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Background and objectives: White adipose tissue (WAT) shows marked sex- and diet-dependent differences. However, metabolic studies, especially on amino acid metabolism, are considerably limited. In the present study we wanted to compare the effects of sex and a obesogenic diet on amino acid metabolism in different WAT sites, centered on the uirea cycle and paths related to ammonium handling. **Experimental design:** Adult female and male rats were maintained under standard conditions. After killing under isoflurane anesthesia. WAT main sites were dissected and weighed. Subcutaneous, perigonadal, retroperitoneal and mesenteric WAT were analyzed for amino acid metabolism gene expression and enzyme activities. **Results:** There was a considerable stability of the urea cycle activities and expressions, irrespective of sex, and with only limited influence of site. Urea cycle operation being more resilient to change than other specialized site metabolic pathways. The robust control of WAT urea cycle was related, probably, to a function as provider of arginine/ citrulline. These data support a generalized, probably essential, role of WAT in overall amino-N handling. In contrast, sex affected deeply WAT ammonium-centered amino acid metabolism in a site-related fashion, with relatively higher emphasis in females' mesenteric WAT, contrasting with the opposite trend in males' subcutaneous WAT. **Conclusions:** We found an active amino acid metabolism in WAT, both in diversity and flow of pathways. Amino acid metabolism was lower than those of glucose-lipid interactions, but the differences were quantitatively lower than usually assumed. The effects of sex were limited on urea cycle expression and activity despite high variation in other metabolic pathways, which points to a centralized control of its operation affecting the adipose organ as a whole.

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ABSTRACT

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Key words: urea cycle, adipose tissue, citrulline, ammonium, urea, lipogenesis, adipose organ

INTRODUCTION

The influence of sex on adipose tissue distribution and function, as well as its implication in metabolic syndrome have been known for a long time (Mayes & Watson 2004). The influence of estrogen in the maintenance of a full functionality of adipose tissue under oxidative challenge (d'Eon et al. 2005), but also in the limitation of the deleterious effects of hypertrophic growth (Kumar et al. 2012) and inflammation (Stubbins et al. 2012) are key sex-related factors affecting the development and consequences of metabolic syndrome (Antonio et al. 2015). The relative antagonism of sex hormones and glucocorticoids , released in the aftermath of a tissue defensive response against excess nutrient load (Alemany 2012), also show marked sex-related differences. The normal distribution of fat in gynoid and android shapes of adult humans are, too, a consequence of the close interrelationship of adipose tissue with androgens and estrogens (Kotani et al. 1994). In addition, the severity of metabolic syndrome is also associated to the predominantly visceral distribution of fat in men as compared with the predominant subcutaneous distribution of premenopausal women (Demerath et al. 2007).

These differences are often overlooked when analyzing the regulation and metabolic responses of adipose tissue, especially under conditions of inflammation. There are clear differences between females and males in site distribution and metabolic responses (Porter et al. 2004), but most studies are limited to a single site and are focused on the responses to inflammation (Revelo et al. 2014).

The metabolic peculiarities of adipose tissue, especially those of white adipose tissue (WAT) are progressively being uncovered, showing a fair uniformity in metabolism and overall regulation (Romero et al. 2014), as well as a deep reinterpretation of the assumed –mainly triacylglycerol storage as energy reserve— main function of WAT (Galic et al. 2010; Romacho et al. 2014). There is a growing consensus on the participation of WAT as a "large" (albeit disperse) combined "organ" in the whole-body energy partition and handling of substrates (Giordano et al. 2014; Romero et al. 2014). The immune, repair, energy control and endocrine-paracrine function of WAT compounds our interest for this unique organ (Eringa et al. 2012; Ferrante 2013; Giordano et al. 2014). However, and in spite of extensive and extensive investigation, the level of knowledge of its metabolism other than the control of lipid synthesis and storage has been largely neglected.

We have found, recently, that WAT (in different main storage sites) contains a full urea cycle (unpublished results), which role we attributed to the extra-splanchnic production of citrulline. Arginine and citrulline are critical factors for muscle function (Ventura et al. 2013) and inter-organ N handling; but WAT is also a net producer of 3C fragments (Arriarán et al. 2015), including alanine (Snell & Duff 1977). It is also a net exporter of glutamine (Kowalski & Watford 1994), and can use branched-chain amino acids for energy and lipogenesis (Herman et al. 2010). The organ size, variety of known amino acid metabolic pathways and variety of physiological functions hint at WAT as a potentially important site for peripheral amino acid metabolism. The limited amount of available information, we found only a couple of earlier studies (Kowalski et al. 1997; López-Soriano & Alemany 1986b), is a serious limitation for a full understanding of whether amino acids should join the well-established role of WAT in the handling of lipids and glucose

This newly uncovered interest for WAT role on amino acid metabolism is largely limited by our almost nil knowledge of the role of sex on these factors. In general terms, androgens favor protein deposition (Griggs et al. 1989), and males tend to consume spontaneously more protein than females (Radcliffe & Webster 1978); on the other hand, estrogens lower body weight (Bryzgalova et al. 2008), in spite of women having –normally—a higher body fat percentage than men. Women and female rats alike, show a powerful resistance to fattening (Meyer et al. 2011), marking –in that point—a considerable difference with the males at the cell level because of estrogen protective effects (Stubbins et al. 2012). However, after menopause, this estrogenic protection wanes (Cagnacci et al. 2007).

In our studies, we wanted to check whether the gross differences in WAT distribution and resilience to change had a robust biochemical basis to translate the hormonal impulses into metabolic function. Thus, we analyzed whether rats WAT urea cycle and related amino acid catabolic processes showed differences modulated by sex. To get a wider picture we used four main WAT sites and we included in the study (for comparison) a number of key paths: genes/enzymes involved in the control of lipogenesis from glucose and in lipolysis.

MATERIALS AND METHODS

Experimental design and animal handling

All animal handling procedures and the experimental setup were in accordance with the animal handling guidelines of the corresponding European and Catalan Authorities. The Committee on Animal Experimentation of the University of Barcelona specifically authorized the procedures used in the present study.

Nine week old female and male Wistar rats (Harlan Laboratory Models, Sant Feliu de Codines, Spain) were used. The rats (N=6 per group) were housed in pairs (same sex) in solid-bottom cages with wood shards for bedding. They had free access to water and ate normal rat chow (type 2014, Harlan). They were kept in a controlled environment (lights on from 08:00 to 20:00; 21.5-22.5 ºC; 50-60% humidity) for at least one month prior to the experiment.

The rats were killed, under isoflurane anesthesia, at the beginning of a light cycle by aortic exsanguination, using dry-heparinized syringes, then, were rapidly dissected, taking samples of WAT sites:

mesenteric (ME), perigonadal (epididymal in males, periovaric in females, PG), retroperitoneal (RP) and subcutaneous (SC, inguinal fat pads). The samples were blotted and frozen with liquid nitrogen; after weighing, they were ground under liquid nitrogen and stored at -80ºC until processed. Later, the dissection of the rats continued, extracting the remaining WAT in ME, EP and RP sites; the rats were skinned, and the whole subcutaneous WAT was dissected. The weights of the recovered WAT were computed to establish the total mass of each WAT site.

Blood plasma parameters

The blood obtained from the aorta was centrifuged to obtain plasma, which was frozen and kept at -80ºC until processed. Plasma samples were used to measure glucose (kit #11504), triacylglycerols (kit #11828), total cholesterol (kit #11505) and urea (kit # 11537), all from Biosystems, Barcelona Spain). Lactate was measured with (ref. #1001330; Spinreac, Sant Esteve de Bas, Spain). Amino acids were analyzed individually using an amino acid analyzer (Pharmacia-LKB-Alpha-plus, Uppsala, Sweden) from plasma samples deproteinized with acetone (Arola et al. 1977). Since the method used did not provide fair analyses for glutamine (Gowda et al. 2015) and other amino acids (Trp, Cys, Asn), we decided to present only the partial sum of the other amino acids as a single indicative value.

Preparation of tissue homogenates

Frozen tissue samples were homogenized, using a tissue disruptor (Ultraturrax IKA-T10, Ika Werke, Staufen, Germany), in 5 volumes of chilled 70 mM hepes buffer pH 7.4 containing 1 mM dithiothreitol (Sigma, St Louis MO USA), 50 mM KCl, 1g/L Triton X-100 (Sigma) and 1 g/L lipid-free bovine serum albumin (Sigma). In homogenates to be used for carbamoyl-P synthase 2 estimation, the concentration of Triton X-100 was halved to decrease foaming. The homogenates were centrifuged for 10 min at 5,000xg; the floating fat layers and gross debris precipitates were discarded. The clean homogenates were kept on ice, and used for enzymatic analyses within 2 h of their preparation.

Tissue protein content was estimated with the Lowry method (Lowry et al. 1951). After development of color, fat droplet-generated turbidity was eliminated with the addition of small amounts of finely powdered solid MgO before reading the absorbance. In the measurements of homogenate protein content, homogenization buffer (which containined albumin) was used as blank. Enzyme activities were expressed in nkat/g protein.

Enzyme activity analyses

Carbamoyl-P synthase was estimated from the incorporation of ^{14}C-bicarbonate (Perkin Elmer, Bad Neuheim, Germany) into carbamoyl-P using a method previously described by us (Arriarán et al. 2012). No significant carbamoyl-P 1 activity was detected (and its gene was not expressed either in WAT). Thus, only carbamoyl-P synthase 2 was measured.

All other enzyme activities (ornithine carbamoyl-transferase, argininosuccinate synthase, arginino-succinate lyase and arginases 1 and 2) were estimated following recently developed methods, which are presented in detail in a Supplementary annex with some detail both to justify their adequacy and to allow others to employ a methodology developed for adipose tissue.

148 *Gene expression analysis*

149 Total tissue RNA was extracted from frozen tissue samples using the Tripure reagent (Roche Applied
150 Science, Indianapolis IN USA), and was quantified in a ND-100 spectrophotometer (Nanodrop
151 Technologies, Wilmington DE USA). RNA samples were reverse transcribed using the MMLV reverse
152 transcriptase (Promega, Madison, WI USA) system and oligo-dT primers. These data were also used to
153 determine the total RNA content of the tissue (per g of weight or g of protein) in order to establish
154 comparisons between the quantitative importance of gene expressions.

155 Real-time PCR (RT-PCR) amplification was carried out using 10 µL amplification mixtures containing
156 Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA USA), 4 ng of reverse-
157 transcribed RNA and 150 nmol of primers. Reactions were run on an ABI PRISM 7900 HT detection system
158 (Applied Biosystems) using a fluorescent threshold manually set to 0.15 for all runs.

159 A semi-quantitative approach for the estimation of the concentration of specific gene mRNAs per unit
160 of tissue/ RNA or protein weight was used (Romero et al. 2007). *Rplp0* was the charge control gene (Eagni
161 et al. 2013). We expressed the data as the number of transcript copies per gram of protein in order to obtain
162 comparable data between the groups. The genes analyzed and a list of primers used is presented in Table
163 1.

164 Figure 1 depicts a scheme of the relationships between the amino acid metabolism-related enzymes
165 which gene expressions have been analyzed in this study. This Figure also shows acronyms or
166 abbreviations of the names of the enzyme-genes used in Figures 2 and 3.

167 *Statistics*

168 One- and two-way ANOVA comparisons between groups, correlations and curve fitting (including Vi
169 estimations). were analyzed with the Prism 5 program (GraphPad Software, San Diego CA USA).

170

171 **RESULTS**

172 *Basic parameters*

173 Table 2 shows the body and main adipose tissue sites weights of the undisturbed female and male
174 animals used in the experiment. When aged about 13 weeks under the standard diet chow, female rats
175 weighed about 62% of their male counterparts. The males accumulated more fat than females, both in
176 individual sites and as a whole. However, the sum of the four sites analyzed showed almost identical
177 proportions vs. body weight, *c.* 8%. There were differences in total protein and RNA proportions (per g of
178 fresh tissue) between the different sites, but there was no global effect attributable to the variable "sex".

179 The main plasma parameters studied are presented in Table 3. Plasma glucose levels were higher
180 in males than in females. However, these data are influenced by isoflurane anesthesia (RA), and are
181 presented only as a general indication of normalcy. No differences were observed for lactate and total
182 cholesterol. Triacylglycerol levels were similar between both sex-groups, the values being, however,
183 significantly higher in females (albeit in the limit of statistical significance). Both plasma urea and the sum
184 of amino acids were also higher in female than in male rats.

185 *Urea cycle enzymes*

Figure 2 depicts both urea cycle enzyme activities and the expression of their corresponding genes in four main WAT sites of male and female rats. In both cases, activity and gene expression, the data were presented per g of tissue protein. The data are displayed on a log scale to allow a visual comparison of the site patterns of enzyme activities and expressions. The data used in this representation are also tabulated in numeric form in the Supplemental Tables 1 and 2. There was a considerable coincidence in the patterns of enzyme activity distribution (and male-female similarities) in enzyme activities for all four sites. This pattern was not parallelled by that of the corresponding gene expression data, which also showed a considerable uniformity in their patterns across the WAT sites. The statistical analysis of the data in Figure 2 showed significant differences for "site" for all enzyme expressions except for arginino-succinate synthase. The site-related differences in enzyme activities, however, were limited to arginino-succinate lyase and carbamoyl-P synthase.

Subcutaneous WAT showed more differences between sexes than other locations, affecting carbamoyl-P synthase 2 (both activity and gene expression) and arginino-succinate lyase (activity). Arginino-succinate synthase activity showed differences between females and males in mesenteric WAT, and higher expression in periovaric WAT.

Other amino acid metabolism-related gene expressions

Figure 3 shows the gene expressions of the non-urea cycle enzymes presented in Figure 1, as well as differentiated arginases 1 and 2, which were combined in Figure 2. The data are depicted also on a log scale to facilitate pattern comparison; the corresponding numerical results are shown in Supplemental Table 3.

In all sites, the expression of e-NOS was, at least one order of magnitude higher than arginase; subcutaneous WAT being an exception: despite showing a similar pattern of expressions, the levels of mRNA per g of tissue protein were higher for most genes, in subcutaneous WAT, than in the other three sites. There was a generalized predominance of glutamine synthetase expression over that of glutaminase. The glycine cleavage system (the H protein of this complex) and AMP deaminase showed also a robust expression, at levels comparable to those of alanine transaminases. The two branched-chain amino acid transaminases were also within this range, but the expression of the form 2 was much higher.

The statistical comparisons of the data in Figure 3 show limited effects for sex; overall only nitric oxide synthase, alanine transaminase 2 and glutaminase showed significant overall differences between female and male rats. Paired sex-related differences were concentrated in subcutaneous WAT, with higher male values in the expression of nitric oxide synthase, glutamine synthase (but female-predominant glutaminase), AMP deaminase and alanine transaminase 1. No sex-related differences were found in the other sites, except higher male values in alanine transaminase 2 of perigonadal WAT. The differences between sites, however, were more marked, affecting all genes studied except N-acetyl-glutamate synthase (low expression) and arginase 2, which is expressed only in subcutaneous WAT.

Gene expressions of proteins involved in WAT acyl-glycerol metabolism

Figure 4 presents the gene expressions of the key transporter and enzymes that regulate the lipogenic process from glucose to acetyl-CoA and from that metabolite to acyl-CoA, including the three most important WAT lipases. The data are presented in a log scale and the numerical data are shown in the Supplemental Table 4. In spite of a considerable uniformity in the patterns for all four sites, there were marked differences in the extent of gene expression. In general, subcutaneous WAT values were higher, than the other sites. Again, overall (for mesenteric, retroperitoneal and perigonadal WAT), female expression values tended to be higher than those of the males for genes coding enzymes of glucose utilization (GLUT4, hexokinase), generation of NADPH (glucose-6P dehydrogenase, malic enzyme), and lipogenesis (citrate: ATP lyase, and Acetyl-CoA carboxylase). Fatty acid synthase showed ample differences, which were not significant because of high data variability. Regulation of pyruvate dehydrogenase by its inhibiting kinases, mitochondrial handling of fatty acids (carnitine palmitoleoyl-transferases, acyl-CoA dehydrogenases) and lipolysis (except adipose triacylglycerol lipase) did not show the higher female expression values compared with males. In subcutaneous adipose tissue, however, this pattern was partly reversed, or the differences attenuated.

Subcutaneous WAT showed higher expression values for males in pyruvate dehydrogenase kinase 4, palmitoleoyl.carnitine acyl-transferase (liver), acyl-CoA dehydrogenase and both lipoprotein and adipose triacylglycerol lipases. In mesenteric WAT the only significant difference was for higher malic enzyme expression in females. In retroperitoneal WAT, female expression values were higher for malic enzyme, citrate: ATP lyase and acetyl-CoA carboxylase. Again, in periogonadal WAT female expression values were higher for glucose-6P dehydrogenase. The overall differences for "site" were significant for all genes investigated except for GLUT4, malic enzyme, citrate: ATP lyase and fatty acid synthase.

Comparison of female and male gene expression

Table 4 shows the direct quotients of the mean expression values for each site between female and male rats. Since the relative importance of a given gene is reflected by the proportion of its mRNA vs. total mRNA; total RNA is only an approximation to mRNA, but it may be a better reference for "metabolic activity" than using expressions per g of protein or tissue weight. Consequently, we expressed the data in fmol/µg of total RNA to calculate the female/ male quotients; the data are thus dimensionless, but show major differences between sexes, albeit without the possible aid of the precise statistical quantification of the differences observed in the direct comparisons of Figures 2-4. This Table 4 tries to summarize independently for each site using RNA instead of protein for comparison.

The expression of urea cycle enzymes in mesenteric WAT showed no differences between sexes, and, except for arginase 1 (more expressed in females in most sites), retroperitoneal WAT showed no differences either. Expressions in perigonadal WAT were higher in females. The only enzyme with higher expression in males was ornithine transcarbamylase in subcutaneous WAT.

In contrast with the absence of sex-related changes in mesenteric WAT, most amino acid metabolism-related genes were more expressed in females in this tissue, in over contrast with subcutaneous and perigonadal WAT. The relatively limited differences in amino acid metabolism-related

expressions induced by sex were in marked contrast with the marked predominance in females of lipogenesis-related genes in mesenteric (maximal differences), retroperitoneal and perigonadal WAT sites; the effects were less marked in subcutaneous WAT. In all (except mesenteric) WAT sites, male rats showed higher male gene expressions for pyruvate dehydrogenase kinase 4, critical factor in the control of conversion of pyruvate to acetyl-CoA, hinting at a more active conversion in male than in female rats.

Again, in marked contrast with lipogenesis, fatty acid oxidation did not show a marked female predominance in related gene expressions. In fact, transport of acyl-CoA into the mitochondria via carnitine palmitoleoyl-transferase showed an all-site higher male gene expression, and this difference extended to beta oxidation in subcutaneous WAT. There were only limited changes in lipase expressions in all sites, with a clear difference between subcutaneous and retroperitoneal (male predominance in adipose triacylglycerol lipase) vs. mesenteric (female predominance in hormone sensitive lipase).

DISCUSSION

The results presented support a wide extension of amino acid metabolism in different sites of WAT, with enzyme activities and expressions following similar patterns in all four sites studied. In addition to urea cycle, AMP-deaminase (Arola et al. 1981a), glutamine synthetase (Arola et al. 1981b), glutamate dehydrogenase (Arola et al. 1979) and nitric oxide synthase (Pilon et al. 2000), we found that WAT expresses the glycine cleavage system (at least the critical H protein), so far not described.

The metabolic capabilities of WAT with respect to amino acid metabolism are probably much more extensive than usually assumed. The range of expressions observed for amino acid metabolism-related enzymes in the four WAT sites studied (Figures 2 and 3) was mostly in the 5-500 fmol/g protein. In comparison, the expressions (Figure 4) for lipogenesis, the (assumed) key metabolic function of WAT, and other lipid metabolism-related expressions were in the range of 10-1000 fmol/g protein. The differences between lipogenesis and amino acid metabolism-related gene expressions were not as extensive as expected, and hints at the potential relative importance of amino acid metabolism in WAT.

The considerable uniformity of WAT urea cycle-enzyme activities and expressions, and their marked independence of sex can be interpreted essentially two ways: a) as playing a minimal metabolic role: i.e., a residual, secondary or specialized pathway. Alternatively, b), it can be assumed to be a consequence of a well established and robust homeostatic maintenance of its function. That is, a role critical enough not to be sensibly influenced by some external regulatory factors such as sex hormones. The first possibility may seem the more obvious, but it is insufficient to counter a number of critical arguments: First of all, the unexpectedly high level of expressions and activities. The mere existence of urea cycle in a peripheral, disperse, tissue, not present (as far as we know) elsewhere out of the splanchnic bed. The varying ratios of activity/ expression hinting at posttranslational (uniformized) control. The probable relationship with the peripheral (and critical) synthesis of citrulline (Yu et al. 1996), which complements its conversion by the kidney (Borsook & Dubnoff 1941). The intervention of WAT in substrate cycles, including alanine synthesis (Snell & Duff 1977) and glutamine release (Kowalski & Watford 1994). The actual quantitative importance

of both activity and gene expression compared with those of the mainstay of WAT metabolism: lipogenesis. The lack of sex-related differences in WAT sites of urea cycle compared with lipogenic processes, as shown in Figures 2-4. This analysis is further complicated by the scarce availability of studies of amino acid metabolism in WAT (López-Soriano & Alemany 1986a), and is compounded by the overall large size of the adipose organ (Romero et al. 2014), even taking into account the metabolically inert mass of fat. Taken together, these arguments support a higher importance than usually assumed for WAT role in amino acid metabolism.

Our data suggest, in any case, a clear site-sex interaction that brings up differences in the expression of several amino acid metabolism-related genes other than urea cycle, which remains uncannily undisturbed and globally uniform. In males, subcutaneous WAT shows higher expressions for genes related to transfer to the mitochondria and oxidation of acyl-CoA than females; this is consistent with the possibility of using fatty acids as energy substrate. The higher male inhibition of pyruvate dehydrogenase, by kinases, generalized to most sites, reinforces this trend. On the other side, mainly in female mesenteric WAT, the expression of lipogenic enzyme genes (with respect to total RNA) was much higher than those of males, with ratios higher than 5 for a number of genes. In contrast, lipases showed almost nil differences. The female/male quotient differences in the expressions of four groups of metabolic pathways (urea cycle, other amino acid metabolism, lipogenesis and lipolysis) prove that the limited changes in urea cycle, compared with lipogenesis, are not a consequence of overall lack of effects of sex on WAT. It is, instead, a specific characteristic of the urea cycle as compared to other metabolic pathways.

The higher lipogenic (and lower lipolytic) gene expressions of female mesenteric WAT is counterintuitive when we think of the higher mass of visceral WAT in adult men (Bosch et al. 2015), correlated with metabolic syndrome-related pathologies (Watanabe & Tochikubo 2003), especially insulin resistance (Pascot et al. 2000). Probably, the lower male expressions found here mirror a less active metabolism, in conjunction with the intestine and liver. The discordances in sex-related control of metabolism and fat deposition between humans and rats described above constitute a critical caveat against generalization to humans of what is found using animal models, in spite of shared mechanisms and trends

Only a few studies have been done comparing different WAT sites' metabolism, including the use of isolated adipocytes (Gondret et al. 2008) and morphologic analyses (Pond et al. 1984). The data we present here show that the most commonly used WAT sites, perigonadal (Vydelingum 1987) and retroperitoneal (Woutheres Bortolotto et al. 2007), show the lesser differences and changes elicited by sex; the main sex-related differences on lipid metabolism being centered on the contrast of true visceral (mesenteric) *vs.* subcutaneous WAT.

In addition to the human-rodent question, the main limitations of this study are the lack of previous data with which establish comparisons, given the extreme scarcity of studies on WAT amino acid handling, made even more difficult by our scarce knowledge of amino acid metabolism as a whole. The extensive and interconnected net of pathways that had to be investigated. The most critical handicap, however, is the

lack of a critical mass of scientists and of actualized methodology: specific protein measurement reagents (antibodies), and/or methods (and products) for the estimation of enzyme activities and metabolites. Consequently, the data we present here should be taken as just an initial foray into a highly promising field of study.

Notwithstanding these caveats, the large amount of data gathered all point to a few preliminary conclusions, which could not be yet fully proven with the data we presented, largely because no other results are available for comparison. The potential for lipid handling of WAT sites was strongly modulated by sex, being considerably dependent on the site studied. This part of the study, devised to provide a background comparison for amino acid metabolism showed more extensive differences than expected, and needs to be studied more specifically and deeply before sufficiently based conclusions could be extracted.

There was a considerable stability of the urea cycle activities and expressions, irrespective of sex, and with only limited influence of site. Which we interpret as this cycle operation being more general than the specialized site metabolic peculiarities with robust control of WAT urea cycle, probably related to a possible role as provider of arginine/ citrulline (Beliveau Carey et al. 1993). The resilience to change of urea cycle in a context of plastic adaptability supports a generalized, probably essential, role in overall amino N handling.

In contrast, sex affected deeply WAT ammonium-centered amino acid metabolism in a site-related fashion, with relatively higher emphasis in females' mesenteric WAT, contrasting with the opposite trend (favoring males) in subcutaneous WAT. The data on amino acid catabolism fit with a role of mesenteric WAT as gatekeeper of the portal system, the hypothesis advanced for glucose (Arriarán et al. 2015) can be easily translated to the management of a possible excess of dietary amino acids.

In sum, amino acid metabolism in WAT seems to play a more important role (in diversity of pathways and intensity of their purported functions) than usually assumed. The effects of sex are limited but discernible, which points to a centralized control of its operation affecting the adipose organ as a whole.

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AUTHOR CONTRIBUTION STATEMENT

SAr and SAg did all the animal handling and laboratory work. JAFL and SAr carried out the statistical analyses. XR, JAFL and MA designed the experiments and established the main conclusions. MA conceived the study and wrote the paper. SAr, XR, JAFL and MA participated in the discussion of the results and in the preparation of the final text.

COMPETING FINANCIAL INTERESTS

The Authors declare that they have no conflict of interests

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Table 1(on next page)

Primer sequences used in the analysis of WAT gene expressions

	protein	gene	EC	primer sequence 5' > 3'	primer sequence 3' > 5'	bp
CPS2	glutamine-dependent carbamoyl-phosphate synthase	*Cad*	6.3.5.5	AGTTGGAGGAGGAGGCTGAG	ATTGATGGACAGGTGCTGGT	90
OTC	ornithine carbamoyl transferase	*Otc*	2.1.3.3	CTTGGGCGTGAATGAAAGTC	ATTGGGATGGTTGCTTCCT	126
ASS	arginino-succinate synthase	*Ass1*	6.3.4.5	CAAAGATGGCACTACCCACA	GTTCTCCACGATGTCAATGC	100
ASL	arginino-succinate lyase	*Asl*	4.3.2.1	CCGACCTTGCCTACTACCTG	GAGAGCCACCCCTTTCATCT	104
ARG1	arginase-1	*Arg1*	3.5.3.1	GCAGAGACCCAGAAGAATGG	GTGAGCATCCACCCAAATG	126
ARG2	arginase-2	*Arg2*	3.5.3.1	GCAGCCTCTTTCCTTTCTCA	CCACATCTCGTAAGCCAATG	122
NAGS	N-acetyl-glutamate synthase	*Nags*	2.3.1.1	GCAGCCCACCAAAATCAT	CAGGTTCACATTGCTCAGGA	82
eNOS	nitric oxide synthase, endothelial	*Nos3*	1.14.13.39	CAAGTCCTCACCGCCTTTT	GACATCACCGCAGACAAACA	138
GS	glutamine synthetase	*Glul*	6.3.1.2	AACCCTCACGCCAGCATA	CTGCGATGTTTTCCTCTCG	148
Gase	glutaminase kidney isoform, mitochondrial	*Gls*	3.5.1.2	CCGAAGGTTTGCTCTGTCA	AGGGCTGTTCTGGAGTCGTA	63
GDH1	glutamate dehydrogenase 1, mitochondrial	*Glud1*	1.4.1.3	GGACAGAATATCGGGTGCAT	TCAGGTCCAATCCCAGGTTA	122
GCS	glycine cleavage system H protein, mitochondrial	*Gcsh*	--	AAGCACGAATGGGTAACAGC	TCCAAAGCACCAAACTCCTC	146
AMPD	AMP deaminase 2	*Ampd2*	3.5.4.6	CGGCTTCTCTCACAAGGTG	CGGATGTCGTTACCCTCAG	78
AlaT1	alanine aminotransferase 1	*Gpt*	2.6.1.2	GTATTCCACGCAGCAGGAG	CACATAGCCACCACGAAACC	85
AlaT2	alanine aminotransferase 2	*Gpt2*	2.6.1.2	CATTCCCTCGGATTCTCATC	GCCTTCTCGCTGTCCAAA	146
BCT1	branched-chain-amino-acid aminotransferase, cytosolic	*Bcat1*	2.6.1.42	TGCCCAGTTGCCAGTATTC	CAGTGTCCATTCGCTCTTGA	138
BCT2	branched-chain-amino-acid aminotransferase, mitochondrial	*Bcat2*	2.6.1.42	AGTCTTCGGCTCAGGCACT	ATGGTAGGAATGTGGAGTTGCT	84
GLUT4	solute carrier family 2 (facilitated glucose transporter), member 4	*Glut4*	--	CACAATGAACCAGGGGATGG	CTTGATGACGGTGGCTCTGC	127
HK	hexokinase-2	*Hk2*	2.7.1.1	ATTCACCACGGCAACCACAT	GGACAAAGGGATTCAAGGCATC	113
G6PDH	glucose-6-phosphate 1- dehydrogenase	*G6pdx*	1.1.1.49	GACTGTGGGCAAGCTCCTCAA	GCTAGTGTGGCTATGGGCAGGT	77
ME	NADP-dependent malic enzyme	*Me1*	1.1.1.40	TTCCTACGTGTTCCCTGGAG	GGCCTTCTTGCAGGTGTTTA	131
PDHK2	pyruvate dehydrogenase kinase 2, mitochondrial	*Pdk2*	2.7.11.2	TCACTCTCCCTCCCATCAA	CGCCTCGGTCACTCATTT	75
PDHK4	pyruvate dehydrogenase [acetyl transferring] kinase 4, mitochondrial	*Pdk4*	2.7.11.2	GTCAGGCTATGGGACAGATGC	TTGGGATACACCAGTCATCAGC	137
CATPL	ATP citrate lyase	*Acly*	2.3.3.8	GACCAGAAGGGCGTGACCAT	GTTGTCCAGCATCCCACCAGT	96
ACoAC	acetyl-CoA carboxylase 1	*Acaca*	6.4.1.2	AGGAAGATGGTGTCCGCTCTG	GGGGAGATGTGCTGGGTCAT	145
FAS	fatty acid synthase	*Fasn*	2.3.1.85	CTTGGGTGCCGATTACAACC	GCCCTCCCGTACACTCACTC	163
PCATl	carnitine palmitoyltransferase 1, liver isoform	*Cpt1a*	2.3.1.21	CCGCTCATGGTCAACAGCA	CAGCAGTATGGCGTGGATGG	105
PCATm	carnitine palmitoyltransferase 2, mitochondrial	*Cpt2*	2.3.1.21	TGCTTGACGGATGTGGTTCC	GTGCTGGAGGTGGCTTTGGT	152
ACADH	long-chain acyl-CoA dehydrogenase, mitochondrial	*Acadl*	1.3.8.8	ATGCCAAAAGGTCTGGGAGT	TCGACCAAAAAGAGGCTAATG	148
ATL	adipose triacylglycerol lipase	*Atgl*	3.1.1.3	CGGTGGATGAAGGAGCAGACA	TGGCACAGACGGCAGAGACT	138
HSL	hormone-sensitive lipase	*Lipe*	3.1.1.79	CCCATAAGACCCCATTGCCTG	CTGCCTCAGACACACTCCTG	94
LPL	lipoprotein lipase	*Lpl*	3.1.1.34	GAAGGGGCTTGGAGATGTGG	TGCCTTGCTGGGGTTTTCTT	103
	60S acidic ribosomal protein 0 (*housekeeping gene*)	*Rplp0*	--	GAGCCAGCGAAGCCACACT	GATCAGCCCGAAGGAGAAGG	62

Table 2 (on next page)

Body and WAT site weight and composition of adult male and female Wistar rats.

The data correspond to the mean ± sem of 6 different animals. Statistical significance of the differences between groups was established with a 2-way anova; post-hoc Tuckey test: different superscript letters represent $P<0.05$ differences % BW corresponds to the percentage of body weight accounted for by the four WAT sites.

1

parameter	units	site	male	female	P site	P sex
body weight	g	-	373 ± 6	232 ± 8		<0.0001
WAT weight	g	SC	12.2 ± 0.20	7.02 ± 0.25	<0.0001	<0.0001
		ME	4.94 ± 0.49	3.92 ± 0.33		
		RP	6.29 ± 0.79	2.79 ± 0.35		
		PG	7.34 ± 0.64	4.83 ± 0.39		
		Σ sites	30.8 ± 1.7	18.6 ± 0.9	-	<0.0001
	% BW		8.3 ± 0.5	8.1 ± 0.5		NS
WAT protein	mg/g	SC	63.1 ± 11.6	51.8 ± 3.3	<0.0001	NS
		ME	74.2 ± 7.4	84.2 ± 2.6		
		RP	65.1 ± 6.3	62.9 ± 4.7		
		PG	44.3 ± 1.6	54.4 ± 2.4		
WAT RNA	µg/g	SC	248 ± 51	219 ± 19	<0.0001	NS
		ME	880 ± 84	793 ± 88		
		RP	48.8 ± 4.1	78.4 ± 4.1		
		PG	94.3 ± 6.0	119 ± 10		

2

3

Table 3(on next page)

Main energy plasma parameters of adult female and male Wistar rats

*This value does not include Gln, Asn, Trp and Cys. The data correspond to the mean ± sem of 6 different animals. Statistical significance of the differences between groups was established with a one-way anova.

parameter	units	male	female	P sex
glucose	mM	10.20 ± 0.42	8.64 ± 0.34	0.0162
lactate	mM	3.10 ± 0.29	3.78 ± 0.24	NS
total cholesterol	mM	1.97 ± 0.07	1.98 ± 0.16	NS
triacylglycerols	mM	1.50 ± 0.06	1.69 ± 0.06	0.0491
urea	mM	3.90 ± 0.17	5.13 ± 0.25	0.0023
total amino acids*	mM	3.34 ± 0.08	3.96 ± 0.18	0.0104

1

Table 4(on next page)

Female / male gene expression ratios in WAT sites of Wistar rats

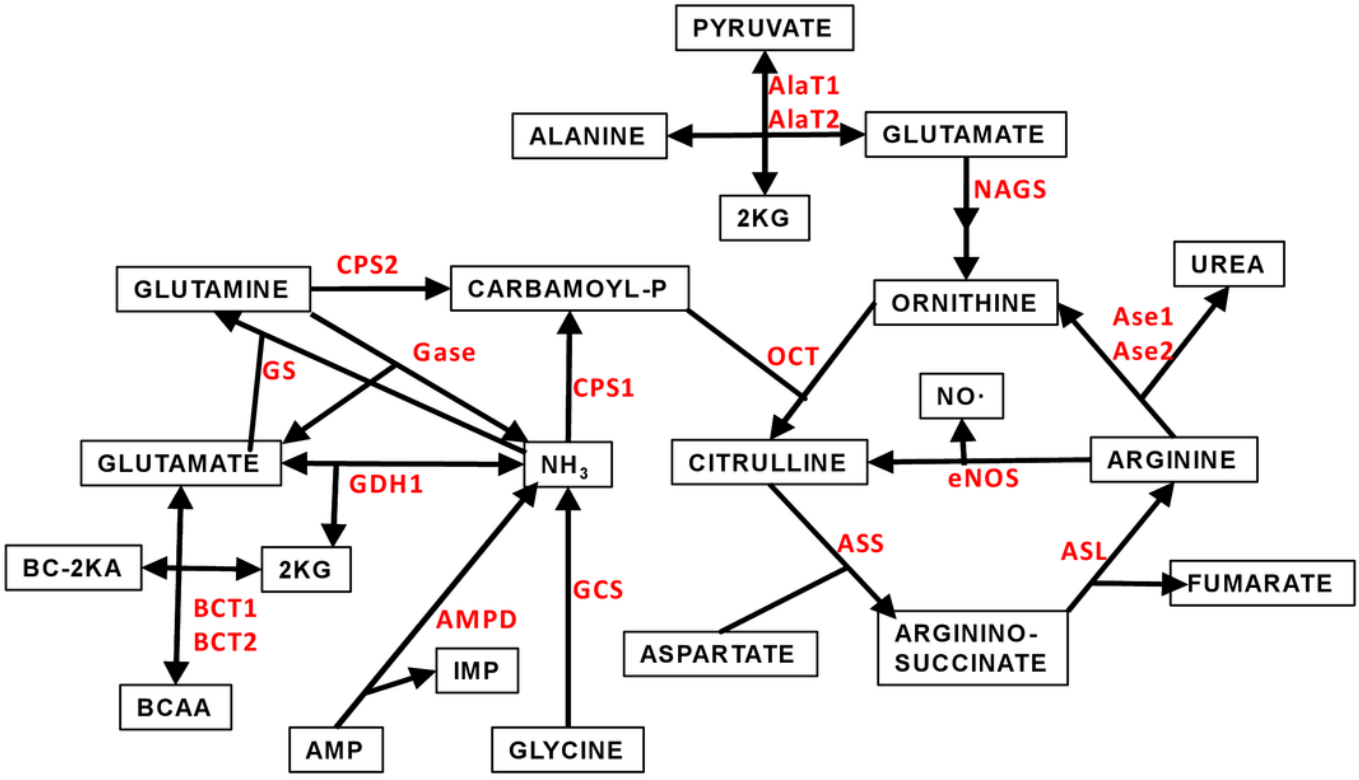
The data shown are the female / male quotients for each gene mRNA (in fmol/µg total RNA) present in the indicated WAT site. The quotients are unique mean values, thus no statistical analysis has been applied. Where the male rat data are predominant, the cell has been tinted in blue, and green when female data were higher. The limits used have been 0.75 and 1.33 (i.e. differences of about ±1/3rd), the data within this interval have been considered not to be different from unity, i.e. it was assumed that no clear differences due to sex existed. A darker shade has been used for differences higher than ±3x (i.e. 0.33 and 3.0)

parameter	SC	ME	RE	PG
urea cycle enzymes				
carbamoyl-P synthase 2	1.46	0.95	1.14	1.09
ornithine carbamoyl -transferase	0.53	1.33	1.03	2.71
argininosuccinate synthase	0.83	1.28	0.79	2.15
argininosuccinate lyase	0.83	1.15	1.02	0.91
arginase 1	1.46	1.16	1.78	1.35
other enzymes of amino acid metabolism				
N-acetyl-glutamate synthase	0.88	1.36	1.41	1.21
glutamate dehydrogenase 1	1.00	1.19	0.92	1.29
glutamine synthetase	0.37	1.36	0.78	0.72
glutaminase	2.04	1.55	0.85	0.91
AMP deaminase	0.66	1.65	0.94	1.08
glycine cleavage system	1.12	1.84	1.09	1.46
alanine transaminase 1	0.50	1.37	0.88	0.81
alanine transaminase 2	0.70	1.16	0.50	0.42
branched-chain amino acid transaminase 1	1.02	1.09	0.68	0.73
branched-chain amino acid transaminase 2	0.82	0.86	0.91	1.19
endothelial nitric oxide synthase	0.52	1.24	0.66	0.75
enzymes (and transporter) related with lipogenesis from glucose				
glucose transporter 4	1.88	2.53	1.63	1.53
hexokinase 2	1.43	2.11	1.75	1.47
glucose-6P dehydrogenase	0.99	1.87	1.73	2.32
malic enzyme	0.77	27.6	4.43	5.01
pyruvate dehydrogenase kinase 2	0.67	1.12	0.82	0.97
pyruvate dehydrogenase kinase 4	0.31	1.26	0.27	0.69
citrate: ATP lyase	2.49	5.39	4.37	2.36
acetyl-CoA carboxylase	2.65	8.19	2.82	1.88
fatty acid synthase	0.75	7.20	2.55	1.95
enzymes (and transporter) related with lipolysis and fatty acid oxidation				
carnitine palmitoleoyl transferase (liver)	0.51	0.73	0.40	0.57
carnitine palmitoleoyl transferase (muscle)	1.33	0.90	0.79	1.19
long-chain acyl-CoA dehydrogenase	0.47	1.28	0.90	1.08
adipose tissue triacylglycerol lipase	0.59	1.11	0.64	0.75
hormone-sensitive lipase	0.93	1.61	0.84	1.25
lipoprotein lipase	0.75	0.89	0.81	0.84

1

Scheme of the core of amino acid metabolism in WAT: urea cycle and ammonium handling

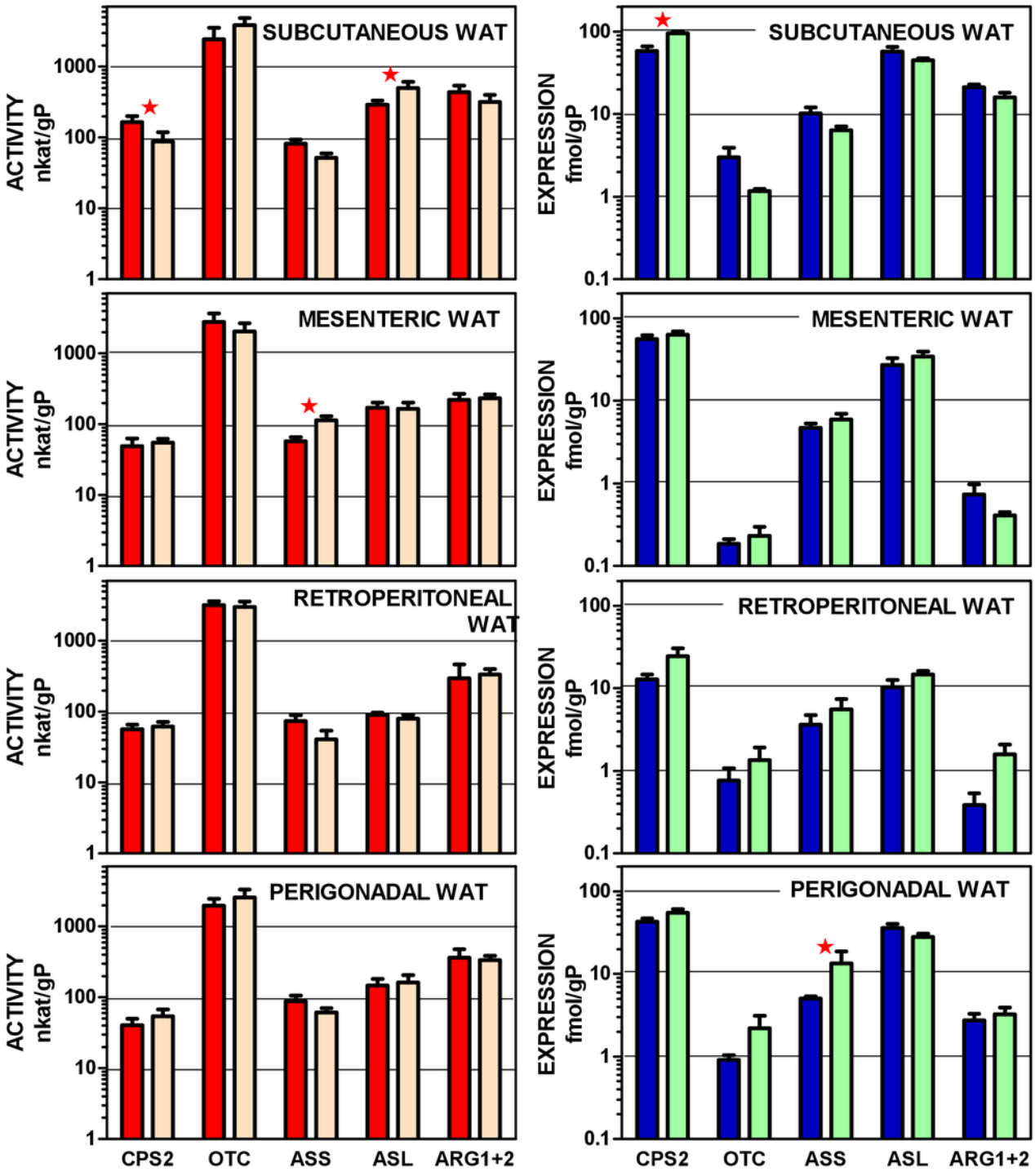
The abbreviations (marked in red) of the enzymes involved in the pathways depicted are the same described in Table 1 and throughout this study



2

Urea cycle enzyme activities and expressions of their coding genes in four WAT sites of female and male rats

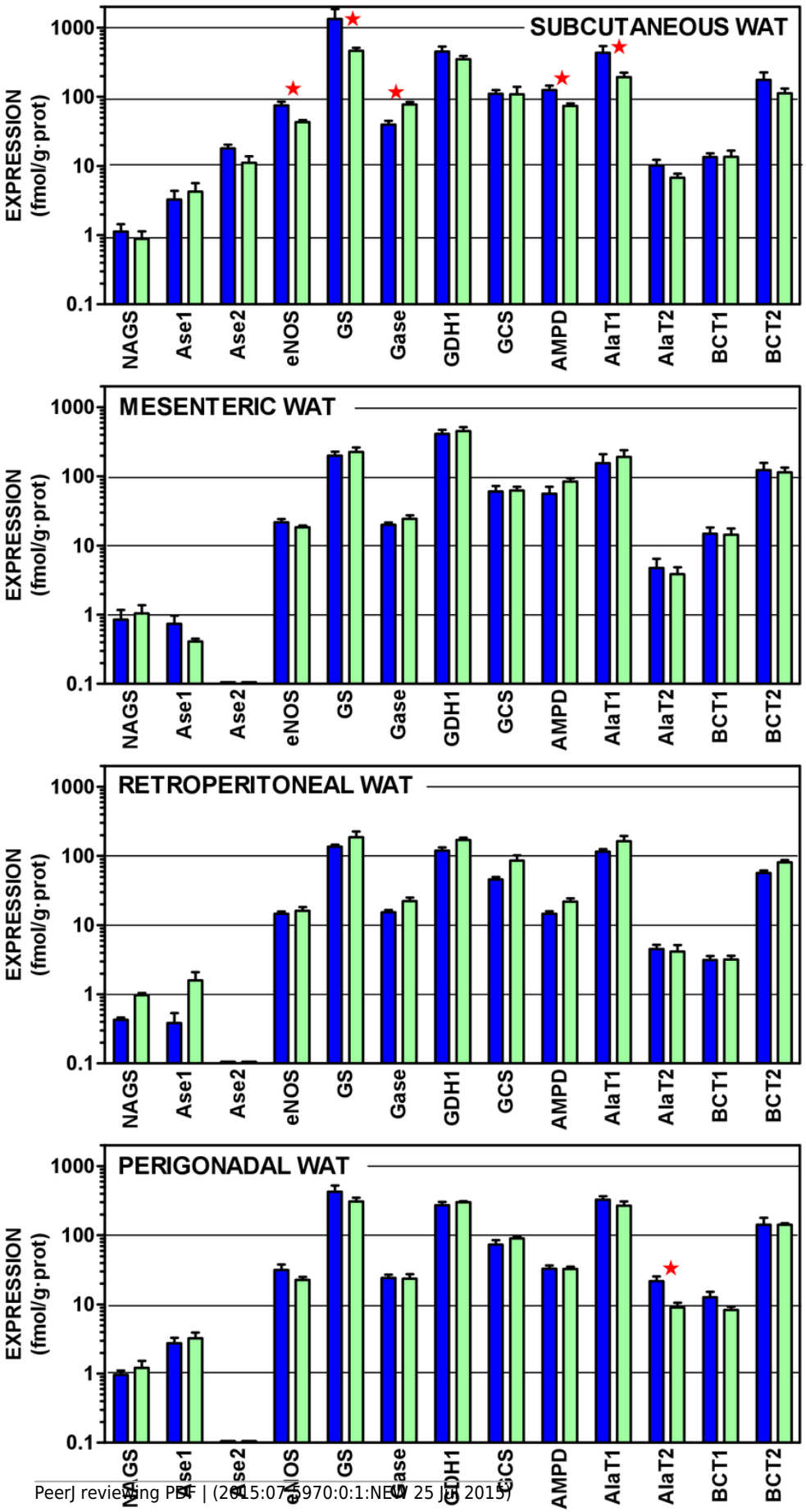
All data are the mean ± sem of 6 animals, and are presented in a log scale. The numerical data are shown in Supplemental Tables 1 and 2. The abbreviations used are the same presented in Figure 1 and Table 1. Left panels: enzyme activities, red columns correspond to males and orange to female rats. Right panels: gene expressions, blue columns represent the males, and green the females. Statistical analysis (2-way anova) of the differences between groups. Activity: There were no significant differences for "sex"; CPS2 and ASL showed $P<0.0001$ for "site". Expression: only CPS2 showed a significant ($P=0.0002$) for "sex": There were significant differences for "site" in CPS2 and ASL ($P<0.0001$), OTC ($P=0.0081$), ARG1+2 ($P<0.0001$); ASS showed no significant differences. The application of post-hoc Tuckey test between male/female pairs are shown in the Figure as red stars.


SUBCUTANEOUS WAT
MESENTERIC WAT
RETROPERITONEAL WAT
PERIGONADAL WAT
ACTIVITY nkat/gP
EXPRESSION fmol/gP
1
10
100
1000
0.1
CPS2
OTC
ASS
ASL
ARG1+2

3

Expression of genes coding for enzymes of amino acid metabolism in WAT sites of male and female rats

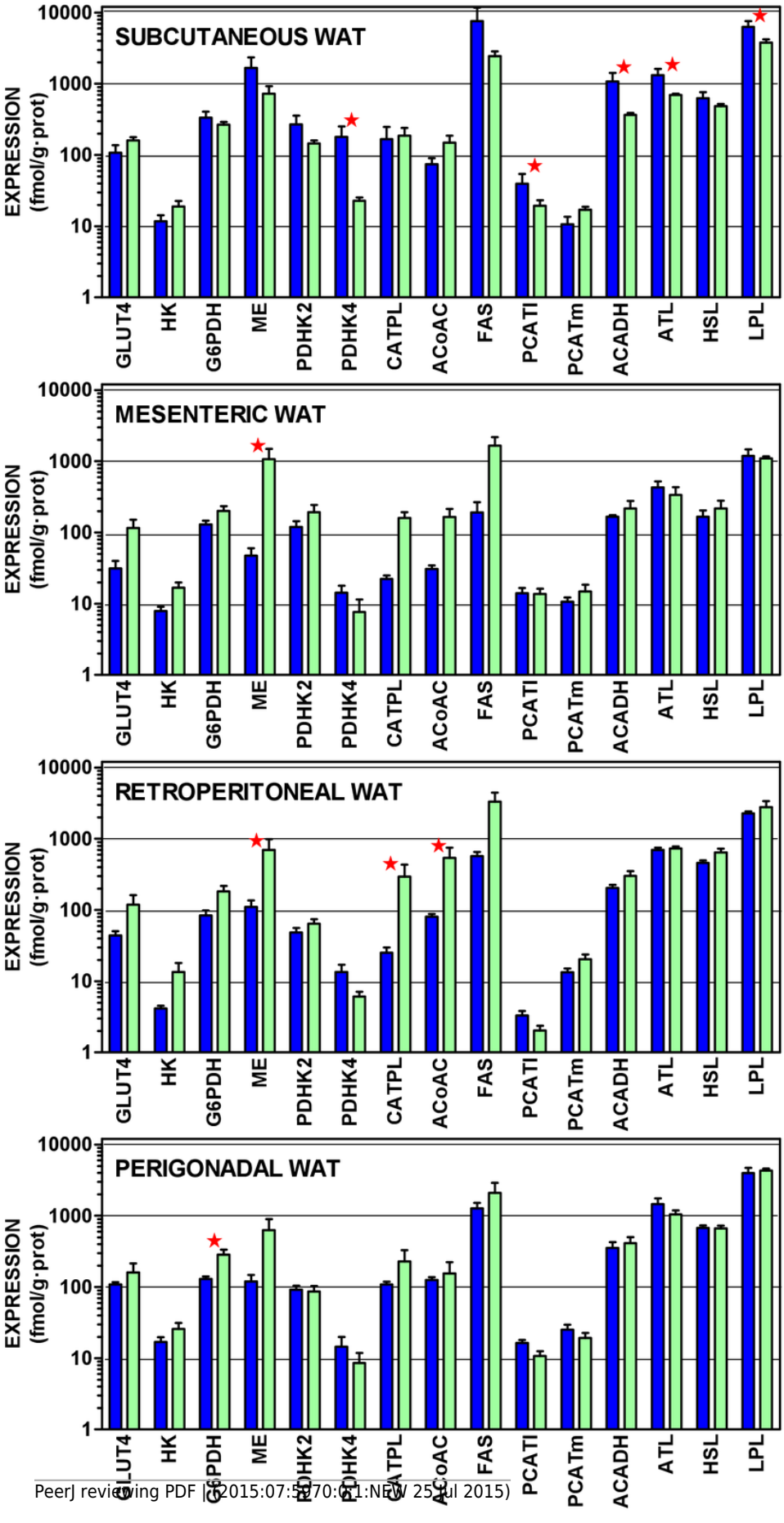
All data are the mean ± sem of 6 animals, and are presented in a log scale. The numerical data are shown in Supplemental Table 3. The abbreviations used are the same presented in Figure 1 and Table 1. Blue columns represent the males, and green the females. Statistical analysis (2-way anova) of the differences between groups. The variable "sex" showed global differences for Gase ($P<0.0001$), eNOS ($P=0.0014$) and AlaT2 ($P=0.0018$). The variable "site" showed significant differences for all genes ($P<0.0001$ for eNOS, Gase, GDH1, AMPD, BCT1 and AlaT2; $P=0.0005$ for Ase1; $P=0.0014$ for GS; $P=0.0023$ for AlaT1, $P=0.024$ for BCT2, and $P=0.039$ for GCS) except N-acetyl-glutamate synthase. The application of post-hoc Tuckey test between male/female pairs are shown in the Figure as red stars between the corresponding columns.


SUBCUTANEOUS WAT
MESENTERIC WAT
RETROPERITONEAL WAT
PERIGONADAL WAT
EXPRESSION (fmol/g·prot)
1000
100
10
1
0.1
NAGS
Ase1
Ase2
eNOS
GS
Gase
GDH1
GCS
AMPD
AlaT1
AlaT2
BCT1
BCT2

4

Expression of genes coding for transporter and enzymes related to lipogenesis from glucose and catabolism of lipid stores in WAT sites of male and female rats

All data are the mean ± sem of 6 animals, and are presented in a log scale. The numerical data are shown in Supplemental Table 4. The abbreviations used are the same presented in Figure 1 and Table 1. Blue columns represent the males, and green the females. Statistical analysis (2-way anova) of the differences between groups. The variable "sex" showed global differences for HK (P=0.0009), AcCoAC (P=0.0035), GLUT4 (P=0.0040), CATPL (P=0.010), G6PDH (P=0.020), PHDK4 (P=0.020), ME (P=0.024) and ATL (P=0.026). The variable "site" showed significant differences for HSL, ATL and LPL (P<0.0001), PCATl (P=0.0004), G6PDH (P=0.0005), PDHK2 (P=0.0013). PDHK4 (P=0.0016), ACADH (0.0022), HK (P=0.0057), PCATm (P=0.015) and AcCoAC (P=0.0384). The application of post-hoc Tuckey test between male/female pairs are shown in the Figure as red stars (P<0.05).


SUBCUTANEOUS WAT
MESENTERIC WAT
RETROPERITONEAL WAT
PERIGONADAL WAT
EXPRESSION (fmol/g·prot)
10000
1000
100
10
1
GLUT4
HK
G6PDH
ME
PDHK2
PDHK4
CATPL
ACoAC
FAS
PCATl
PCATm
ACADH
ATL
HSL
LPL