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# Exercise differentially affects metabolic functions and white adipose tissue in female letrozole- and dihydrotestosterone-induced mouse models of polycystic ovary syndrome

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ABSTRACT

Here we hypothesized that exercise in dihydrotestosterone (DHT) or letrozole (LET)-induced polycystic ovary syndrome mouse models improves impaired insulin and glucose metabolism, adipose tissue morphology, and expression of genes related to adipogenesis, lipid metabolism, Notch pathway and browning in inguinal and mesenteric fat. DHT-exposed mice had increased body weight, increased number of large mesenteric adipocytes. LET-exposed mice displayed increased body weight and fat mass, decreased insulin sensitivity, increased frequency of small adipocytes and increased expression of genes related to lipolysis in mesenteric fat. In both models, exercise decreased fat mass and inguinal and mesenteric adipose tissue expression of Notch pathway genes, and restored altered mesenteric adipocytes morphology. In conclusion, exercise restored mesenteric adipocytes morphology in DHT- and LET-exposed mice, and insulin sensitivity and mesenteric expression of lipolysis-related genes in LET-exposed mice. Benefits could be explained by downregulation of Notch, and modulation of browning and lipolysis pathways in the adipose tissue.



## 1. Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrinopathy in women of reproductive age and is characterized by hyperandrogenism, anovulation and polycystic ovarian morphology (Dumesic et al., 2015). Beyond the reproductive dysfunction, women with PCOS suffers from metabolic dysfunction including insulin resistance, hyperinsulinemia, with an increased risk for type 2 diabetes and cardiovascular diseases (Jayasena and Franks, 2014). Women with PCOS are more likely to be overweight/obese, specifically abdominal obesity (Lim et al., 2012) with accumulation of white adipose tissue (WAT) (Bartelt and Heeren, 2014). Obesity exacerbates the reproductive and metabolic phenotype in PCOS and increase cardiometabolic risks (Jayasena and Franks, 2014,Moran et al., 2015,Karpe and Pinnick, 2015). Along with abdominal fat accumulation, adipose tissue dysfunction, characterized by increased adipocyte size and altered circulating levels of adipokines, is observed in women with PCOS (Spritzer et al., 2015).

Mice and rats exposed to continuous administration of dihydrotestosterone (DHT) or letrozole from pre-pubertal age develop metabolic and reproductive alterations that resemble features

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observed in women with PCOS (van Houten et al., 2012,Kauffman et al., 2015,Maliqueo et al., 2013,Manneras et al., 2007). Previous studies have shown that PCOS mouse model induced by DHT or letrozole presented increased gain of body weight, impaired insulin sensitivity, adipocyte hypertrophy, disrupted estrous cycle, and chronic anovulation (van Houten et al., 2012,Kauffman et al., 2015). However, DHT and letrozole-exposed mice presents different endocrine phenotypes, where DHT-exposed mice presents normal levels of testosterone and high levels of dihydrotestosterone, and letrozole-exposed mice displays high levels of testosterone and unaltered levels of dihydrotestosterone (Caldwell et al., 2014). Both PCOS models are hyperandrogenemic but through different mechanisms; DHT by exogenous administration of androgen, and letrozole by inhibiting the P450 aromatase leading to increased circulating testosterone.

Lifestyle interventions, including diet and exercise, improve PCOS related symptoms (Nybacka et al., 2011,Nybacka et al., 2013). Still, the mechanisms behind the beneficial effects of exercise in women with PCOS are not completely understood. In a PCOS rat model induced by DHT from puberty, exercise decreased adiposity and adipocyte size, improved insulin sensitivity, estrous cyclicity, ovarian morphology, circulating androgens and adipokines levels, and restored the mRNA expression of sympathetic markers in the adipose tissue (Benrick et al., 2013,Manneras et al., 2008,Manneras et al., 2009,Wu et al., 2014).

Exercise can have profound effects on WAT physiology, leading to decreased adipocyte size, and influences the expression of several metabolic proteins, such as glucose transporter 4 (GLUT4) and peroxisome proliferator-activated receptor coactivator 1-alpha (PGC1-α) (Stanford et al., 2015). Moreover, the effects of exercise in WAT differ depending on adipose tissue depot; subcutaneous versus visceral. Exercise is also able to increase the amount of brown-like adipocytes in the WAT, known as “browning” or “beiging”. These brown-like adipose cells dissipate energy as heat through uncoupling protein-1 (UCP-1) activity (Stanford et al., 2015). The browning of WAT has been related to improvements in metabolic health and is a promising strategy to treat metabolic disorders (Bartelt and Heeren, 2014).

Inhibition of the Notch signaling pathway by knockdown of *Notch1* and *Rbpj* genes promotes browning of WAT, improves insulin sensitivity and glucose tolerance, and increases the expression of genes related to brown-like adipocytes activity, such as *Ucp1*, *Ppargc1a* (PGC1-α), *Prdm16* (PR domain containing 16) and *Cidea* (Cell Death-Inducing DFFA-Like Effector A) (Bi et al., 2014). Pharmacological inhibition of Notch pathway with dibenzazepine increases the expression of *Ucp1*, and improves the insulin sensitivity and glucose tolerance of obese mice models, suggesting that this pathway is a potential target for the treatment of obesity and type 2 diabetes (Bi et al., 2014). To our knowledge, the effects of exercise on Notch signaling pathway in adipose tissue have not previously been analyzed.

The Notch pathway has a strong cross-talk with bone morphogenetic proteins (BMPs) (Kluppel and Wrana, 2005). BMP-4 and BMP-7 induces brown-like phenotype in human adipose stem cells (Elsen et al., 2014), and BMP-4 overexpression *in vivo* induces browning and improves glucose and insulin metabolism (Qian et al., 2013). The role of these molecules in adipose tissue of PCOS is not known.

Herein, we tested the hypotheses that 4–5 weeks of voluntary exercise in either DHT- or letrozole (LET)-induced PCOS mice models would improve impaired insulin sensitivity and glucose metabolism, adipose tissue morphology, and expression of genes related to adipogenesis, lipid metabolism, Notch signaling pathway and browning in subcutaneous and mesenteric WAT. Additionally, we investigated the transcriptional expression of BMP family growth factors and markers of sympathetic activity in subcutaneous and mesenteric adipose tissue, and liver triglycerides.

## 2. Materials and methods

### 2.1. Animals

Forty-seven pre-pubertal, three weeks old female mice (C57BL/6JRj) were purchased from Janvier Labs (Le Genest Saint-Isle, France). Animals were housed four to five per cage under controlled temperature and 12 h light/12 h dark cycle. Mice were fed with commercial chow (16.5% protein, 4% fat, 58% carbohydrate, 3.5% fiber, 6% vitamins and minerals, and <12% water; Lantmännen Lantbruk, Malmö, Sweden) and tap water *ad libitum*. This study was approved by the Animal Ethical Committee at Karolinska Institutet, protocol number N259-2014.

### 2.2. Study procedure

At 28 days of age, mice were randomly divided into three groups: control (n = 9), DHT (n = 19), and letrozole (LET) (n = 19), and continuous slow releasing pellets were implanted subcutaneously in the neck region under light anesthesia with isoflurane. The LET group received a 70 days continuous releasing pellet (3.5 mg letrozole; daily dose: 50 μg; Innovative Research of America, Sarasota, USA) (Kauffman et al., 2015) and the DHT group received a 90 days continuous releasing pellet (2.5 mg DHT; daily dose: 27.5 μg; Innovative Research of America) (van Houten et al., 2012). Control animals received a pellet lacking the bioactive molecule. Before start of exercise, at 63 days of age and 5 weeks from pellet implantation, when the DHT- and LET-exposed mice developed the PCOS-like phenotype, body composition was measured by Dual-energy X-ray absorptiometry (DEXA) (Lunar PIXImus, GE Medical Systems, Madison, USA) as described previously (Yang et al., 2015). Exercise started after DEXA examination and groups were divided into: control (n = 9), DHT (n = 10), DHT + exercise (DHT + EX) (n = 9), LET (n = 9), and LET + exercise (LET + EX) (n = 10). At 8 weeks from pellet exposure, after 3 full weeks of exercise, insulin tolerance test (ITT), and 5 days later an oral glucose tolerance test (OGTT), were performed. After 4–5 weeks of exercise, at 10th week from pellet exposure, DEXA measurement was repeated and mice were euthanized after 4 h of fasting (Supplemental Fig. S1). Running wheels were locked 24 h before the euthanasia. Blood was collected by heart puncture and inguinal and mesenteric fat depots were quickly dissected and snap frozen until analyses. Inguinal and mesenteric fat depots were chosen for translational purposes, since these depots are analogous to human fat pads humans (Chusyd et al., 2016).

### 2.3. Voluntary exercise

Mice in the exercise groups were housed as described previously (Goh and Ladiges, 2015), with free access to a low-profile wireless running wheel for mouse (Med Associates, St Albans, USA). Wheel evolutions were registered.

### 2.4. Insulin tolerance test (ITT)

After 2 h fasting and locked running wheels, a basal blood sample from the tail was collected to measure glucose with the One Touch Ultra-2 glucometer (LifeScan, Inc., Milpitas, USA). Then animals received insulin dissolved in saline (0.5U/kg) intraperitoneally. Blood glucose was measured at 15, 30 and 45 min after insulin injection.

### 2.5. Oral glucose tolerance test (OGTT)

After 5–6 h fasting and locked running wheels, a basal blood from the tail was used to measure glucose levels (One Touch). Thereafter, mice received glucose (2 g/kg body weight) by gavage and blood glucose was measured at 15, 30, 60, and 90 min after glucose load. At baseline and 15 min blood (30 μl) was sampled for insulin dosage. Insulin was quantified by the Ultra Sensitive Mouse Insulin ELISA Kit 90080 (Crystal Chem, Inc., Downers Grove, USA). The limit of detection was 0.05 ng/ml. Both intra-assay and inter-assay coefficient of the variations were ≤10%.

### 2.6. Adipocyte size analysis

Adipocyte cell size was analyzed using Multisizer Coulter Counter technology, as previously described (Li et al., 2016,McLaughlin et al., 2007). In short, 8–12 mg of inguinal and mesenteric fat depots was collected separately. Three to four samples per group (obtained from pool of 2–3 animals) were immediately fixed in osmium tetroxide (1% in Collidine Buffer). After 2–3 days of fixation, samples were washed with water, filtered in 250 μm and 25 μm nylon meshes, and stored in saline solution until counting and measurement of cell size in a Multisizer™ 3 Coulter Counter® (Beckman Coulter, Miami, USA) with a 400-μm aperture. The effective cell-size range using this aperture is 20–240 μm. The instrument was set to count 6000 particles.

### 2.7. Quantitative real-time PCR

RNA extraction was performed in inguinal and mesenteric fat depots using RNeasy Lipid Tissue Mini Kit with RNase-Free DNase Set step (QIAGEN GmbH, Hilden, Germany) according to manufactures' instructions. RNA quality was evaluated in all samples with the Experion electrophoresis system (Bio-Rad Laboratories, Hercules, USA). The number of good quality RNA samples selected for cDNA synthesis were: control (n = 9), DHT (n = 10), DHT + EX (n = 9), LET (n = 9), and LET + EX (n = 9) for inguinal fat; and control (n = 9), DHT (n = 9), DHT + EX (n = 9), LET (n = 5), and LET + EX (n = 7) for mesenteric fat. cDNA synthesis was performed using iScript kit (Bio-Rad Laboratories, Hercules, USA). Taqman® assays (primers and probes) inventoried by Life technologies were selected to analyze the expression of *Notch1*, *Rbpj*, *Hes1*, *Hey1*, *Bmp2*, *Bmp4*, *Bmp7*, *Ucp1*, *Ppargc1a*, *Prdm16*, *Cidea*, *Pparg*, *Adrb3*, *Ngf*, *Ngfr*, *Adipoq*, *Slc2a4*, *Serbf1*, *Cebpa*, *Acaca*, *Lpl* and *Lipe*. qRT-PCR reactions were performed for individual samples in duplicate in a total volume of 10 μL (including 6.25 ng of cDNA, 5 μL of Taqman Gene Expression Master Mix and 0.5 μL of the assays) and carried out on Applied Biosystems® ViiA 7 RUO System (Life technologies). The cycle conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Expression of *G0s2*, *Pnpla2*, *Plin1*, *Cgi58*, *Mgll*, and *Fsp27* were evaluated by SYBR® Green system (Life technologies) (3.125 ng of cDNA, 0.3 μL (5 μM) of forward primer, 0.3 μL (5 μM) of reverse primer, and Power SYBR® Green PCR Master Mix) under the same cycle conditions described above. *Gapdh* was selected as endogenous control after analysis on Expression Suite Software v1.0.4 (Life Technologies, USA). Details of probes and primers used in this study are described in Supplemental Table S1. The data were analyzed using ViiA 7 RUO Software (version 1.2.3) and Ct values were transformed to quantities using the comparative Ct method (ΔΔCt), then the fold change for each target gene was calculated and used for statistical analysis.

### 2.8. Quantification of liver triglycerides

Approximately 100 (mean: 103.66; SEM: 0.48) mg of liver tissue was placed in 1 ml of 5% NP40 (EMD Millipore Corp., Billerica, USA) solution and homogenized using Tissue Lyser II (QIAGEN) at 28 Hz for 2 min. Samples were heated at 85 °C for 5 min, cooled at room temperature for 10 min, and heated again at 85 °C for 5 min. Then, samples were centrifuged at 16000×g for 2 min. Triglycerides were quantified in the supernatant using Randox TRIGS kit (Randox Laboratories, Crumlin, UK) according to manufactures' instructions. The detection range of this kit is 22.9–1172 mg/dl. For each sample, triglyceride levels were normalized by the weight (mg) of liver tissue taken.

### 2.9. Statistical analyses

Statistical analyses were performed with IBM SPSS Statistics (version 22.0; IBM Corp., Armonk, USA) and GraphPad Prism (version 6.05; GraphPad Software, La Jolla, USA). The normal distribution of the data was tested by Shapiro-Wilk test. Differences among groups were analyzed by one-way ANOVA followed by Bonferroni post hoc test for data with normal distribution, and by Kruskal-Wallis with Dunn's post hoc test to correct for multiple comparisons test for skewed data. Repeated measures ANOVA followed by post hoc Bonferroni test was performed to evaluate body weight development. T-student or Mann-Whitney *U* test was used to analyze running-wheel activity data. Correlations were performed using Pearson test. Results are presented as mean ± SEM.

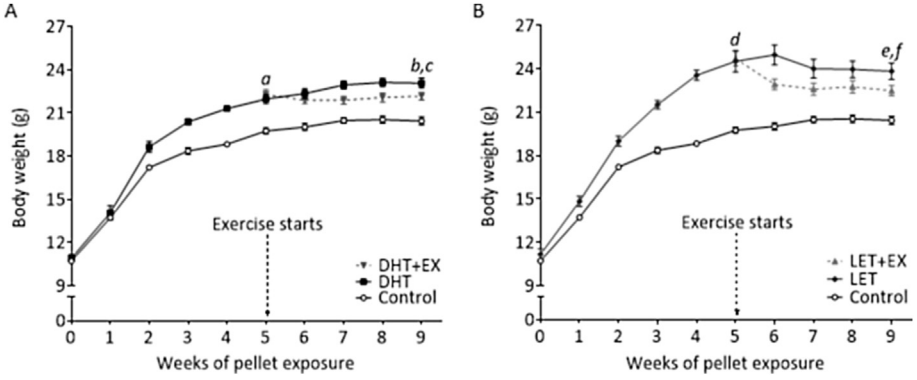


**Fig. 1.** Body weight development. (A) Body weight development in the DHT, DHT + EX and control group, and (B) in the LET, LET + EX and control group. DHT, dihydrotestosterone; LET, letrozole; EX, exercise. *a* – Control vs DHT before exercise ($P < 0.0001$), *b* – Control vs DHT after exercise ($P < 0.001$), *c* – Control vs DHT + EX after exercise ($P < 0.05$), *d* – Control vs LET before exercise ($P < 0.0001$), *e* – Control vs LET after exercise ($P < 0.0001$), *f* – Control vs LET + EX after exercise ($P < 0.001$). Values are shown as mean ± SEM. Repeated measures ANOVA followed by Bonferroni *post hoc* test was performed for evaluation of body weight.

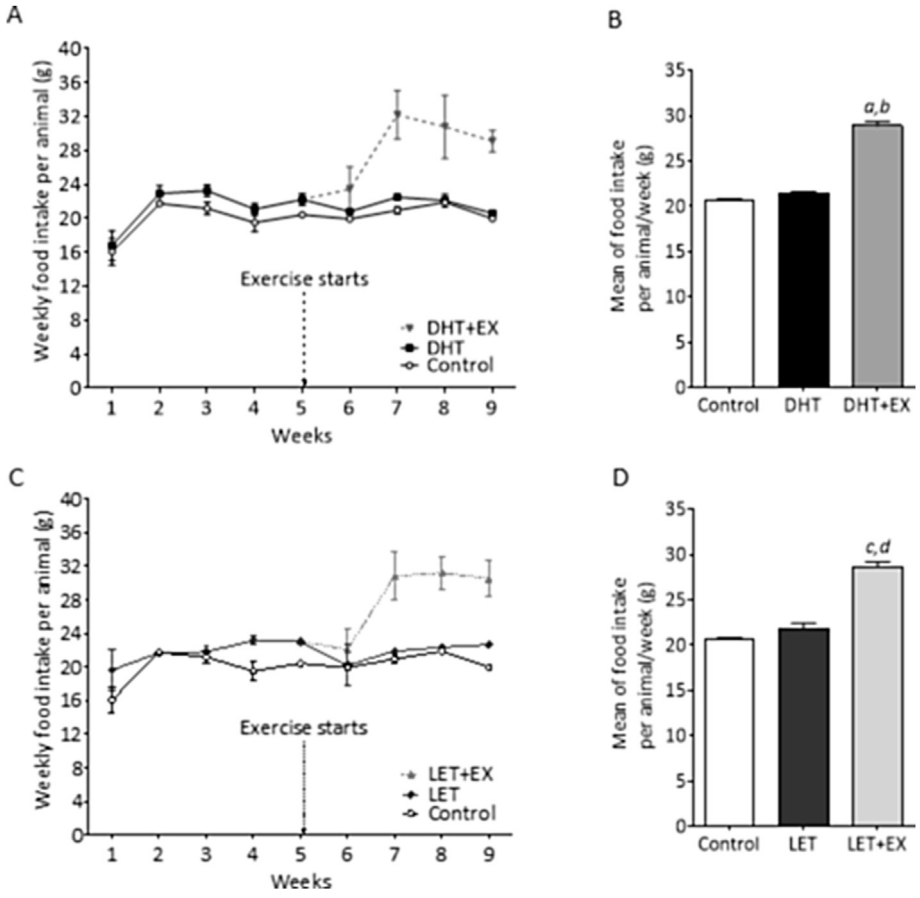


**Fig. 2.** Food intake among experimental groups. (A) Food intake per week and (B) mean of food intake during exercise period in DHT, DHT + EX and control group. (C) Food intake per week and (D) mean of food intake during exercise period in LET, LET + EX and control group. DHT, dihydrotestosterone; LET, letrozole; EX, exercise. *a* – Control vs DHT + EX ($P < 0.0001$), *b* – DHT vs DHT + EX ($P < 0.0001$), *c* – Control vs LET + EX ($P < 0.0001$), *d* – LET vs LET + EX ($P < 0.0001$). Values are shown as mean ± SEM. One-way ANOVA followed by Bonferroni *post hoc* test was performed.

$P < 0.05$ was considered to be significant.

## 3. Results

### 3.1. Body weight development and food intake

After 5 and 9 weeks, mice continuously exposed to DHT or LET weighed more than control mice ($P < 0.05$) (Fig. 1A and B). Five weeks of exercise (i.e. 10 weeks of exposure), did not reduce body weight in DHT- or LET-exposed mice (DHT + EX and LET + EX) compared with non-exercised PCOS mice (Fig. 1A and B). Mice from DHT + EX and LET + EX groups had higher food intake compared to controls and the non-exercised PCOS models ($P < 0.0001$) (Fig. 2, A-D).

### 3.2. Running wheel activity

Mice from LET + EX expended more time on the running wheels than DHT + EX at the second and last week of exercise ($P < 0.01$ and $P < 0.05$, respectively) (Supplemental Table S2). The distance performed each week (expressed as mean distance/day), average speed, and peak speed performed by DHT + EX and LET + EX exposed mice were not statistically different between these groups (Supplemental Table S2).

### 3.3. Body composition and organs weight

After 10 weeks of pellet exposure, non-exercised LET-exposed mice had more total and abdominal fat mass than controls ($P < 0.01$) (Table 1). Both exercised and non-exercised DHT- or LET-exposed mice had more lean mass than controls ($P < 0.0001$) (Table 1).

Four to five weeks of exercise resulted in reduced total and abdominal fat mass, and fat/lean mass ratio in both LET + EX and DHT + EX groups compared to the LET and DHT groups, respectively ($P < 0.05$) (Table 1). Additionally, DHT + EX mice had decreased fat/lean mass ratio and abdominal fat mass compared with controls ($P < 0.01$) (Table 1).

The heart and kidney weighed more in the DHT and DHT + EX groups than in the control group ($P < 0.0001$ and $P < 0.0001$) (Table 1).

LET + EX group had increased kidney weight ($P < 0.01$) compared to controls (Table 1). Body composition after 5 weeks of pellet exposure (before exercise) is described in Supplemental Table S3.

### 3.4. Insulin tolerance test

Neither DHT exposure nor exercise affected the fasting glucose levels (Fig. 3A) or area under the curve (AUC) during the ITT (Fig. 3B) compared with the control group.

**Table 1**
Body composition and organs weight among groups after exercise.

| | Control (n = 9) | DHT (n = 10) | DHT + EX (n = 9) | LET (n = 9) | LET + EX (n = 10) | *P*-value (ANOVA) |
|---|---|---|---|---|---|---|
| *Body composition - DEXA* | | | | | | |
| Body weight (g) | 20.76 ± 0.36 | 23.24 ± 0.37[a] | 22.63 ± 0.24[a] | 24.43 ± 0.61[a] | 23.06 ± 0.39[a] | $P < 0.0001$ |
| Total fat mass (g) | 2.30 ± 0.07 | 2.69 ± 0.12 | 1.93 ± 0.12[b] | 3.13 ± 0.27[a] | 2.38 ± 0.12[c] | $P < 0.0001$ |
| Abdominal fat mass (g) | 1.84 ± 0.06 | 2.12 ± 0.11 | 1.49 ± 0.07[a,b] | 2.40 ± 0.22[a] | 1.80 ± 0.11[c] | $P < 0.0001$ |
| Lean mass (g) | 12.56 ± 0.32 | 14.42 ± 0.18[a] | 14.34 ± 0.61[a] | 15.57 ± 0.32[a] | 15.1 ± 0.53[a] | $P < 0.0001$ |
| Fat/lean mass ratio | 0.18 ± 0.01 | 0.19 ± 0.01 | 0.13 ± 0.004[a,b] | 0.20 ± 0.02 | 0.16 ± 0.004[c] | $P < 0.01$ |
| BMD (g/cm$^2$) | 0.0512 ± 0.0006 | 0.0515 ± 0.0007 | 0.0507 ± 0.0010 | 0.0521 ± 0.0005 | 0.0514 ± 0.0005 | NS |
| *Organs weight* | | | | | | |
| Kidney (mg) | 107.89 ± 2.54 | 145.00 ± 5.25[a] | 154.66 ± 5.11[a] | 121.58 ± 4.32 | 131.8 ± 6.89[a] | $P < 0.0001$ |
| Heart (mg) | 115.78 ± 3.48 | 130.73 ± 2.69[a] | 140.73 ± 3.73[a] | 126.42 ± 2.99 | 130.32 ± 5.85 | $P < 0.0001$ |

Values are described as Means ± SEM. One-way ANOVA followed by Bonferroni post hoc test was performed. BMD, bone mineral density; NS, not significant.
[a] – vs Control, $P < 0.05$.
[b] – vs DHT, $P < 0.05$.
[c] – vs LET, $P < 0.05$.

LET-exposed mice had higher glucose levels during the ITT ($P < 0.05$) (Fig. 3C) and AUC glucose compared with the control group ($P < 0.05$) (Fig. 3D), and five weeks of exercise lowered glucose levels ($P < 0.05$) and AUC of glucose during the ITT ($P < 0.05$) compared with non-exercised LET mice (Fig. 3C and D), and did not differ from controls. However, the baseline glucose levels were lower in LET-exposed mice with and without exercise.

### 3.5. Oral glucose tolerance test

There were no differences between the groups during the OGTT related to: glucose levels (Fig. 3), E and I, insulin levels (Fig. 3, G and K), AUC of glucose (Fig. 3, F and J), or AUC of insulin (Fig. 3, H and L), although there was a tendency towards lower AUC of insulin levels after five weeks of exercise in DHT-exposed mice compared to the non-exercised DHT group (ANOVA: $P < 0.05$, Bonferroni: $P = 0.051$).

### 3.6. Adipocyte size distribution and expression of genes related to adipocyte differentiation and lipid metabolism

*Inguinal fat.* DHT-exposed mice had a shift from larger to smaller inguinal adipocytes compared to controls, and this shift was more marked in trained DHT-exposed mice (Fig. 4A). Regarding expression of genes related to adipogenesis, mRNA expression of *Bmp2* was upregulated in the inguinal fat depot in DHT-exposed mice compared to controls ($P < 0.05$) (Fig. 4B).

On the other hand, LET-exposed mice presented a slight shift from larger to smaller adipocytes and increased percentage of small adipocytes compared to controls (Fig. 4C). These changed patterns were also observed in exercised LET-exposed mice, where the changes were more pronounced (Fig. 4C).

Representative samples and graphs with SEM of inguinal adipocyte size distribution are shown in Supplemental Fig. S2A-L.

The mRNA expression of genes related to adipocyte metabolism did not differ (Fig. 4D). Five weeks of exercise in the LET-exposed mice downregulated the mRNA expression of *Srebf1* compared to control and LET (both $P < 0.05$), and of *Lpl* and *Lipe*, compared to LET group ($P < 0.05$) (Fig. 4D).

*Mesenteric fat.* Mesenteric adipocytes shifted to larger sizes in DHT-exposed mice compared to controls (Fig. 4E). The voluntary exercise in DHT-exposed mice reduced the size of the mesenteric adipocytes compared to non-exercised DHT-exposed mice, being even smaller than controls (Fig. 4E). The mRNA expression of genes related to adipocyte metabolism did not differ between DHT-exposed mice and controls (Fig. 4F). Five weeks of exercise in DHT-exposed mice increased the mRNA expression of *Slc2a4*, *Lpl* and *Lipe* compared to controls and non-exercised DHT mice ($P < 0.05$) (Fig. 4F).

LET-exposed mice presented higher frequency of small mesenteric adipocytes compared to controls (Fig. 4G). Five weeks of exercise tended to restore altered mesenteric adipocyte size distribution in LET-exposed mice (Fig. 4G).

Representative samples and graphs with SEM of mesenteric adipocyte size distribution are shown in Supplemental Fig. S3A-L.

The mRNA expression of *Slc2a4*, *Lpl* and *Lipe* were upregulated compared to controls ($P < 0.01$, $P < 0.01$ and $P < 0.001$, respectively) (Fig. 4H). Five weeks of exercise in LET-exposed mice restored the expression of *Lpl* and *Lipe* and did not differ from controls, whereas *Slc2a4* remained upregulated ($P < 0.01$) (Fig. 4H).

Since non-exercised LET-exposed mice and exercised DHT-exposed mice presented increased expression of genes related to lipolysis (*Lpl* and *Lipe*), we further investigated genes involved in lipolysis to evaluate if there are possible different mechanisms in the lipolytic pathway between those LET- and DHT-exposed mice. The expression of *G0s2* was increased in the DHT + EX compared to non-exercised DHT and control mice ($P < 0.01$) (Supplemental Fig. S4A). No significant changes were observed in other lipolysis-related genes in LET-exposed mice compared to controls (Supplemental Fig. S4B).

### 3.7. Expression of genes related to Notch pathway, BMP family, browning of WAT, and sympathetic activity

Next, we investigated expression of genes known to promote browning of white adipose tissue, and improve insulin sensitivity and glucose tolerance. Five weeks of exercise in DHT-exposed mice decreased the expression of *Notch1* and increased the expression of *Cidea* in the inguinal fat depot compared to non-exercised DHT mice (both $P < 0.05$) and did not differ from controls (Fig. 5A). Compared to controls, five weeks of exercise in the DHT-exposed mice resulted in higher mRNA expression of *Ppargc1a*, *Prdm16*, *Cidea* and *Ucp1* ($P < 0.05$, $P < 0.01$, $P < 0.001$ and $P < 0.05$, respectively) (Fig. 5A). In LET-exposed mice, *Prdm16* mRNA expression was increased compared with controls ($P < 0.05$), and five weeks of exercise decreased the expression of *Notch1* compared to non-exercised LET-exposed mice ($P < 0.01$) (Fig. 5B).

Expression of browning markers *Cidea* and *Ucp1* was negatively correlated to the expression of *Notch1* in the inguinal fat of exercised DHT-exposed mice ($r = -0.74$ and $r = -0.71$, respectively; both $P < 0.05$) (Supplemental Fig. 5). In exercised LET-exposed mice, expression of *Ucp1* and another browning marker, *Ppargc1a*, were also negatively correlated to the expression of *Notch1* in inguinal fat

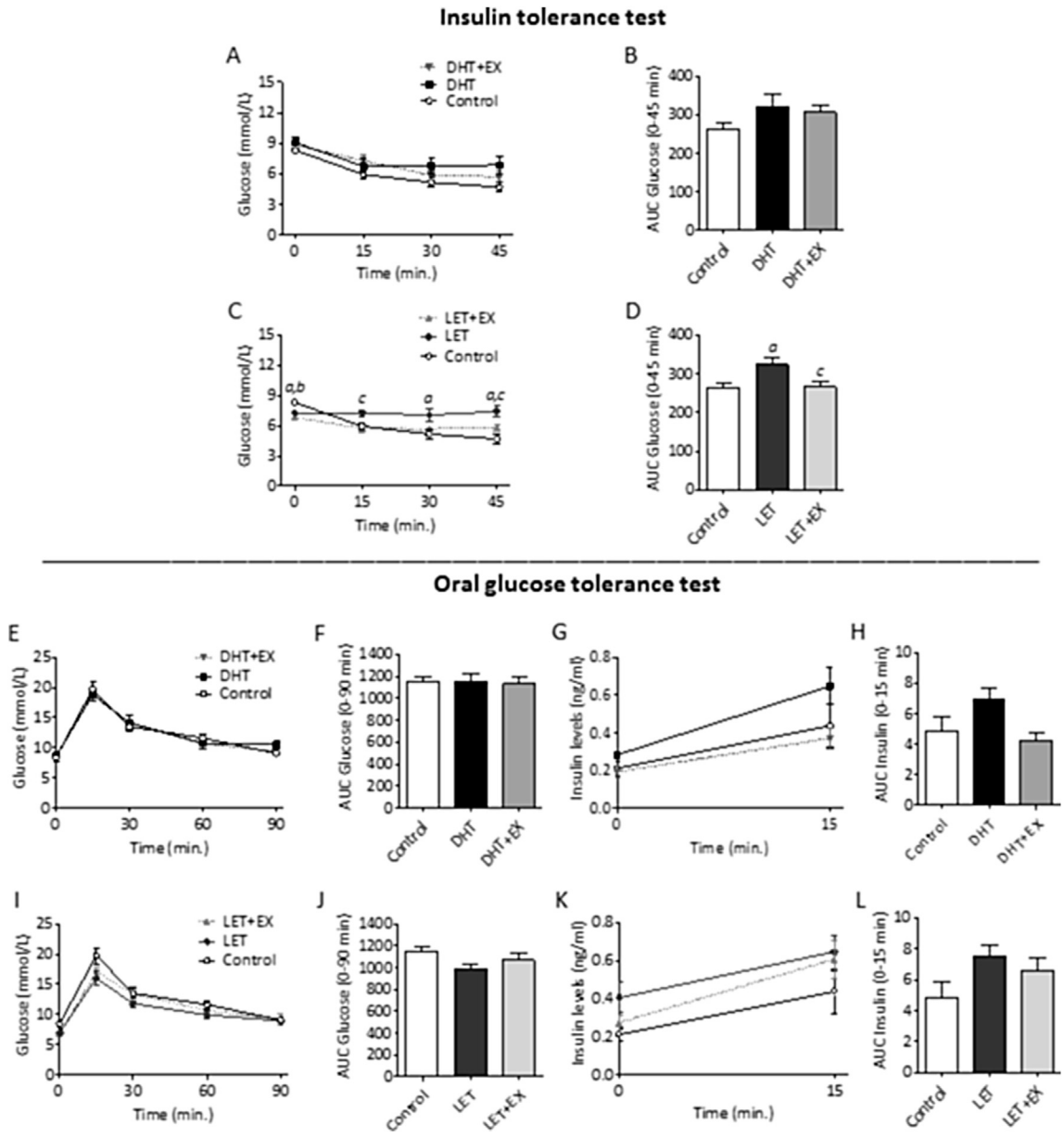


**Fig. 3.** Insulin tolerance test and glucose tolerance test. (A) Blood glucose levels during insulin tolerance test (ITT); and (B) AUC of glucose in DHT, DHT + EX and control. (C) Blood glucose levels during ITT; and (D) AUC of glucose in LET, LET + EX and control. (E) Blood glucose levels during oral glucose tolerance test (OGTT); (F) AUC of glucose levels; (G) plasma insulin levels at baseline and 15 min after glucose load; and (H) AUC of insulin levels in DHT, DHT + EX and control. (I) Blood glucose levels during OGTT; (J) AUC of glucose levels; (K) Plasma insulin levels at baseline and 15 min after glucose load; and (L) AUC of insulin levels in LET, LET + EX and control. AUC, area under the curve, DHT, dihydrotestosterone; EX, exercise. *a* – Control vs LET ($P < 0.05$), *b* – Control vs LET + EX ($P < 0.05$), *c* – LET vs LET + EX ($P < 0.05$). Values are shown as mean ± SEM. One-way ANOVA followed by Bonferroni *post hoc* test were used.

($r = -0.74$ and $r = -0.71$, respectively; both $P < 0.05$) (Supplemental Fig. S5). There were no significant correlations between expression of browning markers and Notch1 in control, DHT and LET groups (Supplemental Fig. S5).

In the mesenteric fat depot, DHT-exposed mice, with and without exercise presented lower mRNA expression of *Notch1* ($P < 0.05$ and $P < 0.001$, respectively). Exercise in DHT exposed mice increased the expression of *Bmp7* and *Cidea* compared to controls ($P < 0.001$ and $P < 0.05$, respectively), and decreased the expression of *Rbpj* ($P < 0.01$) and increased *Bmp7* compared with non-exercised DHT mice ($P < 0.05$) (Fig. 5C). Non-exercised LET mice had increased expression of *Bmp7* and *Cidea* in the mesenteric fat depot compared to controls (both $P < 0.05$), and the expression of *Rbpj* was downregulated in LET + EX group compared to LET and did not differ from controls ($P < 0.05$) (Fig. 5D).

### 3.8. Liver triglycerides content

Triglyceride content in the liver was lower after five weeks of exercise in DHT-exposed mice compared to the non-exercised DHT mice ($P < 0.05$) and controls ($P < 0.001$) (Fig. 6A). There was no significant difference in liver triglyceride content in LET-exposed mice (Fig. 6B).

The results are summarized in Fig. 7.

## 4. Discussion

Herein, we demonstrate that exercise has distinct effects on

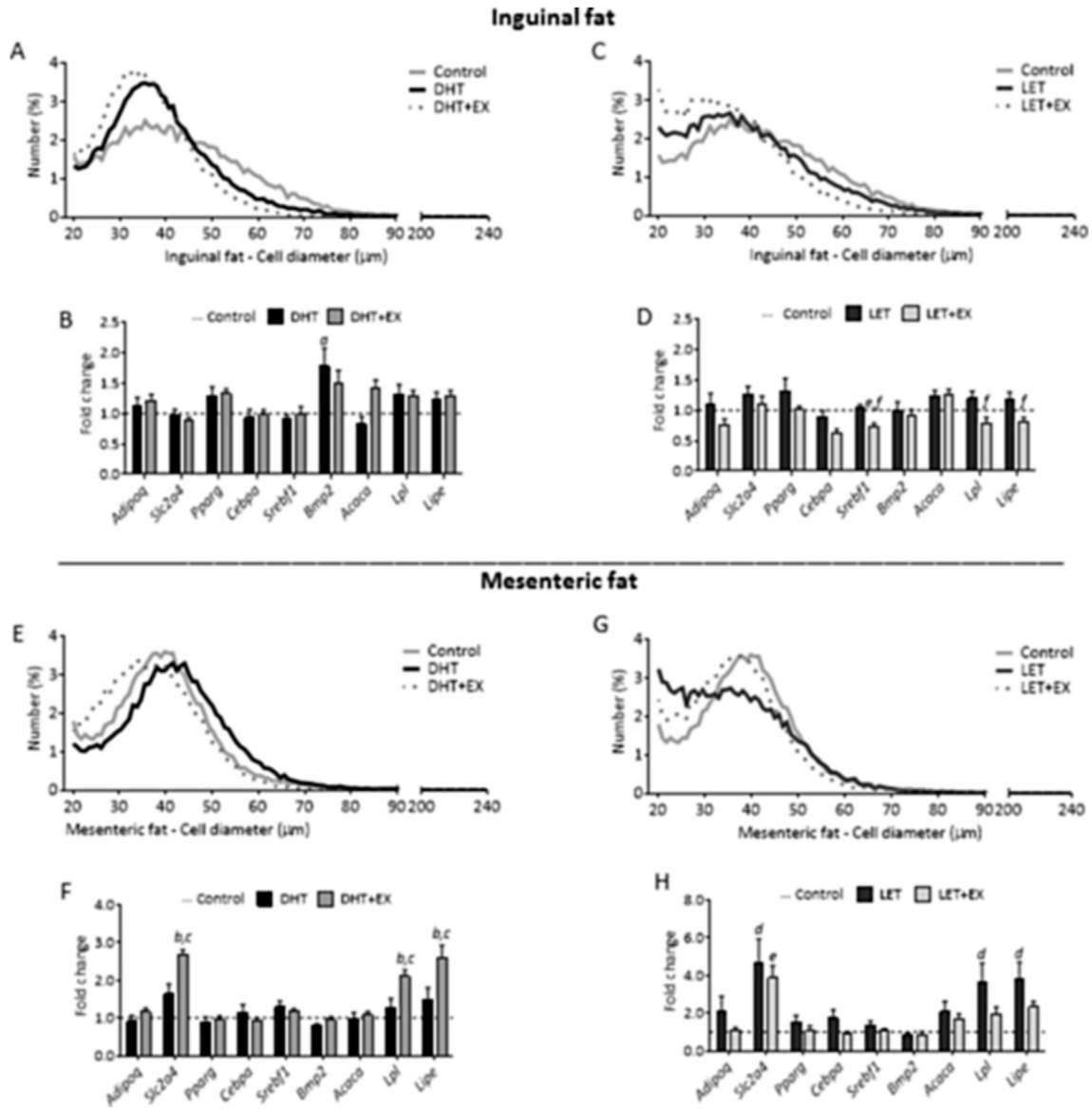


**Fig. 4.** Adipocyte size distribution and expression of genes related to adipogenesis and lipid metabolism in inguinal and mesenteric fat. (A) Adipocyte size distribution; and (B) expression of genes related to adipogenesis and lipid metabolism in inguinal fat of the control, DHT, and DHT + EX groups. (C) Adipocyte size distribution; and (D) Expression of genes related to adipogenesis and lipid metabolism in inguinal fat of the control, LET, and LET + EX groups. (E) Adipocyte size distribution; and (F) expression of genes related to adipogenesis and lipid metabolism in mesenteric fat of the control, DHT, and DHT + EX groups. (G) Adipocyte size distribution; and (H) Expression of genes related to adipogenesis and lipid metabolism in mesenteric fat of the control, LET, and LET + EX groups. DHT, dihydrotestosterone; LET, letrozole; EX, exercise. *a* – Control vs DHT ($P < 0.05$), *b* – Control vs DHT + EX ($P < 0.05$), *c* – DHT vs DHT + EX ($P < 0.05$), *d* – Control vs LET ($P < 0.05$), *e* – Control vs LET + EX ($P < 0.05$), *f* – LET vs LET + EX ($P < 0.05$). Values for adipocyte size distribution are shown as mean and values for mRNA expression are shown as mean ± SEM. ANOVA followed by Bonferroni *post hoc* test was performed to evaluate mRNA expression data.

insulin sensitivity, and adipose tissue morphology and function in two different mice model of PCOS. Furthermore, we show that exercise downregulates the Notch signaling pathway in subcutaneous and visceral adipose tissue in both DHT- and LET-induced PCOS mice models, indicating this signaling pathway can be a target of exercise.

It is well known that women diagnosed with PCOS are more frequently overweight or obese (Dumesic et al., 2015), and also rats and mice exposed to DHT (van Houten et al., 2012,Manneras et al., 2007) or LET (Kauffman et al., 2015,Manneras et al., 2007) have a significant weight gain. In agreement with data obtained from human (Dumesic et al., 2015), both DHT- and LET-exposed mice weighed more than controls, but only LET-exposed mice had an increased total and abdominal fat mass and impaired insulin sensitivity. Whether women with PCOS have an increased amount of visceral fat is still controversial although some data support this concept (Huang et al., 2013). Nevertheless, exercise intervention in women with PCOS decreases central fat accumulation and improves insulin sensitivity (Huber-Buchholz et al., 1999), which is in agreement with our results in PCOS mouse models. Also, the exercising mice presented reduced fat/lean mass ratio compared to the non-exercised PCOS mouse models. Some metabolic benefits promoted by exercise could be attributed to the increased muscle mass and increased energy expenditure.

Adipocyte hypertrophy has been linked with insulin resistance in women with PCOS (Manneras-Holm et al., 2011), and both DHT-

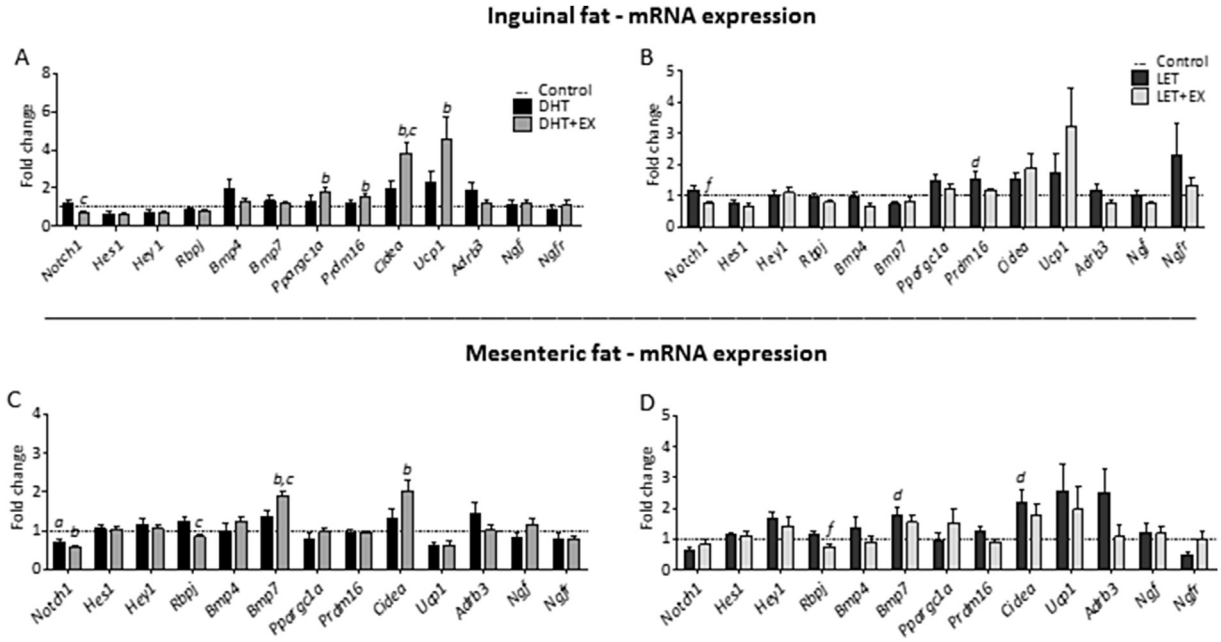


**Fig. 5.** Expression of genes related to Notch pathway, BMP family, browning and sympathetic activity in inguinal and mesenteric fat. (A) mRNA expression in inguinal fat of control, DHT, DHT + EX, (B) LET, and LET + EX groups; and in (C) mesenteric fat of control, DHT, DHT + EX, (D) LET, and LET + EX groups. DHT, dihydrotestosterone; LET, letrozole; EX, exercise. *a* – Control vs DHT ($P < 0.05$), *b* – Control vs DHT + EX ($P < 0.05$), *c* – DHT vs DHT + EX ($P < 0.05$), *d* – Control vs LET ($P < 0.05$), *e* – Control vs LET + EX ($P < 0.05$), *f* – LET vs LET + EX ($P < 0.05$). Values are shown as mean ± SEM. One-way ANOVA followed by Bonferroni post hoc test for data with normal distribution and Kruskal-Wallis followed by Dunn's test for skewed data were performed.

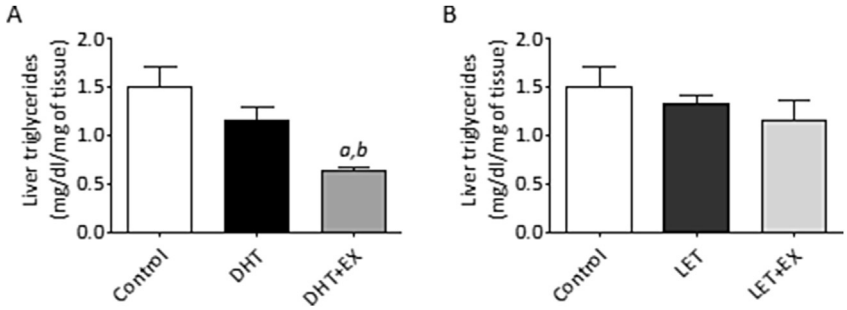


**Fig. 6.** Liver triglycerides content in the experimental groups. (A) Liver triglycerides content in DHT, DHT + EX and control group. (B) Liver triglycerides content in LET, LET + EX and control group. DHT, dihydrotestosterone; LET, letrozole; EX, exercise. *a* – Control vs DHT + EX ($P < 0.001$), *b* – DHT vs DHT + EX ($P < 0.05$). Values are shown as mean ± SEM. One-way ANOVA followed by Bonferroni *post hoc* test was performed.

and LET-induced PCOS mice models have enlarged inguinal and visceral adipocyte size (van Houten et al., 2012,Kauffman et al., 2015,Caldwell et al., 2014). However, those previous studies with DHT-an LET-induced PCOS mice models used different methods to quantify adipocyte size. The existing methods to assess adipocyte size vary regarding accuracy and limitations, but the use of technologies with osmium tetroxide-fixed adipocytes has becoming a powerful tool (Parlee et al., 2014). The use of the osmium tetroxide-fixed adipocytes with Coulter Counter Multisizer technique (McLaughlin et al., 2007) allows a precise analysis of cell distribution, and we found DHT-exposed mice presented more larger mesenteric adipocytes compared to controls. On the other hand, four to five weeks of exercise decreased the diameter of large adipocytes in the mesenteric adipose tissue of DHT-exposed mice, which was accompanied by increased expression of genes related to lipolysis (*Lipe* and *Lpl*). Running wheel exercised mice show increased lipolysis (Monleon et al., 2014), and the decrease in adipocyte size mirrors the effect of the exercise observed in both healthy and obese rat models, which also contribute to reduce adiposity (Gollisch et al., 2009). Adipose tissue lipolysis is an important event during prolonged exercise, since it provides fuel to the working skeletal muscles (Horowitz, 2003). Also, impaired adipogenesis and increased proportion of small adipocytes have been related to insulin resistance (McLaughlin et al., 2007,McLaughlin et al., 2014). Our data show an increased proportion of small adipocytes in mesenteric fat in LET-exposed mice compared to controls and exercise tended to restore this dysfunctional mesenteric fat morphology. Together, based on the differences in adipocyte size distribution comparing LET- and DHT-exposed animals, we suggest the treatments have distinct effects on adipose tissue morphology.

LET-exposed mice, in addition to increased adiposity, had increased expression of genes involved in lipolysis (*Lipe* and *Lpl*) in the mesenteric but not in the inguinal fat. These data fit with the lipolysis pattern observed in obesity, with increased basal lipolysis which is more pronounced in visceral than in subcutaneous fat

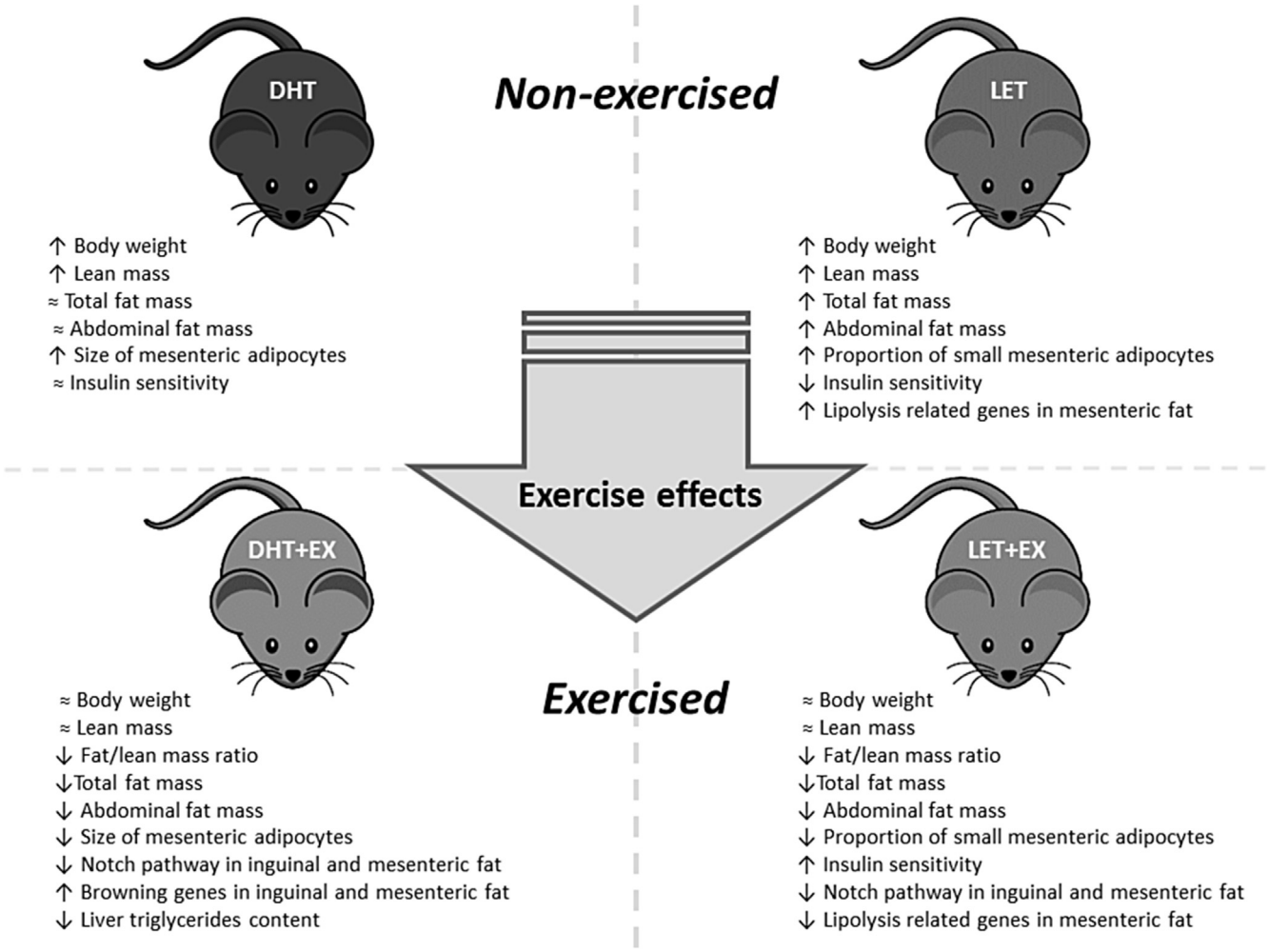


**Fig. 7.** Summary of metabolic phenotypes and exercise effects on letrozole- and dihydrotestosterone-exposed mice. Phenotypes from non-exercised DHT- and LET-exposed mice are summarized in the superior left and right quadrants, respectively. The results from non-exercised mice are compared to control mice. Effects of exercise on DHT- and LET-exposed mice are summarized in the inferior left and right quadrants, respectively. The results from exercised DHT- and LET-exposed mice are compared to respective non-exercised DHT- and LET-exposed mice. DHT, dihydrotestosterone; LET, letrozole; EX, exercise; ↑, increased; ↓ decreased; ≈ unaltered.

(Morigny et al., 2015). Increased lipolysis in obesity seems to be related to an impaired antilipolytic effect of insulin due to insulin resistance in adipocytes (Duncan et al., 2007). Impairment of insulin's antilipolytic action has been noticed in adipose tissue of women with visceral obesity (Johnson et al., 2001,Albu et al., 1999). Of note, weight loss restores the sensitivity to insulin in adipose tissue, which consequently restores the antilipolytic effects mediated by insulin in this tissue (Lofgren et al., 2005). The mRNA expression of *Lipe* and *Lpl* in the LET + EX group did not differ from controls, indicating a restored lipolytic dysfunction in visceral fat by exercise, likely due to improved adipose tissue insulin sensitivity.

Continuous letrozole and DHT exposure has previously been shown to induce insulin resistance, evaluated by euglycemic-hyperinsulinemic clamp, in rats (Maliqueo et al., 2013,Manneras et al., 2007). In our study, letrozole-induced PCOS mice displayed the most impaired metabolic phenotype, demonstrated by impaired glucose regulation during an ITT and increased adiposity. One likely explanation is that inhibition of P450 aromatase activity worsens the metabolic condition in the LET-exposed mice. Interestingly, the LET-exposed mouse models, at the chosen dose, does not present decreased serum estradiol levels compared healthy mice (Kauffman et al., 2015), indicating the metabolic disturbances found in this mouse model are not attributed to circulating estrogen deficiency.

Contrary to previous studies (van Houten et al., 2012,Kauffman et al., 2015), neither letrozole nor DHT exposure shifted the response during a glucose tolerance test. This could be due to the administration of glucose; we have used the oral glucose tolerance test, which represents the most physiological method of glucose administration (Ayala et al., 2010), whereas previous studies used the intraperitoneal (i.p.) glucose administration (van Houten et al., 2012,Kauffman et al., 2015). For example, the i.p. glucose tolerance test has elevated glucose levels compared to OGTT due to absence of incretin response (Andrikopoulos et al., 2008).

We have shown that exercise downregulates Notch signaling pathway, which suggests browning of WAT. Recently, Notch signaling has been recognized as an important player in metabolism. In fact, mice with adipose tissue-specific knockdown of *Notch1* and *Rbpj* displayed increased browning of WAT, reduced adiposity, improved glucose and insulin sensitivity, and were protected against high fat diet-induced obesity (Bi et al., 2014). Also, Notch-ligand blockade in mice has been shown to improve insulin and glucose metabolism; prevent weight gain; reduce fat mass, adipocyte size; and increase mRNA expression of GLUT-4 in adipose

tissue (Fukuda et al., 2012). In our study, exercise downregulated expression of *Notch1* in the inguinal fat depot and *Rbpj* in mesenteric fat in both LET- and DHT-exposed mice. In DHT-exposed mice, exercise increased the mRNA expression of browning markers in inguinal and mesenteric fat. Also, the expression of *Notch1* was negatively correlated to the expression of browning markers in inguinal fat in both exercised LET- and DHT-induced PCOS mice models. Together, these observations suggest that exercise may promote metabolic health via inhibition of Notch signaling pathway in the adipose tissue of the LET and DHT-induced mice PCOS models. This is, to our knowledge, the first study to show that exercise downregulates Notch signaling pathway in both subcutaneous and visceral adipose tissue of two different PCOS mouse models. However further studies are needed to elucidate the mechanisms of the exercise on the Notch signaling pathway in the adipose tissue of DHT- and LET-exposed mice.

In conclusion, four to five weeks of voluntary exercise tended to restore mesenteric adipocytes morphology in DHT- and LET-exposed mice, and restored insulin sensitivity and mesenteric expression of lipolysis-related genes in LET-exposed mice. Benefits could be explained, at least in part, by downregulation of Notch signaling pathway, and modulation of browning and lipolysis pathways in the adipose tissue.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.mce.2017.03.025.

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