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1 ***Short communication***

2 **Metabolomic analysis of oviduct fluid on day 3 post-estrus in Holstein heifers**

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11

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14 **Competing interest:** none.

15 **Short title:** Oviduct fluid metabolome in cattle

16 **Abstract**

17 The metabolites in the oviduct fluid (OF) of both oviducts were analyzed by proton nuclear  
18 magnetic resonance ( $^1\text{H-NMR}$ ) in Holstein heifers on day 3 after synchronized estrus.

19 Twenty-six metabolites were quantified, among which lactate, glycine and myoinositol were  
20 the most abundant. Six metabolites including glycine and myoinositol varied in amount  
21 according to the proximity to the corpus luteum. Glucose and histidine were among the most  
22 variable metabolites among heifers while threonine and lactate were among the most stable  
23 ones, suggesting different mechanisms of homeostasis in the OF.

24 **Keywords:** Oviduct, fallopian tube, metabolomics, embryo, cattle.

25

## 26 **1. Introduction**

27 In cattle breeding, the transfer of embryos into the uterus of female recipients is widely used  
28 for genetic purposes in North America, South America and Europe. Since 2016, the number  
29 of *in-vitro* produced (IVP) embryos exceeds that of their *in vivo* conceived counterparts [1].  
30 However, cattle embryos developed *in vitro*, i.e. by bypassing the oviduct, are of lower  
31 quality than *in-vivo* conceived embryos in terms of morphology, gene expression, methylation  
32 profiles and developmental competence [2]. In order to meet the requirements of the embryo  
33 and improve the quality of IVP blastocysts, a precise knowledge of the early oviduct  
34 microenvironment and its individual variation is highly desirable.

35 The bovine embryo develops in the oviduct fluid (OF) ipsilateral to the ovulation for  
36 approximately 4-5 days after estrus [3]. A crucial step for embryo development is the major  
37 activation of the embryonic genome at the 8- to 16-cell stage that occurs around day 3 post-  
38 estrus [3, 4]. The OF is a complex and dynamic fluid made of proteins, lipids, hormones,  
39 electrolytes and metabolites [5]. In a previous study, proton nuclear magnetic resonance  
40 spectroscopy (<sup>1</sup>H-NMR) was used successfully to monitor the metabolome of the bovine OF  
41 across the estrous cycle [6]. However, the OF samples collected at the post-ovulatory phase of  
42 cycle were pooled in groups of 3-10 cows on days 1 to 5 post-ovulation, precluding any  
43 evaluation of individual variability on a specific day in the cycle. We hypothesize that various  
44 mechanisms of homeostasis in the oviduct may lead to individual changes in OF metabolite  
45 concentrations despite similar genetics, age and breeding conditions. Therefore, the objective  
46 of this study was to compare the metabolome of the bovine OF between Holstein heifers on  
47 day 3 post-estrus.

## 48 **2. Materials and methods**

49 The protocol of this study was approved by the regional 'Centre-Val-de-Loire' ethics  
50 committee (agreement number #22926-2019112513501450 v4). All animals were housed  
51 indoors in straw bedded pens and were fed a diet consisting of straw *ad libitum* supplemented  
52 with 5 kg of concentrate per day distributed in 2 meals. The estrous cycles of nulliparous  
53 Holstein heifers (n=7) of similar age (average  $32.4 \pm 0.7$  months) and live weight (average  
54  $609 \pm 17$  kg) were synchronized using a 9-day progesterone-releasing intravaginal insert  
55 (PRID delta, Ceva Santé Animale, Libourne, France) combined with an injection of 0.5 mg of  
56 a prostaglandin analog (cloprostenol, Estrumate, MSD Animal Health, Beaucouzé, France)  
57 the day before removal of the vaginal insert. Behavioral signs of estrus were monitored using  
58 the automated neck-mounted Heatime HR system (SCR Engineers Ltd., Netanya, Israël).  
59 Heatime HR is an accelerometer which translates each female's activity into index values  
60 representing weighted standard deviations from the female's own basal activity and transfers  
61 data wirelessly to a registering station every 2 h. The maximal index value (or peak of  
62 activity) observed after PRID removal was considered as the time of estrus (Day 0) [7].  
63 Heifers were slaughtered 6 days after PRID removal in a local slaughterhouse (Vendôme,  
64 France). The time interval (h) between estrus (Day 0) and oviduct collection was calculated  
65 retrospectively.

66 The OF samples were collected and prepared as described earlier [6]. Briefly, both oviducts  
67 and ovaries from individual cows were placed on ice within less than 15 min after death and  
68 transported to the laboratory within 3 h after the first cow. One early hemorrhagic corpus  
69 luteum (in the side of ovulation) was observed in all animals. The entire oviducts ipsilateral  
70 and contralateral to ovulation were cleaned of surrounding tissues and vessels and after  
71 elimination of the infundibulum, their content (OF + mucosa cells) was collected by a single  
72 evenly gentle squeeze with a glass slide. Then the OF was separated from oviductal cells and

73 cell debris by two successive centrifugations (2000 g, 5 min then 12 000 g, 10 min) at 4°C.  
74 The OF samples (30-50 µL/oviduct) were stored at -80°C until analyzed.  
75 Metabolites were quantified in OF samples using proton nuclear magnetic resonance (<sup>1</sup>H-  
76 NMR) as previously described [6]. Briefly, 10 µL of OF were diluted in 190 µL of 0.2 M  
77 potassium phosphate buffer in deuterium oxide (D<sub>2</sub>O) (pH 7.4 ± 0.5). The resulting solution  
78 was transferred to conventional 3-mm NMR tubes and <sup>1</sup>H-NMR spectra were then recorded at  
79 298K on a Bruker Ascend 600-MHz spectrometer (Bruker, Sadis, Wissembourg, France),  
80 equipped with a TCI cryoprobe. The intra-assay (sample-to-sample variations) and inter-assay  
81 (assay-to-assay variations) coefficients of variation were 6.0% and 4.6%, respectively.  
82 Metabolite quantifications using the Electronic Reference To access In vivo Concentrations  
83 (ERETIC) peak as a quantitative reference were obtained using the Bruker TopSpin 3.2  
84 program (Bruker daltonik, Karlsruhe, Germany). Spiking experiments were used to validate  
85 metabolite assignments. Spectral assignments were done using the free version of ChenomX  
86 7.1 software (ChenomX, Edmonton, Canada) and based on the human metabolome database  
87 (HMDB).  
88 To obtain an overview of metabolomic data across the various animals, a principal component  
89 analysis of all data was performed using the FactoMineR package (version 1.34) of the  
90 RStudio software (version 1.1.456). A statistical analysis of data was then performed using  
91 the GraphPad software (Prism 7.0; GraphPad Software Inc., San Diego, CA, USA).  
92 Comparisons between sides relative to ovulation were assessed using the non-parametric  
93 Wilcoxon test for paired comparisons. The variability between individuals was assessed by  
94 the coefficients of variation (CV) expressed as percentages (SD/Mean × 100). The correlation  
95 between metabolite concentrations and CV on each side was assessed by Pearson tests. A P-  
96 value lower than 0.05 was considered significant. Chemical classes and biological functions of

97 metabolites were obtained from “The bovine metabolome database” (www.bovinedb.ca/) [8]. All data  
98 are presented as means  $\pm$  SD.

### 99 **3. Results and discussion**

100 The Heatime system was reported previously as highly accurate for estrus detection (positive  
101 predictive value  $\geq$  84%) [7]. Coupled with estrus synchronization, this monitoring system  
102 allowed us to time precisely the collection of oviducts between 72 h (3.0 days) and 88 h (3.7  
103 days) after the peak of estrous activity (average:  $79.1 \pm 2.2$  h *i.e.*  $3.3 \pm 0.1$  days after the peak  
104 of estrus), *i.e.* at the time when cattle embryos reach the 8-cell or early morula stage after  
105 fertilization [3].

106 This is the first time  $^1\text{H-NMR}$  was used to exhaustively identify OF metabolites in Holstein  
107 heifers on day 3 post-estrus. A total of 39 metabolites, including 14 amino acids, were  
108 identified in the OF. Of those, 13 metabolites could not be quantified: 10 (3-hydroxybutyrate,  
109 creatinine, ethanolamine, isobutyrate, methionine, niacinamide, nicotinurate, phenylalanine,  
110 proline and tyrosine) due to low signals and three of them (acetylcholine, glutamate and  
111 glutamine) due to a large overlap of their signals with others.

112 Table 1 shows the average concentrations and CV of the 26 metabolites quantified in both  
113 sides relative to ovulation. Principal component analysis of metabolomic data did not  
114 evidence any separation between animals or ipsilateral *vs.* contralateral sides of ovulation (see  
115 supplementary material). Overall, the amino acids glycine and alanine and the energy  
116 substrates lactate and myoinositol were the most abundant metabolites, with concentrations  
117 above 5 mM (Figure 2A). The concentration of other metabolites ranged from less than 100  
118  $\mu\text{M}$  (succinate) up to 4500  $\mu\text{M}$  (Figure 2 B-C). This is in accordance with earlier reports of  
119 amino acid detection using high performance liquid chromatography (HPLC) or  $^1\text{H-NMR}$  in  
120 the post-ovulatory bovine OF [6, 9-11]. Lactate was also the most abundant energy substrate  
121 in previous studies quantifying glucose, pyruvate and lactate in the post-ovulatory OF of

122 cyclic cows [9-11]. However, absolute concentrations of metabolites vary between studies  
123 which may be due to differences in animal breed, nutrition, parity and/or in methods of OF  
124 collection (post-mortem vs. after oviduct cannulation) and quantification. These metabolites  
125 are known to be involved in a wide range of biological functions (Table 1). Furthermore,  
126 important roles of lactate, pyruvate, glucose, succinate and various amino acids in the  
127 development of the early bovine embryo have been reported (reviewed in [4]).

128 A significant effect of the proximity to the corpus luteum was evidenced for glycine, which  
129 was more abundant in the side of ovulation compared to the other side (Figure 1A). On the  
130 opposite, myoinositol, creatine, carnitine, acetylcarnitine and choline were less abundant on  
131 the side of ovulation compared to the contralateral oviduct (Figure 1A-C). These data are in  
132 keeping with those from Lamy et al. [6] who reported the same patterns of differences  
133 between sides during the post-ovulatory period. Significantly higher concentrations of  
134 progesterone were measured in the oviduct ipsilateral to ovulation compared to the  
135 contralateral one [12, 13]. Furthermore, a systemic supplementation of progesterone on day 3  
136 of the cycle led to an increase in glycine concentrations in the OF of cows [11], in line with  
137 the present results. These data highlight the local regulatory role of ovarian steroid hormones  
138 in the metabolome of the oviduct.

139 In this study, OF samples were collected at the same amount of time post-estrus from animals  
140 of similar genetics, age, weight with identical diet and living conditions, which were assumed  
141 to limit the individual variability in OF metabolites. A very high CV was recorded for G1P  
142 due to its non-detection in 4 out of 14 samples (138.4% and 98.1% in ipsilateral and  
143 contralateral oviducts, respectively). The average CVs for the remaining 25 metabolites were  
144 20.6% (range: 9.8% - 49.1%) and 19.1% (range: 8.1% - 43.2%) in the ipsilateral and  
145 contralateral OF, respectively, with no correlation between CV and metabolite abundance  
146 (Pearson's test not significant). In the ipsilateral side of ovulation, the most stable metabolites



147 included phosphocholine, malonate, threonine and alanine (CVs between 9.8 and 12.4%)  
148 while the most variable metabolites included glucose, glycine, histidine and succinate (CV  
149 between 29.0 and 49.1%; see Table 1). This is in accordance with previous studies in which a  
150 high individual variability (high SEM values) was reported for glucose, lactate and many  
151 amino acids in the OF from crossbred heifers [9, 10, 14]. These data have to be taken with  
152 caution due to the low number of animals in previous studies as well as in this one. It is likely  
153 that metabolites with low CV are essential for oviduct homeostasis and for support of vital  
154 functions in gametes and embryos. On the other hand, metabolites with high CV may vary in  
155 the circulating blood and OF in response to environmental factors. For instance, blood  
156 concentrations of glucose were shown to increase in stress-related situations like surgery [15],  
157 which could explain differences in the response of animals to the stress of transport and  
158 slaughterhouse environment prior to collection of oviducts.

159 In conclusion, 26 metabolites were quantified in the bovine OF at the time of early embryo  
160 development, giving new insights into the embryonic maternal environment. The mechanisms  
161 underlying a relative homeostasis for some OF metabolites and on the opposite a high  
162 individual variability for some others including glucose and glycine, remain to be elucidated.

163 **Author contributions:** MSD and PS conceived and designed the experiments; PS and OD managed  
164 the animals and the collection of biological material; PS, MSD, OD and DLB collected and prepared  
165 the materials; JG performed the metabolomic analyses; CM and MSD performed the analysis of  
166 metabolomic data; PM provided guidance and resources; MSD and PS managed the project; MSD and  
167 CM wrote the paper; all authors read and approved the manuscript.

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213

214

215

216 **Figure legends**

217 **Figure 1:** Metabolites in the oviductal fluid of cyclic Holstein heifers on day 3 post-estrus with  
218 average concentrations above 5 mM (A), between 1 and 5 mM (B) and below 1 mM (C). Data are  
219 indicated as means  $\pm$  SD. Asterisks indicate significant differences between ipsilateral and  
220 contralateral sides relative to ovulation. \*P < 0.05.

221 **Supplementary material:** Principal component analysis (PCA) of metabolites measured in  
222 the oviduct fluid in the ipsilateral (ipsi, red dots) and contralateral (contra, black dots) sides of  
223 ovulation in cyclic Holstein heifers on day 3 post-estrus. Numbers refer to individual cows.

224

225 **Table 1.** Mean concentrations, coefficients of variation (CV) and biological functions of metabolites  
 226 quantified in the oviductal fluid of Holstein heifers on day 3 post-estrus (n = 7). Metabolites are  
 227 classified in decreasing order of abundance.

	Chemical class	<i>Ipsilateral side</i>		<i>Contralateral side</i>		Biological function
		Mean ± SD (µM)	CV (%)	Mean ± SD (µM)	CV (%)	
Lactate	Alpha hydroxy acids and derivatives Energy substrate	34,498.0 ± 4,970.2	14.4	35,843.4 ± 4,616.4	12.9	Biochemical processes. Produced by the muscle during intense activity.
Glycine	Amino acids, peptides, and analogues Non-essential amino acids Proteinogenic	21,399.9 ± 6210.7	29.0	15,009.6 ± 2,726.2	18.2	Participate in metabolic pathways and production of DNA, phospholipids and collagen.
Myoinositol	Alcohols and polyols	10,998.3 ± 1,715.4	15.6	12,431.6 ± 1,963.3	15.8	Involved in the inositol and galactose metabolism.
Alanine	Amino acids, peptides, and analogues Non-essential amino acids Proteinogenic	5,911.2 ± 735.2	12.4	5,647.4 ± 921.0	16.3	Protein biosynthesis. Source of energy.
Threonine	Amino acids, peptides, and analogues Essential amino acids Proteinogenic	4,491.5 ± 556.8	12.4	4,591.3 ± 500.7	10.9	Protein biosynthesis.
Phosphocholine	Quaternary ammonium salts	3,742.0 ± 367.5	9.8	3,843.6 ± 310.6	8.1	Phosphatidylcholine biosynthesis. Metabolic pathways.
Creatine	Amino acids, peptides, and analogues Endogenous amino acid	3,741.9 ± 541.6	14.5	4,232.0 ± 755.4	17.8	Participate in energy metabolism for muscle contractions

Isoleucine	Amino acids, peptides, and analogues Essential amino acids Proteinogenic	3,155.0 ± 728.4	23.1	3,026.0 ± 473.9	15.7	Protein synthesis. Stress, energy and muscle metaboslim.
Glucose	Carbohydrates and carbohydrate conjugates Energy substrate	1,921.0 ± 905.0	47.1	1,781.4 ± 815.6	45.8	Primary source of energy.
Carnitine	Quaternary ammonium salts Non-proteinogenic	1,501.8 ± 268.0	17.8	1,856.5 ± 429.0	23.1	Provide energy for muscles. Vitamin.
Citrate	Tricarboxylic acids and derivatives	1,180.8 ± 175.1	14.8	1,140.5 ± 166.8	14.6	Energy production. Precursor of ROS and prostaglandins.
Aspartate	Amino acids, peptides, and analogues Proteinogenic	1,169.7 ± 211.2	18.1	1,279.1 ± 322.8	25.2	Precursor of cell signaling compound. Involved in gluconeogenesis. Neurotransmitter.
Acetate	Carboxylic acids	1,152.7 ± 183.4	15.9	1,111.6 ± 136.2	12.3	Participate in the metabolism of carbohydrates and fats when associated with coenzyme A.
Malonate	Dicarboxylic acids and derivatives	931.2 ± 113.9	12.2	928.5 ± 108.2	11.7	Fatty acid biosynthesis pathway.
Leucine	Amino acids, peptides, and analogues Essential amino acids Proteinogenic	906.8 ± 176.4	19.5	869.6 ± 123.5	14.2	Protein synthesis. Stress, energy and muscle metaboslim. Stimulation on insulin signaling.
Valine	Amino acids, peptides, and analogues Essential amino acids Proteinogenic	749.5 ± 133.3	17.8	715.4 ± 98.5	13.8	Stress, energy and muscle metabolism.
Mannose	Carbohydrates and carbohydrate conjugates	541.3 ± 119.3	22.0	604.6 ± 172.6	28.5	Protein quality control. Involved in the innate immune system.

Acetylcarnitine	Fatty acid esters Acyl carnitines	494.5 ± 89.5	18.1	546.4 ± 109.7	20.1	Involved in the oxidation of fatty acids.
Choline	Quaternary ammonium salts Essential vitamin	494.5 ± 89.5	18.1	546.4 ± 109.7	20.1	Phospholipids biosynthesis. Converted into the neurotransmitter Acetylcholine
Creatine phosphate	Amino acids, peptides, and analogues	466.9 ± 81.7	17.5	468.7 ± 49.7	10.6	Participate in energy metabolism for muscle contractions
Histamine	Amines	386.5 ± 53.5	13.8	380.4 ± 58.3	15.3	Involved in several metabolic and enzymatic pathway and gastric acid secretion. Neurotransmitter.
Histidine	Amino acids, peptides, and analogues Proteinogenic	227.0 ± 107.4	47.3	250.6 ± 81.1	32.3	Protein biosynthesis. Metabolic pathways. Ammonia recycling.
Pyruvate	Alpha-keto acids and derivatives	178.8 ± 35.2	19.7	167.0 ± 24.5	14.7	Participate in the gluconeogenesis, glycolyse and several metabolic pathways.
Fumarate	Dicarboxylic acids and derivatives	113.4 ± 17.9	15.8	118.1 ± 18.0	15.2	Energy production (TCA cycle). Metabolic pathways.
Succinate	Dicarboxylic acids and derivatives	75.0 ± 36.8	49.1	73.2 ± 31.6	43.2	Involved in the TCA cycle, respiratory chain, metabolic pathways and signal during inflammation.
Glucose-1-phosphate	Carbohydrates and carbohydrate conjugates Energy substrate	65.9 ± 91.1	138. 4	114.5 ± 112.3	98.1	Converted into Glucose-6-phosphate in the glycolyse and gluconeogenesis.

Figure 1A

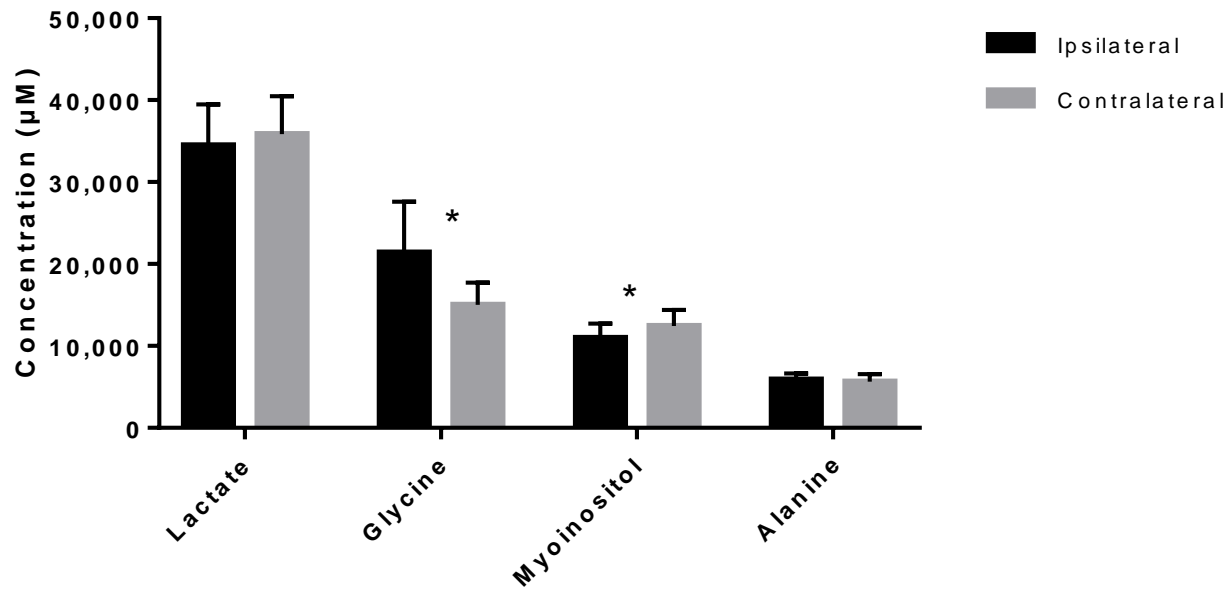


Figure 1B

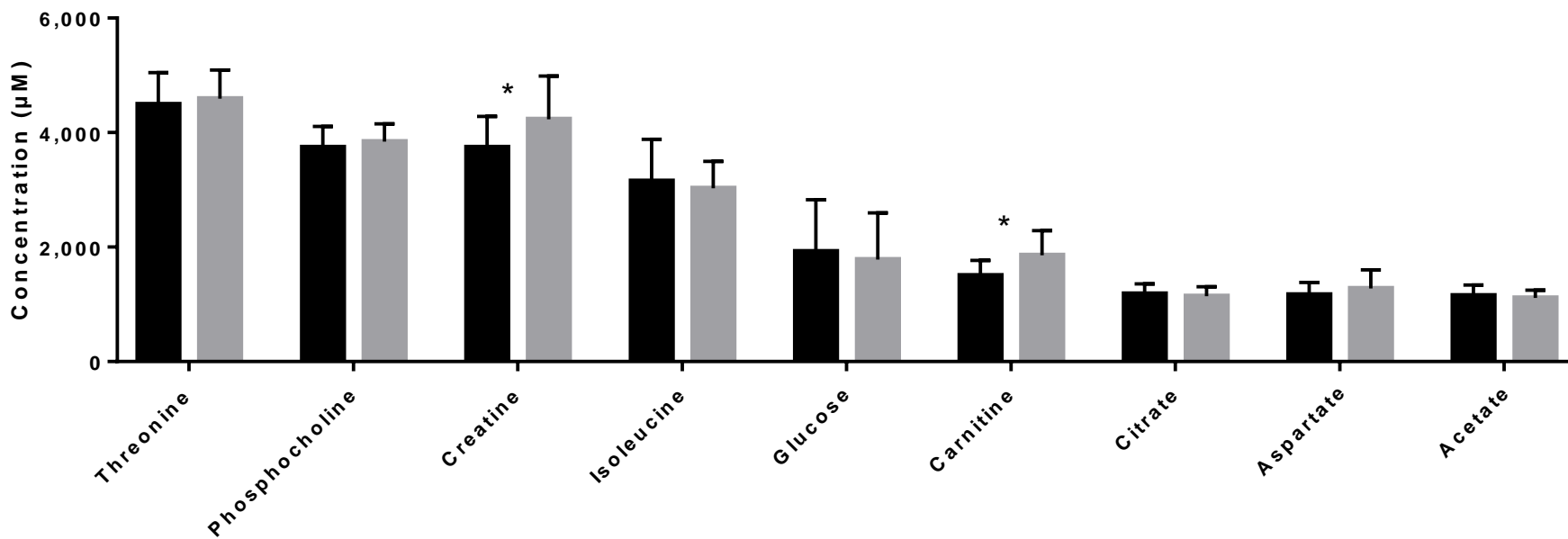


Figure 1C

