solution was analyzed by gas chromatography using a Shimadzu GC–MS QP2010 UltraGas Chromatograph Mass Spectrometer (Shimadzu, Kyoto, Japan), equipped with a capillary column BPX70 (0.25 mm internal diameter, 0.25 μm film thickness, 30 m long, SGE, Austin, TX, USA). The

injector temperature was 250 ºC, and 1 µl of each sample was injected with a split ratio of 1:80. Helium was used as the carrier gas, and the linear velocity was 35 cm/s. The initial column temperature was 155 ºC, followed by a heating rate of 1 ºC/min up to 170 ºC, 4 ºC/min up to 220 ºC and 40 ºC/min until reaching 250 ºC, maintained for 5 min. Linear velocity was 35 cm/s, interface temperature: 250 ºC, ion source temperature: 225 ºC, mass range: 45-500 and event time: 0.3 s. All the experimental measurements were repeated three times and the average values reported. Fatty acids were identified by retention time and fragmentation profile and quantified by the internal standard procedure. Results were expressed as a percentage of total fatty acids.

Lipid peroxidation, activity of antioxidant enzymes and activity of mitochondrial enzyme complexes

A single testis from each animal was homogenized in 2 mL of ice-cold extraction buffer [160 mM sucrose, 10 mM Tris–HCl, pH 7.4] supplemented with a protease inhibitors cocktail (#B14001, Bimake, Munich, Germany) (1:10, p/v)] using a glass-Teflon Potter Elvejhem (Kimble, Millville, NJ, USA). The resulting homogenate was split into two fractions: one was used for adenine nucleotides (ATP, ADP, and AMP) extraction and quantification by high-performance liquid chromatography (HPLC), as described (40); the other was fractionated by differential centrifugation to obtain a mitochondria-free cytosolic fraction and a mitochondria-enriched fraction, as described (38, 40). The final protein content of each fraction was determined by the BCA method (61). The mitochondria-enriched fraction was used to assess the activity of the mitochondrial enzymes

(complexes I, II and IV, V and citrate synthase), while the mitochondria-free cytosolic fraction was used to evaluate the activity of the enzymes of the antioxidant defense system [catalase (CAT), copper-zinc superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione-disulfide reductase (GSR)] and to assess the levels of lipid peroxidation.

Catalase (CAT) activity was polarographically determined following oxygen production resulting from H_2O_2 decomposition using a Clark-type oxygen electrode (Hansatech, Norfolk, UK) (13). Superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione-disulfide reductase (GSR) activities were evaluated in 96-well plates, at 37 ºC, as described (46). The reaction was conducted in 930 µL of 100 mM potassium phosphate buffer (100 mM KH_2PO_4, 5 mM EDTA, pH 7.4) with 50 µl of testicular cytosol extract, and initiated by adding 20 µl of H_2O_2 (2 mM). CAT activity was expressed in nmol O_2/min/mg protein.

SOD activity was evaluated by mixing 50 µl testicular cytosolic fraction with 170 µl potassium phosphate buffer, supplemented with 2 mM nitro blue tetrazolium (NBT) and 0.05 U Xanthine Oxidase (#X1875-10UN, Sigma-Aldrich/Merck KGaA, Darmstadt, Germany). After addition of 10 µl hypoxanthine (2 mM), absorbance at 560 nm was measured every 20 seconds for 5 minutes. Results were expressed as U/min/mg protein, where U is the enzyme activity that inhibited the reduction of NBT to blue formazan by 50%.

GR activity was evaluated by the rate of NADPH oxidation associated with the reduction of GSSG. Assays were carried out in 170 µl potassium phosphate buffer, supplemented with 10 mM NADPH and 10 mM GSSG. After adding 300 µL of testicular cytosolic fraction, NADPH oxidation was recorded by the

fluorescence decrease at 450 nm (Excitation: 360 nm) every 20 seconds for 5 minutes. NADPH oxidation not associated with GSSG reduction was assessed in assays carried out in the absence of GSSG. GR activity was determined by difference between the rate of NADPH oxidation in the presence and absence of GSSG and expressed in nmol/min/mg of protein.

GPx activity was evaluated by the rate of NADPH oxidation associated with H_2O_2 reduction. Assays were performed in 170 µl potassium phosphate buffer, supplemented with 10 mM GSH and 1-3U Glutathione Reductase (#G3664-500UN, Sigma-Aldrich/Merck KGaA, Darmstadt, Germany), and 50 µl of testicular cytosol fraction. Reaction was initiated by the addition of 10 µl H_2O_2 (0.6 mM), and fluorescence at 450 nm (Excitation: 360 nm) was measured every 20 seconds for 5 minutes. GPx activity was determined by the rate of NADPH consumption in nmoles/min/mg of protein.

Testicular lipid peroxidation levels were evaluated by the production of thiobarbituric acid reactive species (TBARS assay). Shortly, 50 µl testicular cytosolic fraction were mixed with 600 µl of Reaction Solution [thiobarbituric acid 0.38% (m/V), trichloroacetic acid 37% and 2,6-ditertbutyl-4-methylphenol 0.02% (m/V) (38)] and incubated at 95 ºC for 30 minutes. Malondialdehyde (MDA) formation was measured by colorimetric methods (ε = 156 $mM^{-1}.cm^{-1}$), and results were expressed in nmol MDA/mg protein.

Activities of the mitochondrial complexes I, II and IV, and citrate synthase (CS) were assessed, at 37 ºC, in 96-well plate by adapting previously described protocols (40). CS was determined spectrophotometrically, by monitoring the reduction of DTNB. The reaction mixture consisted of 200 mM Tris-HCl buffer (pH 8.0), 0.02% Triton X-100, 10M DTNB, 1 mM oxaloacetate, and 20 µl of

testicular mitochondrial fraction. The reaction was initiated by adding 0.37 mM acetyl-CoA, and the absorbance at 412 nm was recorded every 30 seconds for 10 minutes. CS activity was calculated by DTNB (ε = 13.6 $mM^{-1}.cm^{-1}$) reduction rate, determined in the linear range of the plot, and expressed as nmol/min/mg protein.

Complex I activity was assessed following NADH oxidation. The reaction was performed in a potassium phosphate buffer (25 mM KH_2PO_4, 10 mM $MgCl_2$; pH 7.4) supplemented with 1 mM KCN, 162.5 µM decylubiquinone, 3.0 µM rotenone (or equal volume of buffer) and 20 µl of testicular mitochondrial fraction. The reaction was triggered by the addition of NADH (50 µM), and fluorescence at 450 nm (excitation: 366 nm) was measured every 20 seconds for 15 minutes. Complex I activity was determined by the difference between NADH oxidation rate in rotenone-inhibited wells and non-inhibited wells. This value was expressed in nmol NADH/min/mg.

Complex II activity was assessed monitoring 2,6-dichlorophenolindo-phenol (DCPIP) reduction at 600 nm (ε = 20.7 $mM^{-1}.cm^{-1}$) in a potassium phosphate buffer, supplemented with 2 mM KCN, 6.5 µM rotenone, 6.5 µM antimycin A, 0.05 mM DCIP, 0.1 mM decylubiquinone and 20 µl of testicular mitochondrial fraction. Negative control wells were further supplemented with 0.5 mM oxaloacetate (complex II inhibitor). The reaction was initiated by adding 20 mM succinate and absorbance measure every 30 seconds for 20 minutes. Complex II activity was expressed as the DCPIP reduction rate (corrected for spontaneous reduction) in nmol DCPIP/min/mg protein.

Complex IV activity was evaluated by the cytochrome c (CytC) oxidation rate. CytC (50 mM) was fully reduced by the addition of small volumes of saturated

sodium dithionite solution. CytC oxidation reaction was performed in extraction buffer, supplemented with 3 µM rotenone, 0.1 µM antimycin A (inhibitor of complex III), 2 mM KCN (specific inhibitor of complex IV) or equal volume of buffer, and 20 µl of testicular mitochondrial fraction. The reaction was initiated by adding 15 µM reduced CytC, and the absorbance at 550 nm was recorded every 15 seconds for 5 minutes. Complex IV activity was calculated by CytC (ε = 29.5 $mM^{-1}.cm^{-1}$) oxidation rate, corrected by the spontaneous oxidation rate obtained in the KCN-inhibited wells, and expressed as nmol CytC/min/mg protein.

Maximal activity of the mitochondrial complex V was measured using a MitoCheck Complex V Activity Assay kit (Cayman Chemical, Ann Arbor, MI, USA). All colorimetric and fluorometric readings described in this section were obtained using a Biotek Synergy H1 plate reader (Winooski, VT, USA).

Determination of testicular adenosine nucleotides levels by HPLC

Testicular adenosine nucleotides levels were assessed in a Waters 600 HPLC system (Waters, Milford, MA, USA) equipped with 2487 dual-λ Absorbance Detector. ATP, ADP, and AMP were separated on a reverse-phase chromatography column (Lichrospher® RP-18 HPLC Column, 5 µm particle size, L x I.D. 25 cm x 4.6 mm), using a gradient mobile phase that consisted of Phase A (100 mM KH_2PO_4 buffer with 1.2% methanol v/v, pH = 7.0) and Phase B (100 mM KH_2PO_4 buffer with 10% methanol v/v, pH =7.0) The elution program was the following: 100% of A from zero to 20 min followed by a linear gradient up to 100% of B until 25 min, and from 25 to 28 min down to 0% B (initial conditions). The flow was 1 ml/min, and the column temperature was

maintained at 25 °C during the run. Chromatograms were recorded at 254 nm, and analyzed using the Waters Millennium32 (Waters, Milford, MA, USA). Peaks were identified by their retention times, comparing them with samples of standard compounds. ATP, ADP, and AMP levels were quantified using standard curves obtained with a serial of known concentrations of each of the adenine nucleotides run on the same day and conditions of the samples. The results were expressed as mol/g wet tissue.

Statistical and exploratory factor analysis

Different statistical methods were applied depending on the objective and the nature of the analyzed data. Kolmogorov-Smirnoff test was used to test data normality. Whenever normality was ensured, one-way ANOVA with Tukey's post-hoc test was set as the default test. Whenever this assumption was violated, extreme values of more than 3 standard deviations from the mean were omitted. Otherwise, the non-parametric Kruskal-Wallis test was performed, using the Mann-Whitney test as a post-hoc test. The overall distribution of sperm defects between groups was further tested using the χ^2 test. The non-parametric Spearman correlation was used to correlate fertility and sperm parameters. The parametric Pearson correlation (r coefficients) was used to correlate the different sets of variables in the study (sperm parameters, endocrine and metabolic function, mitochondrial function, bioenergetics and antioxidant system), after variables were successfully tested for normality using the Kolmogorov-Smirnoff test, and as part of exploratory factor analysis. The correlation strength was classified according to ranks (67). Variables with significant ($p < 0.05$) linear correlation with sperm parameters were then

considered for Multivariate Analysis based on Principal Components Analysis (PCA). Although PCA is an unsupervised multivariate method, it was used as a sparse and supervised method considering the variable selection criteria based on linear (Pearson) correlation. Forced factor extraction was performed to extract 2 Principal Components (PCs), based on the interpretability criterion, and using Varimax with Keiser's Normalization as Rotation Method. Regression factors were used to plot sample distribution in the two-dimensional Euclidean space. All methods were performed using IBM SPSS Statistics v25 (Armonk, NY, USA). Independently of the statistical method used, significance was considered whenever $p < 0.05$.

RESULTS

Hormonal balance is not affected by high-fat diets, and glucose homeostasis is recovered after dietary correction

In our previous study (12) we found that dietary switch from a fat-rich diet to a standard chow was able to normalize biometric parameters (Figure SF1, panels A and B) and glucose homeostasis (Figure 1). Hereby, we further characterized the model by studying the endocrine function, including insulin and reproductive hormones such as FSH, LH, 17β-estradiol, and testosterone (Table 1). As observed before, the severe impairment in all the parameters related to metabolic homeostasis (HOMA2), significantly improved after diet reversal (Figure 1). HFD mice had a β-cell function approximately 3 and 2-fold higher than those of CTRL and HFD_t (Figure 1a). Influence on glucose sensitivity (%S) (Figure 1b) and insulin resistance (IR) (Figure 1c) was even more pronounced -

10 and 5-fold increase was observed in HFD animals when compared to CTRL and HFD_t, respectively. Plasma insulin levels were found to be increased in HFD mice and notably, diet correction restored plasma insulin levels (Table 1).

Dietary correction in adulthood after HFD in early life does not restore the decreased sperm quality

We collected epididymal sperm immediately after sacrifice. Sperm motility and concentration were promptly assessed according to standard protocols. Sperm viability and morphology were evaluated after specific staining techniques and using optical microscopy. We have found no differences in sperm counts between groups (Figure 2). However, mice fed HFD, even if transiently, displayed reduced sperm motility (CTRL: 81.6 ± 4.6 %; HFD: 69.1 ± 7.4 %; HFD_t: 65.9 ± 14.5 %) and sperm viability (CTRL: 48.2 ± 8.1 %; HFD: 38.1 ± 7.2 %; HFD_t: 34.4 ± 6.6 %). Regarding sperm morphology (Table 2), we used three different classification systems: descriptive (6 defects), categorical (3 categories) and binomial (normal/abnormal). Based on the χ^2 test, we found that sperm morphology distribution is different between groups. Notably, recurring to ANOVA and Tukey's HSD, we have found differences in the prevalence of pinhead, flattened head, bent neck and overall head defects, especially relative to HFD_t. Despite the observed changes in sperm parameters, diet did not influence reproductive outcomes (Table S2). No significant correlations were found between sperm and fertility parameters (data not shown).

Testicular mitochondrial function and bioenergetics are not affected by high-fat diets

Mitochondria were isolated from a whole testis to evaluate relevant parameters related to its activity and physiology. Particularly, relative protein expression of mitochondrial OXPHOS complexes was assessed by western blot, and the activities of complexes I, II, IV and citrate synthase were evaluated by colorimetric/fluorometric assays (Table S3). Testicular bioenergetics were evaluated based upon testicular content of adenine nucleotides (ATP, ADP and AMP), quantified by HPLC (Table S4). These nucleotides were extracted from the same testis used to isolate the mitochondria-rich fraction. There were no differences between any of the experimental groups.

A high-fat diet promotes a decrease in the activity of antioxidant defenses in testis

Lipid peroxidation and activity of enzymes of the antioxidant defense system were evaluated in mitochondria-free cytosolic fraction obtained from a whole-testis homogenate. The dietary regime did not influence testicular levels of lipid peroxidation products (Figure 3a). Additionally, activities of the cytosolic GPx and SOD were similar in the testes of the mice from the different groups (Figure 3b and 3c). Conversely, mitochondria-free cytosolic fraction obtained from the testes of HFD group mice exhibited a decrease in CAT (CTRL: 747.66 ± 141.46 nmol O_2 . min^{-1} . mg $protein^{-1}$; HFD: 552.41 ± 91.51 O_2 . min^{-1} . mg $protein^{-1}$; HFD_t: 670.02 ± 107.21 O_2 . min^{-1} . mg $protein^{-1}$) and GSR activities (CTRL: 133.14 ± 11.31 nmol NADPH . min^{-1} . mg $protein^{-1}$; HFD: 114.00 ± 11.53 nmol

409 NADPH . min^{-1} . mg $protein^{-1}$; HFD_t: 120.44 ± 9.14 nmol NADPH . min^{-1} . mg
410 $protein^{-1}$).

411

412 *Testicular lipid metabolism is irreversibly affected by a HFD during early life*

413 Metabolites were obtained from a whole-testis using a protocol based on
414 chloroform-methanol-water extraction, resulting in a polar and apolar fractions.
415 In the polar fraction, analyzed by ^{1}H-NMR, various phospholipid-related
416 metabolites and lipid precursors were detected (Table 3). HFD had a marked
417 influence on the composition of polar lipids as observed in the levels of
418 phospholipid head groups like myo-inositol, choline or glycerol, or choline
419 related glycerophosphocholine (GPC). Reversal from HFD to normal diet
420 tended to normalize metabolite levels.

421 The apolar fraction of the testicular extract was characterized by GC-MS after
422 transmethylation. The identified fatty acids (FAs) were quantified and their
423 relative abundance is displayed in Table S5. Dietary intervention promoted a
424 meta-state between CTRL and HFD conditions for most of the identified FAs
425 (*e.g*: palmitic acid and stearic acid). The only exception was vaccenic acid
426 (C18:1n-7), which is twice more abundant in testes of HFD_t mice than in CTRL.
427 After grouping FAs by degree of unsaturation and the position of the double
428 bonds (Table S5 and Figure 4), we observed that the most abundant FA family
429 in the testis of CTRL and HFD_t groups are the saturated fatty acids (SFAs)
430 (55.79% and 41.83%, respectively), while polyunsaturated fatty acids (PUFAs)
431 are the most abundant in the testis of mice from the HFD group (44.21%). HFD
432 and HFD_t mice had also increased testicular relative abundance of

monosaturated fatty acids (MUFAs) (26% and 21%, respectively), with increased accumulation of oleic acid (C18:1n-9). Moreover, Table S5 also shows indirect measures for the anti-inflammatory/pro-inflammatory potential via lipid mediators (C22:6n-3/C20:4n-6 ratio), the combined activity of Δ5- and Δ6-desaturases (D5D and D6D) (C20:4n-6/C18:2n-6 ratio), and the activity of Δ4-desaturases (D4D) (C22:5n-6/C20:4n-6 ratio).

Male reproductive dysfunction caused by HFD is correlated to lipid dysmetabolism in testes

Our main aim was to evaluate whether a dietary intervention in early adulthood can prevent damage to male reproductive health. However, as in previous studies (12, 25), groups were not considered when testing for correlations. Assuming that all mice are equivalent before being fed by a specific diet regimen, our test hypothesis is that some pairs of variables vary proportionally in response of HFD. Those pairs reflect the most relevant (discriminant) variables, therefore the variables that must be included in the multivariate model. In a correlation matrix (Figure 5A) we tested variables of mitochondrial function, bioenergetics, endocrine function and antioxidant defenses against sperm parameters. We found a significant negative correlation between Complex I activity and the prevalence of pin head sperm defect ($r = -0.577$; $p = 0.024$). Regarding bioenergetics, the increase in testicular content in ATP ($r = 0.517$; $p = 0.034$) and ATP/ADP ratio ($r = 0.592$; $p = 0.012$) was correlated with elevated sperm counts. AMP ($r = -0.638$; $p = 0.011$) and AMP/ATP ratio ($r = -0.703$; $p = 0.003$) were inversely correlated to sperm head defects, particularly

457 decapitated sperm (r = -0.627; p = 0.012 and r = -0.689; p = 0.005). Conversely,
458 testicular energy charge was positively correlated to both parameters (r = 0.733;
459 p = 0.002 and r = 0.633; p = 0.011). Concerning endocrine function, most
460 correlations were found in glucose homeostasis. Elevated serum levels of
461 insulin (r = -0.425; p = 0.043), HOMA2-%B (r = -0.523; p = 0.010) and HOMA2-
462 IR (r = -0.438; p = 0.037) were correlated with lower sperm viability. HOMA2-
463 %S behaved contrarily to the former (r = 0.424; p = 0.044). FSH showed a
464 protective effect against sperm head defects (r = -0.464; p = 0.039), namely pin
465 (r = -0.505; p = 0.023) or flattened head (r = -0.673; p = 0.001) defects.
466 Concerning antioxidant defenses, only SOD activity presented a significant
467 inverse correlation to sperm concentration (r = -0.527; p = 0.030). Significantly
468 correlated variables were selected for PCA, to evaluate their predictive power
469 (*i.e.,* if samples were clustered according to their diet using these variables as
470 predictors). These correlations promote a significant clustering of samples
471 according to their experimental group, and there is no overlap between groups,
472 considering 2 PCs (Figure 5B).

473 In the second correlation matrix (Figure 6A), sperm parameters were correlated
474 against testicular lipid metabolite levels. Testicular content in SFAs and PUFAs
475 had a mirror effect over the prevalence of bent neck and coiled tail sperm
476 defects: while SFAs were negatively correlated with bent sperm neck defects (r
477 = -0.668; p = 0.035) and positively correlated with coiled sperm tail defects (r =
478 0.649; p = 0.042). MUFAs had the opposite relation to bent sperm neck (r =
479 0.744; p = 0.014) and to coiled sperm tail (r = -0.663; p = 0.037). Globally, lipid
480 precursors correlated positively with sperm viability. Testicular glycerol (r =
481 0.612; p = 0.020) and myo-inositol (r = 0.592; p = 0.026), however, displayed a

positive correlation with the prevalence of bent neck sperm. Choline is positively correlated to normal morphology of sperm ($r = 0.609$; $p = 0.021$), whilst negatively correlated to tail defects ($r = 0.614$; $p = 0.019$). The 2-dimensional projection of the PCs extracted by PCA (Figure 6B) reveal a clear separation of the samples according to their group.

DISCUSSION

Overweight and obesity have reached pandemic proportions worldwide. Simultaneously, the prevalence of childhood obesity is increasing, leading to the onset of obesity-associated comorbidities, such as T2DM, at younger ages. Reproductive function depends on metabolic homeostasis. Several reports link the prevalence of metabolic disease with lower sperm quality (14, 19, 51). In this study, we used a rodent model to investigate whether a dietary intervention to replace an HFD with a balanced diet in early adulthood, is effective in reversing or preventing the negative effects of childhood obesity in fat accumulation, metabolic homeostasis, and sperm quality. Particularly, we evaluated if diet intervention is effective in normalizing testicular metabolic and lipid balance, and sperm quality later in life. We further integrated all data of metabolomics and lipidomics to elucidate the importance of lipids in mechanisms involved in the onset and/or recovery of the fertility phenotypes induced by HFD in early life.

We have previously demonstrated that this model induces phenotypic traits related to obesity and metabolic syndrome, based on biometric and physiological tests for glucose intolerance (ipGTT) and insulin resistance (ipITT)

(12). We have shown that HFD promoted fat deposition in white adipose tissue pads and liver, which were positively correlated with poorer performance in ipGTT and ipITT. In this report we calculated HOMA2 indexes based on fasting glycemia and fasting insulin levels (30). Although HOMA2 application to animal models is often criticized (68) as it has been developed based on a large human database, it has shown an acceptable goodness of fit for mouse and other mammals (4, 5), and has a high correspondence to ipITT, ipGTT and their respective AUCs (1). As its precursor, HOMA, it is based upon serum fasting glycemia and insulinemia, therefore providing a better physiological characterization of glucose homeostasis. The methodology for quantifying hormonal serum concentrations at the sacrifice was ELISA, for all selected hormones. Insulin was the only hormone measured found to be altered when comparing hormonal levels in the serum of mice from the different groups. HOMA2 showed that dietary intervention is not capable to completely reverse the effects of a HFD during early life in β-cell function and insulin sensitivity, despite total recovery of insulin resistance index. Despite these results not being novel (12), it is relevant to state the high correspondence degree with our previous results, obtained using different methods for glucose homeostasis assessment.

Although testosterone aromatization into estradiol has been widely reported in patients with obesity, our results support recent research showing that this effect may be negligible (6, 52). Although another study suggests marked changes in sex hormone levels in HFD rodents (8), circulating levels of fertility-related hormones were not influenced by dietary regimes in this particular study (Table 1). Yet, endocrine function is strongly correlated with sperm quality,

especially glucose homeostasis and FSH secretion (3, 11, 55) (Figure 5A). In this context, Sertoli Cells (SCs) may be the mediator between both factors and sperm quality. SCs nurse differentiating germ cells during spermatogenesis, and their function is modulated by metabolic (26, 56) and endocrine (34-36, 54) factors, with significant impact on mature spermatozoa (49). Fat deposition in the abdominal area, in humans, is correlated with poor sperm quality (15, 21), likely due to increased testicular temperature (17, 24) and local inflammation (32). In our previous work (12), we also reported reduced sperm quality which was associated with fat deposition. Moreover, there is a decrease in the activity of antioxidant enzymes (Cat and GSR) in the testes of life-long HFD fed mice. Therefore, it is possible that extra lipid intake, notably in PUFAs, is the cornerstone in testicular antioxidant balance. This hypothesis is supported by the differences in lipid fractions between groups (Figure 4), the differences in the correlations of lipid fractions against sperm parameters (Figure 6A), and the sample separation achieved by the corresponding PCA (Figure 6B). Nonetheless, more studies will be needed to confirm this hypothesis, particularly by using a model in which HFD is replaced by a high-fat, low-PUFA diet. Moreover, we have not considered the potential contamination of testicular lipid profile by serum. Yet, testes were decapsulated before homogenization to hinder this issue. Besides, the interference of serum lipid profile is unlikely considering blood to testis volume (27) and a previous study comparing testicular and serum lipid profiles (44).

HFD is the culprit for testicular lipid dysmetabolism. The proportion of SFAs is the lowest in testes of mice from the HFD group, and the highest in testis from mice of the CTRL group. Contrastingly, MUFA and PUFA fractions were more

abundant in HFD than in the CTRL group. As lard, one of the main components of the diet formulations, is rich in oleic acid (18:1n9), it is not surprising that HFD and HFDt testes are enriched in this fatty acid. Yet, the relative content of MUFAs compared to PUFAs, in both groups, suggest that oleic acid and other MUFAs are unsaturated into PUFAs. Besides, the detected n-3 and n-6 long-chain PUFAs can only be synthesized after their precursors, the linoleic and linolenic acid are obtained by diet, as they cannot be synthesized *de novo* in mammals (39). According to Koeberle et al. (28), mammals store large amounts of PUFAs in testes during puberty, by the action of the lysophosphatidic acid acyltransferase 3, resulting in a unique FA profile. For instance, docosapentaenoic acid (C22:5n-6, n-6 DPA) is more abundant in testis than in other mammalian tissues (7, 74). Although several studies describe n-6 DPA-enrichment in various tissues due to HFD (16, 43, 50), testicular content of DPA was unchanged in our model, but we observed testicular enrichment in other n-6 FAs (Table S5).

Regarding PUFAs, the n-3/n-6 ratio in testis supports an amelioration in testicular metabolism after ceasing HFD feeding at early adulthood. The Δ6 desaturase (D6D) has been associated with the deleterious effects of HFD, particularly regarding glucose resistance (23). The dietary n-3/n-6 ratio intake, for humans, is ideally close to 1, and lower ratios (n-6 enrichment) are associated with health deterioration including increased cardiovascular risk (59). In human testis, a lower n-3/n-6 ratio has been reported in oligo and asthenozoospermia (73). Herein, we found a much lower n-3/n-6 ratio (0.22) in testicular FA content, in the CTRL group. Yet, this ratio was significantly lower (0.18) in HFD-fed mice during their lifetime. Considering the critical role of

581 docosahexaenoic acid (C22:6n-3, DHA) in spermatogenesis, the detrimental
582 content of n-3 in testis may be associated with the observed phenotype, as it
583 cannot balance the pro-inflammatory environment induced by HFD. Effectively,
584 DHA supplementation was showed to improve testicular n-3/n-6 ratio,
585 concomitantly improving antioxidant balance (63). However, in our study, sperm
586 parameters did not correlate to the testicular n-3/n-6 ratio.

587 Although the HFD formulation had similar relative amounts of SFAs, MUFAs
588 and PUFAs comparing to the standard chow, the C18:3n-3/C18:2n-6 molar ratio
589 is an order of magnitude higher in HFD (according to the manufacturer
590 information). Interestingly, testicular cells responded to this difference by
591 accumulating even more n-6 PUFAs, without changing their n-3 PUFA content
592 (Figure 4/Table S5). Moreover, n-6 PUFA changes are distinguished not only by
593 an increase in the relative abundance of dietary available linoleic acid (C18:2n-
594 6) but also by an increase of dihomo-*γ*-linolenic acid (C20:3n-6), and
595 arachidonic acid (C20:4n-6, AA), which are obtained from linoleic acid by an
596 enzyme-catalyzed desaturation−elongation process. This may reflect an overall
597 inhibition of the D4Ds and D5D induced by HFD, notably by the relative
598 enrichment in the dietary n-3. D6D is considered the rate-limiting enzyme in the
599 desaturation-elongation processes in mammalian cells, but according to the
600 relative abundance of n-6 PUFAs to n-3 PUFAs, and the C20:4n-6/C18:2n-6
601 ratio, HFD does not change its activity nor it is overwhelmed by the extra dietary
602 n-3. Therefore, testicular metabolic pathways involving n-6 PUFA remodeling
603 and metabolism exhibit high sensitivity to the excess of dietary fat (Figure 4).

604 The structural importance of phospholipids and its FA composition to cell
605 membrane fluidity should not be overlooked (31). Testicular SFA content is

606 negatively correlated with bent neck defects, whilst positively correlated with
607 coiled tail defects. Interestingly, MUFAs present mirrored correlations. This
608 observation may be linked to the different membrane composition of the
609 midpiece (neck) and the tail, or even to different membrane lipid needs of germ
610 cells at different spermatogenic stages (70). However, due to the limited
611 availability of testicular samples, it was not possible to estimate the detailed
612 testicular content of neutral lipid and phospholipid classes, besides the total lipid
613 profile. In this context, it is noteworthy that FAs from the same lipid saturation
614 family can result in opposite outcomes in testicular function. DHA is reduced in
615 testes of acyl-CoA synthetase isoform 6 (ACSL6) KO mice, leading to an
616 enrichment in the AA (20). This change fosters a pro-inflammatory environment,
617 as AA is an n-6 PUFA and precursor of pro-inflammatory eicosanoids, whereas
618 DHA is an n-3 PUFA and precursor of resolvins. According to those authors,
619 ACSL6-KO mice suffered from subfertility, with hypogonadism,
620 oligozoospermia, reduced number of germ cells, and morphological
621 abnormalities of the seminiferous epithelium. Our data show that HFD group
622 presented overlapping phenotypes, such as AA accumulation in testes and
623 poorer sperm parameters, although without DHA depletion in testes, likely due
624 to its dietary intake. Particularly, testicular AA content is positively correlated to
625 sperm pin head defects. However, Hale et al. (20) further reported that DHA-
626 rich and AA-rich phospholipids are delocalized in testes of ACSL6-KO mice. So,
627 the relative testicular content in those FAs is crucial to maintain a normal
628 testicular ultrastructure and spermatogenesis although some caution must be
629 taken, as the characterization of FAs was not quantitative. Despite the different

proportions in lipid fractions between diet regimens, it is not possible to state whether absolute testicular FA content is enriched after HFD.

HFD promotes a positive feedback towards a pro-inflammatory state in testis. Both n-3/n-6 and C22:6n-3/C20:4n-6 reflect a shift towards pro-inflammatory pathways in the testis of mice continuously fed by HFD (Table S5). Interestingly, the same group presented lower CAT and GSR activity and lower potential to prevent oxidative damage. The testicular enrichment in C18:1n-9, in HFD mice, may promote cell membrane fluidity in testicular cells and, consequently, their vulnerability to peroxidation and phospholipase A2. Ultimately, it leads to increased release of PUFAs, particularly AA, a central precursor of pro-inflammatory pathways (leukotrienes, thromboxanes and prostaglandins). Overall, the present data indicate that HFD shifts the cellular redox environment towards a more pro-oxidant and pro-inflammatory state, enhancing testes susceptibility for oxidative stress and inflammatory processes development. Thus, the absence of changes in lipid peroxidation (TBARS) and in the activity of mitochondrial Electron-Transfer Chain activity complexes suggest that HFD by itself seems not to be enough stimulus to trigger oxidative stress. It was reported that HFD causes an increase in antioxidant metabolites in testis, notably GSH and betaine (12). Although we cannot rule out that the decreased activity of Cat and GSR can emerge from post-translational changes (e.g. acetylation, phosphorylation and ubiquitination), it was reported that HFD has not only a negative impact (decrease) on the expression of testicular antioxidant enzymes (18, 32), but also promotes an increase in antioxidant metabolites in testes, notably GSH and betaine (12). Thus, the increased testicular GSH and

654 betaine levels may be enough to keep the redox balance in testis despite the
655 decreased enzymatic antioxidant protection.

656 In addition to this, lipolysis can be another contributor to the observed
657 differences in lipid fractions, and a critical process to mediate membrane
658 integrity and fluidity of all cells in testicular tissue. It is well known that dietary
659 MUFAs promote lipolysis (48). Changes in testicular glycerol, choline, myo-
660 inositol and glycerophosphocholine in HFD (and partially in HFD_t) are likely due
661 to lipolysis and indicate increased phospholipid turnover and/or degradation.
662 Nevertheless, lipolysis raises another threat to seminiferous tubules stability.
663 Glycerol accumulates in the testes of HFD and HFD_t mice. Glycerol destabilizes
664 thight-junctions and desmosomes between SCs, causing a leaky blood-testis
665 barrier (10). We also observed an accumulation of the organic osmolytes
666 glycerophosphocholine and myo-inositol in testes, after HFD. Previously, we
667 reported an accumulation of betaine, glutamine and glutamate in the same
668 condition (12). Interestingly, this phenomenon has been previously reported in
669 kidney medullar cells, as a response against hypertonic stress (45). FA
670 oxidation requires a hydration step; therefore, the extra dietary intake of FAs
671 can induce hypertonic stress in testes, triggering this response in testicular
672 cells, notably SCs. Our data also suggests a protective effect of choline against
673 sperm tail defects. A recent study linked choline (and betaine) supplementation
674 with lipolysis activation, due to elevated succinate concentration (60) in plasma.
675 Interestingly, we have obtained opposite results in testes, after dietary
676 intervention (12). Yet, our data support a promotion of lipolysis in testes by HFD
677 in early adult life. Again, SCs are likely involved in this outcome, as their
678 metabolic activity has been described to be remodeled towards lipolysis as a

679 response to a high-energy environment, as it is the case of obesity (56). In our
680 model, we have observed that AMP and AMP/ATP ratio inversely correlated
681 with sperm head defects suggesting that energy-consuming pathways can be
682 beneficial to spermatozoa. Conversely, increased testicular energy charge
683 promoted sperm head defects. Additionally, sperm motility was reduced in
684 groups where our data suggest lipolysis overactivation (HFD and HFD_t groups)
685 as supported by the changes observed in metabolites related to phospholipids.
686 An exaggerated accumulation of lipid droplets in testis leads to lower sperm
687 motility, but a proper FA acid supply is needed for sperm capacitation in the
688 epididymis (37). Thus, different metabolic pathways related to different lipid
689 classes have different roles and importance not only in different testicular cells,
690 but also in different parts of the spermatozoon (66).

691 In sum, our data demonstrates that a HFD during early life akin to childhood
692 and puberty causes an excessive accumulation of unsaturated FAs is testes. A
693 diet intervention, replacing HFD for a balanced diet was proven effective in
694 protecting/preventing metabolic dysfunction. However, a HFD during early life
695 caused irreversible metabolic remodeling in testes, with long-term sperm
696 defects. Dietary intervention in early adulthood promotes lipolysis in testes,
697 particularly from unsaturated FAs, towards the CTRL state, but this process is
698 apparently too slow to recover normal sperm parameters. Mechanistically, our
699 data suggests that HFD promotes a pro-inflammatory state in testis, aggravated
700 by a positive feedback system that favors the accumulation of n-6 PUFAs,
701 precursors of inflammatory response signaling molecules. Our model did not
702 allow us to verify whether testicular lipid composition and normal sperm quality
703 could be achieved later in life, but we must also consider that sperm quality

declines with age, even in rodents (25). Epigenetic modifications are likely involved in the observed phenotypes after HFD, especially those which have not been reversed by diet switch. Indeed, the prepubertal period is critical for epigenetic remodeling of germ cells (47, 58), and we plan to investigate the influence those mechanisms in our model in future work. Our findings highlight the importance of preventing childhood obesity, to avoid irreversible damage for the reproductive health of the fathers of tomorrow, with unpredicted effects to their progeny.

Declaration of interest

The authors declare no conflict of interest.

Funding

This work was supported by the Portuguese Foundation for Science and Technology: L. Crisóstomo (SFRH/BD/128584/2017), M.G. Alves (IFCT2015 and PTDC/MEC-AND/28691/2017), P.F. Oliveira (IFCT2015), UMIB (UID/Multi/00215/2019) and QOPNA (UID/QUI/00062/2019) co-funded by FEDER funds (POCI/COMPETE 2020); by the Portuguese Society of Diabetology: L. Crisóstomo and M.G. Alves (“Nuno Castel-Branco” research grant and Group of Fundamental and Translational Research); and by the Croatian Science Foundation: K. Starčević (IP-2016-06-3163).

Author Contributions

L.C., P.F.O., M.G.A. and R.L.B. contributed to study design, analysis and interpretation of data. L.R., I.J., K.S., T.M., R.A.V. and L.C. performed experimental work. L.C. edited the images and tables, performed the statistics and contributed to the analysis and interpretation of data. R.L.B., J.F.R. and R.A.V. critically reviewed the manuscript and suggested modifications. All the authors contributed to manuscript writing/editing and approved the final version.

Acknowledgments

We acknowledge the Portuguese Nuclear Magnetic Resonance Network (REDE/1517/RMN/2005) for access to their facilities. We thank Prof. Pedro N. Oliveira (ICBAS-UP) for his advice on the statistical methods.

Supplemental Data

Supplemental tables S1 – S5 and the supplemental figure SF1 are available as Supplemental Data:

URL: https://figshare.com/s/785af8effa28e8bc2935

DOI: https://doi.org/10.6084/m9.figshare.12302171

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FIGURE LEGENDS

Figure 1: HOMA2 indexes and Disposition Index of mice fed standard chow (CTRL) or life-long high-fat diet (HFD) or subjected to diet correction after 60 days (HFD_t). Fasting glycemia and insulin were used as inputs for the iHOMA2 calculator (University of Oxford, UK). Results are expressed as Tukey's whisker boxes (median, 25^{th} to 75^{th} percentiles ± 1.5 IQR). Extreme values are represented individually (● CTRL – standard chow; ■ HFD – high-fat diet; ▲ HFD_t – transient high-fat diet). Data was tested by one-way ANOVA with Tukey's HSD for group comparison. Significance was considered when $p < 0.05$. $^{+}$ group average; * vs. CTRL; $^{\#}$ vs. HFD. * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001.

Figure 2: Epididymal sperm parameters of mice fed standard chow (CTRL) or life-long high-fat diet (HFD) or subjected to diet correction after 60 days (HFD_t). Results are expressed as mean ± standard deviation. Sperm count is expressed as million spermatozoa per milliliter (M/mL), while other parameters are expressed as the % of total sperm cells. Individual values are represented (● CTRL – standard chow; ■ HFD – high-fat diet; ▲ HFD_t – transient high-fat diet). Data was tested by one-way ANOVA with Tukey's HSD for group comparison. Significance was considered when $p < 0.05$. * vs. CTRL; $^{\#}$ vs. HFD. * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001.

Figure 3: Lipid peroxidation (TBARS assay) and activity of the four main enzymes of the antioxidative defense metabolism – SOD, GSR, GPx and CAT

in the testis of mice standard chow (CTRL) or life-long high-fat diet (HFD) or subjected to diet correction after 60 days (HFD_t). Two outliers were excluded from the analysis resulting in n=5 for every group. Results are expressed as Tukey's whisker boxes (median, 25^{th} to 75^{th} percentiles ± 1.5 IQR). Data was tested by one-way ANOVA with Tukey's HSD for group comparisons. Significance was considered when $p < 0.05$. $^{+}$ group average; * vs. CTRL; $^{\#}$ vs. HFD. * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001.

Figure 4: Relative abundance of lipids in the apolar fraction of testicular extracts of mice fed standard chow (CTRL) or life-long high-fat diet (HFD) or subjected to diet correction after 60 days (HFD_t), grouped by saturation. CTRL – standard chow; HFD – high-fat diet; HFD_t – transient high-fat diet. The results were tested by one-way ANOVA with Tukey's Honest Significance Difference (HSD) for post-hoc group comparisons. Significance was considered when $p < 0.05$. * vs. CTRL; $^{\#}$ vs. HFD. * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001. Abbreviations: SFA – Saturated Fatty Acids; MUFAs – Monounsaturated Fatty Acids; PUFAs – Polyunsaturated Fatty Acids.

Figure 5: Correlations between sperm parameters to endocrine, antioxidant, mitochondrial and bioenergetic parameters. **A)** Individual scores for the previously assessed parameters were correlated using the parametric Pearson correlation (n = 24). Values are represented as Pearson r coefficients, according to the color scale, when $p < 0.05$. **B)** Sample distribution according to 2PCs extracted by PCA, and based on correlations between sperm parameters

to endocrine, antioxidant, mitochondrial and bioenergetic parameters (n = 10). [a]After base-10 logarithmic transformation. Abbreviations: CS – Citrate Synthase; C I – Complex I activity; C II - Complex II activity; C IV - Complex IV activity; C V - Complex V activity; Pool – Total adenosine nucleotide pool; Glycaemia – Fasting glycemia; FSH – Serum FSH; LH – Serum LH; E_2 – Serum 17β-estradiol; T – Serum testosterone; TBARS – Thiobarbituric Acid reactive species assay (Lipid peroxidation); SOD – Superoxide dismutase activity; GPx – Glutathione Peroxidase activity; CAT – Catalase activity; GSR – Glutathione-disulphide Reductase activity.

Figure 6: Correlation of sperm parameters to testicular lipids and lipid precursors. **A)** Individual scores for the previously assessed parameters were correlated using the parametric Pearson correlation (n = 14). Values are represented as Pearson r coefficients, according to the color scale, when $p < 0.05$. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. **B)** Sample distribution according to 2PCs extracted by PCA, and based on correlations between sperm parameters to testicular lipids and lipid precursors (n = 11). [a] Spearman rho coefficients, as the normality assumption was violated for this variable. Abbreviations: EtAmine – Ethanolamine; PhosEtAmine – Phosphoethanolamine; 3OH-But – 3'-hydroxybutyrate; PhosCholine – Phosphocholine; GlyPhosCholine – Glycerophosphocholine; SFAs – Saturated Fatty Acids; MUFAs – Monounsaturated Fatty Acids; PUFAs – Polyunsaturated Fatty Acids.

SUPPLEMENTAL DATA

Table S1: ^{1}H NMR resonance assignment of polar testicular lipid precursors (s: singlet, d: doublet, dd: doublet of doublets, t: triplet, m: multiplet).

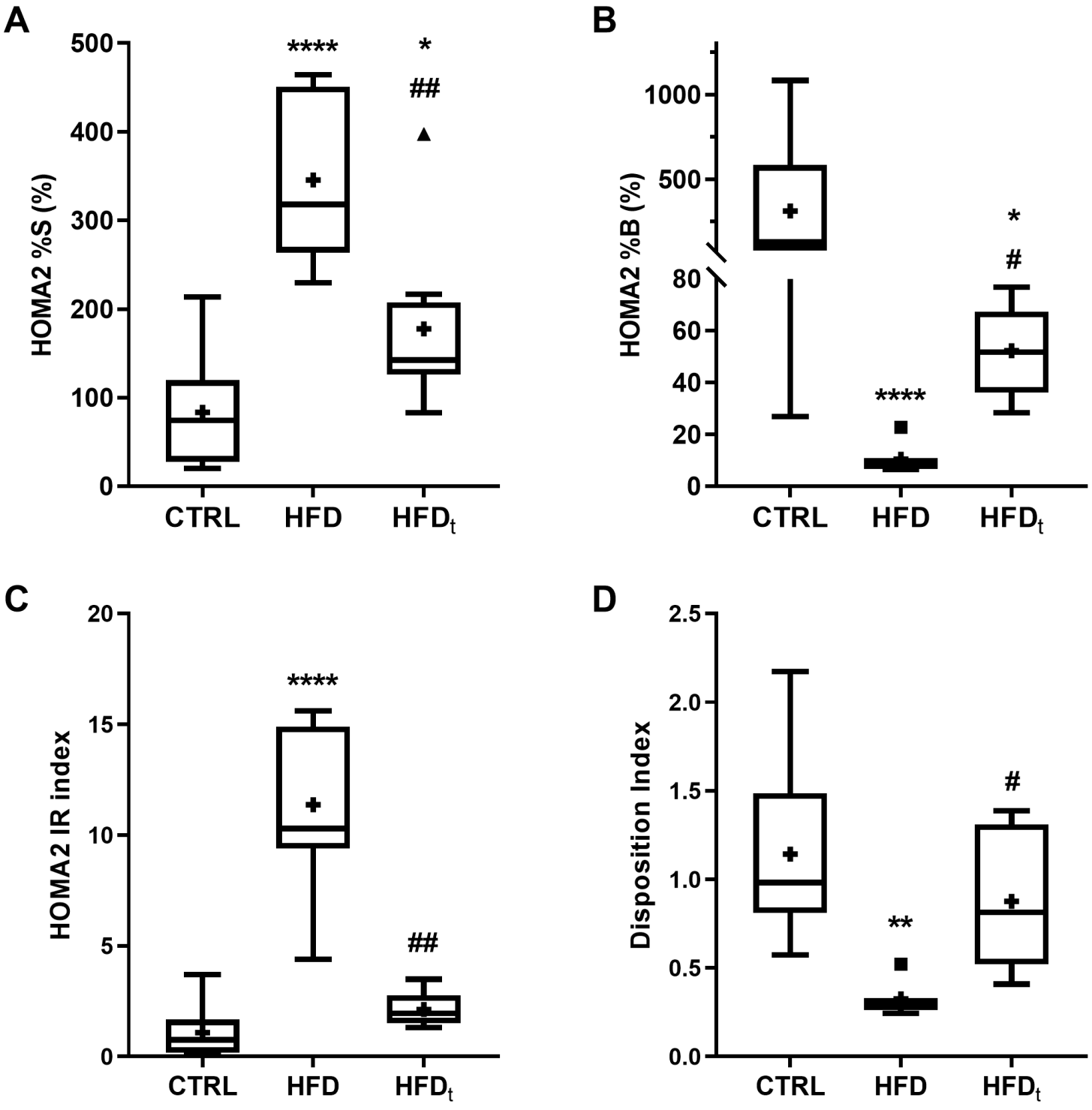
Table S2: Reproductive parameters. Results are expressed as the mean (%) ± standard error of the mean. Data tested using χ^2 test, based on the number of successful matings to the number of attempts (success rate), and on the number of male offspring to the litter size (male pups).

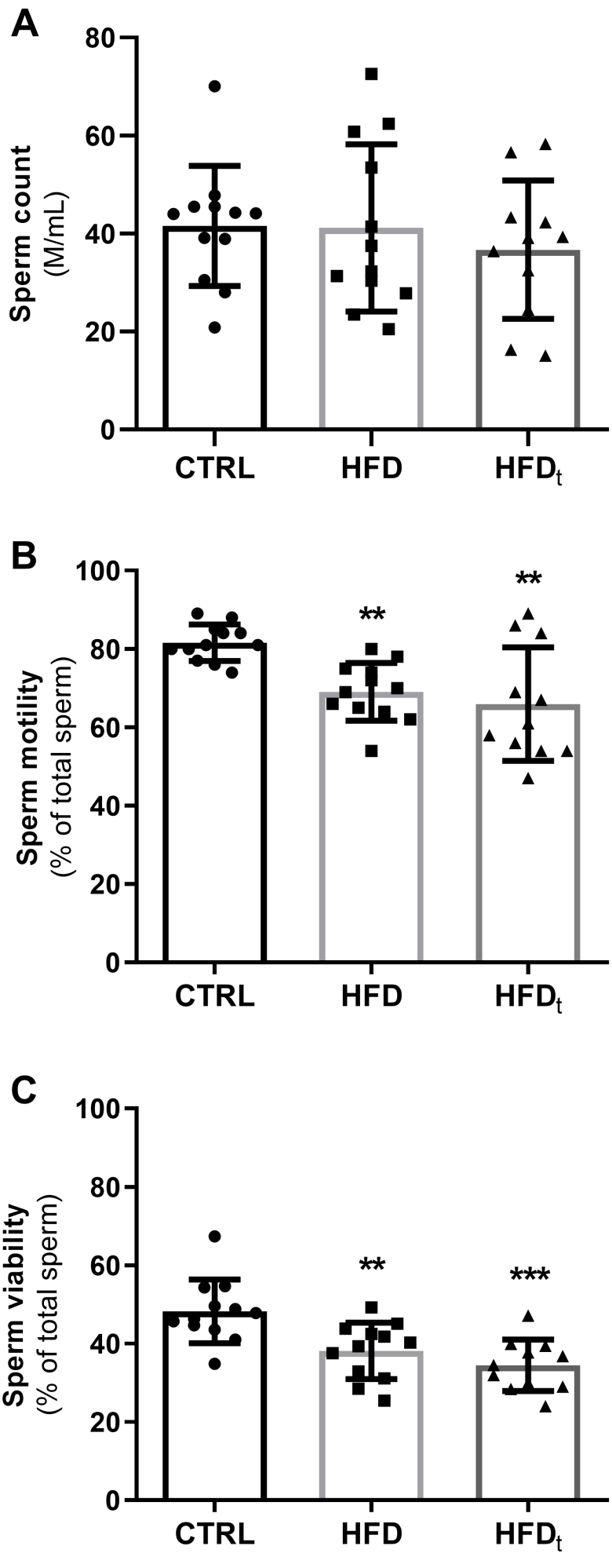
Table S3: Enzymatic activity of mitochondrial complexes in testes of mice fed standard chow (CTRL), life-long high-fat diet (HFD), and those subjected to diet correction after 60 days (HFD_t). Results are expressed as mean ± standard deviation. Experimental groups were compared by one-way ANOVA with Tukey's HSD. Significance was considered when $p < 0.05$. * vs. CTRL; # vs. HFD. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

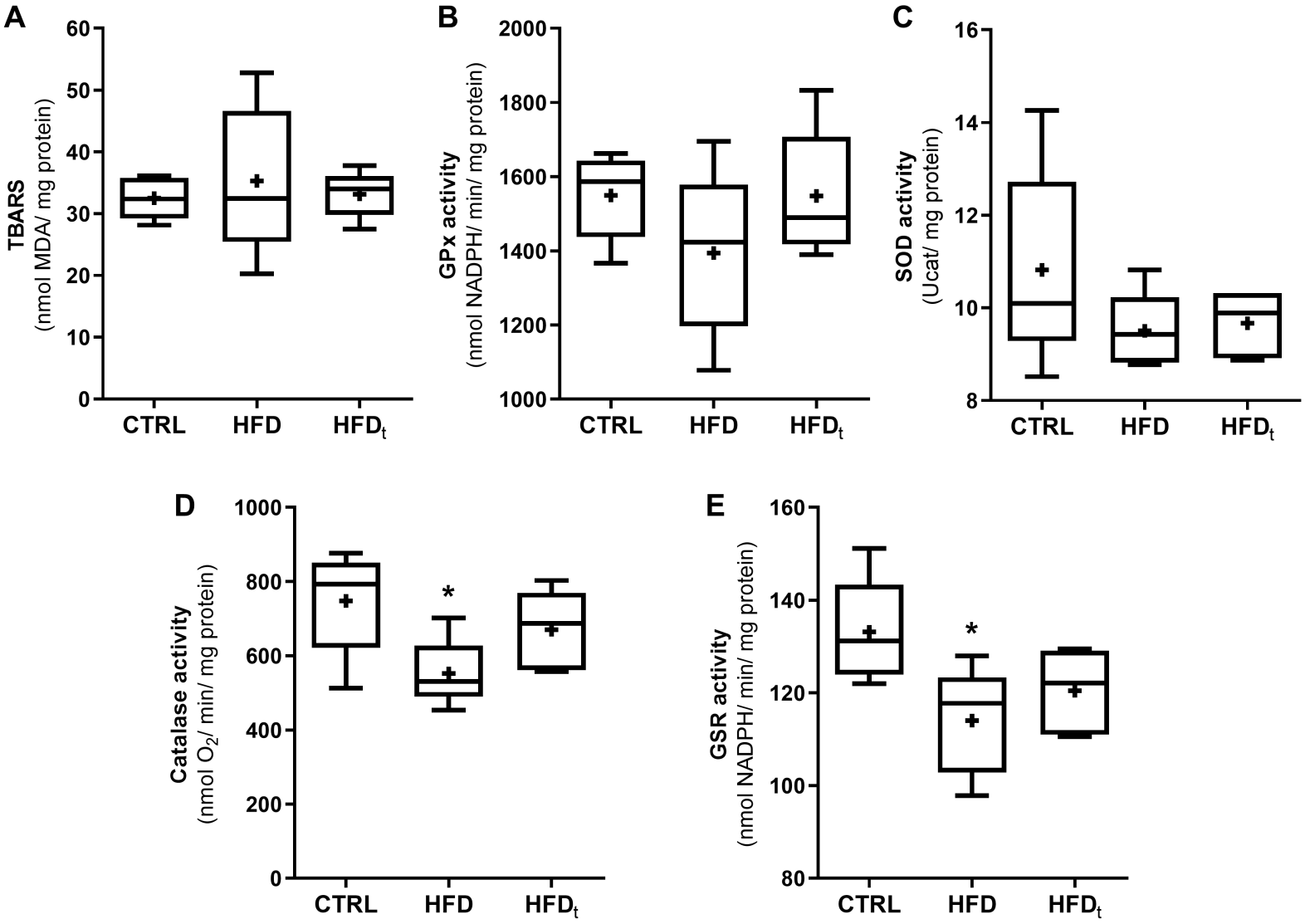
Table S4: Testicular bioenergetics of mice fed standard chow (CTRL), life-long high-fat diet (HFD), and those subjected to diet correction after 60 days (HFD_t). Results are expressed as mean ± standard deviation. Experimental groups were compared by one-way ANOVA with Tukey's HSD. Significance was considered when $p < 0.05$. * vs. CTRL; $^{\#}$ vs. HFD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

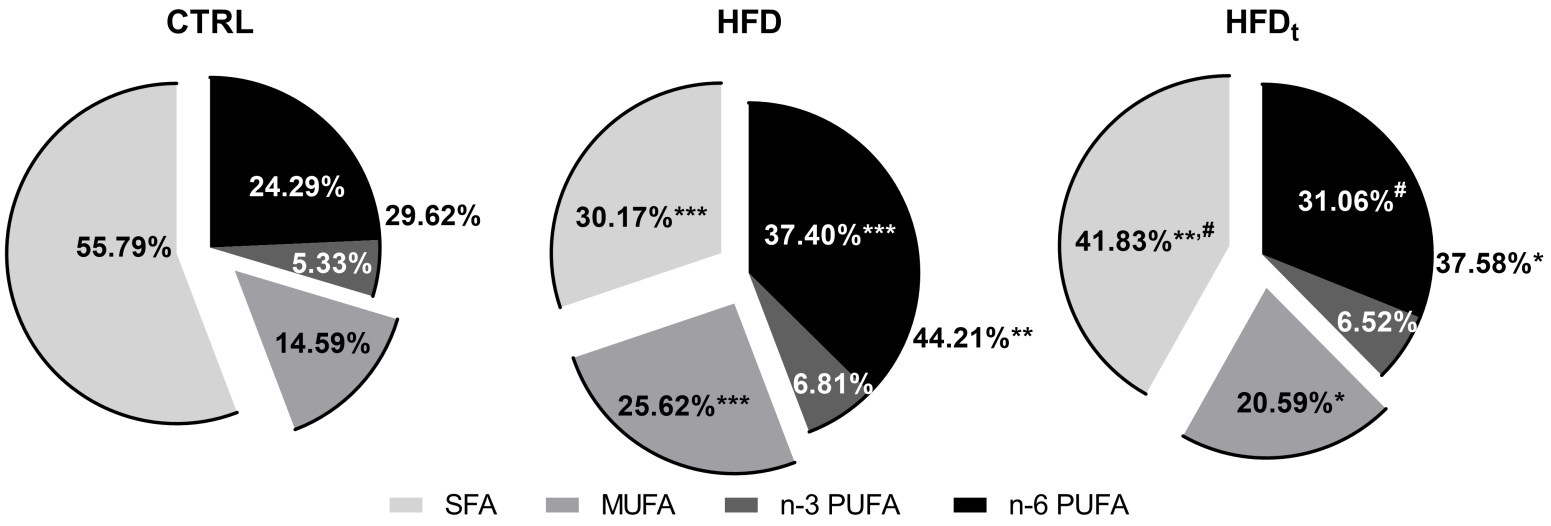
Table S5: Fatty acid composition of the apolar testicular extracts of mice fed standard chow (CTRL), life-long high-fat diet (HFD), and those subjected to diet correction after 60 days (HFD_t). FAs are presented as: Common name (Lipid numbers). Results are expressed as the mean (% of total lipids) ± standard deviation. Results were tested by one-way ANOVA with Tukey's HSD. Significance was considered when $p < 0.05$. * vs. CTRL; $^{\#}$ vs. HFD. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. Abbreviations: SFAs – Saturated Fatty Acids; MUFAs – Monounsaturated Fatty Acids; PUFAs – Polyunsaturated Fatty Acids.

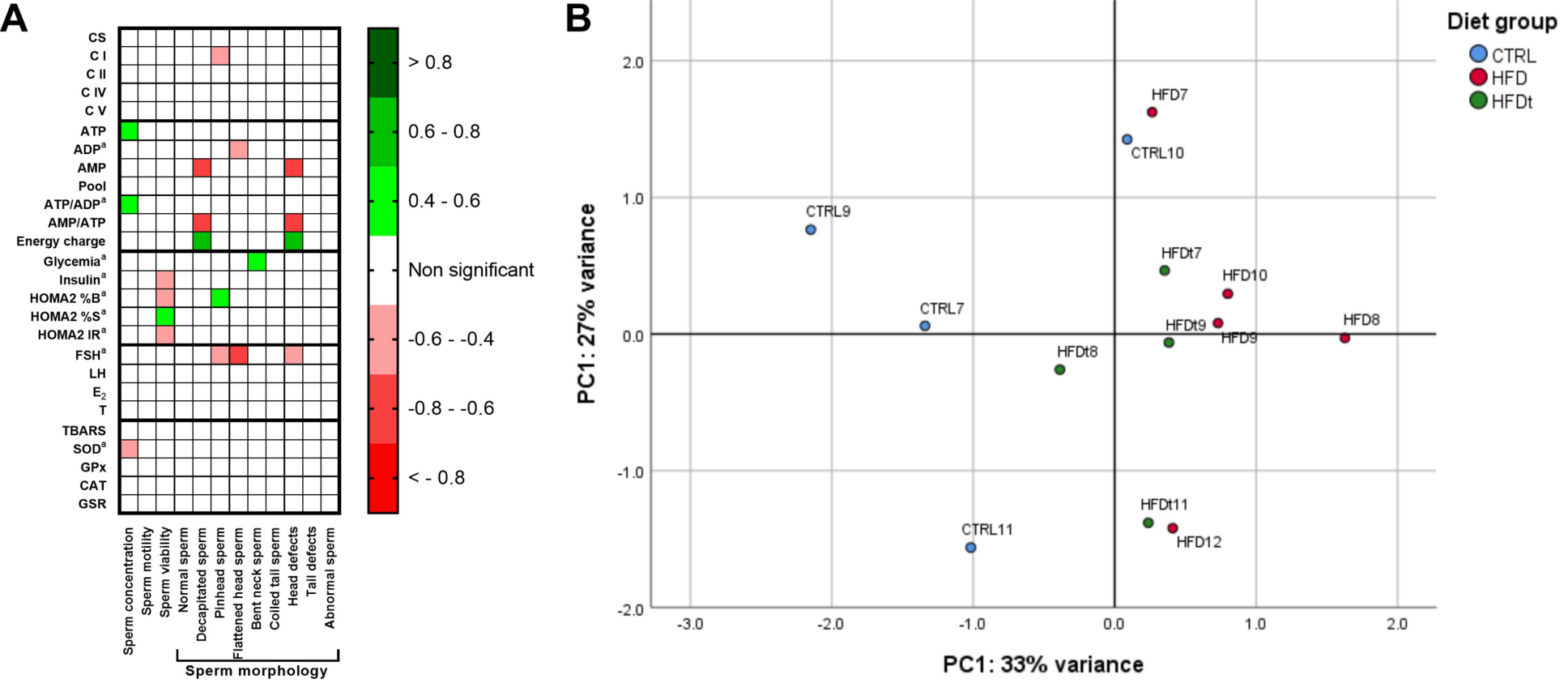
Figure SF1: Body weight and food/water intake monitoring throughout the experiment. A) Average body weight 60 days after weaning, when diet reversion was performed to HFD_t group. B) Average body weight at the end of the experiment, 200 days after weaning. C) Cumulative food intake per mouse. D) Cumulative water intake per mouse. Results are expressed as mean ± standard deviation. Data were tested using one-way ANOVA corrected by Tukey's HSD. Significance was considered when $p < 0.05$. * vs. CTRL; $^{\#}$ vs. HFD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.


A
HOMA2 %S (%)
500
400
300
200
100
0
****
*
##
CTRL
HFD
HFD$_t$
B
HOMA2 %B (%)
1000
500
80
60
40
20
0
****
*
#
CTRL
HFD
HFD$_t$
C
HOMA2 IR index
20
15
10
5
0
****
##
CTRL
HFD
HFD$_t$
D
Disposition Index
2.5
2.0
1.5
1.0
0.5
0.0
**
#
CTRL
HFD
HFD$_t$


A
Sperm count
(M/mL)
80
60
40
20
0
CTRL
HFD
HFDt
B
Sperm motility
(% of total sperm)
100
80
60
40
20
0
**
**
CTRL
HFD
HFDt
C
Sperm viability
(% of total sperm)
100
80
60
40
20
0
**
***
CTRL
HFD
HFDt


A
TBARS
(nmol MDA/ mg protein)
60
50
40
30
20
10
0
CTRL
HFD
HFDt
B
GPx activity
(nmol NADPH/ min/ mg protein)
2000
1800
1600
1400
1200
1000
CTRL
HFD
HFDt
C
SOD activity
(Ucat/ mg protein)
16
14
12
10
8
CTRL
HFD
HFDt
D
Catalase activity
(nmol O2/ min/ mg protein)
1000
800
600
400
200
0
*
CTRL
HFD
HFDt
E
GSR activity
(nmol NADPH/ min/ mg protein)
160
140
120
100
80
*
CTRL
HFD
HFDt


CTRL
HFD
HFD$_t$
24.29%
29.62%
55.79%
5.33%
14.59%
30.17%***
37.40%***
44.21%**
25.62%***
6.81%
41.83%**,#
31.06%#
37.58%*
6.52%
20.59%*
SFA
MUFA
n-3 PUFA
n-6 PUFA


A
CS
C I
C II
C IV
C V
ATP
ADP[a]
AMP
Pool
ATP/ADP[a]
AMP/ATP
Energy charge
Glycemia[a]
Insulin[a]
HOMA2 %B[a]
HOMA2 %S[a]
HOMA2 IR[a]
FSH[a]
LH
E2
T
TBARS
SOD[a]
GPx
CAT
GSR
Sperm concentration
Sperm motility
Sperm viability
Normal sperm
Decapitated sperm
Pinhead sperm
Flattened head sperm
Bent neck sperm
Colled tail sperm
Head defects
Tail defects
Abnormal sperm
Sperm morphology
> 0.8
0.6 - 0.8
0.4 - 0.6
Non significant
-0.6 - -0.4
-0.8 - -0.6
< - 0.8
B
Diet group
CTRL
HFD
HFDt
PC1: 27% variance
PC1: 33% variance
HFD7
CTRL10
CTRL9
HFDt7
HFD10
CTRL7
HFDt9
HFD8
HFD9
HFDt8
HFDt11
CTRL11
HFD12
2.0
1.0
0.0
-1.0
-2.0
-3.0
-2.0
-1.0
0.0
1.0
2.0

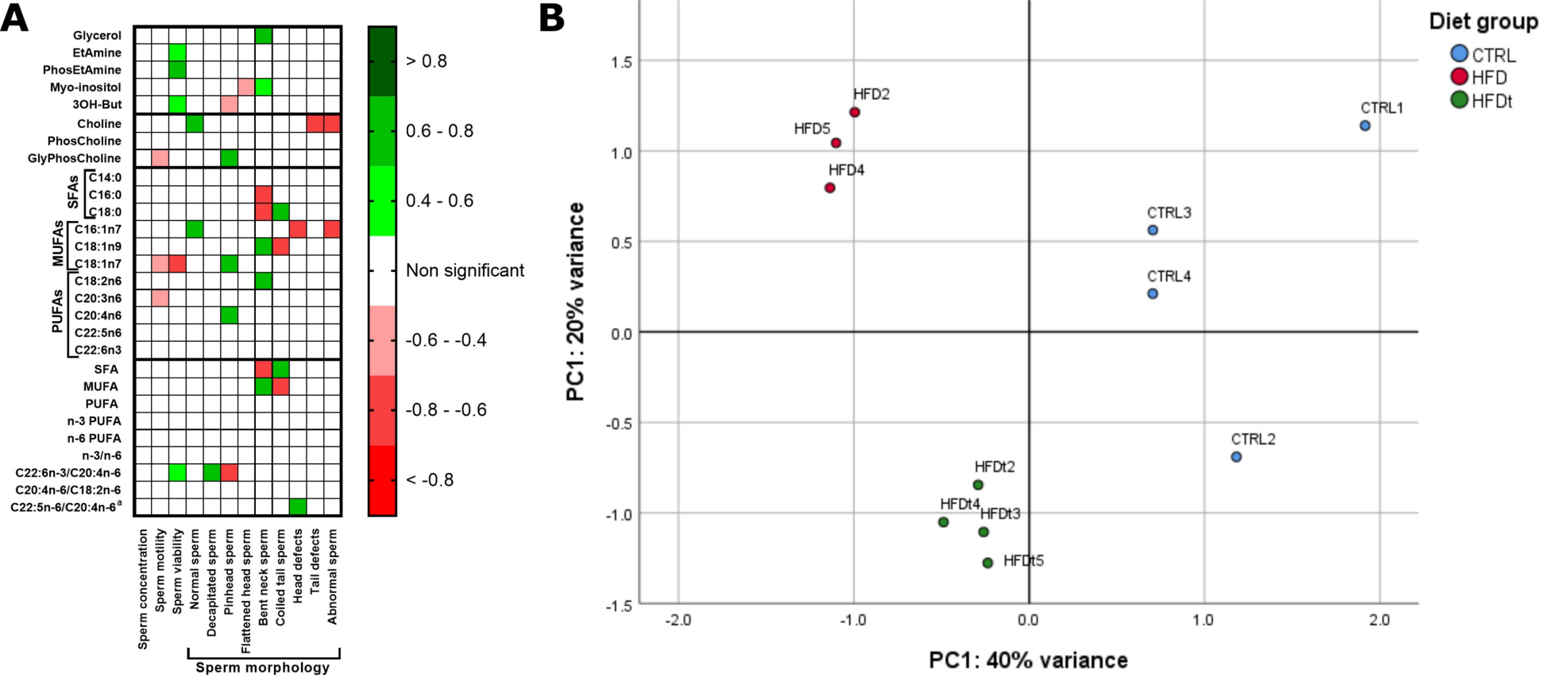

A
Glycerol
EtAmine
PhosEtAmine
Myo-inositol
3OH-But
Choline
PhosCholine
GlyPhosCholine
SFAs
C14:0
C16:0
C18:0
MUFAs
C16:1n7
C18:1n9
C18:1n7
PUFAs
C18:2n6
C20:3n6
C20:4n6
C22:5n6
C22:6n3
SFA
MUFA
PUFA
n-3 PUFA
n-6 PUFA
n-3/n-6
C22:6n-3/C20:4n-6
C20:4n-6/C18:2n-6
C22:5n-6/C20:4n-6[a]
Sperm concentration
Sperm motility
Sperm viability
Normal sperm
Decapitated sperm
Pinhead sperm
Flattened head sperm
Bent neck sperm
Coiled tail sperm
Head defects
Tail defects
Abnormal sperm
Sperm morphology
> 0.8
0.6 - 0.8
0.4 - 0.6
Non significant
-0.6 - -0.4
-0.8 - -0.6
< -0.8
B
Diet group
CTRL
HFD
HFDt
PC1: 20% variance
PC1: 40% variance
HFD2
HFD5
HFD4
CTRL1
CTRL3
CTRL4
CTRL2
HFDt2
HFDt4
HFDt3
HFDt5
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-1.0
0.0
1.0
2.0

Table 1: Fasting glycemia and serum concentrations of insulin, FSH, LH, 17β-estradiol and testosterone in mice fed standard chow (CTRL), life-long high-fat diet (HFD), and those subjected to diet correction after 60 days (HFD_t). Fasting glycemia was evaluated using a glucometer (One Touch Ultra Lifescan-Johnson, Milpitas, CA, USA). Hormones were quantified by ELISA assay kits. Results are expressed as mean ± standard deviation (units disclosed in the table). CTRL – standard chow; HFD – high-fat diet; HFD_t – transient high-fat diet. Data was tested by one-way ANOVA with Tukey's HSD for group comparison. Significance was considered when $p < 0.05$. * vs. CTRL; # vs. HFD. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

Analyte	**CTRL (n=8)**	**HFD (n=8)**	**HFD_t (n=8)**
Fasting glucose (mg/dL)	94.50 ± 7.94	116.08 ± 15.26 ***	84.91 ± 9.91 ####
Insulin (nmol/mL)	0.49 ± 0.68	3.56 ± 1.75 ****	0.67 ± 0.25 ####
FSH (nmol/mL)	6.60 ± 2.83	4.25 ± 3.18	3.77 ± 2.67
LH (nmol/mL)	435.99 ± 79.50	406.89 ± 61.62	470.30 ± 28.34
17β-estradiol (pmol/mL)	348.89 ± 46.55	335.46 ± 114.04	413.87 ± 108.97
Testosterone (nmol/mL)	15.88 ± 0.58	15.88 ± 0.48	15.23 ± 0.97

Table 2: Sperm morphology distribution, according to different classifications, in mice fed standard chow (CTRL), life-long high-fat diet (HFD), and those subjected to diet correction after 60 days (HFD_t). Results are expressed as mean (% of total sperm cells) ± standard deviation. CTRL – standard chow; HFD – high-fat diet; HFD_t – transient high-fat diet. Sperm count distributions across groups were tested using the χ^2 test. Differences between groups for each morphological classification were tested by one-way ANOVA with Tukey's HSD. For both methods, significance was considered when $p < 0.05$. * vs. CTRL; # vs. HFD. * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001.

Sperm Morphology	CTRL (n=9)	HFD (n=12)	HFD_t (n=9)	χ^2 test
Normal (%)	39.79 ± 6.01	33.82 ± 6.74	36.51 ± 4.08	106.83 ****
Decapitated (%)	12.10 ± 5.27	11.19 ± 3.61	12.18 ± 3.11	
Pinhead (%)	3.70 ± 1.30	6.20 ± 1.26**	6.68 ± 1.94**	
Flattened head (%)	3.92 ± 0.76	4.62 ± 1.20	6.02 ± 0.86***,#	
Bent neck (%)	6.49 ± 0.97	9.38 ± 3.64	5.30 ± 1.97##	
Coiled tail (%)	34.00 ± 5.73	34.77 ± 7.06	33.32 ± 2.46	
Normal (%)	39.79 ± 6.01	33.82 ± 6.74	36.51 ± 4.08	48.57 ****
Head defects (%)	19.72 ± 3.82	22.03 ± 4.52	24.88 ± 2.45*	
Tail defects (%)	40.49 ± 5.58	44.15 ± 6.41	38.61 ± 3.00	
Normal (%)	39.79 ± 6.01	33.82 ± 6.74	36.51 ± 4.08	26.16 ****
Abnormal (%)	60.21 ± 6.01	66.18 ± 6.74	63.49 ± 4.08	

Table 3: Polar lipid metabolites detected by ^{1}H-NMR in testicular extracts of mice fed standard chow (CTRL), life-long high-fat diet (HFD), and those subjected to diet correction after 60 days (HFD_t). Results are expressed as mean (arbitrary units) ± standard deviation. CTRL – standard chow; HFD – high-fat diet; HFD_t – transient high-fat diet. Results were tested by one-way ANOVA with Tukey's HSD, except where stated otherwise. Significance was considered when $p < 0.05$. * vs. CTRL; # vs. HFD. * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001.

	Metabolite	CTRL (n=6)	HFD (n=6)	HFD_t (n=5)
Lipid intermediaries	*Glycerol*	$7.50x10^{-3} \pm 8.48x10^{-4}$	**$9.26x10^{-3} \pm 2.58x10^{-4}$ ****	**$7.82x10^{-3} \pm 7.14x10^{-4}$ \#\#**
	Ethanolamine [a]	$1.75x10^{-3} \pm 2.21x10^{-4}$	$1.74x10^{-3} \pm 1.45x10^{-4}$	**$1.48x10^{-3} \pm 1.31x10^{-4}$ *, \#**
	Phosphoethanolamine	$2.01x10^{-2} \pm 1.75x10^{-3}$	$2.01x10^{-2} \pm 5.93x10^{-4}$	$1.99x10^{-2} \pm 9.14x10^{-4}$
	Myo-inositol	$4.94x10^{-3} \pm 4.85x10^{-4}$	**$5.54x10^{-3} \pm 2.69x10^{-4}$ ***	**$4.58x10^{-3} \pm 3.16x10^{-4}$ \#\#**
	3-Hydroxybutyrate	$6.68x10^{-2} \pm 1.08x10^{-2}$	$5.70x10^{-2} \pm 3.40x10^{-3}$	$5.97x10^{-2} \pm 6.44x10^{-3}$
Choline metabolism	*Choline*	$1.32x10^{-2} \pm 1.56x10^{-3}$	**$0.98x10^{-2} \pm 1.29x10^{-3}$ ****	$1.12x10^{-2} \pm 1.32x10^{-3}$
	Phosphocholine	$2.01x10^{-2} \pm 1.75x10^{-3}$	$2.01x10^{-2} \pm 5.93x10^{-4}$	$1.99x10^{-2} \pm 9.14x10^{-4}$
	Glycerophosphocholine	$3.00x10^{-3} \pm 3.39x10^{-4}$	**$4.26x10^{-3} \pm 3.70x10^{-4}$ ******	**$3.81x10^{-3} \pm 2.67x10^{-4}$ ***

[a] Kruskal-Wallis test, unadjusted p-value.