

Metabonomic study of the biochemical profiles of heterozygous myostatin knockout swine

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Abstract Myostatin is a transforming growth factor- β family member that normally acts to limit skeletal muscle growth. Myostatin gene (*MSTN*) knockout (KO) mice show possible effects for the prevention or treatment of metabolic disorders such as obesity and type 2 diabetes. We applied chromatography and mass spectrometry based metabonomics to assess system-wide metabolic response of heterozygous *MSTN* KO (*MSTN*^{+/-}) swine. Most of the metabolic data for *MSTN*^{+/-} swine were similar to the data for wild type (WT) control swine. There were, however, metabolic changes related to fatty acid metabolism, glucose utilization, lipid metabolism, as well as BCAA catabolism caused by monoallelic *MSTN* depletion. The statistical analyses suggested that: (1) most metabolic changes were not significant in *MSTN*^{+/-} swine compared to WT swine; (2) only a few metabolic properties were significantly different between KO and WT swine, especially for lipid metabolism. Significantly, these minor changes were most evident in female KO swine and suggested differences in gender sensitivity to myostatin.

Keywords myostatin, transforming growth factor- β family, skeletal muscle, metabolic disorders, chromatography, mass spectrometry, metabonomics

1 Introduction

Metabonomics is a systems approach for studying *in vivo* metabolic profiles, which promises to provide information

on drug toxicity, disease processes and gene function at several stages in the discovery and development process. This approach can also be readily adapted to investigate the functional consequences of genetic variation and transgenesis^[1]. Metabolic changes are directly linked to phenotypic changes, whereas gene expression changes are not; they merely indicate the potential for an end-point change. As such, metabonomics provides a useful connection between the ‘omics’ platforms and actual tissue histology and physiology.

Nuclear magnetic resonance, liquid chromatography-gas chromatography (LC-MS) and gas chromatography-mass spectrometry (GC-MS) are now routinely applied for determination of the changes in metabolite profiles associated with toxicity^[2–8], human disease^[9–11], pharmaceutical drugs^[12–15], genetic modification^[1,16–20], and influencing factors such as age, strain, gender, dietary difference and diurnal variations in rodents^[21–30].

Myostatin, also called growth differentiation factor-8 (GDF-8) previously, is a transforming growth factor- β (TGF- β) family member essential for the regulation of muscle development or function^[31,32]. The predicted myostatin protein includes an N-terminal signal sequence, a dibasic proteolytic processing site, and a C-terminal domain following the processing site. The N-terminal fragment following proteolytic processing has been most commonly referred to as the propeptide, which is presumed to be essential for the proper folding of the C-terminal domain into a cystine knot structure. In the C-terminal region, the myostatin sequence shows significant homology to other family members, and is also capable of binding the activin type II receptors, ActRIIA and ActRIIB *in vitro*^[32–34]. Loss of myostatin activity has been studied in mice^[35–39], cattle^[40–43], sheep^[44], dogs^[45], humans^[46], and swine (unpublished data). The results indicate that the function of myostatin appears to have been conserved across these species. Significantly, myostatin gene (*MSTN*)

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knockout (KO) mice have beneficial effects on fat and glucose metabolism and this approach may possibly be effective for the prevention or treatment of obesity and type 2 diabetes^[33,38]. This study aimed to research the metabolic effects in heterozygous *MSTN* KO (*MSTN*^{+/-}) swine by LC-MS and GC-MS.

2 Materials and methods

2.1 Animals

All experiments were conducted in accordance with the guidelines of China on the Use and Care of Laboratory Animals. Swine were housed in the same conditions and fed food and water *ad libitum*. *MSTN*^{+/-} Large White pigs were produced by homologous recombination and somatic cell nuclear transfer according to our previously published methods^[47,48].

2.2 Sample collection

Blood of 26 F₁ *MSTN*^{+/-} pigs (Table 1) were obtained in the morning following an overnight fast to minimize metabolic effects of inter-individual variations of food intake. Blood was centrifuged and plasma was taken and divided into two parts, one part (about 1 mL) was sent to the veterinary hospital of China Agricultural University, Beijing, China for clinical laboratory analysis. Another part (about 500 µL) was stored at -80°C until metabolomics analyses.

2.3 Sample preparation

Plasma samples (100 µL) were extracted using an automated MicroLab STAR[®] system (Hamilton Company, UT, USA) and maintained at 4°C throughout the extraction process. Recovery standards were added prior to the first step in the extraction process for quality control (QC) purposes. The samples were extracted using a proprietary series of organic and aqueous extractions according to the methods of Lawton and colleagues^[49]. The resulting extract was divided into two equal aliquots, one for LC/MS and one for GC/MS. Aliquots were placed on a TurboVap[®] (Zymark, Runcorn, UK) to remove the organic solvent, frozen and dried under vacuum overnight. Samples were then prepared for the appropriate instrument,

either LC/MS or GC/MS.

2.4 LC/MS and GC/MS analysis

LC/MS was conducted using Waters Acquity UPLC (ultra performance liquid chromatography) equipment (Waters, Milford, MA) coupled to an LTQ mass spectrometer (Thermo Fisher Scientific, Waltham, MA) equipped with an electrospray ionization (ESI) source and linear ion-trap (LIT) mass analyzer. Two separate UPLC/MS injections were performed on each sample which contained 11 or more injection standards at fixed concentrations. One aliquot was analyzed using acidic positive ion optimized conditions and the other using basic negative ion optimized conditions in two independent injections using separate dedicated columns. Extracts reconstituted in acidic conditions were gradient-eluted using water and methanol both containing 0.1% formic acid, while the basic extracts, which also used water/methanol, contained 6.5 mmol·L⁻¹ ammonium bicarbonate. The MS analysis alternated between MS and data-dependent MS² scans using dynamic exclusion.

The derivatized samples destined for GC/MS were analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole MS (ThermoElectron Corporation) using electron impact ionization. The GC column was 5% phenyl, the initial oven temperature was 40°C and then ramped to 300°C for a 16 min. period. The samples were re-dried under vacuum desiccation for a minimum of 24 h before being derivatized under dried nitrogen using bistrimethyl-silyl-trifluoroacetamide (BSTFA). GC/MS was tuned and calibrated daily for mass resolution and mass accuracy^[50].

2.5 Compound identification

Compounds were identified by automated comparison to Metabolon's reference library entries using Metabolon's proprietary software (<http://www.metabolon.com>) developed for creating library entries from known chemical entities and then automatically fitting those spectra to experimentally derived spectra. As of writing, more than 1000 commercially available purified standard compounds had been acquired and registered in the laboratory information management system for distribution to both the LC and GC platforms for determination of their analytical characteristics. The combination of chromato-

Table 1 Experimental animals

Group	Sample	Bodyweight (mean±SD)/kg	Generation	Description
WT	Male (<i>n</i> = 11)	114.36±4.02	F ₁	Wild type adult swine (<i>MSTN</i> ^{+/+})
	Female (<i>n</i> = 3)	113.92±5.40		
KO	Male (<i>n</i> = 7)	113.74±5.35	F ₁	Heterozygous <i>MSTN</i> KO adult swine (<i>MSTN</i> ^{+/-})
	Female (<i>n</i> = 5)	114.43±5.24		

graphic properties and mass spectra give an indication of a match to the specific compound or an isobaric entity. Additional entities could be identified by virtue of their recurrent nature (both chromatographic and mass spectral). These compounds have the potential to be identified by future acquisition of a matching purified standard or by classical structural analysis.

2.6 Data normalization

For studies spanning multiple days, a data normalization step was performed to correct variation resulting from instrument inter-day tuning differences. Essentially, each compound was corrected in run-day blocks by adjusting medians to 1.0 and normalizing each data point proportionately (i.e., block correction, Fig. 1). For studies that did not require more than one day of analysis, no normalization was necessary, other than for purposes of data visualization.

2.7 Statistical analysis

Welch's *t*-tests and random forest analyses were used for significance tests and classification analysis, respectively. Welch's *t*-test is an adaptation of Student's *t*-test intended for use with two samples having possibly unequal variances^[51]. Random forest is a supervised classification technique based on an ensemble of decision trees^[52,53]. In contrast to a *t*-test, the random forest method tests whether the unknown means for two populations are different or not. An ANOVA was also performed with sex factor in this study. Statistical analyses are performed with the program R (<http://cran.r-project.org/>).

3 Results

3.1 Clinical laboratory analysis

As shown in Table 2, most clinical laboratory data revealed no significant differences between the WT and KO swine, indicating that the two groups of swine had a similar health status. The plasma level of alanine transaminase and lactic dehydrogenase (LDH) was significantly lower in KO male swine ($P < 0.05$), and the plasma LDH level was very significantly lower in KO female swine ($P < 0.01$). These minor differences in clinical data suggest that there may have been minor metabolic differences between the two group of swine.

3.2 Metabolomic analysis

Using LC/MS and GC/MS analysis, 300 compounds matched a named structure in Metabolon's reference library; these compounds came from the following super metabolic pathways: amino acid, peptide, energy, lipid, nucleotide, xenobiotics, cofactors and vitamins. Of these, 43 biochemicals were different significantly ($P \leq 0.05$) between the experimental groups. The statistical changes were calculated by the ratio of their group means and are listed in Table 3. These data suggest that loss of myostation has minimal effects on the biochemical profile of *MSTN*^{+/-} swine. Significantly, these minor changes were most evident in female *MSTN* KO plasma (female to male ratio of 35:10, Table 3) and may reflect gender specific differences in sensitivity to myostatin. Furthermore, the total number of significant changes observed in male *MSTN* KO plasma (10) was below the number expected by

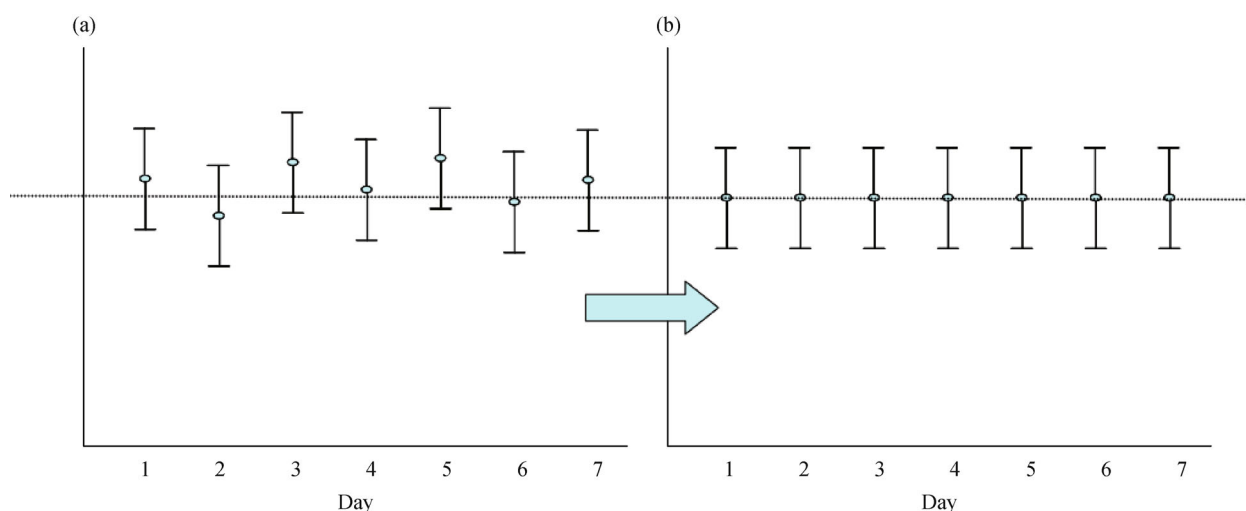


Fig. 1 Visualization of data normalization. (a) Before correction; (b) after correction.

Table 2 Clinical laboratory analysis of KO (*MSTN*^{+/-}) and WT (*MSTN*^{+/+}) swine

Item	KO (<i>MSTN</i> ^{+/-})		WT (<i>MSTN</i> ^{+/+})		<i>P</i>	
	Male	Female	Male	Female	Male	Female
WBC/($\times 10^9 \cdot L^{-1}$)	20.8 (4.8)	23.5 (1.0)	26.1 (6.8)	25.4 (7.4)	0.0923	0.6984
RBC/($\times 10^{12} \cdot L^{-1}$)	6.3 (0.8)	6.9 (0.5)	6.9 (0.5)	6.0 (0.7)	0.1446	0.1387
HGB/(g·L ⁻¹)	106 (12.8)	120.2 (7.8)	117.8 (3.8)	107.0 (15.9)	0.0742	0.2838
HCT/%	34.7 (4.4)	39.1 (2.9)	38.1 (2.0)	34.3 (4.1)	0.1221	0.1666
MCV/fL	54.9 (1.2)	56.4 (2.5)	55.2 (2.4)	56.7 (1.0)	0.7692	0.8638
MCH/pg	16.8 (1.0)	17.4 (1.1)	17.1 (1.2)	17.6 (0.7)	0.5846	0.7050
MCHC/(g·L ⁻¹)	307 (22.9)	308 (10.3)	310.3 (15.2)	312.0 (15.0)	0.7272	0.7094
PLT/($\times 10^9 \cdot L^{-1}$)	228 (119.7)	321 (103.7)	329.1 (103.2)	351.7 (92.4)	0.1933	0.6870
TBIL/($\mu\text{mol} \cdot L^{-1}$)	3.57 (2.44)	4.80 (3.11)	3.10 (1.91)	1.00 (0)	0.6773	0.0525
DBIL/($\mu\text{mol} \cdot L^{-1}$)	12.14 (1.46)	12.80 (2.17)	12.60 (1.17)	11.33 (0.58)	0.5067	0.2135
ALT/(U·L ⁻¹)	56.43 (11.31) ^a	69.60 (9.91)	71.70 (9.15) ^a	58.00 (7.21)	0.0128	0.1091
AST/(U·L ⁻¹)	113.00 (83.66)	83.20 (7.76)	121.40 (63.12)	71.33 (34.02)	0.8265	0.6091
TP/(g·L ⁻¹)	63.03 (8.08)	69.82 (2.19)	73.24 (5.75)	71.23 (9.38)	0.0164	0.8200
ALB/GLO	1.51 (0.32)	1.60 (0.19)	1.43 (0.17)	1.27 (0.24)	0.5586	0.1297
GGT/(U·L ⁻¹)	37.14 (9.42)	110.80 (40.54)	40.90 (16.35)	40.33 (19.09)	0.5587	0.0164
ALP/(U·L ⁻¹)	19.43 (17.92)	10.00 (7.91)	11.40 (7.03)	5.33 (3.21)	0.2956	0.2895
BUN/(mmol·L ⁻¹)	4.06 (1.45)	4.61 (0.45)	4.55 (0.90)	4.85 (1.25)	0.4515	0.7780
CRE/($\mu\text{mol} \cdot L^{-1}$)	156 (17.72)	152 (10.92)	145 (19.46)	152 (28.36)	0.2551	0.9972
GLU/(mmol·L ⁻¹)	4.27 (1.81)	3.43 (0.67)	4.62 (1.32)	3.99 (0.43)	0.6700	0.2018
TG/(mmol·L ⁻¹)	0.56 (0.24)	0.52 (0.13)	0.40 (0.09)	0.36 (0.12)	0.1367	0.1372
CHO/(mmol·L ⁻¹)	1.94 (0.33)	2.33 (0.15)	2.08 (0.19)	2.10 (0.15)	0.3294	0.0899
CK/(U·L ⁻¹)	2215 (750.5)	2014.00 (1121.3)	1726 (769.7)	758 (321.9)	0.2122	0.0659
LDH/(U·L ⁻¹)	731 (221.3) ^a	894 (78.3) ^b	1170 (531.3) ^a	475 (42.0) ^b	0.0362	0.0001
AMY/(U·L ⁻¹)	2442 (962.5)	3160 (1126.1)	2179 (1028.3)	1857 (976.0)	0.5989	0.1463

Note: Data are mean (SD). WBC, white blood cell; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, platelets; TBIL, total bilirubin; DBIL, direct bilirubin; ALT, alanine transaminase; AST, aspartate transaminase; TP, total protein; ALB, albumin; GLO, globulin; GGT, gamma-glutamyl transpeptidase; ALP, alkaline phosphatase; BUN, blood urea nitrogen; CRE, creatinine; GLU, glucose; TG, triglyceride; CHO, cholesterol; CK, creatine kinase; LDH, lactic dehydrogenase; AMY, amylase. a, significantly different between two groups ($P < 0.05$); b, very significantly different between two groups ($P < 0.01$).

Table 3 Significantly altered biochemicals between the experimental groups

Biochemicals	KO/WT	KO-M/WT-M	KO-F/WT-F
Total ($P \leq 0.05$)	43	10	35
Fold of change ≥ 1.00	29	2	25
Fold of change < 1.00	14	8	10

Note: KO, (*MSTN*^{+/-}); WT, (*MSTN*^{+/+}); M, male; F, female.

random chance (15) and therefore suggests the metabolic profile of *MSTN* KO male and wild-type porcine is similar. This overlap between WT and *MSTN* KO male biochemical profiles is further revealed by principal component analysis (Fig. 2). Nominal changes observed between WT and *MSTN* KO female plasma are highlighted below.

3.2.1 Fatty acid metabolism

As shown in Appendix A (Table S1), an accumulation of

essential, dicarboxylate and long-chain fatty acids was observed in KO swine plasma compared to WT. Importantly, these differences were predominantly observed in myostatin deficient females, but not males. Elevated free fatty acid levels in the blood are often suggestive of an increase in lipolysis. Lipolysis is the process by which triglycerides are hydrolyzed into free fatty acids that can then be further degraded through beta oxidation. Similarly, free carnitine levels were decreased in *MSTN* KO plasma, while carnitine conjugated lipids such

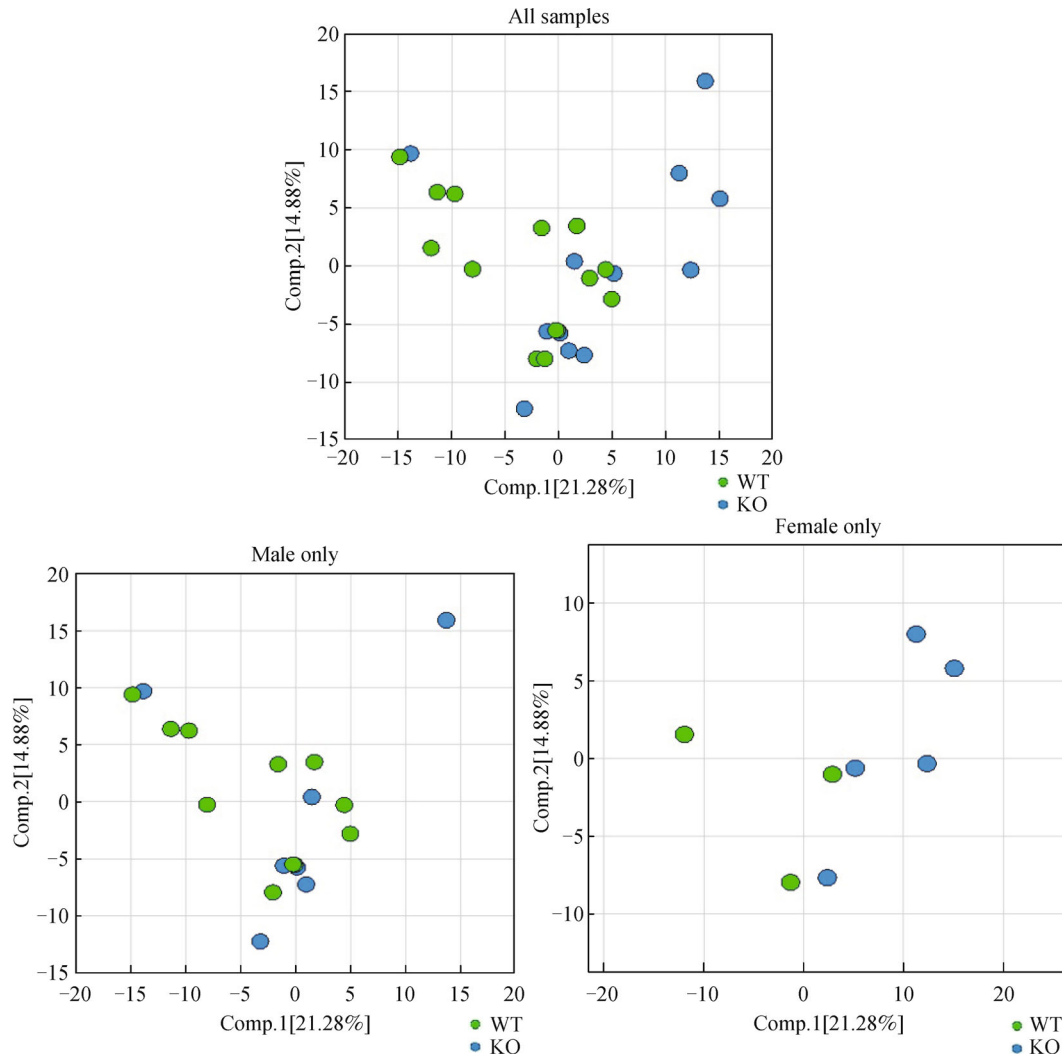


Fig. 2 Principle component analysis (PCA) for the two experimental groups

as palmitoylcarnitine were modestly elevated (Fig. 3). This suggests that the conjugation of long-chain free fatty acids to carnitine prepared it for transport into the mitochondria and subsequent oxidation. In agreement with markers of lipid oxidation, modest elevations in glycerol and 3-hydroxybutyrate (BHBA) was also observed in *MSTN* KO female plasma (Fig. 3). While glycerol can be utilized for the synthesis of triacylglycerides, it is also a catabolic product that is generated during their degradation. 3-hydroxybutyrate is a ketone body that is generated from excess acetyl-CoA that often results from enhanced fatty acid oxidation. Together, these results suggest the absence of myostatin in female swine may selectively result in increased fatty acid oxidation.

3.2.2 Glucose utilization

Both the levels of glucose and glycolytic intermediates,

such as glucose 6-phosphate, 3-phosphoglycerate and lactate, failed to differ in comparison to WT plasma (Fig. 4). Similarly, significant changes were not observed in the sorbitol pathway, which can increase in the presence of excess glucose, or pentose phosphate pathway.

3.2.3 Branched chain amino acid catabolism

In addition to protein synthesis, branched chain amino acids (BCAA) are important in replenishing the tricarboxylic acid cycle (TCA) cycle through the generation of acetyl-CoA and succinyl-CoA. In KO female plasma, the BCAA catabolic products 4-methyl-2-oxopentanoate, 3-methyl-2-oxobutyrate, and 3-hydroxyisobutyrate were elevated compared to WT (Fig. 5). Considering that glucose and lipid metabolism should be sufficient to fuel the TCA cycle, the accumulation of BCAA intermediates may be indicative of muscle turnover.

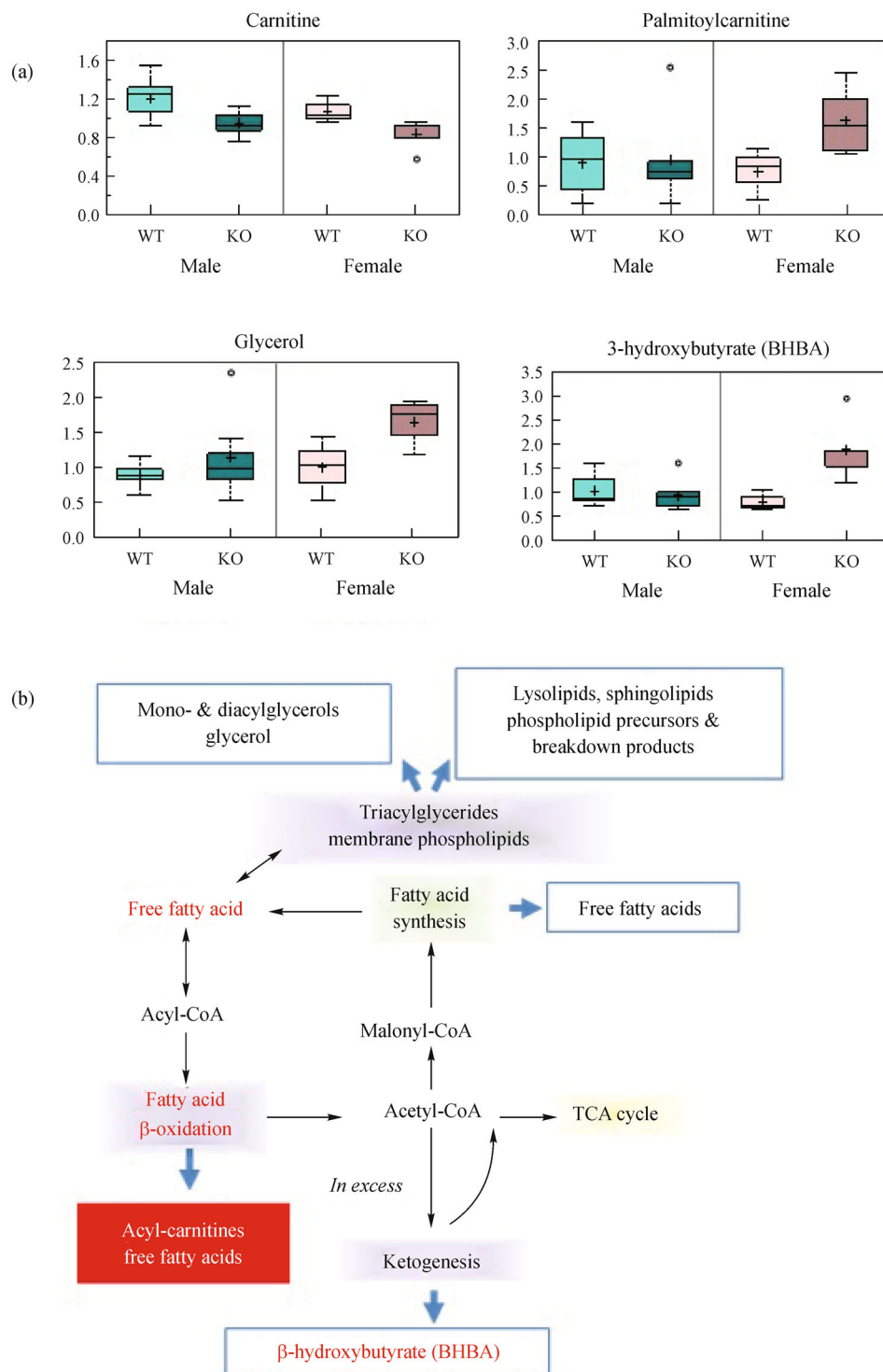


Fig. 3 Lipid metabolism. (a) Box plots of representative biochemicals between groups, median scaled values are presented on the y-axis; (b) lipid metabolic pathway.

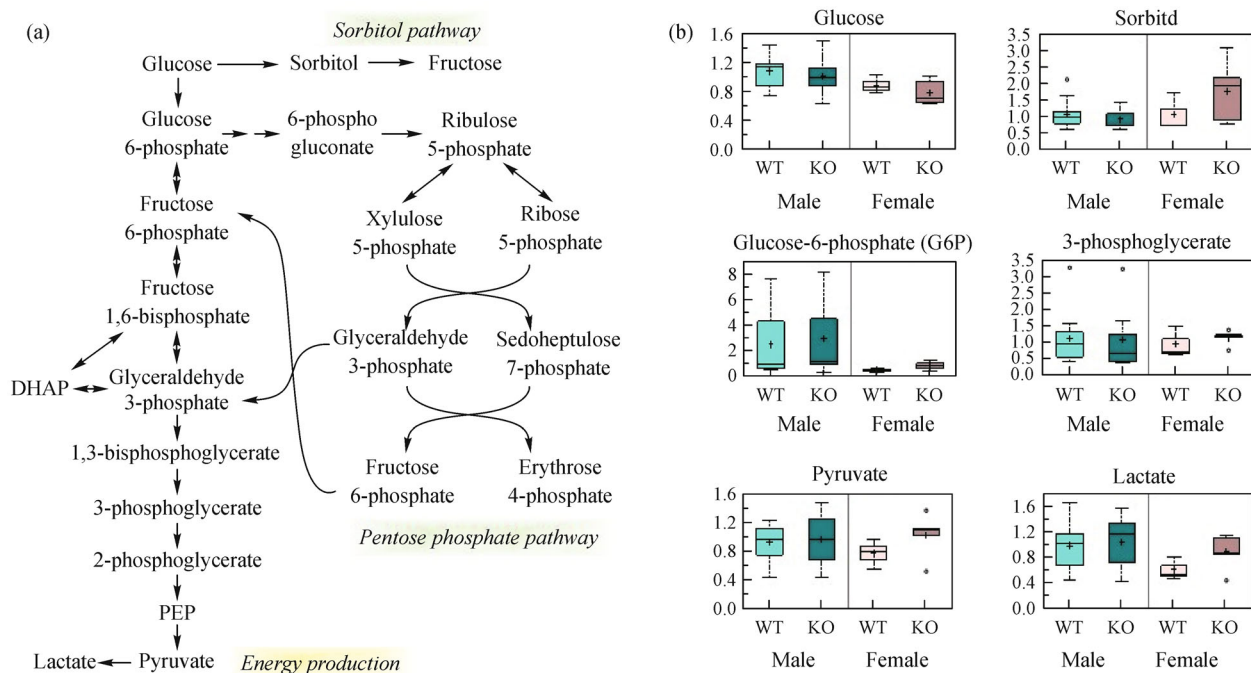


Fig. 4 Glucose metabolism. (a) Glucose metabolic pathway; (b) box plots of representative glucose and glycolytic intermediates between groups.

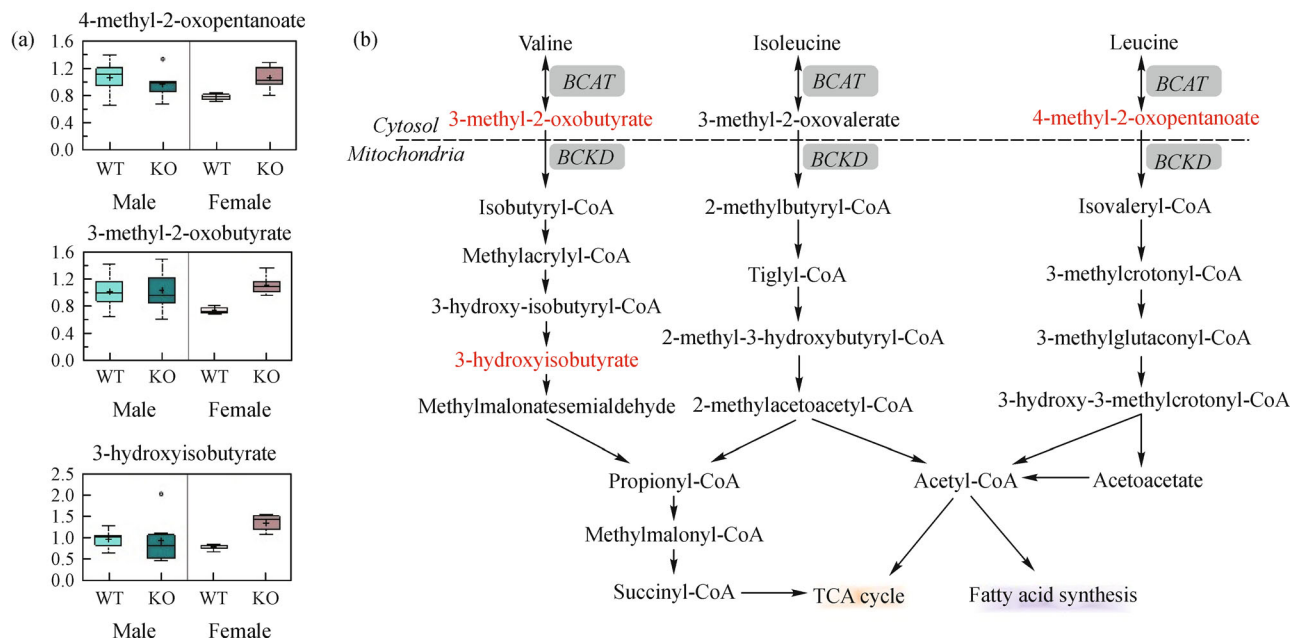


Fig. 5 BCAA catabolism. (a) Box plots of the BCAA catabolic key products, there were significant differences ($P < 0.05$) only between females; (b) BCAA catabolic pathway.

3.2.4 Other metabolic pathway

Besides the above pathways, this work also compared

metabolites of other pathways between KO and WT swine, such as amino acid, peptide, carbohydrate, energy, nucleotide, xenobiotics, cofactors and vitamins. Although

minor differences in metabolites were present, by and large, there were no significant metabolic differences between the two groups (data not shown).

4 Discussion

4.1 Influencing factors in metabolite profile

Bollard et al. found by metabonomics that time of sampling (day and night) markedly affected the metabolites profiles^[26]. In the case of C57BL10J mice, urine samples collected in the morning were found to contain higher concentrations of creatin, hippurate, trimethylamine, succinate, citrate and α -ketoglutarate, and decreased amounts of taurine, trimethylamine-*N*-oxide, spermine and 3-hydroxy-iso-valerate compare with samples collected in the afternoon^[27,54]. Except for the diurnal variation, there are many other factors, such as age, strain, diet and gender, that also affect metabolites profiles^[23,24,30,55]. This study attempted to minimize the impact of such factors on the metabolic results, by sampling in the morning (8:00–10:00), after overnight fasting, feeding the same forage, selecting age and gender matched controls and half-sibling animals.

4.2 Metabolomic analysis

Published studies have clearly indicated that adipogenesis decreased in *MSTN* KO mice^[33,38]. This study showed that fatty acid oxidation increased in *MSTN*^{+/-} female swine, it also indicated that adipose may be decreased in *MSTN*^{+/-} female swine. Together with reduced adipose and increased fatty acid oxidation, the lipid profile in *MSTN* null animals was improved. Adult male homozygous *MSTN* KO (*MSTN*^{-/-}) mice had significantly lower serum cholesterol and triglyceride levels compared to WT mice^[38,56,57]. This study has shown higher long chain fatty acids and glycerol levels in KO swine, and that KO swine had lower triglyceride levels compared with WT swine. However, no significant difference in cholesterol between KO and WT swine was found.

As found in fatty acid oxidation, this study also observed difference in the *MSTN* KO female in BCAA catabolism. Similar to Lee's findings^[39], this study also indicated a maternal effect on metabolic reaction results, most likely from prenatal transfer of myostatin or some other mediator from mother to fetus.

Circulating glucose levels often serve as an indicator of systemic metabolic status. However, no significant difference in glucose metabolism was observed in KO swine in this study. Together with lipid metabolism, the results suggests that glucose utilization was unaltered in *MSTN*^{+/-} swine, which differs from published reports of increased glycolysis and decreased lipid oxidation in myostatin deficient skeletal muscle in rodents^[37–39].

The published studies, mentioned above, have also demonstrated: (1) loss of myostatin activity can have beneficial metabolic effects in two genetic models of obesity and type 2 diabetes, and (2) myostatin inhibitors may be useful agents for the prevention or treatment of metabolic disorders such as obesity and type 2 diabetes^[33,38]. While the current study did not find any strong evidence for the prevention or treatment of obesity and type 2 diabetes in swine by lipid and glycolysis metabolism, there were minor changes in lipid and BACC metabolism. We conclude that the effect may not be obvious in *MSTN*^{+/-} animals, and we will confirm and improve these results when enough *MSTN*^{-/-} swine are produced in future.

5 Conclusions

This study demonstrated a few significant differences between WT and *MSTN*^{+/-} swine plasma. In the absence of myostatin, lipid oxidation, but not glucose utilization, was selectively elevated in female plasma. In contrast, the metabolic profile of WT and *MSTN* KO males were indistinguishable. Ultimately, these findings have important implications for livestock development, suggesting *MSTN*^{+/-} swine may be metabolically similar to WT swine.

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Supplementary materials The online version of this article at <http://dx.doi.org/10.15302/J-FASE-2015045> contains supplementary material (Appendix A).

Compliance with ethics guidelines Jianxiang Xu, Dengke Pan, Jie Zhao, Jianwu Wang, Xiaohong He, Yuehui Ma and Ning Li declare that they have no conflict of interest or financial conflicts to disclose.

All applicable institutional and national guidelines for the care and use of animals were followed.

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