

1 **Metformin attenuates lipid accumulation in hepatocytes of blunt snout bream**
2 **(*Megalobrama amblycephala*) via activation of AMP-activated protein kinase**

3

4 Wenhao Zhou^{a,†}, Samad Rahimnejad^{a,†}, Douglas R. Tocher^b, Kangle Lu^{a,*}, Chunxiao
5 Zhang^a, Yunzhang Sun^a

6

7 ^aXiamen Key Laboratory for Feed Quality Testing and Safety Evaluation, Fisheries
8 College, Jimei University, Xiamen 361021, China

9 ^bInstitute of Aquaculture, Faculty of Natural Sciences, University of Stirling, Stirling
10 FK9 4LA, Scotland, UK

11

12 [†]These authors contributed equally to this work

13 *Corresponding author: Kangle Lu, Fisheries College, Jimei University, Xiamen
14 361021, China. E-mail: lukangle@jmu.edu.cn, Tel: 86-0592-6181054.

Accepted refereed manuscript of:

Zhou W, Rahimnejad S, Tocher DR, Lu K, Zhang C & Sun Y (2019) Metformin attenuates lipid accumulation in hepatocytes of blunt snout bream (*Megalobrama amblycephala*) via activation of AMP-activated protein kinase. *Aquaculture*, 499, pp. 90-100.

DOI: <https://doi.org/10.1016/j.aquaculture.2018.09.028>

© 2018, Elsevier. Licensed under the Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International <http://creativecommons.org/licenses/by-nc-nd/4.0/>

15 **Abstract**

16 Currently, there is a trend to use high-fat diets in intensive aquaculture that is
17 accompanied with incidence of fatty liver when dietary lipid level surpasses an upper
18 limit. So, it is necessary to develop appropriate strategies to reduce the risk of fatty liver
19 in commercial fish farming. Studies in mammals have revealed a correlation between
20 fatty liver and AMP-activated protein kinase (AMPK) activity, which has been
21 recognized as a key modulator of lipid metabolism. Considering the frequent
22 occurrence of fatty liver in blunt snout bream farming, an *in-vitro* study was designed
23 to evaluate the efficiency of metformin, as a stimulator of AMPK, in activation of
24 AMPK and its subsequent effects on lipid metabolism in primary hepatocytes. Fish
25 hepatocytes were seeded at a density of $1 \times 10^6 \text{ ml}^{-1}$ in 6-well tissue culture plates and
26 treated with three different media including: 1) Leibovitz's L-15 medium [L15] as
27 control, 2) high-fat medium [L15+400 μM oleic acid], and 3) metformin medium
28 [L15+400 μM oleic acid+200 μM metformin]. After 48 h of culture, the cells and
29 supernatant were collected for analysis. The results showed significant ($P < 0.05$)
30 enhancement of cell triglyceride and total cholesterol concentrations in the high-fat
31 medium group over control, and metformin addition significantly reduced the values.
32 Also, the high-fat medium group exhibited significantly higher aspartate
33 aminotransferase activity than both control and metformin groups. The lowest AMPK
34 and phospho-AMPK protein expression was found in the high-fat medium group while
35 metformin addition significantly up-regulated their expression levels. Mitochondrial
36 and peroxisomal oxidation rates in the high-fat medium group were significantly lower
37 than control while similar oxidation rates were observed for metformin treated and
38 control groups. The high-fat medium group showed significantly lower CPT I activity
39 than control, and metformin inclusion increased the activity. Expression of genes

40 associated with lipid metabolism such as PPAR α , CPT I, AOX, PGC-1 α and TFAM
41 was suppressed in the high-fat medium group, and metformin supplementation up-
42 regulated their expression levels. The opposite trend was true for the expression of
43 ACC2 gene. Also, the results showed down-regulation of FAS and SREBP-1C genes in
44 the high-fat medium group, and metformin addition resulted in further reduction of their
45 expression level. The lowest activities of mitochondrial complexes (I-III) were found
46 in the high-fat medium group and metformin prevented high-fat-induced reduction of
47 mitochondrial complexes activity. Notably increased concentrations of reactive oxygen
48 species and malondialdehyde were found in the high-fat medium group, and metformin
49 treatment reduced their concentrations. Moreover, metformin group exhibited higher
50 glutathione peroxidase activity than the high-fat medium group. The findings in this
51 study showed clearly that metformin activated AMPK in blunt snout bream hepatocytes,
52 which contributed to enhanced lipid metabolism and attenuated lipid deposition in the
53 cells incubated with high-fat medium.

54 **Key words:** blunt snout bream; fatty liver; metformin; AMP-activated protein kinase;
55 lipid metabolism

56 **1. Introduction**

57 It is well known that adequate levels of non-protein energy sources should be
58 incorporated in fish diet in order to maximize protein utilization for growth (Wilson and
59 Halver, 1986). Dietary lipids have been extensively used as a source of concentrated
60 energy for saving protein and increasing feed efficiency in economical fish farming
61 practices (Boujard et al., 2004; Hillestad et al., 1998). Use of high-fat (high energy)
62 diets has become a common practice in the aquaculture industry since the discovery of
63 the protein sparing effects of lipids by Lee and Putnam (1973). However, it become
64 apparent that dietary lipid content could be increased up to a certain level but, thereafter,
65 undesirable impacts could be achieved such as reduced growth performance and
66 unwanted lipid accumulation that can subsequently result in hyperlipidemia, fatty liver,
67 and lipid peroxidation (Du et al., 2005, 2008; Ji et al., 2011; Jin et al., 2013; Li et al.,
68 2012; Lu et al., 2013a). Fatty liver, which is characterized by excessive triglyceride
69 accumulation in hepatocytes (Bolla et al., 2011; Lu et al., 2013), is not only a sign of
70 wasted dietary energy but also has detrimental effects on fish health (Lu et al., 2013b;
71 Nanton et al., 2003). Poor growth performance and high mortality caused by fatty liver
72 have been reported in several farmed fish species leading to substantial economic losses
73 (Du et al., 2014). Accordingly, it is vital to recognize the nutritional factors and
74 mechanisms involved in the development of fatty liver.

75 The complex metabolic regulation systems in animals enable them to adapt to
76 nutritional modifications (Soengas, 2014). As such, in most animals increasing dietary
77 lipid is accompanied with alterations in lipid metabolism including suppression of

78 lipogenic enzymes (Clarke and Hembree, 1990; G lineau et al., 2001; Hillgartner et al.,
79 1995) and increased fatty acid oxidation (Kim et al., 2004). Therefore, an appropriate
80 approach for cost effective fish production could be developing strategies to inhibit
81 excessive fat deposition in liver, and instead increase the utilization of dietary lipid to
82 promote edible biomass production. Research in mammals has revealed a correlation
83 between fatty liver and AMP-activated protein kinase (AMPK) activity (You and
84 Rogers, 2009). It has been demonstrated that AMPK plays a key role in regulating lipid
85 metabolism, and is now known as a metabolic master switch for modification of cellular
86 energy charge (J ger et al., 2007). Stimulation of AMPK leads to suppression of
87 anabolic processes such as fatty acid and cholesterol synthesis, and activation of
88 catabolic processes like fatty acid oxidation (Fryer and Carling, 2005; Hardie et al.,
89 2006; Kahn et al., 2005). For instance, AMPK phosphorylates acetyl-CoA-carboxylase-
90 2 (ACC-2) leading to reduction of malonyl-CoA (an inhibitor of carnitine
91 palmitoyltransferase I, CPT I), which subsequently results in increased fatty acid
92 oxidation in mitochondria (Merrill et al., 1997; Vavvas et al., 1997). Knockout of the
93 AMPK gene is linked to higher incidence of obesity and fatty liver (Viollet et al., 2003).
94 On the other hand, its over-expression attenuates fatty liver through activation of
95 enzymes associated with fatty acid oxidation (J rgensen et al., 2007; Winder et al.,
96 2000). Thus, AMPK has received attention as a novel target for treatment of fatty liver
97 in mammals (Lin et al., 2007). Although the existence of AMPK has been reported in
98 several fish species including blunt snout bream (*Megalobrama amblycephala*) (Xu et
99 al., 2017), rainbow trout (*Oncorhynchus mykiss*) (Polakof et al., 2011b) and turbot

100 (*Scophthalmus maximus*) (Zeng et al., 2016), its role in regulating fish lipid metabolism
101 has not yet been investigated.

102 Blunt snout bream is an herbivorous freshwater fish native to China, and has been
103 a favored aquaculture species in China due to its fast growth, tender flesh and high
104 disease resistance. However, occurrence of fatty liver has been a common issue when
105 reared in captivity due to its lower hepatosomatic index compared to other farmed fish
106 species (Lu et al., 2014a). Metformin has been identified as one of the most commonly
107 known agents for activation of AMPK and its potency in treatment of fatty liver has
108 been established in mammals. To better understand the role of AMPK in regulating
109 lipid metabolism in fish, effects of metformin on AMPK activation, lipid metabolism
110 and lipid accumulation were investigated in primary hepatocytes of blunt snout bream.

111 **2. Materials and methods**

112 2.1. Experimental fish

113 Blunt snout breams (averaging 100 g) were purchased from a private farm and
114 transported to the aquaculture laboratory of Jimei University. The fish were stocked
115 into a 1000-L tank supplied with aerated fresh water in a recirculating system and fed
116 twice daily (8:30 and 16:30) with a commercial diet (Tongwei, Suzhou, China) (35%
117 protein and 5% lipid) for two weeks. The average water temperature was 28 ± 1.5 °C
118 and the photoperiod was maintained on a 12:12 light:dark schedule. The fish were
119 fasted for 24 h prior to sampling.

120 2.2. Isolation of hepatocytes

121 Prior to isolation of hepatocytes, fish were anesthetized with MS-222 (tricaine
122 methanesulfonate; Sigma, USA) (100 mg l^{-1}) and bled by cutting the gill arches. Then,

123 liver was rapidly isolated and washed several times in ice-cold phosphate buffered
124 saline (PBS) containing antibiotic (100 IU ml⁻¹ penicillin G sodium and 100 IU ml⁻¹
125 streptomycin). After removal of PBS by sterile pipette, the samples were cut into small
126 pieces (about 1 mm³) and digested with pancreatin at 28 °C for 30 min. Thereafter cell
127 suspension was centrifuged at 500 ×g for 10 min and washed twice. The harvested cell
128 pellets were re-suspended in Leibovitz's L-15 medium (L15 medium) (HyClone™,
129 USA) with 15% fetal bovine serum (Biological Industries, USA) at a density of 1×10⁶
130 ml⁻¹. For each test three different fish were used and each time the livers were pooled
131 to make a single sample.

132 2.3. Cell treatment

133 Two milliliter of isolated hepatocytes was seeded in each well of 6-well culture
134 plates. After 24 h, all cells attached and cultured in 2 ml of the following media: control
135 medium (L15), high-fat medium (L15+400 μM oleic acid), and metformin medium
136 (L15+400 μM oleic acid+200 μM metformin). After 48 h, the cells and supernatant
137 were collected for analysis. The supernatant was collected by sterile pipette. Then, cells
138 were harvested by trypsinization (0.25% trypsin–EDTA) at 25 °C in 5 min. All the tests
139 were performed in three replicates. Each replicate was made up by pooling six wells
140 for Western blotting, and two wells for the rest of the tests.

141 2.4. Biochemical parameters

142 Cell triglyceride and total cholesterol (TC) concentrations were determined by
143 colorimetric enzymatic methods using commercial kits (Beijing BHKT Clinical
144 Reagent Co., Ltd, China) as described previously (Lu et al., 2016a). Activities of
145 aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in supernatant
146 were measured through enzymatic colorimetric methods according to Reitman and

147 Frankel (1957).

148 2.5. Gene expression

149 Total RNA was extracted from the hepatocytes using RNAiso Plus (Takara Co. Ltd,
150 Japan) according to the protocol provided by the manufacturer. Isolated RNA was
151 quantified using the NanoDrop ND-2000 spectrophotometer, and its integrity was
152 confirmed by agarose gel electrophoresis. RNA samples were treated by RQ1 RNase-
153 Free DNase prior to RT-PCR (Takara Co. Ltd, Japan) to avoid genomic DNA
154 amplification. cDNA was generated from 500 ng DNase-treated RNA using ExScript™
155 RT-PCR kit (Takara Co. Ltd, Japan), and the mixture consisted of 500 ng RNA, 2 µl
156 buffer (5×), 0.5 µl dNTP mixture (10 mM each), 0.25 µl RNase inhibitor (40 U µl⁻¹),
157 0.5 µl dT-AP primer (50 mM), 0.25 µl ExScript™ RTase (200 U µl⁻¹), and total volume
158 made up to 10 µl with DEPC-treated H₂O. The reaction conditions were as follows:
159 42 °C for 40 min, 90 °C for 2 min, and 4 °C thereafter.

160 Real-time PCR was employed to determine mRNA levels based on the SYBR®
161 Green I fluorescence kit (Takara Co. Ltd, Japan). Primer characteristics used for real-
162 time PCR are listed in Table 1, according to the MIQE Guidelines (Bustin et al., 2011).
163 Real-time PCR was performed in a Mini Option real-time detector (BIO-RAD, USA).
164 The fluorescent quantitative PCR reaction solution consisted of 12.5 µl SYBR® premix
165 Ex Taq™ (2×), 0.5 µl PCR forward primer (10 µM), 0.5 µl PCR reverse primer (10
166 µM), 2.0 µl RT reaction (cDNA solution), and 9.5 µl dH₂O. The reaction conditions
167 were as follows: 95 °C for 3 min followed by 45 cycles consisting of 95 °C for 10 s and
168 60 °C for 20 s. The fluorescent flux was then recorded, and the reaction continued at
169 72 °C for 3 min. The dissolution rate was measured between 65 and 90 °C. Each
170 increase of 0.2 °C was maintained for 1 s, and the fluorescent flux was recorded. All
171 amplicons were initially separated by agarose gel electrophoresis to ensure that they

172 were of correct size. A dissociation curve was determined during the PCR program to
173 make sure that specific products were obtained in each run. All reactions were
174 performed in three technical replicates. The gene expression levels were normalized
175 towards mean of the reference gene (β -actin). The gene expression was calculated by
176 using the comparative ($2^{-\Delta\Delta C_t}$) method (Livak and Schmittgen, 2001).

177 2.6. Western blotting

178 Western blots were carried out following the protocols described by Lau and
179 Richards (2011) with slight modifications. Briefly, cell pellets (about 10^8 cells) were
180 lysed in ice-cold lysis buffer (Cell Signaling, Danvers, MA, USA) and centrifuged at
181 $12000 \times g$ for 5 min, and then the resulting supernatants were stored at -80°C . Total
182 protein was determined according to the methods outlined by Bradford (Bradford,
183 1976). Aliquots of each sample were added to an equal volume of SDS-sample buffer
184 (Laemmli, 1970), boiled for 5 min, and $20 \mu\text{g}$ of total protein was loaded into each well,
185 separated by SDS-PAGE for 1-2 h at 100 V using a Mini-Protean system (BioRad,
186 Spain) and transferred to a polyvinylidene fluoride (PVDF) membranes (Millipore,
187 Massachusetts, USA). Subsequently, the membrane was blocked with blocking buffer
188 (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20, pH 7.6) containing 5% (w/v) non-
189 fat dry milk for 1 h. The membrane was then incubated with rabbit polyclonal
190 antibodies against GAPDH blots (Cell Signaling Technology, USA), anti-AMPK α (Cell
191 Signaling Technology, USA) and antiphospho-AMPK α (#2535, Cell Signaling
192 Technology, USA) at 4°C overnight. After washing, membranes were incubated with
193 anti-rabbit secondary antibody. Bands were visualized by an electro-
194 chemiluminescence (ECL) system (GE Healthcare, Buckinghamshire, UK) and
195 quantified by the densitometry band analysis tool in ImageJ 1.44p.

196 2.7. Mitochondria

197 The activities of respiratory chain complexes (I, II, III) and citrate synthase were
198 determined using commercial kits (Nanjing JianCheng Bioengineering Institute, China)
199 as described previously (Lu et al., 2017). Thiobarbituric-acid-reactive substances
200 assays were performed with a malondialdehyde (MDA) kit (Nanjing JianCheng
201 Bioengineering Institute) as described by Rueda-Jasso et al. (2004). Mito tracker green
202 was used for mitochondrial labeling. Hepatocytes were incubated in phenol red-free
203 Dulbecco's Modified Eagle's Medium (DMEM) containing 10% foetal calf serum and
204 labeled with 80 nM Mito Tracker Green FM (Beyotime C1048-50 μ g; Nantong, China),
205 and incubated for 30 min at 28 °C, in a humidified atmosphere, 5% CO₂ in air,
206 subsequently washed with DMEM medium, and examined and photographed by
207 fluorescence microscopy (450-490 nm excitation light, 520 nm barrier filter; Olympus).

208 2.8. Fatty acid oxidation rate

209 Mitochondrial and peroxisomal β -oxidation of hepatocytes were determined using
210 radiolabelled [1-¹⁴C] palmitate (16:0) as a substrate, as described previously (Lu et al.,
211 2014b). Palmitate oxidation rates were measured at 28 °C using two media as described
212 by Frøyland et al. (1995), the first allowing the total (mitochondrial and peroxisomal)
213 activities to occur (13.2 mM HEPES [pH 7.3], 16.5 mM MgCl₂, 82.5 mM KCl, 13.2
214 mM dithiothreitol, 6.6 mM ADP, 0.2 mM NAD⁺, 100 mM-CoA and 0.7 mM EDTA),
215 the second allowing the peroxisomal activity only (the medium only differing by the
216 presence of 73 mM antimycin and 10 mM rotenone to block the mitochondrial
217 respiratory chain). Palmitate oxidation was measured with 115 μ M [1-¹⁴C] palmitate
218 supplemented with 1.2 mM L-carnitine. The samples were incubated for 60 min at
219 28 °C, then reactions were stopped by addition of 1.5 M KOH; fatty acid-free bovine
220 serum albumin (BSA, 100 mg ml⁻¹) was added to the suspension in order to bind

221 unoxidized substrates and then 4 M HClO₄ was added to precipitate unoxidized
222 substrates bound to BSA. The total solution was then centrifuged at 1880 ×g for 15
223 min. Aliquots of 200 µl were transferred to a scintillation tube containing 4 ml of liquid
224 scintillation cocktail and assayed for radioactivity in a LS6500 liquid scintillation
225 analyzer (Beckman, USA).

226 2.9. Oxidative status

227 Cell suspensions were incubated at 37 °C for 30 min with 10 µM 2',7'
228 dichlorofluorescein diacetate (DCFH/DA, Nanjing Jiancheng Bioengineering Institute,
229 China). To measure the intracellular reactive oxygen species (ROS), the fluorescence
230 of DCF was excited by a 15mW laser tuned to 488 nm and the emitted fluorescence
231 was measured with 530/30 band pass filter in a FACScalibur Becton Dickinson flow
232 cytometer. The conditions for data acquisition and data analysis were established using
233 negative and positive controls with the CellQuest Program of Becton Dickinson and
234 these conditions were maintained during all the experiments. Total superoxide
235 dismutase (SOD) activity was measured using a commercial kit (Nanjing Jiancheng
236 Bioengineering Institute, China) according to Nakano (1990). Glutathione peroxidase
237 (GPX) activity was measured using the method of Dabas et al. (2012). Thiobarbituric-
238 acid-reactive substances assays were performed with amalondialdehyde (MDA) kit
239 (Nanjing Jiancheng Bioengineering Institute, China) as described by Rueda-Jasso et al.
240 (2004).

241 2.10. Statistical analysis

242 Data were analyzed by one-way ANOVA using the SPSS 16.0 for Windows.
243 Duncan's test was used for the multiple comparisons. The level of significance was set
244 at $P < 0.05$. All data were presented as means ± SE.

245 **3. Results**

246 3.1. Cellular lipid accumulation

247 Hepatocytes cultured with high-fat medium had significantly ($P < 0.05$) higher TG
248 and TC contents than the control group, and metformin addition to high-fat medium
249 significantly reduced their values (Fig. 1A, B). Also, significantly higher AST activity
250 was detected in the high-fat medium group and a significant reduction was obtained by
251 metformin supplementation (Fig. 1C). A similar trend was observed for ALT activity
252 although no significant differences were found between high-fat and metformin groups
253 (Fig. 1D).

254 3.2. AMPK activity

255 Western blot analyses revealed significant reduction in expression of AMPK and
256 phospho-AMPK proteins in the high-fat medium group, while metformin inclusion
257 significantly increased their expression levels (Fig. 2).

258 3.3. Fatty acid β -oxidation

259 Both mitochondrial and peroxisomal fatty acid oxidation rates were significantly
260 lower in the high-fat medium group than in the control, while their values were
261 significantly improved by metformin addition and comparable values to those of
262 control group were achieved (Fig. 3A, B). Similarly, CPT I activity was significantly
263 lower in the high-fat medium group and this was reversed by supplementing metformin
264 to the culture medium (Fig. 3C).

265 3.4. Expression of lipid metabolism-related genes

266 Expression of the genes associated with lipid metabolism including PPAR α ,
267 PPAR β , CPT I, AOX, PGC-1 α , PGC-1 β , ACC2, TFAM, FAS and SREBP-1C are
268 presented in Fig. 4. The results showed that metformin supplementation significantly

269 up-regulated the expression of PPAR α , CPT I, AOX, PGC-1 α and TFAM compared to
270 the high-fat medium group. An opposite trend was true in the case of ACC2 gene, where
271 the high-fat medium treated group exhibited dramatically higher expression level of
272 ACC2 than the other groups. Expression of FAS and SREBP-1C genes were down-
273 regulated in high-fat medium group and a further reduction was observed by metformin
274 addition. Expression of other investigated genes including PPAR β and PGC-1 β were
275 unchanged.

276 3.5. Mitochondrial status

277 Activities of citrate synthase and mitochondrial complexes are presented in Fig. 5.
278 Activities of mitochondrial complex I, II and III were significantly suppressed in high-
279 fat medium group compared to control. Metformin supplementation significantly
280 increased activities of mitochondrial complex I and II, however, the values were still
281 significantly lower than those of the control group. A similar increase was observed for
282 complex III activity but the difference was not significant. However, citrate synthase
283 activity was not significantly influenced. Mito-Tracker Green, a fluorescent molecular
284 probe with high affinity for mitochondrial membranes, was used to identify
285 mitochondria in living cells. As shown in Fig. 6, the fluorescence intensity in the high-
286 fat medium group was weaker than the other treatments.

287 3.6. Oxidative status

288 The results showed significant enhancement of ROS in the high-fat medium group
289 and inclusion of metformin significantly reduced ROS concentration in cells incubated
290 in high-fat medium (Fig. 7A). Likewise, significantly higher MDA concentration was
291 detected in the high-fat treatment and an intermediary value was achieved with the
292 metformin-treated group (Fig. 7B). High-fat medium showed significantly lower SOD

293 and GPX activities than control, and metformin addition resulted in significant
294 enhancement of GPX activity and numeral increase of SOD activity (Fig. 7C, D).

295 **4. Discussion**

296 Metformin has long been used as an anti-diabetic drug capable of modulating
297 muscle and liver metabolism and controlling hyperglycemia in mammals (Alengrin,
298 1995; Lin et al., 2000; Panserat et al., 2009; Zhou et al., 2001). These functions have
299 also been investigated in fish and the results have revealed the mammalian-like effects
300 of metformin (Magnoni et al., 2012; Polakof et al., 2011a; Xu et al., 2018). It has been
301 suggested that metformin exerts its therapeutic effects through activation of AMPK
302 (Zou et al., 2004) which is linked to reduced glucose production and facilitated fatty
303 acid oxidation in hepatocytes (Zhou et al., 2001). In addition, studies in humans showed
304 that metformin can ameliorate liver dysfunction in patients with fatty liver (Garinis et
305 al., 2010). Lipid accumulation has been reported in chronic metabolic diseases such as
306 obesity and type 2 diabetes, and it has been demonstrated that AMPK activation inhibits
307 excessive nutrient-induced hepatic lipid accumulation (Li et al., 2014). To our
308 knowledge, this is the first report to evaluate the regulatory effects of metformin on
309 lipid metabolism in fish with a particular emphasis on its efficacy in AMPK activation.

310 The results achieved for TG and TC concentrations in hepatocytes demonstrated
311 clearly that metformin could attenuate lipid accumulation in fish hepatocytes subjected
312 to “high-fat” via supplementation with oleic acid. In agreement with our results, Xu et
313 al. (2018) showed that metformin supplementation in both low and high carbohydrate
314 diets for blunt snout bream resulted in reduced lipid accumulation in liver and adipose
315 tissues and decreased plasma triglyceride concentration. The authors attributed these
316 results to increased fatty acid oxidation and/or inhibited fatty acid synthesis through
317 activation of AMPK that ultimately resulted in reduced lipid accumulation (Zang et al.,

318 2004). Also, Kim et al. (2010) reported that metformin lowered liver TG and TC
319 contents and prevented fat accumulation in liver of mice fed a high-fat diet. These
320 authors suggested that such effects of metformin were mediated through AMPK
321 activation, which eventually results in decreased expression of sterol regulatory
322 element binding protein-1C (SREBP-1C) and fatty acid synthase (FAS). Similarly, in
323 the current study metformin treatment down-regulated the expression of both SREBP-
324 1C and FAS genes. SREBPs play key roles in both lipogenesis and cholesterol
325 homeostasis (Horton et al., 1998; Kim et al., 1998; Pai et al., 1998; Shimano et al.,
326 1996). It is believed that SREBP-1 is particularly involved in activation of the genes
327 that control fatty acid metabolism and *de novo* lipogenesis (Horton et al., 1998; Pai et
328 al., 1998). Furthermore, it has been suggested that modulation of FAS by SREBP-1c is
329 dependent on upstream stimulatory factors (Griffin et al., 2007; Latasa et al., 2003;
330 Yoshikawa et al., 2001). SREBP-1c is the key modulator of hepatic triglyceride
331 synthesis, making it a target for the inhibition and/or therapy of steatosis in hepatocytes.

332 AST and ALT levels are often used as indicators of liver disease including non-
333 alcoholic fatty liver (Krakoff et al., 2010). The results of a study on mice showed
334 enhancement of serum AST and ALT activities following administration of a high-fat
335 diet, and metformin supplementation suppressed the activity of both enzymes (Kim et
336 al., 2013). Krakoff et al. (2010) used serum ALT activity as a marker for non-alcoholic
337 fatty liver disease (NAFLD) in humans, and their results showed a significant reduction
338 of ALT activity in metformin-treated patients. Furthermore, it has been reported that
339 metformin lowered aminotransferases level and decreased liver fat content in mice with
340 NAFLD (Lin et al., 2000). Likewise, in the current study, the high-fat medium group
341 showed significantly higher AST and ALT activities than control, and treatment with
342 metformin led to reduced activity of both enzymes although the difference was not

343 statistically significant in the case of ALT activity.

344 The results of a recent *in vivo* study revealed the induction of AMPK
345 phosphorylation by metformin administration in fish (Xu et al., 2018). It has been
346 reported that metformin phosphorylates AMPK via stimulation of LKB1 kinase activity
347 (Shaw et al., 2005). As such, the results of Western blot analysis in the present study
348 showed clearly that metformin can up-regulate the expression of AMPK and P-AMPK
349 proteins in hepatocytes. Similarly, Kim et al. (2010) showed that metformin enhanced
350 AMPK and P-AMPK expression in mice compared to mice receiving a high-fat diet.
351 However, some *in vitro* studies showed no significant alteration of AMPK
352 phosphorylation in fish hepatocytes (Polakof et al., 2011b). These inconsistent results
353 could be due to differences in the duration of treatment of hepatocytes with metformin;
354 in the current study hepatocytes were treated with 200 μ M metformin for 48 h while
355 Polakof et al. (2011b) incubated the rainbow trout hepatocytes with metformin for 16
356 h.

357 It has been shown that there is a correlation between liver fatty acid oxidation and
358 fatty liver (Smith et al., 2016); where enhancement of oxidation rate reduced fatty liver
359 (Perry et al., 2015; Stefanovic-Racic et al., 2008) while suppressed oxidation facilitated
360 development of fatty liver (Zhang et al., 2007). AMPK, as a cellular “energy sensor”,
361 plays a key role in lipid metabolism (Hardie and Sakamoto, 2006). Once fatty acids are
362 taken up across the plasma membrane and activated to fatty acyl-CoA, they are either
363 directed towards oxidation or storage. At this stage, AMPK plays a role by determining
364 the fate of the absorbed fatty acids as it is known to phosphorylate and inactivate acetyl-
365 CoA carboxylase (ACC) leading to reduced malonyl-CoA, which is an inhibitor of CPT
366 I (McGarry et al., 1978; Saha and Ruderman, 2003). CPT I is considered as the
367 mitochondrial gateway for fatty acid entry into the matrix, and is the main modulator

368 of hepatic mitochondrial β -oxidation flux (Bartlett and Eaton, 2004; Lu et al., 2016b).
369 Attenuated β -oxidation capacity resulting from reduced catalytic efficiency of CPT I
370 has been recognized as one of the main causes of fatty liver in fish (Lu et al., 2014b).
371 Our previous research showed that reduction of fatty acid β -oxidation in blunt snout
372 bream is closely linked to the occurrence of fatty liver (Lu et al., 2014b). In the current
373 study, the metformin-treated group exhibited significantly higher AMPK protein
374 expression level and β -oxidation capacity than the high-fat medium group indicating
375 that metformin increased fatty acid oxidation in hepatocytes through AMPK activation.
376 This notion was also supported by enhanced CPT I activity in the metformin-treated
377 group over high-fat medium group.

378 ACC has two different isoforms, ACC1 and ACC2, with the only difference
379 between the two forms being the presence of an extra NH₂-terminal extension of 146
380 amino acids in ACC2, localizing the enzyme to mitochondria (Abu-Elheiga et al., 1995,
381 2000). Such localization has been suggested to be related to the regulatory effects of
382 ACC2 on fatty acid oxidation, as malonyl-CoA is produced in close proximity to CPT
383 I (Iverson et al., 1990). The results of early experiments in mammals showed that
384 AMPK inhibits ACC2 activity by phosphorylating at Ser-221 (Ahu-Fjheiga and Wakil,
385 1997). It has been shown that ACC2 exhibits similar phosphorylation in fish and these
386 serine residues are conserved (Cheng et al., 2011; He et al., 2014). Although in the
387 current study malonyl-CoA content was not measured, the expression of ACC2 gene in
388 liver was down-regulated by metformin indicating that inhibition of ACC2 by AMPK
389 activation contributed to increased CPT I activity and fatty acid β -oxidation. Moreover,
390 it has been reported that expression of CPT I mRNA is influenced by PPARs as CPT I
391 has a PPAR responsive component (Rao and Reddy, 2001). All the mammalian isotypes
392 of PPAR have also been recognized in several fish species although their function has

393 been shown to be different (Leaver et al., 2005). In fish, PPAR α activates lipid
394 catabolism through transcriptional control of target genes encoding enzymes involved
395 in peroxisomal and mitochondrial β -oxidation mainly in the liver (Michung, 2009). Up-
396 regulation of PPAR α was correlated with increased CPT I activity (Morais et al., 2007).
397 Meanwhile, previous studies have revealed that AMPK activation is accompanied by
398 increased PPAR α expression (Baar, 2004; Lee et al., 2006). So, up-regulation of PPAR α
399 by AMPK activation in this study could be another contributing factor to enhanced CPT
400 I activity and fatty acid β -oxidation. Lu et al. (2014b) reported the down-regulation of
401 AOX gene expression in blunt snout bream following administration of a high-fat diet.
402 AOX is believed to catalyze the first rate-limiting step in peroxisomal β -oxidation
403 (Morais et al., 2007). The authors attributed the reduced AOX activity to the decreased
404 peroxisomal β -oxidation. Likewise, in the current study both AOX gene expression and
405 β -oxidation rate decreased in the high-fat medium group.

406 In addition to CPT I activity, both quantity and quality of mitochondria are
407 considered as critical factors in determining β -oxidation capacity (Du et al., 2006;
408 Morash et al., 2008). There are several reports indicating that drastic decrease in
409 mitochondrial protein content of liver as well as the impairment of mitochondria leads
410 to reduced metabolic activity and oxidative capacity in fish (Du et al., 2006; Lu et al.,
411 2014b). In the present study, Mito-Tracker Green, which is a molecular probe with high
412 affinity for mitochondrial membranes, was used to identify mitochondria in the living
413 cells. The results demonstrated lower abundance of mitochondria in the high-fat
414 medium group compared to the other groups. This is consistent with previous findings
415 in fish that showed the administration of high-fat diets reduced mtDNA copies and
416 mitochondrial protein (Liao et al., 2016; Lu et al., 2014a). Furthermore, our results
417 indicated enhanced abundance of mitochondria following metformin supplementation.

418 This could be due the fact that AMPK is involved in mitochondrial biogenesis (Zong et
419 al., 2002). In mammals, PGC-1 α is considered as a key regulator of mitochondrial
420 biogenesis through stimulating the expression of mitochondrial transcriptional factor A
421 (TFAM) (Lehman et al., 2000; Puigserver et al., 1998; Wu et al., 1999). AMPK
422 activation has been identified as a prerequisite for increased expression of PGC-1 α
423 (Zong et al., 2002). Accordingly, it could be suggested that AMPK activation by
424 metformin in the present study up-regulated PGC-1 α expression leading to subsequent
425 enhancement of mitochondrial biogenesis. We found a notable increase in expression
426 of complex I, II and III by metformin inclusion. Although the precise underlying
427 mechanism is still unclear, we suggest that this could be associated with enhanced
428 mitochondrial biogenesis as newly generated mitochondria exhibit improved biological
429 function.

430 There are numerous studies indicating that lipid accumulation in liver adversely
431 affects liver function and induces oxidative stress in fish (Lu et al., 2016a). Since
432 mitochondria are the main site of ROS formation, mitochondria dysfunction could be
433 taken as one of the main causes of oxidative stress. Likewise, in the current study the
434 high-fat medium group exhibited the highest concentrations of ROS and MDA and the
435 lowest SOD and GPX activities, and metformin supplementation could reduce ROS
436 and MDA concentrations and enhance GPX activity. It is believed that mitochondrial
437 complex I is involved in scavenging ROS in the inner mitochondrial membrane (Bottje
438 and Carstens, 2009), and that the lower activity of complex I results in over production
439 of ROS (Lu et al., 2016a). Therefore, reduction of ROS and MDA concentrations by
440 metformin in the present study could be attributed to enhanced complex I activity.

441 In conclusion, the findings in the present study showed that metformin activated
442 AMPK in hepatocytes of blunt snout bream, and this was accompanied by enhanced

443 fatty acid β -oxidation via AMPK/ACC2/CPT I and AMPK/PPAR α /CPT I pathways.
444 Furthermore, AMP activation up-regulated the expression of PGC-1 α and TFAM,
445 which are involved in mitochondrial biogenesis. Furthermore, metformin decreased
446 hepatic ROS and MDA concentrations via enhancing mitochondrial complexes activity.
447 Overall, activation of AMPK by metformin could attenuate lipid accumulation and
448 oxidative stress in hepatocytes mainly due to elevation of fatty acid β -oxidation and
449 mitochondrial function.

450 **Acknowledgments**

451 This work was funded by National Nature Science Foundation of China
452 (31602171), Nature Science Foundation of Fujian Province (2017J05056) and China
453 Postdoctoral Science Foundation Grant (2017M620269).

454 **References**

- 455 Abu-Elheiga, L., Jayakumar, A., Baldini, A., Chirala, S.S., Wakil, S.J., 1995. Human
456 Acetyl-CoA Carboxylase: Characterization, Molecular Cloning, and Evidence for
457 Two Isoforms. P. NATL. ACAD. SCI. USA 92, 4011-4015.
- 458 Abu-Elheiga, L., Brinkley, W.R., Zhong, L., Chirala, S.S., Woldegiorgis, G., Wakil, S.J.,
459 2000. The subcellular localization of acetyl-CoA carboxylase 2. P. NATL. ACAD.
460 SCI. USA 97, 1444-1449.
- 461 Abu-Elheiga, L., Wakil, S.J., 1997. Human acetyl-CoA carboxylase 2. J. Biol. Chem.
462 272, 10669-10677
- 463 Alengrin, F., 1995. Metabolic Effects of Metformin in Non-Insulin-Dependent Diabetes
464 Mellitus. N. Engl. J. Med. 333, 550-554.
- 465 Baar, K., 2004. Involvement of PPAR gamma co-activator-1, nuclear respiratory factors
466 1 and 2, and PPAR alpha in the adaptive response to endurance exercise. Proc.
467 Nutr. Soc. 63, 269-273.
- 468 Bartlett, K, Eaton, S., 2004. Mitochondrial β -oxidation. FEBS J. 271, 462-469.
- 469 Beamish, F.W.H., Medland, T.E., 1986. Protein sparing effects in large rainbow trout,
470 *Salmo gairdneri*. Aquaculture 55, 35-42.
- 471 Bolla, S., Nicolaisen, O., Amin, A., 2011. Liver alterations induced by long term
472 feeding on commercial diets in Atlantic halibut (*Hippoglossus hippoglossus* L.)
473 females. Histological and biochemical aspects. Aquaculture 312, 117-125.
- 474 Bottje, W.G., Carstens, G.E., 2009. Association of mitochondrial function and feed
475 efficiency in poultry and livestock species. J. Anim. Sci. 87, 48-63.

476 Boujard T., Gelineau, A., Coves, D., Corraze, G., Dutto, G., Gasset, E. Kaushik, S. 2004.
477 Regulation of feed intake, growth, nutrient and energy utilisation in european sea
478 bass (*Dicentrarchus labrax*) fed high fat diets. *Aquaculture* 231, 529-545.

479 Bradford M.M., 1976. A rapid and sensitive method for the quantitation of protein
480 utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248-254.

481 Cheng, H.L., Ji, N.J., Peng, Y.X., Shen, X., Xu, J.H., Dong, Z.G., Wu, C.C., 2011.
482 Molecular characterization and tissue-specific expression of the acetyl-CoA
483 carboxylase α gene from Grass carp, *Ctenopharyngodon idella*. *Gene* 487, 46-51.

484 Clarke, S.D., Hembree, J. 1990. Inhibition of triiodothyronine's induction of rat liver
485 lipogenic enzymes by dietary fat. *J. Nutr.* 120, 625-630.

486 Du, Z.Y., 2014. Causes of fatty liver in farmed fish: a review and new perspectives.
487 *Journal of Fisheries of China* 38, 1628-1638. (Chinese with English abstract)

488 Du, Z.Y., Clouet, P., Zheng, W.H., Degrace, P., Tian, L.X., Liu, Y.J., 2006. Biochemical
489 hepatic alterations and body lipid composition in the herbivorous grass carp
490 (*Ctenopharyngodon idella*) fed high-fat diets. *Br. J. Nutr.* 95, 905-915.

491 Du, Z.Y., Liu, Y.J., Tian, L.X., Wang, J.T., Wang, Y., Liang, G.Y., 2005. Effect of dietary
492 lipid level on growth, feed utilization and bodycomposition by juvenile grass carp
493 (*Ctenopharyngodon idella*). *Aquac. Nutr.* 11:139-146.

494 Frøyland, L., Asiedu, D., Vaagenes, H., Garras, A., Lie, Ø., Totland, G., Berge, R., 1995.
495 Tetradecylthioacetic acid incorporated into very low density lipoprotein: changes
496 in the fatty acid composition and reduced plasma lipids in cholesterol-fed hamsters.
497 *J. Lipid Res.* 36, 2529-2540.

498 Fryer, L.G., Carling, D., 2005. AMP-activated protein kinase and the metabolic
499 syndrome. *Biochem. Soc. T.* 33, 362-366.

500 Garinis, G.A., Fruci, B., Mazza, A., De, S.M., Abenavoli, S., Gulletta, E., et al., 2010.
501 Metformin versus dietary treatment in nonalcoholic hepatic steatosis: a
502 randomized study. *I. J. Obesity* 34, 1255-1264.

503 G lineau, A., Corraze, G., Boujard, T., Larroquet. L., Kaushik, S.. 2001. Relation
504 between dietary lipid level and voluntary feed intake, growth, nutrient gain, lipid
505 deposition and hepatic lipogenesis in rainbow trout. *Reprod. Nutr. Dev.* 41, 487-
506 503.

507 Griffin, M.J., Wong, R.H., Pandya, N., Sul, H.S., 2007. Direct interaction between USF
508 and SREBP-1c mediates synergistic activation of the fatty-acid synthase promoter.
509 *J Biol Chem.* 282, 5453-5467.

510 Hansen, J. ., Berge, G.M., Hillestad, M., Krogdahl,  ., Galloway, T.F., Holm, H.,
511 Holm, J., Ruyter, B., 2008. Apparent digestion and apparent retention of lipid and
512 fatty acids in Atlantic cod (*Gadus morhua*) fed increasing dietary lipid levels.
513 *Aquaculture* 284, 159-166.

514 Hardie, D.G., Hawley, S.A., Scott, J.W., 2006. AMP-activated protein kinase-
515 development of the energy sensor concept. *J. Physiol.* 574, 7-15.

516 Hardie, D.G., Sakamoto, K., 2006. AMPK: a key sensor of fuel and energy status in
517 skeletal muscle. *Physiology* 21, 48-60.

518 He, A.Y., Liu, C.Z., Chen, L.Q., Ning, L.J., Zhang, M.L., Li, E.C., Du, Z.Y., 2014.
519 Identification, characterization and nutritional regulation of two isoforms of acyl-

520 coenzyme A oxidase 1 gene in Nile tilapia (*Oreochromis niloticus*). Gene 545, 30-
521 35.

522 Hillestad, M., Johnsen, F., 1994. High-energy/low protein diets for Atlantic salmon:
523 effects on growth, nutrient retention and slaughter quality. Aquaculture 124, 109–
524 116.

525 Horton, J.D., Shimomura, I., Brown, M.S., Hammer, R.E., Goldstein, J.L., Shimano,
526 H., 1998. Activation of cholesterol synthesis in preference to fatty acid synthesis
527 in liver and adipose tissue of transgenic mice overproducing sterol regulatory
528 element-binding protein-2. J Clin Invest. 101, 2331-2339.

529 Iverson, A.J., Bianchi, A., Nordlund, A.C., Witters, L.A., 1990. Immunological analysis
530 of acetyl-CoA carboxylase mass, tissue distribution and subunit composition.
531 Biochem. J. 269, 365.

532 Jäger, S., Handschin, C., St-Pierre, J., Spiegelman, B.M., 2007. AMP-activated protein
533 kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-
534 1alpha. P. NATL. ACAD. SCI. USA 104, 12017-12022.

535 Ji, H., Li, J., Liu, P., 2011. Regulation of growth performance and lipid metabolism by
536 dietary n-3 highly unsaturated fatty acids in juvenile grass carp,
537 *Ctenopharyngodon idellus*. Comp. Biochem. Physiol. 159B, 49–56.

538 Jin, M., Zhou, Q.C., Zhang, W., Xie, F.J., Shentu, J.K., Huang, X.L., 2013. Dietary
539 protein requirements of the juvenile swimming crab, *portunus trituberculatus*.
540 Aquaculture 414, 303-308.

541 Jørgensen, S.B., Trebak, J.T., Viollet, B., Schjerling, P., Vaulont, S., Wojtaszewski, J.F.,

542 Richter, E.A., 2007. Role of AMPK α 2 in basal, training-, and AICAR-induced
543 GLUT4, hexokinase II, and mitochondrial protein expression in mouse muscle.
544 Am. J. Physiol. Endocrinol. Metab. 292, E331-339.

545 Kahn, B.B., Alquier, T., Carling, D., Hardie, D.G., 2005. AMP-activated protein kinase:
546 Ancient energy gauge provides clues to modern understanding of metabolism. Cell
547 Metab. 1, 15-25.

548 Kim, D., Lee, J.E., Jung, Y.J., Lee, A.S., Lee, S., Park, S.K., Kim, S.H., Park, B.H.,
549 Kim, W., Kang, K.P., 2013. Metformin decreases high-fat diet-induced renal injury
550 by regulating the expression of adipokines and the renal AMP-activated protein
551 kinase/acetyl-CoA carboxylase pathway in mice. Int J Mol Med. 32, 1293-1302.

552 Kim, J.B., Sarraf, P., Wright, M., Yao, K.M., Mueller, E., Solanes, G., Lowell, B.B.,
553 Spiegelman, B.M., 1998. Nutritional and insulin regulation of fatty acid synthetase
554 and leptin gene expression through ADD1/SREBP1. J Clin Invest 101, 1-9.

555 Kim, S., Shin H.J., Kim, S.Y., Kim, J.H., Lee, Y.S., Kim, D.H., Lee, M.O., 2004.
556 Genistein enhances expression of genes involved in fatty acid catabolism through
557 activation of PPAR α . Mol. Cell. Endocrin. 220, 51-58.

558 Kim, Y.H., Lee, Y.J., Jeong, Y.Y., Kim, Y.W., Park, S.Y., Doh, K.O., Kim, J.Y., 2010.
559 The Effect of Metformin on Liver Lipid Accumulation in Mice Fed a High-fat Diet.
560 J. Korean Soc. Appl. Biol. Chem. 53(2), 198-205.

561 Krakoff, J., Clark, J.M., Crandall, J.P., Wilson, C., Molitch, M.E., Brancati, F.L.,
562 Edelstein, S.L., Knowler, W.C., 2010. Effects of Metformin and Weight Loss on
563 Serum Alanine Aminotransferase Activity in the Diabetes Prevention Program.

564 Obesity (Silver Spring) 18(9): 1762–1767. doi:10.1038/oby.2010.21.

565 Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head
566 of bacteriophage T4. *Nature* 227, 680-685.

567 Latasa, M.J., Griffin, M.J., Moon, Y.S., Kang, C., Sul, H.S., 2003. Occupancy and
568 function of the -150 sterol regulatory element and -65 E-box in nutritional
569 regulation of the fatty acid synthase gene in living animals. *Mol Cell Biol.* 23,
570 5896-5907.

571 Lau, G.Y., Richards, J.G., 2011. AMP-activated protein kinase plays a role in initiating
572 metabolic rate suppression in goldfish hepatocytes. *J. Comp. Physiol. B* 181, 927-
573 939.

574 Leaver, M.J., Boukouvala, E., Antonopoulou, E., Diez, A., Favrekrey, L., Ezaz, M.T.,
575 Bautista, J.M., Tocher, D.R., Krey, G., 2005. Three peroxisome proliferator-
576 activated receptor isotypes from each of two species of marine fish. *Endocrinology*
577 146, 3150-3162.

578 Lee, W.J., Kim, M., Park, H.S., Kim, H.S., Jeon, M.J., Oh, K.S., Koh, E.H., Won, J.C.,
579 Kim, M.S., Oh, G.T., 2006. AMPK activation increases fatty acid oxidation in
580 skeletal muscle by activating PPARalpha and PGC-1. *Biochem. Biophys. Res. Co.*
581 340, 291-295.

582 Lehman, J.J., Barger, P.M., Kovacs, A., Saffitz, J.E., Medeiros, D.M., Kelly, D.P., 2000.
583 Lehman, J. J. et al. Peroxisome proliferator-activated receptor coactivator-1
584 promotes cardiac mitochondrial biogenesis. *J. Clin. Invest.* 106, 847-856.

585 Li, H., Min, Q., Ouyang, C., Lee, J., He, C., Zou, M.H., Xie, Z., 2014. AMPK activation

586 prevents excess nutrient-induced hepatic lipid accumulation by inhibiting
587 mTORC1 signaling and endoplasmic reticulum stress response. *Biochimica et*
588 *Biophysica Acta* 1842, 1844–1854.

589 Li, X., Jiang, Y., Liu, W., Ge, X., 2012. Protein-sparing effect of dietary lipid in practical
590 diets for blunt snout bream (*Megalobrama amblycephala*) fingerlings: effects on
591 digestive and metabolic responses. *Fish Physiol. Biochem.* 38, 529-541.

592 Liao, K., Yan, J., Mai, K., Ai, Q., 2016. Dietary lipid concentration affects liver
593 mitochondrial DNA copy number, gene expression and DNA methylation in large
594 yellow croaker (*Larimichthys crocea*). *Comp. Biochem. Phys. B* 193, 25-32.

595 Lin, C.L., Huang, H.C., Lin, J.K., 2007. Theaflavins attenuate hepatic lipid
596 accumulation through activating AMPK in human HepG2 cells. *J. Lipid Res.* 48,
597 2334-2343.

598 Lin D., Mao, Y.S., Cai, F.S., 1990. Nutritional lipid liver disease of grass carp
599 (*Ctenopharyngodon idullus*). *Chinese Journal of Oceanology & Limnology* 8,
600 363-373.

601 Lin, H.Z., Yang, S.Q., Chuckaree, C., Kuhajda, F., Ronnet, G., Diehl, A.M., 2000.
602 Metformin reverses fatty liver disease in obese, leptin-deficient mice. *Nat. Med.*
603 9, 998-1003.

604 Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using
605 real-time quantitativePCR and the 2- $^{-\Delta\Delta CT}$ Method. *Methods*
606 2001;25:402–408.

607 Lu, K.L., Xu, W.N., Li, J.Y., Li, X.F., Liu, W.B., 2013. Alterations of liver histology

608 and blood biochemistry in blunt snout bream *Megalobrama amblycephala* fed
609 high-fat diets. Fish. Sci. 79, 661-671.

610 Lu, K.L., Wang, L.N., Zhang, D.D., Liu, W.B., Xu, W.N., 2016a. Berberine attenuates
611 oxidative stress and hepatocytes apoptosis via protecting mitochondria in blunt
612 snout bream *Megalobrama amblycephala* fed high-fat diets. Fish Physiol.
613 Biochem. 43, 1-12.

614 Lu, K.L., Xu, W.N., Liu, W.B., Wang, L.N., Zhang, C.N., Li, X.F., 2014a. Association
615 of mitochondrial dysfunction with oxidative stress and immune suppression in
616 Blunt Snout Bream *Megalobrama amblycephala* fed a high-fat diet. J. Aquat.
617 Anim. Health 26, 100-112.

618 Lu, K.L., Xu, W.N., Wang, L.N., Zhang, D.D., Zhang, C.N., Liu, W.B., 2014b. Hepatic
619 β -Oxidation and Regulation of Carnitine Palmitoyltransferase (CPT) I in Blunt
620 Snout Bream *Megalobrama amblycephala* Fed a High Fat Diet. Plos One 9,
621 e93135.

622 Lu, K.L., Zhang, D.D., Wang, L.N., Xu, W.N., Liu, W.B., 2016b. Molecular
623 characterization of carnitine palmitoyltransferase IA in *Megalobrama*
624 *amblycephala* and effects on its expression of feeding status and dietary lipid and
625 berberine. Comp. Biochem. Phys. B 191, 20.

626 Magnoni, L.J., Vraskou, Y., Palstra, A.P., Planas, J.P., 2012. AMP-Activated Protein
627 Kinase Plays an Important Evolutionary Conserved Role in the Regulation of
628 Glucose Metabolism in Fish Skeletal Muscle Cells. Plos One 7(2): e31219.
629 doi:10.1371/journal.pone.0031219.

630 Malin, S.K., Kashyap, S.R., 2014. Effects of metformin on weight loss: potential
631 mechanisms. *Curr. Opin. Endocrinol. Diabetes Obes.* 21, 323-329.

632 McGarry, J.D., Takabayashi, Y., Foster, D.W., 1978. The role of malonyl-coa in the
633 coordination of fatty acid synthesis and oxidation in isolated rat hepatocytes. *J Biol*
634 *Chem.* 253, 8294–8300.

635 Merrill, G.F., Kurth, E.J., Hardie, D.G., Winder, W.W., 1997. AICA riboside increases
636 AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat
637 muscle. *Am. J. Physiol.* 273, 1107-1112.

638 Michung, Y., 2009. The role of PPAR α in lipid metabolism and obesity: focusing on the
639 effects of estrogen on PPAR α actions. *Pharmacol. Res.* 60, 151-159.

640 Mootha, V.K., Handschin, C., Dan, A., Xie, X., Pierre, J.S., Sihag, S., Yang, W.,
641 Altshuler, D., Puigserver, P., Patterson, N., 2004. Err α and Gabpa/b specify PGC-
642 1 α -dependent oxidative phosphorylation gene expression that is altered in diabetic
643 muscle. *P. NATL. ACAD. SCI. USA* 101, 6570-6575.

644 Morais, S., Knoll-Gellida, A., André, M., Barthe, C., Babin, P.J., 2007. Conserved
645 expression of alternative splicing variants of peroxisomal acyl-CoA oxidase 1 in
646 vertebrates and developmental and nutritional regulation in fish. *Physiol.*
647 *Genomics* 28, 239-252.

648 Morash, A.J., Kajimura, M., McClelland, G.B., 2008. Intertissue regulation of carnitine
649 palmitoyltransferase I (CPTI): mitochondrial membrane properties and gene
650 expression in rainbow trout (*Oncorhynchus mykiss*). *BBA - Biomembranes* 1778,
651 1382-1389.

652 Nanton, D.A., Lall, S.P., Ross, N.W., and McNiven, M.A., 2003. Effect of dietary lipid
653 level on fatty acid β -oxidation and lipid composition in various tissues of haddock,
654 *Melanogrammus aeglefinus* L. *Comp. Biochem. Physiol. B*, 135, 95–108.

655 Pai, J.T., Guryev, O., Brown, M.S., Goldstein, J.L., 1998. Differential stimulation of
656 cholesterol and unsaturated fatty acid biosynthesis in cells expressing individual
657 nuclear sterol regulatory element-binding proteins. *J Biol Chem.* 273, 26138-
658 26148.

659 Panserat, S., Skiba-Cassy, S., Seiliez, I., Lansard, M., Plagnes-Juan, E., Vachot, C.,
660 Aguirre, P., Larroquet, L., Chavernac, G., Medale, F., Corraze, G., Kaushik, S.,
661 Moon, T.W., 2009. Metformin improves postprandial glucose homeostasis in
662 rainbow trout fed dietary carbohydrates: a link with the induction of hepatic
663 lipogenic capacities? *Am J Physiol Regul Integr Comp Physiol.* 297, R707–R715.
664

665 Perry, R.J., Camporez, J.P., Kursawe, R., Titchenell, P.M., Zhang, D., Perry, C.J.,
666 Jurczak, M.J., Abudukadier, A., Han, M.S., Zhang, X.M., Ruan, H.B., Yang, X.,
667 Caprio, S., Kaech, S.M., Sul, H.S., Birnbaum, M.J., Davis, R.J., Cline, G.W.,
668 Petersen, K.F., Shulman, G.I., 2015. Hepatic acetyl CoA links adipose tissue
669 inflammation to hepatic insulin resistance and type 2 diabetes. *Cell* 160, 745–758.

670 Polakof, S., Moon, T.W., Aguirre, P., Skiba-Cassy, S., Panserat, S., 2011a. Glucose
671 homeostasis in rainbow trout fed a high-carbohydrate diet: metformin and insulin
672 interact in a tissue-dependent manner. *Am. J. Physiol. -Reg. I.* 300, R166-174.

673 Polakof, S., Panserat, S., Craig, P.M., Martyres, D.J., Plagnesjuan, E., Savari, S.,

674 Arisbrosou, S., Moon, T.W., 2011b. The Metabolic Consequences of Hepatic
675 AMP-Kinase Phosphorylation in Rainbow Trout. Plos One 6, e20228.

676 Puigserver, P., Wu, Z., Park, C.W., Graves, R., Wright, M., Spiegelman, B.M., 1998. A
677 cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis.
678 Cell 92, 829-839.

679 Rao, M.S., Reddy, J.K., 2001. Peroxisomal beta-oxidation and steatohepatitis. Semin.
680 Liver Dis. 21, 43.

681 Reitman, S., Frankel, S., 1957. A colorimetric method for the determination of serum
682 glutamic oxalacetic and glutamic pyruvic transaminases. Am. J. Clin. Pathol. 28,
683 56.

684 Ruedajasso, R., Lec, C., Dias, J., Wde, C., Gomes, E., Rees, J.F., Soares, F., Dinis, M.T.,
685 Sorgeloos, P., 2004. Effect of dietary non-protein energy levels on condition and
686 oxidative status of Senegalese sole (*Solea senegalensis*) juveniles. Aquaculture
687 231, 417-433.

688 Saha, A.K., Ruderman, N.B., 2003. Malonyl-CoA and AMP-activated protein kinase:
689 an expanding partnership. Mol. Cell. Biochem. 253, 65-70.

690 Sergio, P., Stéphane, P., Craig, P.M., Martyres, D.J., Elisabeth, P.J., Sharareh, S.,
691 Stéphane, A.B., Moon, T.W., 2011. The Metabolic Consequences of Hepatic
692 AMP-Kinase Phosphorylation in Rainbow Trout. Plos One 6, e20228.

693 Shaw, R.J., Lamia, K.A., Vasquez, D., Koo, S.H., Bardeesy, N., Depinho, R.A., et al.,
694 2005. The Kinase LKB1 Mediates Glucose Homeostasis in Liver and Therapeutic
695 Effects of Metformin. Science 310, 1642.

696 Shimano, H., Horton, J.D., Hammer, R.E., Shimomura, I., Brown, M.S., Goldstein, J.L.,
697 1996. Overproduction of cholesterol and fatty acids causes massive liver
698 enlargement in transgenic mice expressing truncated SREBP-1a. *J Clin Invest.* 98,
699 1575-1584.

700 Smith, B.K., Marcinko, K., Desjardins, E.M., Lally, J.S., Ford, R. J., Steinberg, G.R.,
701 2016. Treatment of nonalcoholic fatty liver disease: role of AMPK. *Am J Physiol*
702 *Endocrinol Metab.* 311, E730–E740.

703 Soengas, J.L.,2014. Contribution of glucose- and fatty acid sensing systems to the
704 regulation of food intake in fish. a review. *Gen. Comp. Endocr.* 205, 36-48.

705 Stefanovic-Racic, M., Perdomo, G., Mantell, B.S., Sipula, I.J., Brown, N.F., O’Doherty,
706 R.M., 2008. A moderate increase in carnitine palmitoyltransferase 1a activity is
707 sufficient to substantially reduce hepatic triglyceride levels. *Am J Physiol*
708 *Endocrinol Metab.* 294, E969–E977.

709 Vavvas, D., Apazidis, A., Saha, A.K., Gamble, J., Patel, A., Kemp, B.E., Witters, L.A.,
710 Ruderman, N.B., 1997. Contraction-induced changes in acetyl-CoA carboxylase
711 and 5'-AMP-activated kinase in skeletal muscle. *J. Biol. Chem.* 272, 13255.

712 Viollet, B., Andreelli, F., Jørgensen, S.B., Perrin, C., Flamez, D., Mu, J., Wojtaszewski,
713 J.F., Schuit, F.C., Birnbaum, M., Richter, E., 2003. Physiological role of AMP-
714 activated protein kinase (AMPK): insights from knockout mouse models.
715 *Biochem. Soc. T.* 31, 216-219.

716 Wilson, R.P. and Halver, J.E., 1986. Protein and amino acid requirements of
717 fishes.*Annu. Rev. Nutr.* 6, 225-244.

718 Winder, W.W., Holmes, B.F., Rubink, D.S., Jensen, E.B., Chen, M., Holloszy, J.O.,
719 2000. Activation of AMP-activated protein kinase increases mitochondrial
720 enzymes in skeletal muscle. *J. Appl. Physiol.* 88, 2219-2226.

721 Wu, Z., Puigserver, P., Andersson, U., Zhang, C., Adelmant, G., Mootha, V., Troy, A.,
722 Cinti, S., Lowell, B., Scarpulla, R.C., 1999. Mechanisms Controlling
723 Mitochondrial Biogenesis and Respiration through the Thermogenic Coactivator
724 PGC-1. *Cell* 98, 115-124.

725 Xu, C., Liu, W.B., Zhang, D.D., Wang, K.Z., Xia, S.L., Li, X.F., 2017. Molecular
726 characterization of AMP-activated protein kinase $\alpha 2$ from herbivorous fish
727 *Megalobrama amblycephala* and responsiveness to glucose loading and dietary
728 carbohydrate levels. *Comp. Biochem. Physiol. A* 208, 24-34.

729 Xu, C., Liu, W.B., Zhang, D.D., Cao, X.F., Shi, H.J., Li, X.F., 2018. Interactions
730 between dietary carbohydrate and metformin: Implications on energy sensing,
731 insulin signaling pathway, glycolipid metabolism and glucose tolerance in blunt
732 snout bream *Megalobrama amblycephala*. *Aquaculture* 483, 183-195.

733 Yoshikawa, T., Shimano, H., Amemiya-Kudo, M., Yahagi, N., Hastay, A.H., Matsuzaka,
734 T., Okazaki, H., Tamura, Y., Iizuka, Y., Ohashi, K., Osuga, J., Harada, K., Gotoda,
735 T., Kimura, S., Ishibashi, S., Yamada, N., 2001. Identification of liver X receptor-
736 retinoid X receptor as an activator of the sterol regulatory element-binding protein
737 1c gene promoter. *Mol Cell Biol.* 21, 2991-3000.

738 You, M., Rogers, C.Q., 2009. Adiponectin: a key adipokine in alcoholic fatty liver. *Exp.*
739 *Biol. Med.* 234, 850-859.

740 Zang, M.W., Zuccollo, A., Hou, X.Y., Nagata, D., Walsh, K., Herscovitz, H., Brecher,
741 P., Ruderman, N.B., Cohen, R.A., 2004. AMP-activated protein kinase is required
742 for the lipid-lowering effect of metformin in insulin-resistant human HepG2 cells.
743 J. Biol. Chem. 279, 47898–47905.

744 Zeng, L., Liu, B., Wu, C.W., Lei, J.L., Xu, M.Y., Zhu, A.Y., Zhang, J.S., Hong, W.S.,
745 2016. Molecular characterization and expression analysis of AMPK α subunit
746 isoform genes from *Scophthalmus maximus* responding to salinity stress. Fish
747 Physiol. Biochem. 42, 1595-1607.

748 Zhang, D., Liu, Z.X., Choi, C.S., Tian, L., Kibbey, R., Dong, J., Cline, G.W., Wood,
749 P.A., Shulman, G.I., 2007. Mitochondrial dysfunction due to long-chain Acyl-CoA
750 dehydrogenase deficiency causes hepatic steatosis and hepatic insulin resistance.
751 Proc Natl Acad Sci USA. 104, 17075–17080.

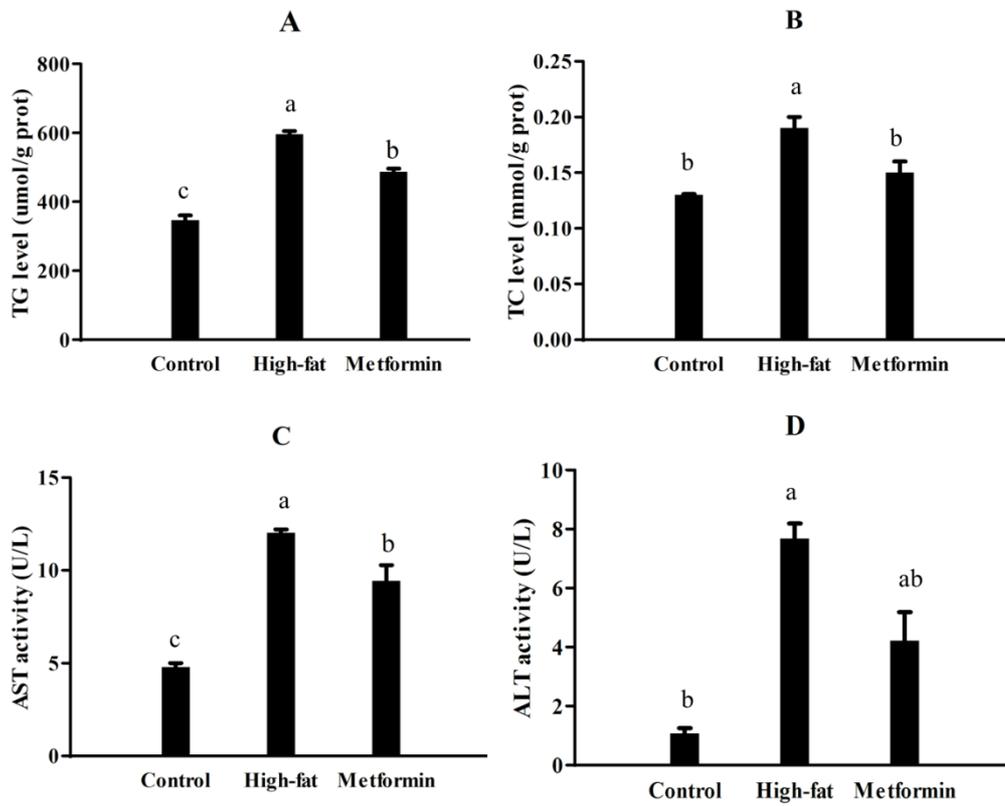
752 Zhou, G., Myers, R., Li, Y., Chen, Y., Shen, X., Fenyk-Melody, J., Wu, M., Ventre, J.,
753 Doebber, T., Fujii, N., 2001. Role of AMP-activated protein kinase in mechanism
754 of metformin action. J. Clin. Invest. 108, 1167-1174.

755 Zong, H., Ren, J.M., Young, L.H., Pypaert, M., Mu, J., Birnbaum, M.J., Shulman, G.I.,
756 2002. AMP kinase is required for mitochondrial biogenesis in skeletal muscle in
757 response to chronic energy deprivation. P. NATL. ACAD. SCI. USA 99, 15983-
758 15987.

759 Zou, M.H., Kirkpatrick, S.S., Davis, B.J., Nelson, J.S., Wiles, W.G., Schlattner, U., et
760 al., 2004. Activation of the amp-activated protein kinase by the anti-diabetic drug
761 metformin in vivo role of mitochondrial reactive nitrogen species. J. Biol. Chem.

762 279, 43940-43951.

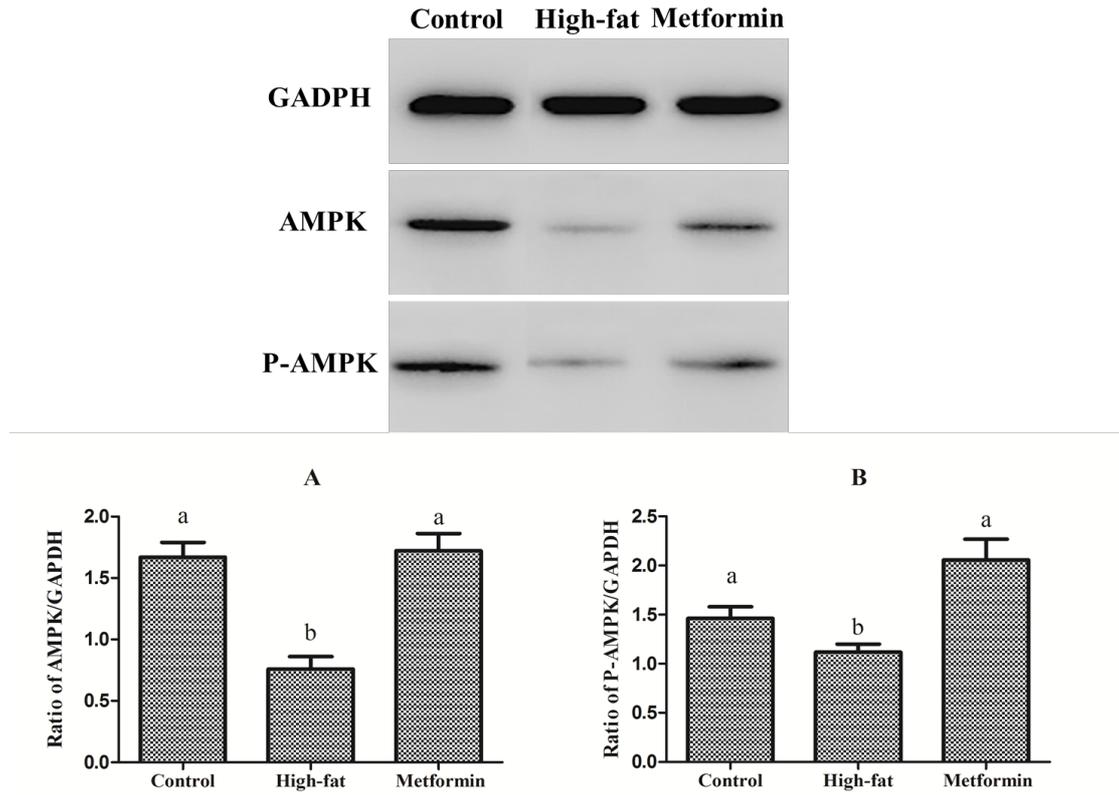
763



765

766 **Fig.1.** Concentrations of triglyceride (TG: A) and total cholesterol (TC: B), and
 767 activities of aspartate aminotransferase (AST: C) and alanine aminotransferase (ALT:
 768 D) in primary hepatocytes of blunt snout bream. Bars with different letters are
 769 significantly different ($P < 0.05$).

770



771

772 **Fig.2.** AMPK (A) and phospho-AMPK (B) expression levels as determined by Western
 773 blot in primary hepatocytes of blunt snout bream. Bars with different letters are
 774 significantly different ($P < 0.05$).

775

776

777

778

779

780

781

782

783

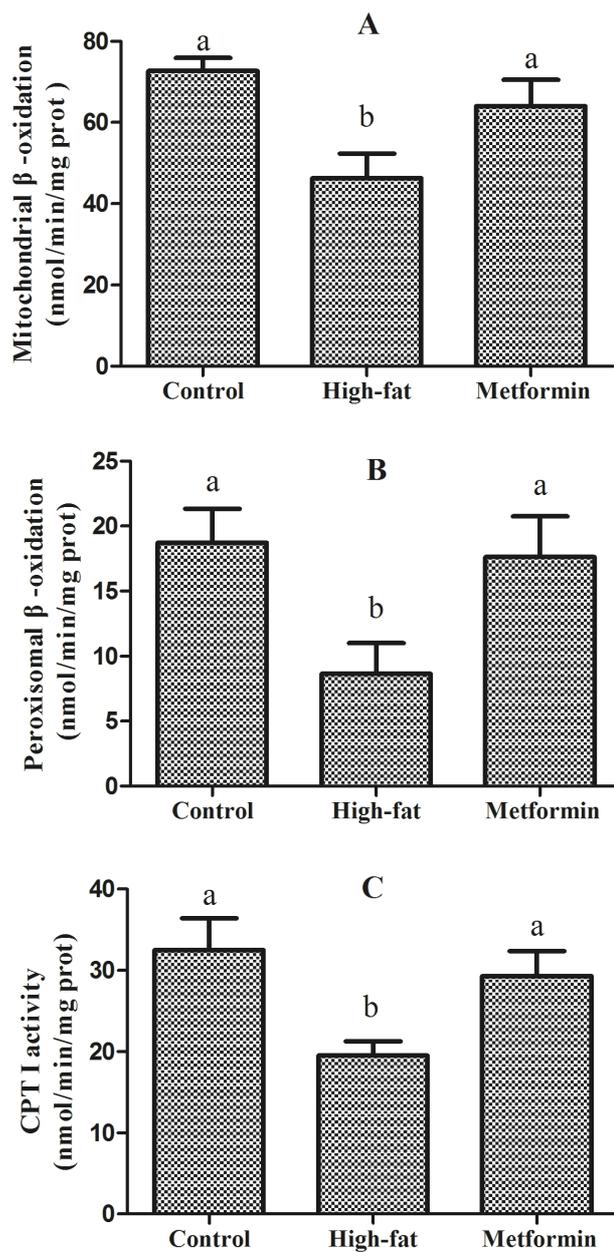
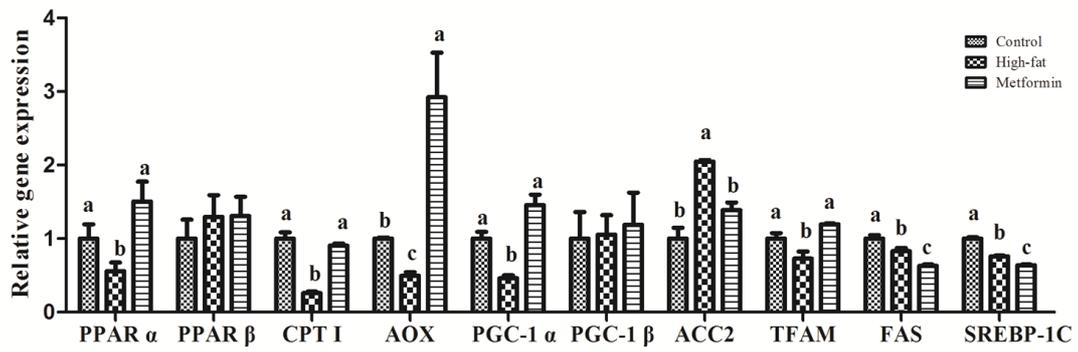


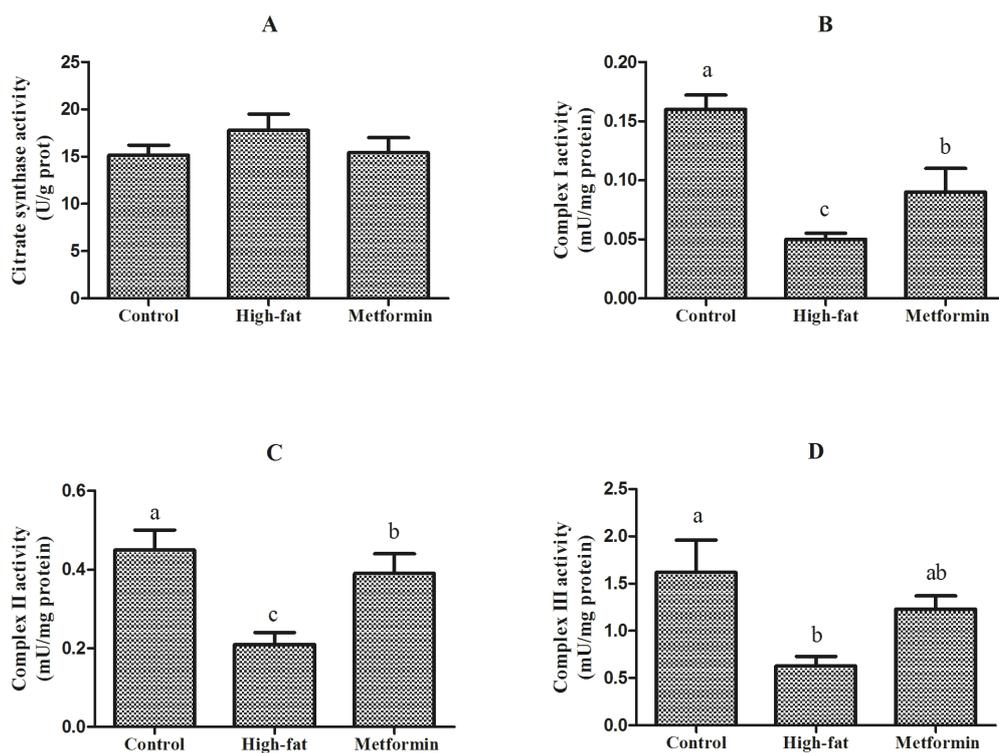
Fig.3. Mitochondrial (A) and peroxisomal (B) β -oxidation, and CPT I (C) activity in primary hepatocytes of blunt snout bream. Bars with different letters are significantly different ($P < 0.05$).



822

823 **Fig.4.** Relative expression of lipid metabolism related genes in primary hepatocytes of
 824 blunt snout bream. Bars with different letters are significantly different ($P < 0.05$).

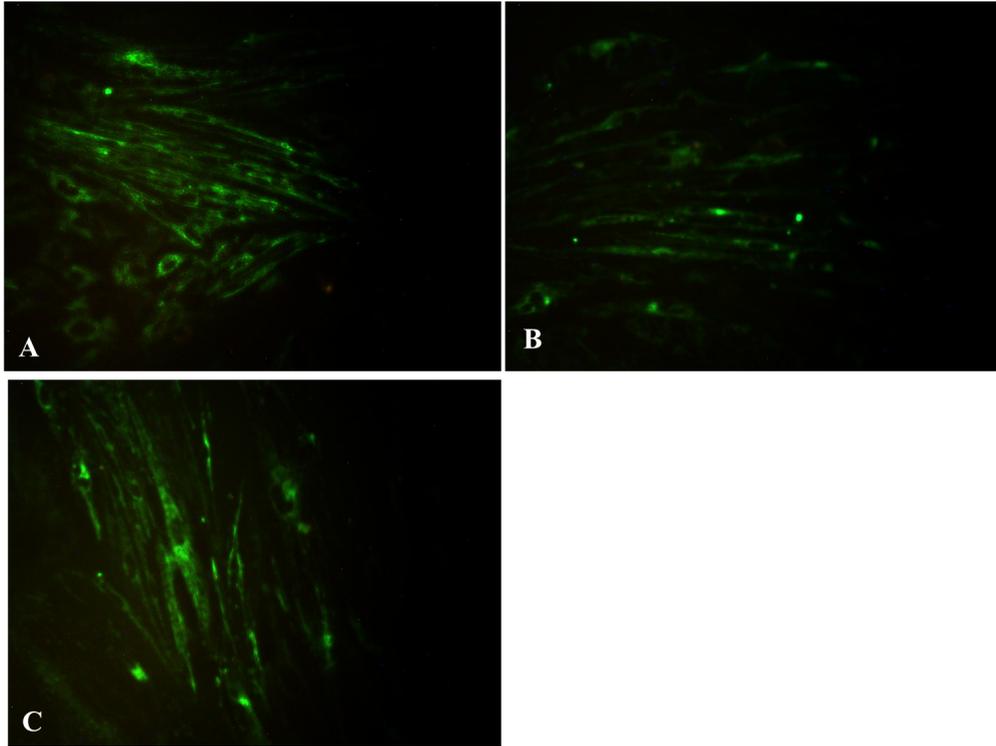
825



826

827 **Fig.5.** Activities of citrate synthase (A) and mitochondrial complexes (I: B, II: C, III:
 828 D) in primary hepatocytes of blunt snout bream. Bars with different letters are
 829 significantly different ($P < 0.05$).

830

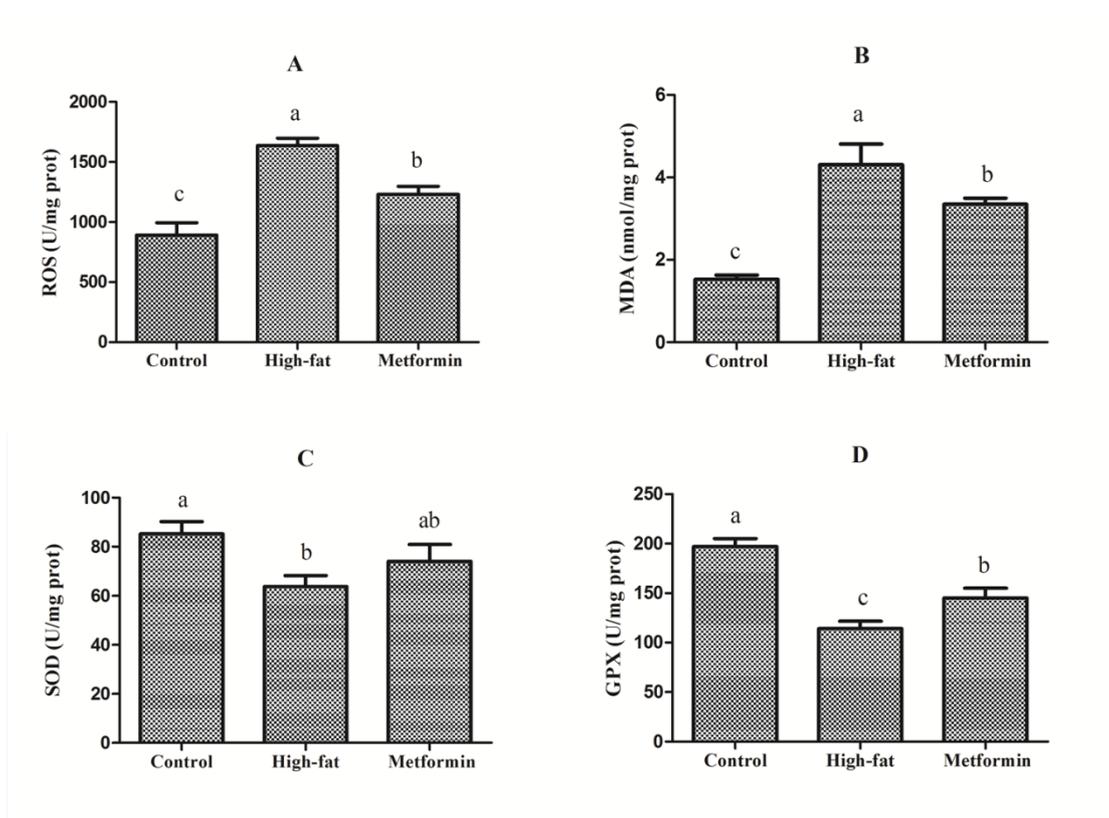


831

832 **Fig.6.** Mitochondria abundance identified by Mito-Tracker Green in hepatocytes of
833 blunt snout bream (A: control, B: high-fat, C: metformin). The fluorescence intensity
834 is indicative of mitochondria abundance.

835

836



837

838

839

840

841

842

Fig.7. Concentrations of reactive oxygen species (ROS: A) and malondialdehyde (MDA: B), and activities of superoxide dismutase (SOD: C) and glutathione peroxidase (GPX: D) in primary hepatocytes of blunt snout bream. Bars with different letters are significantly different ($P < 0.05$).

843 **Table 1.** Sequences of primers used for RT-PCR in this study.

Target genes	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')	Annealing temperature (°C)
β -Actin	CGGACAGGTCATCACCATTG	CGCAAGACTCCATACCCAAGA	60
PPAR α	GAGGAACCGAAACAAGTGCCAATA	GCTCAGTCACCGTCTCAACC	60
PPAR β	GGACTCACTATGGCAGGCAGAA	CACTGGCAGCGGTAGAAGACAT	60
CPT I	TACTTCCAAAGCGGTGAG	AGAGGTATTGTCCGAGCC	60
AOX	GCTCAACCCTGGCATACT	TCATCACACCCATTCGCT	60
PGC-1 α	TGCCCTCGGTTTCATTGTC	GATTTCTGATTGGTCGCTGTA	60
PGC-1 β	CTCTAAGGGTGAATCGCAACG	TCCTCCGCCACTTCCACAT	60
ACC2	CGGAGTTATCAAGCCAAGAGC	ACAGCAGTCGCCGCAA	60
TFAM	CTTTGGTATCCAGGGAGCAGT	GTTGAATCGCATCCAGTCGT	60
FAS	TTGTTCCCTCATCCACCCC	TGCCTCAAGCACTCCACG	60
SREBP-1C	AGAACAGAGGAGTGCGAGAT	CCGCTGCCTAGTTTGATG	60

844 PPAR α : Peroxisome proliferator-activated receptor α

845 PPAR β : Peroxisome proliferator-activated receptor β

846 CPT I: Carnitine palmitoyltransferase I

847 AOX :Acyl-Co A Oxidase

848 PGC-1 α : Peroxisome proliferators activated receptor γ coactivator-1 α

849 PGC-1 β : Peroxisome proliferators activated receptor γ coactivator-1 β

850 ACC2: Acetyl CoA carboxylase 2

851 TFAM: Mitochondrial transcription factor A

852 FAS: Fatty acid synthesis

853 SREBP-1C: sterol regulatory element binding protein-1C

