TITLE: A switch from high-fat to normal diet does not restore sperm quality but prevents metabolic syndrome

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ABSTRACT

In recent decades, the prevalence of metabolic diseases has concomitantly increased with a decline on fertility rates and sperm quality. High-fat diets (HFD) are seldom considered part of the problem, but the molecular mechanisms underlying its effects on male fertility remain poorly understood. Herein we postulated that HFD alter sperm quality. We evaluated the effects of switching from a HFD to a normal diet in early adulthood on metabolic disease onset, testicular metabolism and sperm quality. Thirty-six male C57BL6/J mice were divided in: a control group fed with standard chow; a group fed with HFD for 200 days; and a group fed with HFD for 60 days and then with standard chow (HFD_t). Biometric data and whole-body metabolism were assessed. Epididymal sperm was studied for concentration, motility, viability and morphology. ^{1}H-NMR metabolomics approach was performed on testicular extracts to trace the metabolic changes. Diet switch reduced body weight and fat mass, preventing metabolic syndrome onset. However, sperm viability, motility and morphology were deteriorated by HFD consumption and not restored by diet switch. HFD induced irreversible changes in pyruvate and glutamate metabolism, ethanol degradation and ammonia recycling in testis. Furthermore, HFD_t changed purine and cysteine metabolism, urea cycle, and glutathione content. Overall, HFD caused irreversible changes in testicular metabolism even after switching to normal diet. HFD feeding until early adulthood decreases sperm quality, which cannot be restored by diet switch or weight loss, even when development of metabolic syndrome is avoided.

INTRODUCTION

Since the second half of the 20th century, the prevalence of metabolic disease has increased worldwide. Obesity and Type 2 Diabetes Mellitus (T2DM) are the most prevalent metabolic diseases, and are regarded as global epidemics by the World Health Organization (World Health Organization 2000). Obesity and T2DM, are largely caused by lifestyle, particularly sedentary behaviours and poor dietary habits, such as high-fat diets (HFD). Interestingly, during the same period, several studies report a decline in human fertility rates, and particularly in male reproductive parameters (Hamilton and Ventura 2006, Skakkebaek et al. 2016). Indeed, couples with overweight and obesity attending fertility clinics have worse reproductive outcomes. Energy balance is crucial for spermatogenesis and overweight men are reported to present poorer sperm quality amongst other reproductive problems (Oliveira et al. 2017). Similarly, men with T2DM suffer from several reproductive problems, such as microvascular disease. Men attending fertility clinics are often requested to change their eating habits and lose weight to improve their sperm parameters. Nevertheless, few studies report the molecular basis linking food habits, overweight and poor male fertility. So, the efficiency of dietary intervention such as reduced intake of dietary fats and weight loss upon male fertility is unknown.

Male reproductive function demands a considerable amount of energy to be sustained. Thus, metabolic regulation of testicular cells is crucial to maintain spermatogenesis (Rato et al. 2012). The elevated energy content of HFD (and hypercaloric diets) registers a metabolic signature in the body. The first visible effect of hypercaloric diets is the storage of excess energy as fat in white adipose tissue (WAT) pads. This accumulation promotes the onset of metabolic disease,

translated into glucose intolerance, insulin resistance and insulin deficiency. The rodent model has been long used to carry out experimental designs and test hypotheses that would be impossible to test or take too long in humans, and to avoid the typical confounding factors of human subjects (Dutta and Sengupta 2016).

In this work, we propose to study the molecular mechanisms by which HFD and transient high-fat diet (HFD_t) can alter male reproductive health, such as glucose homeostasis and metabolic changes in testes that compromise sperm quality. We used a C57BL6/J mice model fed with HFD or HFD_t and compared it with mice fed with a standard diet. The biometric data and the whole-body metabolic function were characterised. In addition, epididymal sperm parameters and testicular metabolic fingerprints were studied. The effect of switching from HFD to standard chow was given special attention. We integrated the data under a metabolomics-based approach to establish a testicular metabolic profile and determine how this profile correlates with sperm quality, in animals fed with HFD and HFD_t.

MATERIALS & METHODS

Animal Model

Mus musculus C57BL6/J 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 they were subjected to the same random *in utero* stimuli. Mice from

the CTRL group were fed with a standard chow (#F4031, BioServ, USA – Carbohydrate: 61.6%, Protein: 20.5%, Fat: 7.2%). Mice from the HFD group received a fat-enriched diet (#F3282, BioServ USA – Carbohydrate: 35.7%, Protein: 20.5%, Fat: 36.0%) during 200 days after weaning. The mice from HFD_t group were fed with a transient high-fat diet (#F3282, BioServ, New Jersey, USA) for 60 days after weaning, ~~which was~~ 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. Reproductive success rate, litter size and male ratio was assessed. Animals were killed by cervical dislocation 200 days after weaning, and tissues were collected for further analysis. Total body weight, water and food intake were monitored weekly from weaning to sacrifice. The animal model is compliant with the ARRIVE guidelines and was licensed by the Portuguese Veterinarian and Food Department (0421/000/000/2016).

Glucose tolerance test and insulin resistance test

One week before sacrifice (190-193 days after weaning), animals were subjected to intraperitoneal Glucose Tolerance Test (ipGTT) and intraperitoneal Insulin Tolerance Test (ipITT) as described by the NIH Mouse Metabolic Phenotyping Center (MMPC) Consortium guidelines (Ayala et al. 2010). Briefly, 16–18 h before the test, access to food was removed from the animals (overnight fasting). For the ipGTT, an intraperitoneal injection with 6 mL glucose 30% (w/v) per kg of body weight was given to each animal and blood glucose levels were measured every 30 min during 2 h. For the ipITT, an intraperitoneal insulin injection (0.75 U

per kg body weight) was given to each animal (McGuinness et al. 2009) and blood glucose levels were measured every 30 min during 2 h.

Evaluation of epididymal sperm parameters

Epididymis 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 (Rato et al. 2013).

NMR spectroscopy

A combined extraction of polar and nonpolar metabolites from testicular tissue was performed as previously described (Alves et al. 2011). The aqueous phase containing polar water-soluble metabolites was lyophilized and analysed by NMR spectroscopy as described (Jarak et al. 2018). Metabolites were identified by comparing recorded spectra with reference spectra and the Human Metabolome Database (HMDB) (Wishart et al. 2007) and according to Metabolomics Standards Initiative (MSI) guidelines for metabolite identification (Sumner et al. 2007). Identification levels are indicated in Table S1 (Supplementary data). After processing, 1H spectra were processed using previously described methods (Jarak et al. 2018). Obtained areas were normalized by total spectral area and analysed by univariate analysis.

Metabolomics integration

Metabolites found to have differences between groups, or to have strong and significant correlations against tested parameters, were selected for metabolomics integration using MetaboAnalyst 4.0 tools (Chong et al. 2018). Pathway enrichment and topology analysis were used for metabolic pathway identification and evaluation of metabolic impact and significance. For Pathway Topology Analysis, we used the SMPDB *Mus Musculus* database, Relative-Betweenness Centrality and Fisher's Exact Test. Pathways with Pathway Impact (PI) > 0.1 and one-tailed unadjusted $p < 0.05$ were considered affected.

Statistical and correlation analysis

Different statistical methods were applied depending on the objective and the nature of data under analysis. One-way ANOVA with Tukey's post-hoc test was used to compare biometric data, glucose metabolism and reproductive data between experimental groups. Normalized areas of testicular metabolites signals were also analysed by ordinary One-way ANOVA followed by Tukey's multiple comparisons test. Two-way ANOVA with Tukey's post-hoc test was used to compare body weight, ipGTT and ipITT. Mixed Factorial ANOVA with Tukey's post-hoc test was applied to compare sperm morphology, in respect to within-subject variance of the counts. Multivariate Analysis based on Spearman's rank correlation (Spearman's r) was used to correlate the different sets of variables in study (testicular metabolite content, sperm parameters, global metabolic state and biometric data). The correlation strength was classified according to ranks (Taylor 1990). Exact p value was calculated when 16 or less pairs were

correlated. Over this number, it was obtained an approximate p value. All methods were performed using GraphPad Prism 7.04 for Windows (GraphPad Software, La Jolla, California, USA). Independently of the statistical method used, significance was considered whenever $p < 0.05$.

RESULTS

Dietary switch from HFD to standard chow normalizes biometric data and whole-body metabolic function

Body weight was monitored throughout the experiment (Fig. 1a). Mice from CTRL group were the heaviest after weaning (CTRL: 13.09 ± 1.31 g; HFD: 6.64 ± 0.23 g; HFD_t: 7.38 ± 0.29 g), but that difference faded in 7 days. By the 35th day after weaning, mice from the HFD and HFD_t groups were heavier than mice from the CTRL group (CTRL: 23 ± 0.7 g; HFD: 24 ± 0.6 g; HFD_t: 26 ± 1.0 g). This difference increased up to 60 days after weaning, when mice fed with HFD and HFD_t weighed, approximately, 20% more than CTRL mice. At this time point, HFD in mice from the HFD_t group was replaced by standard chow. Fourteen days later, HFDt mice had lost weight and reached the mean values of mice from the control (CTRL: 27 ± 0.5 g; HFD_t: 28 ± 0.9 g), while mice fed with the HFD continued to gain weight (34 ± 1 g). At sacrifice, HFD mice were 50% heavier than CTRL and HFD_t mice.

Gonadosomatic index (GSI) (Fig. 1b) was determined as the relative size of gonads to total body mass. Similarly, body fat (Fig. 1c) was calculated as the ratio of adipose tissue to total body weight. GSI of mice fed with a HFD was lower than in mice from CTRL and HFD_t group. Weight gain as WAT is the main source for

body weight increase and lower GSI in HFD (Supplementary data, table S2). Liver was 20% heavier in mice fed with HFD (1.4 ± 0.09 g) than in CTRL mice (1.2 ± 0.02 g) and 27% heavier than in mice from the HFDt group (1.1 ± 0.03 g). Finally, mice fed with a HFD presented higher glycaemia levels during ipGTT (Fig. 1d), during ipITT (Fig. 1e) and after overnight fast (CTRL: 95 ± 2 mg/dL; HFD: 116 ± 4 mg/dL; HFD_t: 85 ± 3 mg/dL) (Fig. 1f). HFD_t mice showed fasting glycaemia levels in line with CTRL group.

Diet switch in mice does not restore the decreased sperm quality induced by consumption of a HFD

We collected sperm from mice epididymis immediately after sacrifice and characterised sperm quality by analysing sperm count, viability, motility and morphology. We found no differences in sperm count in mice from the different groups (Fig. 2a). However, mice fed with a HFD, even transiently, had approximately 12% and 15% less motile spermatozoa than mice from the control group (CTRL: 82 ± 1 %; HFD: 69 ± 2 %; HFD_t: 66 ± 4 %) (Fig. 2b). We have observed the same tendency in sperm viability. Mice from the HFD and HFD_t groups presented one-fifth less viable spermatozoa than mice from the control group (CTRL: 48 ± 2 %; HFD: 38 ± 2 %; HFD_t: 35 ± 2 %) (Fig. 2c). Concerning sperm morphology, smears from mice of the CTRL group displayed 6% more normal spermatozoa (40 ± 2 %) than mice fed with a HFD (34 ± 2 %), while those mice had 6% more (44 ± 2 %) spermatozoa with tail defects than mice fed with HFD_t (39 ± 1 %) (Fig. 2d). In addition, the animals from the HFDt group revealed the highest percentage of spermatozoa with head defects (25 ± 1 %).

Nevertheless, no changes were observed on any of the accessed reproductive parameters (reproductive success ratio, litter size and male ratio) (data not shown).

Diet switch in mice did not restore energy metabolism homeostasis in the testis

Overall, metabolome analysis revealed a unique metabolic signature for each group in mice testes. Regarding the content of metabolites related to energy-producing metabolic pathways, it revealed evident differences between CTRL and groups fed with high fat diets (Fig. 3). The AMP content in testes of mice from HFD and HFD_t groups was increased 4-fold and 3-fold respectively, when compared to the levels detected in the testis of CTRL mice (CTRL: $4.7 \times 10^{-4} \pm 8.5 \times 10^{-5}$ a.u.; HFD: $2.0 \times 10^{-3} \pm 1.9 \times 10^{-4}$ a.u.; HFD_t: $1.4 \times 10^{-3} \pm 3.6 \times 10^{-4}$ a.u.) (Fig. 3a). Conversely, adenosine levels decreased by 30% and 20% in testes of mice from HFD and HFD_t (CTRL: $2.3 \times 10^{-3} \pm 1.9 \times 10^{-4}$ a.u.; HFD: $1.6 \times 10^{-3} \pm 9.6 \times 10^{-5}$ a.u.; HFD_t: $1.8 \times 10^{-3} \pm 2.7 \times 10^{-4}$ a.u.) (Fig. 3b). Notably, the levels of lactate were not affected by diet regime (Fig. 3c), whereas acetate levels were 50% higher in the testis of mice from the CTRL group ($2.3 \times 10^{-3} \pm 7.3 \times 10^{-5}$ a.u.) than in those from mice of the HFD ($1.6 \times 10^{-3} \pm 8.5 \times 10^{-5}$ a.u.) and HFD_t ($1.5 \times 10^{-3} \pm 5.5 \times 10^{-5}$ a.u.) groups (Fig. 3d). Regarding succinate, the content in testes of mice from the HFD_t ($8.0 \times 10^{-3} \pm 1.9 \times 10^{-3}$ a.u.) was more than 2-fold higher than ~~the levels detected~~ in testes of mice from the CTRL group ($3.3 \times 10^{-3} \pm 4.6 \times 10^{-4}$ a.u.), and more than 3-fold the value obtained in testes of mice from the HFD group ($2.4 \times 10^{-3} \pm 4.6 \times 10^{-4}$ a.u.) (Fig. 3e). Creatine remained unchanged in testes of mice from the different experimental groups (Fig. 3f).

Mice testicular content of amino acids remained altered even after diet switch

Testicular levels of six amino acids were analysed: 3 intermediaries of the alanine shuttle (alanine, glutamate and glutamine), and 3 branched chain amino acids (BCAAs) (valine, leucine and isoleucine). No differences were found in the testicular content of alanine (Fig. 4a). Glutamate was increased in testis of mice from the HFD_t group (Fig. 4b), while testicular glutamine levels were higher in mice from the HFD and HFD_t groups (Fig. 4c). Testes from mice fed with HFD_t were 10% richer in glutamate ($1.6 \times 10^{-2} \pm 2.7 \times 10^{-4}$ a.u.) than testes of mice from other groups (CTRL: $1.5 \times 10^{-2} \pm 4.4 \times 10^{-4}$ a.u.; HFD: $1.5 \times 10^{-2} \pm 3.8 \times 10^{-4}$ a.u.). The testicular content of glutamine in mice from HFD and HFD_t groups was respectively 15% ($5.6 \times 10^{-3} \pm 2.1 \times 10^{-4}$ a.u.) and 20% ($5.8 \times 10^{-3} \pm 1.1 \times 10^{-4}$ a.u.) greater than those from the CTRL group ($4.8 \times 10^{-3} \pm 2.4 \times 10^{-4}$ a.u.). Concerning BCAAs, valine (Fig. 4d), leucine (Fig. 4e) and isoleucine (Fig. 4f), we ~~have~~ only observed alterations in the testicular content of leucine. Its levels were 19% lower in testes of mice fed with HFD ($1.5 \times 10^{-3} \pm 1.1 \times 10^{-4}$ a.u.) and 8% lower in testes of mice fed with HFD_t ($1.8 \times 10^{-3} \pm 7.0 \times 10^{-5}$ a.u.) than in testes of mice from CTRL group ($1.9 \times 10^{-3} \pm 4.7 \times 10^{-5}$ a.u.).

Diet switch in mice was not able to restore the oxidative profile in testis

Changes were also observed in the testicular levels of metabolites related to antioxidative defence, in a diet-specific manner (Fig. 5). Our results showed that betaine increased approximately by 20% in testis of mice fed with HFD ($9.5 \times 10^{-2} \pm 3.0 \times 10^{-3}$ a.u.), comparing to the levels detected in the testis of CTRL mice

(7.9 x 10^{-2} ± 4.7 x 10^{-3} a.u.) and mice fed with HFD_t (8.2 x 10^{-2} ± 3.2 x 10^{-3} a.u.) (Fig. 5a). No differences were found in testicular content of taurine (Fig. 5b). Glutathione, the key cellular antioxidative component in mammals, was 60% more abundant in testes from both mice fed with HFD (3.7 x 10^{-3} ± 2.8 x 10^{-4} a.u.) and HFD_t (3.7 x 10^{-3} ± 2.4 x 10^{-4} a.u.), comparing to CTRL mice (2.3 x 10^{-3} ± 4.76 x 10^{-4} a.u.), suggesting increased oxidative stress (Fig. 5c). However, unlike betaine, testicular glutathione levels were not restored by diet switch.

Testicular metabolic profile is associated with glucose (in)tolerance and sperm quality

Individual values for biometric data, glucose homeostasis, sperm parameters and testicular metabolites were mutually correlated (Supplementary data, tables S3 - S10). A strong correlation was found between total body weight and fat mass ($r = 0.8209$, $p < 0.0001$), and individual fat depots; epididymal fat ($r = 0.8793$, $p < 0.0001$), perirenal fat ($r = 0.8959$, $p < 0.0001$) and retroperitoneal fat ($r = 0.7695$, $p < 0.0001$). (Table S3). Strong correlation was observed between total body and liver weight ($r = 0.7015$, $p < 0.0001$). WAT weight and fat mass establish positive correlations with AUC in ipGTT and ipITT (Table S4). WAT weight correlates with sperm defects and notably, retroperitoneal fat was correlated with pin head spermatozoa (a type of head defect) ($r = 0.4338$, $p < 0.05$) (Table S5). Insulin resistance is a risk factor towards abnormal spermatozoa ($r = -0.5029$, $p < 0.005$) (Table S6). Overall, WAT was associated with a decrease in leucine, isoleucine and succinate, while BAT ~~specifically~~ negatively correlates with isoleucine ($r = -0.117$, $p < 0.05$) (Table S7). Total body weight showed a very strong positive

correlation with taurine ($r = -0.8194$, $p < 0.0005$), whereas taurine was negatively correlated to testis weight. Glucose intolerance is correlated with betaine ($r = 0.5180$, $p < 0.05$), conversely to succinate ($r = -0.5059$; $p < 0.05$) and creatine ($r = -0.7285$, $p < 0.005$) (Table S8). A correlation matrix illustrates the relation between testicular metabolites and sperm parameters (Fig. 6a). Amino Acids were negatively correlated with sperm counts (sperm concentration) and sperm viability; *e.g.* glutamine is positively correlated to pin head defects ($r = 0.5771$, $p < 0.05$). Acetate was found to be a protective factor for sperm quality (Table S9).

Male reproductive dysfunction in mice is caused by metabolic interference of HFD

After analysing all the individual correlations between biometric data, glucose homeostasis, sperm parameters and testicular metabolome of our experimental model, we explored how they associate with the differences observed in the first stage of our study (Fig. 6). For mice fed with HFD, four metabolic pathways were found impacted in testes (Fig. 6b). Pyruvate (PI = 0.15, $p = 1.10 \times 10^{-3}$, FDR = 3.60×10^{-2}) and glutamate metabolism (PI = 0.14, $p = 1.46 \times 10^{-3}$, FDR = 3.60×10^{-2}) were the most significantly affected pathways in testes. Ethanol degradation (PI = 0.49, $p = 3.48 \times 10^{-3}$, FDR = 6.89×10^{-2}) and ammonia recycling (PI = 0.22, $p = 9.87 \times 10^{-3}$, FDR = 0.14) were also affected. The same metabolic pathways were affected in mice fed with HFD_t (Fig. 6c). Glutamate metabolism was the most affected metabolic pathway (PI = 0.45, $p = 2.60 \times 10^{-6}$, FDR = 2.57×10^{-4}), 3-fold more impacted than in HFD group. Ammonia recycling (PI = 0.31, $p = 5.75 \times 10^{-4}$, FDR = 1.14×10^{-2}) was 40% more impacted in testes of mice fed with HFD_t. Pyruvate metabolism and ethanol degradation were equally impacted in

testes of mice from HFD and HFD_t. Additionally, HFD_t mice suffered impact in testicular purine metabolism (PI = 0.12, p = 5.25 x 10^{-4}, FDR = 1.14 x 10^{-2}), urea cycle (PI = 0.19, p = 4.55 x 10^{-4}, FDR = 1.14 x 10^{-2}), cysteine metabolism (PI = 0.29, p = 9.93 x 10^{-3}, FDR = 6.15 x 10^{-2}) and glutathione metabolism (PI = 0.41, p = 9.10 x 10^{-4}, FDR = 6.01 x 10^{-2}).

DISCUSSION

The obesity pandemic and the decline of sperm quality share a timeline with a common denominator: high-fat diets (World Health Organization 2000, Skakkebaek et al. 2016). Yet, there is a gap on mechanistic studies on the subject. Clinical studies often have confounding factors ~~of human samples~~. Thus, animal models can be a reliable choice to study the molecular mechanisms of HFD-induced male infertility. Herein, we used NMR-based metabolomics to identify testicular metabolic fingerprints induced by HFD and how weight loss caused by dietary switch from HFD to normal diet affects sperm quality, in a rodent model (*mus musculus*). A number of metabolomics-based studies were already used to unveil the molecular mechanisms associated with male fertility (dys)function (Deepinder et al. 2007, Minai-Tehrani et al. 2016). Recently, we described aging-associated testicular changes using NMR-based metabolomics in a rodent model (Jarak et al. 2018). In the present study, we detected distinct testicular metabolic signatures in animals fed by HFD, even if temporarily. Additionally, changes observed in testicular metabolome were correlated with general metabolic status and parameters of sperm quality ~~parameters~~.

Our group has extensively addressed the changes induced by HFD and related diseases in the male reproductive system. We have described mechanisms by which HFD induce a pre-diabetic state and cause functional changes in the testis (Rato et al. 2013). In addition, we demonstrated how energy homeostasis triggers specific metabolic shifts in Sertoli Cells (Martins et al. 2015, Jesus et al. 2016, Martins et al. 2016, Martins et al. 2019). We further discussed how excess accumulation of lipids due to obesogenic substances impairs sperm maturation (Rato et al. 2017). Besides, deleterious effects associated to pathological states in energy homeostasis impair endocrine function and metabolic balance in testis that can cause abnormal sperm parameters (Rato et al. 2014, Alves et al. 2015, Rato et al. 2015), suggesting functional changes in testicular cells beyond the somatic SCs. In the present work, we add a new level of evidence, based on Multivariate Analysis and Metabolomics, to show that the metabolic fingerprints induced by HFD in testes may not be fully reverted by dietary switch.

Poorer sperm quality was found in mice fed with a HFD. Interestingly, even though mice from the HFD_t group were fed with a balanced diet for the majority of their lives, some metabolic changes and defects in sperm quality caused by HFD and/or previous obesity were not reversed by dietary change/weight loss. The period that mice from the HFD_t group were fed with a HFD (60 days) overlaps with childhood, puberty and the early years of adulthood, a critical age for male reproductive maturation (Dutta and Sengupta 2016). The first two spermatogenic cycles in mice occur during this period (Whittingham and Wood 1983), stressing the importance of testicular environment in this phase for successful reproductive outcomes later in life (Woodruff et al. 2008). This fact is even more interesting considering that diet switch and weight loss were effective in recovering mice

weight to normal level and in preventing the onset of metabolic syndrome. In *Mus Musculus*, a spermatogenic cycle takes approximately 9 days, while spermatogenesis takes 40 days (Costa et al. 2018). Therefore, in mice from the HFD_t group, the time between diet reversion and sacrifice allows the completion of 11 spermatogenic cycles, which are enough to correctly access the impact of the new diet in sperm parameters. Nevertheless, it is interesting that sperm parameters did not lead to poorer reproductive outcomes. No differences were found in pregnancy success rates, litter size nor male ratio, though litter size was smaller than officially reported in this strain (Murray et al. 2010). Our observations have a translational echo in a recent meta-analysis by (Lee et al. 2019), were the researchers report no improvements in sperm quality after metabolic bariatric surgery, despite the improvements on overall metabolic and endocrine function.

Our data shows that WAT is the main source for body weight differences observed between groups. Besides, the differences detected in liver weight underline the role of this organ in mediating the effects induced by HFD. We found a very strong positive correlation between WAT and liver weight, particularly in HFD mice. This correlation has been widely described and reflects steatosis caused by diet (Samuel et al. 2004, Fraulob et al. 2010, Gao et al. 2015, Lai et al. 2015), a condition that may progress to Non-Alcoholic Fatty Liver Disease (NAFLD) and ultimately to cirrhosis. In addition, WAT accumulation and liver enlargement (steatosis) are predictive factors for metabolic dysfunction, towards metabolic disease (Dixon et al. 2001, Kotronen and Yki-Jarvinen 2008). We found poorer performance of mice fed with HFD during ipGTT and ipITT, which reflect metabolic syndrome onset. In fact, WAT deposits are extremely correlated with poorer performance in ipGTT and ipITT, and can be regarded as the link between

HFD and the onset of metabolic disease (Rato et al. 2013). ~~Despite mice fed with HFD_t performed similarly to mice fed with standard chow during the metabolic stress tests, which are favourable prognoses against the onset of metabolic disease after diet switch, those mice were slower to react to insulin injection than mice fed with normal diet, illustrating a certain degree of insulin insensitiveness.~~

Testicular metabolome and ~~the~~ evaluated fertility parameters were distinct in HFD and HFD_t groups, although globally negatively affected by HFD. In both groups, testicular metabolome was enriched in AMP, glutamine and glutathione, whereas acetate content was strikingly reduced. In addition, nucleic acid metabolism was significantly affected by HFD. AMP participates in energy production homeostasis, which is indispensable in highly metabolically active tissue. AMP is involved in the regulation of lipolysis and lipogenesis in response to insulin, thus it is a marker for whole-body metabolic homeostasis (Hajer et al. 2008). The increase in AMP content was correlated with the weight of BAT, WAT and liver, but not total body weight. Also, AMP is involved in spermatogenesis through cAMP activated signalling pathways (AMPK). AMPK signalling is an important regulator of the nutritional support of spermatogenesis by regulating Sertoli cell metabolism (Galardo et al. 2007), thus highlighting the relevance of these signalling pathways and how they respond to fat. However, the metabolic hallmarks observed, as glucose intolerance, are mostly correlated with epididymal fat, which humans lack (Wronska and Kmiec 2012). Thus, caution should be taken when discussing these effects in humans. Yet, the model was able to correlate retroperitoneal fat weight with the prevalence of head and tail defects in sperm of mice (Chen et al. 2013, McPherson and Lane 2015), which was already reported in humans (Martini et al. 2010, Rato et al. 2017).

Concerning testicular metabolome, we found that glutamine and acetate were correlated with sperm quality. Glutamine is an important energetic substrate for the somatic Sertoli cells (Oliveira et al. 2015). Hence, testicular accumulation of glutamine may indicate SC metabolic dysfunction. On the other hand, acetate, an energy substrate obtained from lipolysis (Martins et al. 2015), was reduced in both groups of mice fed with HFD and HFD_t. We may hypothesize that acetate is being redirected for membrane remodelling, possibly due to increased cell turnover in testes (Alves et al. 2012). This assumption is supported by the changes found in antioxidant intermediaries and in purine metabolism. On the other hand, acetate excretion by SC may be inhibited by the effect of leptin (Martins et al. 2015). ~~Thus, acetate and glutamine may be associated with the detected alterations in sperm parameters.~~ Glutathione content was upregulated in testes of mice from both HFD groups, whilst betaine content was only increased in those fed with HFD. Diet switch failed to attenuate oxidative stress as evidenced by the increased levels of glutathione. High levels of glutathione in testes of HFD-fed mice were associated with higher prevalence of sperm pin head defects, lower sperm viability and decreased sperm motility.

The consumption of HFD is associated with local inflammatory process in mice testes (Chen et al. 2013), which leads to glutathione overexpression. Recalling data obtained on testicular acetate content, this increase may be related to a local membrane remodelling process, associated with germ cell turnover by SCs (Martins et al. 2015). Although we should be cautious about this claim, as no apoptotic markers in testes were assessed, this response to HFD has been previously reported (Isidori et al. 1999, Mu et al. 2017). Yet, it is possible to assume that diet, rather than fat deposition, is the major contributor to changes

in sperm viability, and glutathione and acetate testicular content. All those metrics were changed in HFD groups, but did not significantly correlated with fat mass nor fat deposits. Testes of mice fed with HFD were further enriched in betaine, an intermediary of antioxidant pathways that is also associated with glucose intolerance and insulin resistance (Wang et al. 2011). Indeed, we have also found a strong correlation between betaine and the AUC of ipGTT in HFD-fed mice. Testicular betaine content was also strongly correlated with weight gain of WAT pads, which may link the presence of this metabolite in testes to a direct effect of HFD. Decreased levels of testicular leucine is another hallmark of HFD. We found that leucine content in testes was negatively correlated with WAT weight. According to the metabolite correlation matrix (Supplementary data, Table S10), leucine is negatively correlated with AMP, which may indicate an AMPK-related signalling cascade repressing leucine excretion or leucine consumption in testes of mice fed with HFD. Although increased serum concentration of leucine has been correlated with the onset of T2DM in humans (Wang et al. 2011), we have observed an opposite trend in our study: mice fed with HFD, which displayed glucose intolerance and insulin resistance, had lower leucine levels in testes. This tissue-specific difference should not be overlooked and shows a functional specificity of the testis concerning amino acids metabolism, which should be further explored.

The most distinct metabolic feature in testes of mice fed with HFD_t was the enrichment in adenosine, glutamate and succinate. Overall these three metabolites are associated with WAT lipid mobilization, weight loss, decreased percentage of sperm with bent neck, increased percentage of sperm pin head defects and improved glucose tolerance. Succinate has been correlated with

sperm defects and glucose intolerance. A strong correlation was also observed with perirenal and epididymal fat lipid mobilization. Previous studies ~~have~~ reported changes in gene expression of epididymal WAT in response to diet restriction, namely in energy and lipid metabolism, with particular inhibition of genes related to mitochondrial pathways (Duivenvoorde et al. 2011). The authors reported the same phenotypic changes we observed in mice fed with HFD_t, such as weight loss and metabolic normalization. The accumulation of succinate in testes of mice fed with a HFD_t also suggests increased TCA activity and mitochondrial involvement in the irreversible deleterious effects caused by a diet rich in fats. Although lipophagy is primarily described in adipocytes and hepatocytes, with pivotal implications in lipid homeostasis (Liu and Czaja 2012, Christian et al. 2013), we should not overlook the possibility that a similar process occurs in testes, namely in SCs. Lipophagy allows the release of lipids from lipid droplets, which can then be recruited for metabolic functions (Liu and Czaja 2012). This process is another energy-triggered phenomenon and HFD, obesity and hyperinsulinemia inhibit it, prompting lipid accumulation (Liu and Czaja 2012, Christian et al. 2013). Nevertheless, the process is triggered by cytoplasmatic concentrations of FFAs and cholesterol, rather than extracellular availability (Liu and Czaja 2012). In fact, SCs accumulate lipid droplets as a result of phagocytosis of apoptotic germ cells (Wang et al. 2006).

Interestingly, we found that pyruvate metabolism, glutamate metabolism, ethanol degradation and ammonia recycling in testes were irreversibly affected by HFD, as the differences were verified even after dietary switch and weight loss. Furthermore, testes of mice fed with HFD_t presented alterations in purine metabolism, urea cycle, cysteine metabolism and glutathione. Those alterations,

associated with antioxidant defences and metabolic reprograming, are linked to testicular metabolic adaptations to diet switch, posterior to sexual maturation.

Overall, our observations have a translational echo in a recent meta-analysis by Lee et al. (2019), which concluded that there are no improvements in sperm quality after metabolic bariatric surgery, despite the improvements on overall metabolic and endocrine function. In another review, Oliveira et al. (2017) postulates that dietary interventions, such as bariatric surgery, exercise plans or caloric restriction may not present satisfactory results to male fertility, as due to a collateral damages of induced by the sudden weight loss, and prospective accumulation of endogenous toxicants. Our study has, however, some limitations. We have only used polar testicular fractions to quantify metabolites, and there are dissimilarities between animal models and humans. Furthermore, our experimental model does not allow us to differentiate between HFD and weight gain/obesity, because the switch from HFD to normal diet comprises dietary switch plus weight loss. Despite several sperm parameters have been affected by the consumption of HFD, we are cautious to draw conclusions regarding the reproductive success of these mice, as our protocol does not provide a extensive assessment of fertility outcomes. Nevertheless, this is the first study showing that excess of weight/obesity until early adulthood can cause irreversible damage to testicular function and sperm quality even when diet reversion restored normal global metabolic status. The biomarkers reported in this study are potential clinical targets for prevention and treatment of obesity-related infertility in males.

Declaration of interest

The authors declare no conflict of interest.

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Author Contributions

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

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Supplementary Data

Tables S1 – S10 are provided as Supplementary Data.

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FIG. LEGENDS

Fig. 1: Biometric and metabolic characterisation of mice in this experiment, per group.

Results are expressed as mean ± SEM (n = 12 for each group). a) Total body weight (g) evolution throughout the experiment (days). Weight was evaluated individually every seven days. Results were tested by a Repeated Measures (RM) two-way ANOVA with Tukey's Multicomparison test. Fig. 1b) and 1c) show the comparison of Gonadosomatic Index (GSI) and Fat mass mean between experimental groups, respectively. Results are expressed as the mean (%) ± SEM (n = 12 for each group). Results were tested by one-way ANOVA with Tukey's Multicomparison test. Mice glycaemia (mg/dL) during the ipGTT - intraperitoneal Glucose Tolerance Test (Fig. 1d) and ipITT - intraperitoneal Insulin Tolerance Test (Fig. 1e). Results for ipGTT and ipITT were tested by RM two-way ANOVA with Tukey's Multicomparison test. Data for fasting glucose before sacrifice (Fig. 1f) was compared using Ordinary one-way ANOVA with Tukey's Multicomparison test. # - Dietary switch; † – CTRL vs HFD; ‡ – CTRL vs HFDt; § – HFD vs HFDt. * $p < 0.05$; ** $p < 0.005$; *** < 0.0005; **** $p < 0.0001$.

Fig. 2: Characterisation of mice sperm parameters.

Samples were directly obtained from mice epididymis. Results are expressed as the mean ± SEM (n = 12 for each group). Mice sperm concentration (Fig. 2a) and sperm motility (Fig. 2b) were assessed immediately after sample collection. Sperm viability (Fig. 2c) was evaluated in eosin-negrosin dyed smear, while sperm morphology (Fig. 2d) was classified in smears dyed with Diff-Kwik. Sperm

morphology was grouped in three categories: Normal, Head defects (decapitated, pin head, flattened head), and Tail defects (bent neck, coiled tail). Samples with more than 30% decapitated sperm were not considered. The means for sperm counts, motility and viability were tested by one-way ANOVA with Tukey's Multicomparison test. The means for sperm morphology classes were tested by Mixed factorial ANOVA with Tukey's Multicomparison test. Statistical significance was considered when $p \leq 0.05$. Significance marks stand as it follows: † –vs CTRL; ‡ –vs HFD; § – vs HFD_t. * $p < 0.05$; ** $p < 0.005$; *** < 0.0005; **** $p < 0.0001$.

Fig. 3: ^{1}H-NMR quantification of testicular metabolites related to energy-obtaining pathways.

Absolute quantification of a) AMP, b) adenosine, c) lactate, d) acetate, e) succinate and f) creatine, metabolites involved metabolic processes related to energy obtaining, using ^{1}H-NMR. Results are expressed as mean ± SEM (n = 6 for each condition). Results were tested by one-way ANOVA with Tukey's Multicomparison and were considered significant when $p < 0.05$. † –vs CTRL; ‡ –vs HFD; § – vs HFD_t. * $p < 0.05$; ** $p < 0.005$; *** < 0.0005; **** $p < 0.0001$.

Fig. 4: ^{1}H-NMR quantification of testicular AAs.

Absolute quantification of testicular content of the amino acids a) alanine, b) glutamate, c) glutamine, d) valine, e) leucine and f) isoleucine, using 1H-NMR. Results are expressed as mean ± SEM (n = 6 for each condition). Results were tested by one-way ANOVA with Tukey's Multicomparison, and were considered

significant when $p < 0.05$. † –vs CTRL; ‡ –vs HFD; § – vs HFD_t. * $p < 0.05$; ** $p < 0.005$; *** < 0.0005; **** $p < 0.0001$.

Fig. 5: ^{1}H-NMR quantification of testicular antioxidants.

Absolute quantification of a) betaine, b) taurine and c) glutathione, metabolites involved in the antioxidative action, using ^{1}H-NMR. Results are expressed as mean ± SEM (n = 6 for each condition). Results were tested by one-way ANOVA with Tukey's Multicomparison, and were considered significant when $p < 0.05$. † –vs CTRL; ‡ –vs HFD; § – vs HFD_t. * $p < 0.05$; ** $p < 0.005$; *** < 0.0005; **** $p < 0.0001$.

Fig. 6: Metabolomics integration of NMR data.

Summary of main metabolomic correlations drawn from our experimental model in testis. a) Simplified correlation matrix of Sperm parameters vs testicular metabolites. The scale represents Spearman r coefficient values, when $p < 0.05$ (n = 16, Exact p-value). b) MetaboAnalyst 4.0 Pathway Analysis Tool was used to identify the most affected metabolic pathways in HFD mice, using the list of metabolites which presented differences from CTRL group. Hence, input metabolites were: leucine, betaine, AMP, acetate, glutamine and glutathione. 1 – pyruvate metabolism; 2 – glutamate metabolism; 3 – ethanol degradation; 4 – ammonia recycling. c) The same methodology as used to profile the most relevant metabolic changes in HFDt group. Input metabolites were: adenosine, succinate, glutamate, AMP, acetate, glutamine and glutathione. 1 – glutamate metabolism; 2 – purine metabolism; 3 – urea cycle; 4 – ammonia recycling; 5 –

pyruvate metabolism; 6 – cysteine metabolism; 7 – glutathione metabolism; 8 – ethanol degradation.

Table S1: Resonance assignment in the ^{1}H NMR spectra of polar testicular extracts (s: singlet, d: doublet, dd: doublet of doublets, t: triplet, m: multiplet).

	Compound	δ ^{1}H ppm (multiplicity, assignment)
1	acetate[a]	1.92 (s, αCH_3)
2	adenine	8.18 (s, C8H ring), 8.22 (s, C2H ring)
3	adenosine	3.83 (m, ribose), 3.91 (m, ribose), 4.28 (m, ribose), 4.44 (m, ribose), 4.79 (m, ribose), 6.10 (d, C1'H ribose), 8.23 (s, C8H ring), 8.35 (s, C2H ring)
4	alanine	1.48 (d, βCH_3), 3.78 (t, αCH)
5	aspartate	2.66 (dd, βCH), 2.81 (dd, $'CH$), 3.86 (m, αCH)
6	betaine	3.26 (s, CH_3), 3.90 (s, αCH_2)
7	choline	3.20 (s, CH_3), 3.51 (m, NCH_2), 4.06 (m, OCH_2)
8	citrate	2.49 (d, $\alpha,\gamma CH$), 2.69 (d, $\alpha\ ',\gamma' CH$)
9	creatine	3.03 (s, CH_3), 3.93 (s, CH_2)
10	dimethylglycine	2.91 (s, αCH), 3.72 (s, CH_3)
11	ethanolamine	3.14 (m, NCH_2), 3.82 (m, CH_2)
12	formate	8.46 (s, CH)
13	glutamate	2.07 (m, βCH), 2.14 (m, $\beta' CH$), 2.35 (m, γCH_2), 3.76 (m, αCH)
14	glutamine	2.13 (m, βCH_2), 2.45 (m, γCH_2), 3.75 (m, αCH)
15	glutathione	2.16 (q, βCH_2 Gl), 2.56 (m, γCH_2 Glu), 2.95 (m, βCH_2 Cys), 3.79 (m, αCH Gly), 4.59 (m, CH_2 Cys)
16	glycerol	3.56 (dd, $C1H_2$), 3.65 (dd, $C3H_2$), 3.79 (m, C2H)
17	glycerophosphocholine	3.23 (s, CH_3), 3.68 (m, NCH_2), 3.88 (m, αCH_2), 4.33 (m, OCH_2)
18	glycine	3.55 (s, CH_2)
19	3-hydroxybutyrate	1.20 (d, γCH_3), 2.42 (dd, αCH), 4.15 (m, βCH)
20	hypoxanthine	8.19 (s, C2H), 8.21 (s, C8H)
21	IMP/AMP	3.75 (ribose), 4.03 (ribose), 4.36 (ribose), 4.5 (ribose), 6.14 (d, C1'H ribose), 8.26 (s, C8H ring), 8.59 (s, C2H ring)
22	isoleucine	0.94 (t, δCH_3), 1.01 (d, $\beta' CH_3$), 1.26 (m, γCH), 1.49 (m, $\gamma' CH$), 1.98 (m, βCH), 3.67 (m, αCH)
23	lactate	1.33 (d, βCH_3), 4.11 (q, αCH)
24	leucine	0.96 (t, δCH_3), 1.73 (m, $\beta\gamma CH_2$), 3.73 (m, αCH)
25	lysine	1.72 (m, δCH_2), 1.92 (m, βCH_2), 3.01 (m, εCH_2), 3.76 (m, αCH)
26	*myo*-inositol	3.26 (t, C5H), 3.52 (dd, C1H, C3H), 3.62 (m, C4H, C6H), 4.06 (t, C2H)
27	phosphocholine	3.22 (s, CH_3), 3.59 (m, NCH_2), 4.17 (m, OCH_2)
28	phosphoethanolamine	3.21 (M, NCH_2), 3.97 (m, OCH_2)
29	scyllo-inositole	3.35 (s, CH)
30	succinate	2.40 (s, CH_2)
31	taurine	3.26 (t, NCH_2), 3.43 (t, SCH_2)
32	tyrosine	6.87 (d, C3H, C5H ring), 7.18 (d, C2H, C6H ring)
33	uridine	4.33 (m, C2'H ribose), 5.89 (d, C1'H ribose), 5.91 (d, C5H ring), 7.87 (d, C6H ring)
34	valine	0.99 (d, γCH_3), 1.04 (d, $\gamma' CH_3$), 2.26 (m, βCH), 3.62 (m, αCH)

[a] all metabolites are putatively annotated (level 2 of identification according to Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative recommendations)

Table S2: Mice organ and tissue weight. Organs and tissue were collected at sacrifice, 200 days after weaning. Results were tested by one-way ANOVA with Tukey's Multicomparison test. Statistical significance was considered when $p < 0.05$. Results are expressed as mean (g) ± SEM. † –vs CTRL; ‡ –vs HFD; § – vs HFDt. * $p < 0.05$; ** $p < 0.005$; *** < 0.0005; **** $p < 0.0001$.

	L. testis	R. testis	R. epididymis	Liver	Seminal vesicles	Epididymal fat	Perirenal fat	Retroperitoneal fat	BAT
CTRL	0.11 ± 0.00	0.11 ± 0.00	0.06 ± 0.00	1.15 ± 0.02	0.25 ± 0.02	0.73 ± 0.07	0.29 ± 0.04	0.32 ± 0.04	0.14 ± 0.01
HFD	0.12 ± 0.00	0.11 ± 0.00	0.06 ± 0.00	1.38 ± 0.09 †*	0.31 ± 0.01	2.21 ± 0.23 †****	1.56 ± 0.11 †****	2.52 ± 0.21 †****	0.18 ± 0.01
HFD_t	0.11 ± 0.00	0.11 ± 0.00	0.06 ± 0.00	1.08 ± 0.13 ‡***	0.27 ± 0.03	0.63 ± 0.09 ‡****	0.23 ± 0.04 ‡****	0.35 ± 0.06 ‡****	0.16 ± 0.01

Table S3: Correlation factors between testicular mice total body weight and collected tissues. GSI and Fat mass were also considered in this correlation as they are result from weight measurements of gonads and adipose tissue, respectively. The correlation was obtained using the Spearman r with Approximate p-value (n = 35). * $p < 0.05$; ** $p < 0.005$; *** < 0.0005; **** $p < 0.0001$.

	R testis	L testis	R epididymis	Seminal Vesicles	Liver	GSI	BAT	Epididymal fat	Perirenal fat	Retroperiton. fat	Fat mass
r value	0.4021 *	0.0703	0.0241	0.4732 **	0.7015 ****	-0.7964 ****	0.4294 *	0.8793 ****	0.8959 ****	0.7695 ****	0.8209 ****
P (two-tailed)	0.0166	0.6882	0.8907	0.0041	< 0.0001	< 0.0001	0.0100	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Table S4: Correlation factors between biometric data and Area Under Curve (AUC) of ipGTT and ipITT. The correlation was obtained using the Spearman r with Approximate p-value (n = 35). * $p < 0.05$; ** $p < 0.005$; *** < 0.0005; **** $p < 0.0001$.

		Fasting glycaemia	R testis	L testis	R epididymis	Seminal Vesicles	Liver	GSI	BAT	Epididymal fat	Perirenal fat	Retroperiton. fat	Fat mass
AUC ipGTT	r value	0.4700 **	0.1386	-0.1492	0.2386	0.4069 *	0.3553 *	-0.6416 ****	0.2173	0.6457 ****	0.549 ***	0.5722 ***	0.5666 ***
	P (two-tailed)	0.0044	0.4271	0.3922	0.1675	0.0153	0.0362	< 0.0001	0.2099	< 0.0001	0.0006	0,0003	0.0004
AUC ipITT	r value	0.5399 ****	0.1747	-0.1257	0.2035	0.3245	0.2863	-0.5203 **	0.2764	0.5308 **	0.5156 **	0.5637 ***	0.5511 ***
	P (two-tailed)	0.0008	0.3154	0.4717	0.2409	0.0572	0.0955	0.0014	0.1080	0.0010	0.0015	0.0004	0.0006

Table S5: Correlation factors between sperm parameters and biometric data. The correlation was obtained using the Spearman r with Approximate p-value (n = 35). * $p < 0.05$; ** $p < 0.005$; *** < 0.0005; **** $p < 0.0001$.

	R testis	L testis	R epididymis	Seminal Vesicles	Liver	GSI	BAT	Epididymal fat	Perirenal fat	Retroperiton. fat	Fat mass
Sperm concentration	0.2869	**0.4242 ***	-0.2082	0.0058	0.0473	0.1696	-0.0371	0.1406	0.1546	0.1489	0.1927
Sperm viability	0.0527	-0.0785	-0.1004	-0.292	-0.0454	-0.0431	-0.1669	0.0149	-0.0423	-0.1168	-0.044
Sperm motility	0.2542	0.1162	**-0.3837 ***	-0.0629	-0.0007	0.1647	**-0.3798 ***	-0.0599	-0.0192	-0.0501	-0.0792
Normal	-0.0756	0.3157	-0.2495	-0.0425	**-0.4008 ***	0.2987	-0.3354	-0.1499	-0.2671	-0.2648	-0.3013
Decapitated	0.1163	0.0694	0.3448	-0.1378	0.0852	0.1648	-0.0472	-0.1843	-0.1563	-0.2634	-0.1809
Pin head	-0.2398	-0.1684	0.0754	0.1480	-0.0029	-0.2028	**0.4357 ***	0.1462	0.2265	**0.4338 ***	0.3563
Flattened head	-0.2222	-0.0497	-0.2111	0.0246	-0.1914	0.1953	0.0086	-0.142	-0.0804	0.1130	0.0113
Bent neck	-0.1451	-0.1358	0.1985	0.0544	0.2566	**-0.5358 ****	0.1645	**0.4836 ***	**0.3720 ***	**0.3955 ***	**0.4323 ***
Coiled tail	0.1455	-0.1922	0.0120	0.074	0.2426	-0.0664	0.0666	0.0309	0.1410	0.0370	0.0937

Table S6: Correlation factors between Area Under Curve (AUC) of ipGTT and ipITT and sperm parameters. The correlation was obtained using the Spearman r with Approximate p-value (n = 35). * p < 0.05; ** p < 0.005; *** < 0.0005; **** p < 0.0001.

		Sperm concentration	Sperm viability	Sperm motility	Normal	Decapitated	Pin head	Flattened head	Bent neck	Coiled tail
AUC ipGTT	r value	-0.2017	-0.1793	-0.0756	-0.2365	-0.0883	0.1434	-0.1002	**0.5211 ****	-0.0701
	P (two-tailed)	0.2452	0.3028	0.6659	0.2082	0.6425	0.4496	0.5985	0.0031	0.7129
AUC ipITT	r value	-0.1771	-0.1862	0.03712	**-0.5029 ****	0.2450	0.1711	0.0098	**0.4045 ***	-0.0427
	P (two-tailed)	0.3088	0.2843	0.8323	0.0046	0.1919	0.3659	0.9590	0.0266	0.8226

Table S7: Correlation factors between biometric data and Testicular metabolites. The correlation was obtained using the Spearman r with Exact p-value (n = 15). * $p < 0.05$; ** $p < 0.005$; *** < 0.0005; **** $p < 0.0001$.

	Ala	Glu	Gln	Val	Leu	Ile	AMP	Adenosine	Lac	Acetate	Succinate	Creatine	GSH	Betaine	Taurine
R testis	-0.4709	-0.3567	-0.3514	-0.482	-0.3175	-0.4149	0.299	-0.2532	-0.359	0.2807	0.0092	-0.0214	-0.3783	0.3419	**-0.8194 *** **
L testis	**-0.7021 ****	-0.3553	-0.2882	**-0.6478 ***	-0.395	-0.2742	0.3284	-0.2712	-0.4737	0.2501	-0.0060	-0.1206	-0.1778	**0.5035 ***	**-0.7096 ****
R epididymis	-0.0117	0.0273	-0.0399	-0.0563	0.1953	-0.2039	0.0485	0.0893	0.0039	-0.0680	-0.2447	-0.2769	-0.2738	0.0146	-0.2442
Seminal Vesicles	-0.3503	-0.2265	0.3146	**-0.5192 ***	-0.3129	-0.1254	0.441	-0.3112	-0.4542	-0.1947	-0.0501	-0.2974	0.3378	0.476	-0.1345
Liver	0.0944	-0.1152	0.2041	-0.0044	-0.2754	-0.2046	**0.5725 ***	-0.3591	-0.0892	0.1177	-0.2193	-0.2445	0.1148	0.4124	-0.1615
GSI	-0.0414	0.2581	-0.2657	-0.1945	0.2249	0.3508	-0.3847	0.2314	-0.1587	0.2918	**0.6175 ***	0.4196	-0.2845	-0.3968	-0.0879
BAT	0.0885	0.1141	0.1835	-0.1706	-0.3706	**-0.5117 ***	**0.5177 ***	-0.4189	-0.1686	-0.1631	0.2783	-0.1041	0.3561	0.4222	-0.0262
Epididymal fat	-0.4598	-0.3867	0.1487	-0.2794	**-0.6313 ***	-0.4971	**0.5735 ***	-0.4559	-0.2798	-0.2324	**-0.5529 ***	**-0.5416 ***	0.1941	**0.7432 ****	-0.2078
Perirenal fat	-0.403	-0.3806	-0.0022	-0.2548	**-0.5969 ***	**-0.5700 ***	**0.5287 ***	-0.4138	-0.2367	-0.1105	**-0.5287 ***	-0.4024	0.2239	**0.6809 ****	-0.2952
Retroperiton. fat	-0.339	-0.1415	0.0973	-0.2329	**-0.6214 ***	-0.4273	**0.7092 ****	**-0.6054 ***	-0.1241	-0.1395	-0.3531	-0.4499	0.2582	**0.8575 *****	-0.2729
Fat mass	-0.423	-0.3041	0.0206	-0.2735	**-0.7388 ****	**-0.5588 ***	**0.6412 ***	**-0.5353 ***	-0.2253	-0.2235	-0.4176	-0.4268	0.2588	**0.727 ****	-0.2889
Body weight	-0.2552	**-0.5547 ***	0.1186	-0.0662	-0.3166	**-0.5077 ***	0.299	-0.2532	-0.359	0.2807	0.0092	-0.0214	-0.3783	0.3419	**-0.8194 *** **

Table S8: Correlation factors between testicular metabolite content and Area Under Curve (AUC) of IPGTT and IPITT. The correlation was obtained using the Spearman r with Approximate p-value (n = 35). * p < 0.05; ** p < 0.005; *** < 0.0005; **** p < 0.0001.

		Ala	Glu	Gln	Val	Leu	Ile	AMP	Adenosine	Lac	Acetate	Succinate	Creatine	GSH	Betaine	Taurine
AUC ipGTT	r value	-0.1489	0.07085	0.3093	0.02353	-0.262	-0.3294	0.3853	-0.3676	0.1325	-0.4471	**-0.5059 ***	**-0.7285 ****	-0.0382	**0.5180 ***	-0.0486
	P (two-tailed)	0.5797	0.7937	0.2421	0.9345	0.3244	0.2127	0.1414	0.1618	0.6224	0.0844	0.0479	0.0019	0.8910	0.0418	0.8584
AUC ipITT	r value	0.0000	-0.1403	0.3589	-0.0898	-0.0309	-0.4312	0.3473	-0.3974	-0.0737	-0.3297	-0.1810	-0.3586	-0.1781	0.2121	-0.1372
	P (two-tailed)	>0.9999	0.6015	0.1712	0.7400	0.9099	0.0965	0.1866	0.1278	0.7848	0.2112	0.4995	0.1713	0.5066	0.4271	0.6092

Table S9: Correlation matrix of Sperm parameters vs. Testicular metabolites. The correlation was obtained using the Spearman r with Exact p-value (n = 15). * p < 0.05; ** p < 0.005; *** < 0.0005; **** p < 0.0001.

	Ala	Glu	Gln	Val	Leu	Ile	AMP	Adenosine	Lac	Acetate	Succinate	Creatine	GSH	Betaine	Taurine	Creatine
Sperm concentration	**-0.7222 ****	-0.1963	**-0.6421 ***	-0.4059	**-0.574 ***	-0.2706	0.3206	-0.3529	-0.3166	0.2147	-0.0353	-0.0486	-0.1294	0.3238	**-0.6942 ****	-0.0486
Sperm viability	-0.2255	-0.4089	**-0.5626 ***	0.2471	0.2104	-0.1029	-0.3559	0.4118	0.3314	**0.7294 ****	-0.3118	0.1148	**-0.6118 ***	0.0765	-0.4407	0.1148
Sperm motility	-0.2965	-0.3959	-0.4783	0.1251	0.3446	-0.2237	-0.3032	0.3385	0.2417	**0.7314 ****	-0.3797	-0.0604	**-0.6637 ***	0.1311	**-0.5752 ***	-0.0604
Normal	-0.3951	-0.0232	0.0727	0.2090	0.1630	0.2046	-0.2134	0.3102	0.3517	0.2420	-0.2068	-0.1145	-0.1386	0.4736	0.0541	-0.1145
Decapitated	0.0132	-0.0840	-0.2750	0.1077	0.0682	0.2000	-0.2132	0.1648	0.0529	0.1165	0.1253	0.2552	-0.2747	**-0.5765 ***	-0.0794	0.2552
Pin head	0.1678	0.2279	**0.5771 ***	-0.3410	-0.2985	-0.1320	0.462	**-0.5699 ***	-0.3407	**-0.6073 ***	0.1672	-0.3051	**0.7459 ****	0.3106	0.4801	-0.3051
Flattened head	0.0684	0.3050	0.3366	-0.0593	0.0000	0.1473	0.1165	-0.3011	-0.0815	-0.2527	**0.7055 ***	0.3432	0.2571	-0.0330	0.3594	0.3432
Bent neck	-0.1004	0.0597	0.1200	0.2860	-0.3910	-0.4334	0.495	-0.3190	0.3308	-0.2486	**-0.6095 ***	-0.5220	0.2112	**0.6421 ***	-0.1634	-0.5220
Coiled tail	0.2029	-0.1238	-0.1980	-0.4681	0.0704	-0.2308	-0.0681	0.0374	-0.5859 *	0.0462	0.1341	0.0946	-0.0857	-0.2860	-0.1720	0.0946

Table S10: Correlation factors between identified metabolites in the polar fraction of mice testicular extracts. The correlation was obtained using the Spearman r with Exact p-value (n = 15). * p < 0.05; ** p < 0.005; *** < 0.0005; **** p < 0.0001.

	Ala	**Glu**	**Gln**	**Val**	**Leu**	**Ile**	**AMP**	**Adenosine**	**Lac**	**Acetate**	**Succinate**	**Betaine**	**GSH**	**Taurine**	**Creatine**
Ala		**0.5296 ***	0.4657	**0.5129 ***	0.455	0.2476	-0.1489	0.1518	0.3454	-0.0221	0.3346	-0.385	0.1459	**0.6492 ***	0.1792
Glu	**0.5296 ***		0.2018	0.2937	0.0251	0.1683	0.1284	-0.248	0.3082	-0.2007	0.4812	0.0406	0.1077	0.4142	-0.1315
Gln	0.4657	0.2018		0.0471	0.0774	-0.0854	0.3152	-0.2975	-0.0501	-0.486	0.0781	0.185	0.4683	**0.693 ****	-0.3522
Val	**0.5129 ***	0.2937	0.0471		**0.518 ***	0.2382	-0.4265	0.4765	**0.9087 ******	0.2000	-0.0941	-0.1383	-0.2529	0.4068	0.2620
Leu	0.455	0.0251	0.0774	**0.518 ***		0.3267	**-0.6578 ***	**0.6976 ****	0.4296	0.4386	0.0559	-0.4345	-0.4680	0.2080	0.3166
Ile	0.2476	0.1683	-0.0854	0.2382	0.3267		-0.3118	0.3529	0.1458	0.1265	0.1029	-0.4739	0.0500	0.3110	0.2929
AMP	-0.1489	0.1284	0.3152	-0.4265	**-0.6578 ***	-0.3118		**-0.9412 ********	-0.4153	**-0.5206 ***	0.1059	**0.5710 ***	0.3765	-0.1916	-0.4871
Adenosine	0.1518	-0.248	-0.2975	0.4765	**0.6976 ****	0.3529	**-0.9412 ********		0.4433	**0.5647 ***	-0.2176	**-0.5239 ***	-0.3765	0.1444	0.4798
Lac	0.3454	0.3082	-0.0501	**0.9087 ********	0.4296	0.1458	-0.4153	0.4433		0.2018	-0.1267	0.0015	-0.2194	0.2627	0.2004
Acetate	-0.0221	-0.2007	-0.4860	0.2000	0.4386	0.1265	**-0.5206 ***	**0.5647 ***	0.2018		-0.1088	-0.1089	**-0.6441 ***	-0.3125	0.2517
Succinate	0.3346	0.4812	0.0781	-0.0941	0.0559	0.1029	0.1059	-0.2176	-0.1267	-0.1088		-0.2826	0.1176	0.0987	0.4665
Betaine	-0.385	0.0406	0.1850	-0.1383	-0.4345	-0.4739	**0.571 ***	**-0.5239 ***	0.0015	-0.1089	-0.2826		0.0132	-0.2176	**-0.5523 ***
GSH	0.1459	0.1077	0.4683	-0.2529	-0.468	0.05	0.3765	-0.3765	-0.2194	**-0.6441 ***	0.1176	0.0132		0.4908	-0.0353
Taurine	**0.6492 ***	0.4142	**0.6930 ****	0.4068	0.208	0.311	-0.1916	0.1444	0.2627	-0.3125	0.0987	-0.2176	0.4908		-0.0354
Creatine	0.1792	-0.1315	-0.3522	0.262	0.3166	0.2929	-0.4871	0.4798	0.2004	0.2517	0.4665	**-0.5523 ***	-0.0353	-0.0354	