1 # Metformin counteracts glucose-dependent lipogenesis and impairs
2 transdeamination in the liver of gilthead sea bream (*Sparus aurata*)

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12 **Running Head:** Metformin impairs transdeamination in *Sparus aurata*

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19 **ABSTRACT**

20 Metformin is an anti-diabetic drug with a major impact on regulating blood glucose levels by
21 decreasing hepatic gluconeogenesis but also affecting other pathways, including glucose
22 transport and energy/lipid metabolism. Carnivorous fish are considered glucose intolerant, as
23 they exhibit poor ability to using dietary carbohydrates. To increase the current knowledge
24 about the molecular mechanisms by which metformin can improve glucose homeostasis in
25 carnivorous fish, we addressed the effect of intraperitoneal administration of metformin, in
26 the presence or absence of a glucose load, on metabolic rate-limiting enzymes and lipogenic
27 factors in the liver of gilthead sea bream (*Sparus aurata*). Hyperglycemia markedly up-
28 regulated the expression of glycolytic enzymes (glucokinase and 6-phosphofructo-1-kinase,
29 PFK1) 5 h following glucose administration, while at 24 h post-treatment it increased
30 isocitrate dehydrogenase (IDH) activity, a key enzyme of the tricarboxylic acid cycle, and the
31 expression of lipogenic factors (PGC1β, Lpin1 and SREBP1). Metformin counteracted
32 glucose-dependent effects, and down-regulated glutamate dehydrogenase, alanine
33 aminotransferase and mTOR 5 h post-treatment in the absence of a glucose load, leading to
34 decreased long-term activity of PFK1 and IDH. The results of the present study suggest that
35 hyperglycemia enhances lipogenesis in the liver of *S. aurata*, and that metformin may exert
36 specific metabolic effects in fish by decreasing hepatic transdeamination and supressing the
37 use of amino acids as gluconeogenic substrates. Our findings highlight the role of amino acid
38 metabolism in the glucose-intolerant carnivorous fish model.

39

40 **Keywords:** Metformin, lipogenesis, glutamate dehydrogenase, liver, *Sparus aurata*

41 **INTRODUCTION**

42 Metformin (1,1-dimethylbiguanide hydrochloride) is an anti-diabetic drug used for the
43 treatment of type 2 diabetes to enhance glucose homeostasis by improving the insulin
44 sensitivity mainly in the liver and skeletal muscle (44). Metformin reduces the hepatic
45 production of glucose by a mechanism involving inhibition of gluconeogenesis and
46 glycogenolysis, increased insulin sensitivity and peripheral glucose uptake, and reduced
47 intestinal glucose absorption (40, 42). Metformin-dependent reduction of hepatic glucose
48 production involves transitory inhibition of complex I of the mitochondrial respiratory chain,
49 leading to activation of adenosine monophosphate-activated protein kinase (AMPK), an
50 energy sensor involved in glucose and lipid metabolism. AMPK activation stimulates
51 glycolysis while down-regulates the hepatic transcription of gluconeogenic genes (16, 23,
52 36). In addition, metformin represses lipogenesis and triglycerides accumulation in the liver
53 through a mechanism that involves induced activation of AMPK and down-regulation of
54 sterol regulatory element-binding protein (SREBP) 1c, a key transcription factor for *de novo*
55 synthesis of lipids (19, 41, 47). Metformin also improves the glucose control via increasing
56 insulin-stimulated glucose disposal, enhancing insulin receptor tyrosine kinase activity,
57 increasing glycogen synthesis activity, and enhancing activity of glucose facilitative
58 transporter type 4 (GLUT4) in skeletal muscle (6).

59 The molecular action of metformin has been mostly studied in rodents and human-
60 derived cell lines. In fish, metformin reduces blood glucose levels when administrated
61 intraperitoneally, infused using osmotic pumps or included in the food diet (15, 31, 33, 46).
62 However, knowledge of mechanisms underlying metformin action in fish remains limited.
63 Carnivorous fish are considered glucose intolerant mainly due to prolonged hyperglycemia
64 experienced after a glucose load or intake of high carbohydrate diets (34). The molecular
65 basis of glucose intolerance in fish has been mainly attributed to dysregulation of enzyme

66 activities that control the rate of substrate cycling between glucose and glucose-6-phosphate
67 in the liver, glucokinase (GK) and glucose-6-phosphatase (G6Pase). In this regard, lower
68 glucose affinity and postprandial delayed induction of GK expression was reported for
69 gilthead sea bream (*Sparus aurata*) (8). In addition, no significant modulation of G6Pase
70 expression was reported in the liver of rainbow trout (*Oncorhynchus mykiss*) irrespective of
71 the carbohydrate content of the diet (29, 30), while insulin hardly affected the promoter
72 activity of the G6Pase catalytic subunit in the absence of glucose in *S. aurata* primary
73 hepatocytes, suggesting that a reduced capacity of insulin-dependent repression of G6Pase
74 may contribute to insulin resistance in fish (37).

75 In contrast to observations in mammals, albeit dietary metformin reduced postprandial
76 glycemia in rainbow trout supplied with high-carbohydrate diets, unexpected induction of
77 gluconeogenic and lipogenic gene expression by metformin was found in the liver (31).
78 Indeed, metformin counteracts the effects of insulin after intraperitoneal administration of
79 glucose in metformin-infused rainbow trout, especially in the muscle, which lead the authors
80 to conclude that metformin is unable to improve glucose homeostasis under hyperglycemic
81 conditions in rainbow trout (35).

82 Considering that the effect of metformin on the intermediary metabolism of carnivorous fish
83 remains limited to a few species and that the phylogenetic diversity of fish may determine specific
84 metabolic adaptations, the purpose of the present study was to examine the metabolic effects of
85 metformin in *S. aurata*. To this end, we analyzed the effect of intraperitoneal administration of
86 metformin and glucose on the expression of key enzymes involved in hepatic glycolysis-
87 gluconeogenesis: GK, 6-phosphofructo-1-kinase (PFK1) and fructose-1,6-bisphosphatase (FBPase1).
88 Given the major role of amino acids as gluconeogenic substrates and fuel in carnivorous fish and the
89 involvement of metformin on lipogenic gene expression (1, 43, 31), we also studied the effect of
90 metformin on key enzymes of the tricarboxylic acid cycle (isocitrate dehydrogenase, IDH; and α-
91 ketoglutarate dehydrogenase, OGDH), amino acid metabolism (alanine aminotransferase, ALT;

92 aspartate aminotransferase, AST; and glutamate dehydrogenase, GDH), nutrient-sensitive
93 serine/threonine-protein kinase TOR (mTOR) and lipogenic factors (SREBP1; peroxisome
94 proliferator-activated receptor gamma coactivator 1-β, PGC1β; and Lpin1).

95

96 **MATERIALS AND METHODS**

97 *Animals*

98 Gilthead sea bream (*S. aurata*) juveniles obtained from Piscimar (Burriana, Castellón, Spain) were
99 maintained at 20 ºC in 260-L aquaria supplied with running seawater as described (13). The diet,
100 supplied at 30 g/Kg body weight (BW) once a day (10 a.m.), contained 46 % protein, 9.3 %
101 carbohydrates, 22 % lipids, 10.6 % ash, 12.1 % moisture and 21.1 kJ/g gross energy. To study the
102 metabolic effects of metformin on *S. aurata*, four groups of fish were intraperitoneally administered
103 24 h after the last meal with a volume of 10 μl/g BW containing saline (9 g/L NaCl; control group),
104 glucose (2 g/Kg BW), metformin (150 mg/Kg BW) and glucose (2 g/Kg BW) + metformin (150
105 mg/Kg BW), respectively. Five hours and 24 hours following treatment, fish were sacrificed by
106 cervical section, blood was collected and the liver was dissected out, frozen in liquid N_2 and kept at -
107 80 °C until use. To prevent stress, fish were anesthetized with MS-222 (1:12,500) before handling.
108 The University of Barcelona's Animal Welfare Committee approved the experimental procedures in
109 compliance with local and EU legislation.

110

111 *Enzyme activity assays and metabolite determinations*

112 In order to obtain liver crude extracts for determination of enzyme activities, powdered frozen
113 tissue was homogenized (1:5, w/v) in 50 mM Tris–HCl (pH 7.5), 4 mM, EDTA, 50 mM NaF, 0.5 mM
114 phenylmethylsulfonyl fluoride, 1 mM dithiothreitol and 250 mM sucrose using a PTA-7 Polytron
115 (Kinematica GmbH, Littau-Luzern, Switzerland) (position 3, 30 s). After centrifugation at 20,000 *g*
116 for 30 min at 4 °C, the supernatant was collected and used to perform enzyme activity assays. PFK1

117 was assayed after addition of 1 mM ATP to a 200-μl reaction mix containing 100 mM Tris-HCl pH
118 8.25, 5 mM $MgCl_2$, 50 mM KCl, 0.15 mM ammonium sulfate, 4 mM 2-mercaptoethanol, 0.15 mM
119 NADH, 10 mM fructose 6-phosphate, 30 mM glucose 6-phosphate, 0.675 U ml^{-1} aldolase, 5 U ml^{-1}
120 triose phosphate isomerase, 2 U ml^{-1} glycerol 3-phosphate dehydrogenase and 4 μl of crude extract.
121 FBPase1 was monitored in a final volume of 200 μl containing 85 mM imidazole-HCl pH 7.7, 5 mM
122 $MgCl_2$, 0.5 mM NADP, 12 mM 2-mercaptoethanol, 0.05 mM fructose 1,6-bisphosphate, 2.5 U ml^{-1}
123 phosphate glucose isomerase, 0.48 U ml^{-1} glucose 6-phosphate dehydrogenase and 4 μl of extract.
124 GDH was determined by monitoring NADH oxidation in a 250-μl mixture containing 50 mM
125 imidazole-HCl (pH 7.4), 250 mM ammonium acetate, 5 mM α-ketoglutaric acid, 0,1 mM NADH, 1
126 mM ADP and 4 μl crude extract. To assay IDH, $NADP^+$ reduction was assayed after addition of 32
127 μM $NADP^+$ and 3.9 mM $MnSO_4$ to a final volume of 200 μl containing 80 mM triethanolamine buffer
128 (pH 7.5), 42 mM NaCl, 3.7 mM isocitrate and 4 μl crude extract. OGDH activity was determined
129 after addition of 0.12 mM coenzyme A to a final volume of 200 μl containing 50 mM phosphate
130 buffer (pH 7.4), 2 mM $MgCl_2$, 0.6 mM thiamine pyrophosphate, 2 mM NAD^+, 10 mM α-
131 ketoglutarate, 0.2 mM EGTA, 0.4 mM ADP and 4 μl crude extract. ALT and AST were assayed using
132 commercial kits (Linear Chemicals, Montgat, Barcelona, Spain). All enzyme assays were were
133 performed at 30 °C and monitored at 340 nm in a Cobas Mira S analyser (Hoffman-La Roche, Basel,
134 Switzerland). Enzyme activities were expressed per mg of soluble protein (specific activity). One unit
135 of enzyme activity was defined as the amount of enzyme required to transform 1 μmol of substrate
136 per min, except for PFK1 activity, which was defined as the amount of enzyme oxidising 2 μmol of
137 NADH per min. The Bradford method (5) using bovine serum albumin as a standard was adapted for
138 automated determination of total protein in liver crude extracts as described (27). Serum glucose,
139 triglycerides and cholesterol were measured with commercial kits (Linear Chemicals, Montgat,
140 Barcelona, Spain).

141

142 *Quantitative real-time RT-PCR*

143 One μg of total RNA isolated from the liver of *S. aurata* was reverse-transcribed to cDNA using
144 Moloney murine leukaemia virus RT (Life technologies, Carsbad, CA, USA) for 1 h at 37 ºC and
145 random hexamer primers. *S. aurata* GK, PFK1, FBPase1, GDH, mTOR, SREBP1, PGC1β and Lpin1
146 mRNA levels were determined in a Step One Plus Real Time PCR System (Applied Biosystems,
147 Foster City, CA, USA) in a 20-μl mixture containing 0.4 μM of each primer (Table 1), 10 μl of SYBR
148 Green (Applied Biosystems, Foster City, CA, USA), and 1.6 μl of diluted cDNA. The temperature
149 cycle protocol for amplification was 95 °C for 10 min, followed by 40 cycles with 95 °C for 15 s and
150 62 °C for 1 min. A dissociation curve was applied after each experiment to confirm amplification of
151 one product only. Specificity of the amplification was assayed by amplicon sequencing at least once
152 for each gene. Standard curves were generated with serial dilutions of control cDNA to determine the
153 efficiency of PCR reaction for each gene. Amplicon size was checked by agarose gel electrophoresis.
154 The amount of mRNA for the gene of interest in each sample was normalized with *S. aurata*
155 ribosomal subunit 18s, β-actin and elongation factor 1 α (EF1α) as endogenous controls using primer
156 pairs JDRT18S/JDRT18AS, QBACTINF/QBACTINR and AS-EF1Fw/AS-EF1Rv, respectively
157 (Table 1). Variations in gene expression were calculated by the standard $\Delta\Delta C_T$ method (32).

158

159 *Statistics*

160 Analyses were performed with SPSS software Version 24 (IBM, Armonk, NY, USA). Data were
161 submitted to two-way ANOVA with time (5 h and 24 h) and treatment (saline, glucose, metformin
162 and glucose + metformin) as independent variables. Student-Newman-Keuls post hoc test was applied
163 to determine differences among treatments ($p < 0.05$).

164

165 **RESULTS**

166 Five and 24 h following intraperitoneal administration with saline, glucose, metformin, and
167 glucose plus metformin, serum metabolites and the expression of key enzymes, transcriptional
168 coactivators and transcription factors involved in the regulation of intermediary metabolism were

169 analyzed in the liver of *S. aurata*. Data on serum glucose, triglycerides and cholesterol are presented
170 in Figure 1. Plasma glucose levels were significantly affected by sampling time, treatment and their
171 interaction (Fig. 1A). Five h after glucose injection, plasma glucose levels increased from 3.3 mM in
172 control animals (saline) to 49.0 mM. Thereafter it decreased to the control values at 24 h post-
173 treatment. At 5 h after the administration of metformin, glycemia reached 7.3 mM (2.2-fold over
174 control values), while, it promoted a slight hypoglycemia (2.9 mM) 24 h following the treatment. In
175 combination with glucose, metformin prevented the increase in blood glucose levels: mostly at 5 h
176 after the treatment and totally at 24 h following the administration (Fig. 1A). No statistical differences
177 were observed in serum triglycerides and cholesterol concerning the metformin effect. However, both
178 triglycerides and cholesterol exhibited a similar trend to slightly increase as a result of glucose
179 administration at 24 h post-treatment. Such effect was totally prevented by the administration of
180 metformin (Figs. 2B and 2C).

181 The effect of sampling time and treatment on *S. aurata* liver mRNA levels for rate-limiting
182 enzymes in glycolysis-gluconeogenesis is shown in Figure 2. Five h following the treatment, glucose
183 injection significantly increased mRNA levels and enzyme activity for genes involved in glycolysis
184 (GK and PFK1), being GK the most affected enzyme. The administration of metformin alone did not
185 modulate GK expression, while down-regulated PFK1 mRNA levels at 5 and 24 h post-treatment as
186 well as PFK1 activity at 24 h. When administrated with glucose, metformin totally prevented the
187 effect of glucose administration on GK and PFK1 expression (Figs. 2A-2D). The mRNA levels of
188 FBPase1 decreased as a result of metformin administration, while no significant effects were observed
189 by injecting metformin combined with glucose. At the level of enzyme activity no effects were found
190 for FBPase1 in any of the treatments performed (Figs. 2E and 2F).

191 For all treatments, we also analyzed the hepatic activity of two rate-limiting enzymes of the
192 tricarboxylic acid cycle, IDH and OGDH. Glucose administration significantly increased 2-fold IDH
193 activity compared to control fish at 24 h after the treatment. The opposite effects were observed after
194 the administration of metformin alone, while metformin combined with glucose prevented the rise of

195 IDH activity promoted by glucose (Fig. 3A). In contrast to IDH, glucose and metformin did not affect
196 significantly OGDH activity in the liver of *S. aurata* (Fig. 3B).

197 The effect of glucose and metformin in regard of key enzyme activities in amino acid metabolism
198 in the liver is presented in Figure 4. After glucose injection, ALT activity significantly decreased at 24
199 h compare to control animals. Albeit not significant, a similar trend was observed for AST activity.
200 Metformin prevented the glucose-dependent decrease in ALT activity 24 h after the treatment (Figs.
201 4A and 4B). The effect of sampling time and treatment on mRNA levels and enzyme activity of GDH
202 is shown in Figures 4C and 4D, respectively. Glucose injection did not affect GDH expression at any
203 of the sampling times studied. However, metformin significantly down-regulated both GDH mRNA
204 levels (3.3-fold) and activity (2.9-fold) 5 h post-treatment. Twenty-four h after the treatment, GDH
205 expression was not affected by metformin.

206 Given that the multiproteic complex mTORC1 is considered a sensor of nutrient availability and
207 energy status of the cell (18), we analyzed the hepatic mRNA levels of mTOR, a cytosolic
208 serine/threonine kinase included in the mTORC1 complex. A trend to decrease mTOR expression was
209 observed 5 h after the administration of metformin. Glucose alone did not affect mTOR expression
210 and reverted the effect observed with metformin (Fig. 5A). We also addressed the effect of metformin
211 on transcriptional coactivators and transcription factors involved in the control of lipogenesis.
212 Experimental treatments significantly affected PGC1β and SREBP1 mRNA levels. Five h following
213 glucose administration, SREBP1 expression increased 2.7-fold, while at 24 h post-treatment glucose
214 up-regulated PGC1β and SREBP1 mRNA levels about 2-fold compared to control animals (saline), an
215 effect that was at least partially reverted when glucose was administered in combination with
216 metformin. At 5 h after the administration of metformin alone, a tendency to increase PGC1β and
217 SREBP1 mRNA levels was found (Figs. 5B and 5C). Sampling time also affected Lpin1 expression.
218 At 5 h post-treatment, metformin up-regulated Lpin1, while glucose in combination with metformin
219 reverted the effect. At 24 h after the administration, the highest Lpin1 mRNA levels were observed in
220 the group of fish injected with glucose (Fig. 5D).

221

222 **DISCUSSION**

223 Herein we addressed the effect of metformin on key metabolic actors known to play
224 important roles in the control of postprandial glycemia in the liver of *S. aurata*. Five h
225 following glucose administration, blood glucose levels markedly increased and it was totally
226 recovered 24 h post-treatment. Consistent with previous reports in *S. aurata* (9, 13, 27), our
227 findings showed that hyperglycemia led to increased glycolytic rate by stimulating the
228 expression of GK and PFK1 while hardly affected the activity of the gluconeogenic enzyme,
229 FBPase1. In the present study, metformin counteracted the activating effect of glucose on GK
230 and PFK1 expression in the liver of *S. aurata.* When metformin was administered in the
231 absence of glucose, it decreased both PFK1 and FBPase1 mRNA levels. However, only a
232 significant decrease was observed in PFK1 activity 24 h post-treatment, possibly due to
233 allosteric regulation of both PFK1 and FBPase1 activity. Similarly, the expression of GK,
234 PFK1, G6Pase, FBPase1 and phosphoenolpyruvate carboxykinase (PEPCK) decreased in the
235 liver of metformin-infused rainbow trout 6 h following intraperitoneal administration of
236 glucose (35). However, in the same species, no effect of dietary metformin supplied to fish
237 fed high carbohydrate diets was observed on the activity of glycolytic enzymes in the liver
238 and white muscle, while it paradoxically increased the expression of FBPase1 and other
239 gluconeogenic enzymes (31). Differences in the experimental design as well as metformin
240 dosage and administration may explain the results obtained in rainbow trout.

241 Considering rate-limiting enzymes of the tricarboxylic acid cycle (TCA) in the liver of *S.*
242 *aurata*, IDH activity, which catalyzes the oxidative decarboxylation of isocitrate to α-
243 ketoglutarate, appeared to be more sensible than OGDH to experimental treatments: glucose
244 stimulated IDH activity while metformin decreased the enzyme activity. However, OGDH
245 activity was not significantly affected. Possibly, increased availability of fuel in the liver after

246 glucose administration and glucose-dependent enhancement of glycolysis may be responsible
247 of long-term enhancement of IDH activity and therefore enable glucose oxidation in the liver
248 for ATP production. Similarly as in *S. aurata*, metformin inhibits oxidative reaction of IDH
249 in human-derived cancer cells (3, 17).

250 Given that a tendency to increase triglycerides and cholesterol blood levels was observed
251 24 h following glucose administration, our findings support that hepatic lipogenesis seems an
252 efficient metabolic pathway for long-term transformation of excess glucose to lipids in *S.*
253 *aurata*. Consistent with this hypothesis, glucose administration up-regulated the expression
254 of SREBP1, a transcription factor that plays a major role in the transcriptional regulation of
255 genes involved in fatty acid and cholesterol synthesis (39), and, at long-term, Lpin1 and the
256 transcriptional coactivator PGC1β, which are also involved in lipogenesis (10, 12). We
257 previously showed that although *S. aurata* is a carnivorous fish, it tolerates partial
258 replacement of dietary protein by carbohydrates through a mechanism that involves
259 modulation of glycolysis in the liver (8, 13, 25–27). Conceivably, glucose-dependent effects
260 on SREBP1, Lpin1 and PGC1β mRNA levels, which lasted 24 h after the treatment, may
261 exert a major role in lipid synthesis from dietary carbohydrates at long-term in the liver.

262 The administration of metformin prevented glucose-dependent hyperglycemia and the
263 effects on other serum metabolites, the activity of key enzymes in glycolysis and TCA cycle
264 as well as in the expression of SREBP1, Lpin1 and PGC1β. In the absence of a glucose load,
265 a trend to increase SREBP1 and PGC1β mRNA levels was found in the liver of *S. aurata* 5 h
266 following the administration of metformin. However, the fact that *S. aurata* treated with
267 metformin plus glucose presented lower SREBP1, Lpin1 and PGC1β mRNA levels than
268 glucose-treated fish, point to down-regulation of hepatic lipogenesis as a result of metformin
269 action to reduce blood glucose levels in the hyperglycemic state. Metformin-dependent
270 down-regulation of SREBP1 expression and lipogenesis is consistent with previous

271 observations in mammals (19, 28, 41, 47), but contrasts with results reported for rainbow
272 trout that indicate that dietary metformin increases expression of lipogenic enzymes in the
273 liver of rainbow trout fed on high-carbohydrate diets (31). Indeed, the administration of
274 metformin together with insulin up-regulated SREBP1 expression in the liver of rainbow
275 trout fed high-carbohydrate diets, while metformin alone did not affect SREBP1 mRNA
276 levels (33). Different metabolic responses to metformin in *S. aurata* and rainbow trout may
277 result from the fact that although both species are carnivores, they belong to phylogenetically
278 distant orders (*Spariformes* and *Salmoniformes*, respectively).

279 Remarkably, at 5 h post-treatment, metformin administration in the absence of a glucose
280 load down-regulated GDH, which plays a major role in amino acid catabolism by catalyzing
281 reversible oxidative deamination of L-glutamate into α-ketoglutarate, and ALT activity.
282 Carnivorous fish exhibit preferential use of amino acids as gluconeogenic substrates and fuel
283 (1, 43). Indeed, optimal growth of fish requires high levels of dietary protein and amino acids
284 are the most potent insulin secretagogues in fish (34). Therefore, transdeamination, which
285 involves transferring of amino groups from amino acids to α-ketoglutarate to produce
286 glutamate and subsequent glutamate deamination by GDH, is of major importance in fish
287 liver for entering the carbon skeleton of amino acids into the TCA cycle to obtain energy and
288 for biosynthetic purposes (7, 14, 20, 22, 38). The fact that metformin markedly decreased
289 GDH expression and to a lesser extent ALT activity suggests reduced amino acid
290 deamination and transamination capacity in the liver of *S. aurata*, and therefore limited use of
291 amino acid for gluconeogenic purposes, which in turn may be essential for the long-term
292 hypoglycemic effect of metformin in the liver of *S. aurata*. In contrast to ALT, AST activity
293 remained unaffected by metformin. Indeed, although ALT and AST are quantitatively the
294 most important aminotransferases in the fish liver, previous studies indicated greater
295 sensibility of ALT to changes in the nutritional status than AST in the liver of *S. aurata* (2,

13, 27). In addition to GDH and ALT, metformin down-regulated mTOR expression at 5 h post-treatment in the liver of *S. aurata*. Consistently, metformin supplementation suppresses upregulation of hepatic mTOR mRNA levels when feeding the herbivorous cyprinid *Megalobrama amblycephala* with high carbohydrate diets (45). The mTOR pathway is considered a major signaling pathway in fish and as in mammals is sensitive to the dietary protein to carbohydrate ratio (4). As in fish, metformin inhibits mTORC1 signaling in humans (24). Bearing in mind that mTOR is part of the amino acid sensor mTORC1 complex and that knockdown of GDH1 inhibits mTORC1 activity and leucine requires GDH1 for promoting mTORC1 activity in human cells (21), our results suggest that metformin may inhibit mTORC1 signaling by decreasing GDH expression in the liver of *S. aurata*. Conceivably, metformin-dependent reduced transdeamination activity in the liver of *S. aurata* would decrease availability of amino acid carbon skeletons as fuel and determine the low levels of PFK1 and IDH activity observed 24 h after metformin administration in the absence of a glucose load. In this regard, it was proposed that inhibition of mTORC1 improves glucose tolerance by inhibiting hepatic gluconeogenesis in rainbow trout (11).

PERSPECTIVES AND SIGNIFICANCE

The present study addressed for the first time the effect of acute metformin treatment on the intermediary metabolism of *S. aurata*. Our findings suggest that hyperglycemia enhances lipogenesis in the liver and that metformin may improve glucose homeostasis by counteracting the activating effects of glucose on the activity of rate-limiting enzymes in glycolysis and TCA as well as the expression of lipogenic factors. In addition, the present study provides evidence that metformin may reduce the gluconeogenic rate by decreasing hepatic transdeamination and the entrance of amino acids into the TCA cycle in fish. Further

320 studies are needed to better understand the link between metformin action, GDH expression
321 and the use of amino acids as gluconeogenic substrates in carnivorous fish.
322

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326

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330

331 **DISCLOSURES**

332 No conflicts of interest, financial or otherwise, are declared by the authors.
333

334 **AUTHOR CONTRIBUTION**

335 I.M., I.V.B. and J.I.S.-M. conceived and designed research; A.R., L.S. and J.I.S.-M. performed
336 experiments; I.M., J.I.S.-M. and A.R. analyzed data and interpreted results of experiments; A.R. and
337 I.M. prepared figures and drafted manuscript; A.R., J.I.S.-M., L.S., I.M. and I.V.B. edited and revised
338 manuscript, and approved final version of manuscript.
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483

484 **FIGURE LEGENDS**

485 Fig. 1. Effect of glucose and metformin administration on serum metabolite levels in *S. aurata*.
486 Twenty-four h after the last meal, four groups of fish were intraperitoneally administered with saline
487 (control), 2 g/Kg BW glucose, 150 mg/Kg BW metformin and 2 g/Kg BW glucose + 150 mg/Kg BW
488 metformin, respectively. Five and 24 h following the treatment, blood was collected and serum
489 recovered. Serum levels of glucose (A), triglycerides (B) and cholesterol (C) are presented as mean ±
490 SE (n = 6 fish). Statistical significance for independent variables (sampling time and treatment) are
491 indicated as follows: *$P < 0.05$; ***$P < 0.001$; *NS* not significant. Homogeneous subsets for the
492 treatment are shown with different letters ($P < 0.05$).

493

494 Fig. 2. Effect of glucose and metformin administration on the expression of key enzymes in
495 glycolysis-gluconeogenesis in the liver of *S. aurata*. Twenty-four h after the last meal, four groups of
496 fish were intraperitoneally administered with saline (control), 2 g/Kg BW glucose, 150 mg/Kg BW
497 metformin and 2 g/Kg BW glucose + 150 mg/Kg BW metformin, respectively. Five and 24 h
498 following the treatment, the liver was collected and RNA isolated. Hepatic mRNA levels and enzyme
499 activity of GK (A, B), PFK1 (C, D) and FBPase1 (E, F) are presented as mean ± SE (n = 6 fish).
500 Expression levels for each gene were normalized using ribosomal subunit 18s, β-actin and EF1α as
501 housekeeping genes. Statistical significance for independent variables (sampling time and treatment)
502 are indicated as follows: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; *NS* not significant. Homogeneous
503 subsets for the treatment are shown with different letters ($P < 0.05$).

504

505 Fig. 3. Effect of glucose and metformin administration on the activity of key enzymes in the
506 tricarboxylic acid cycle in the liver of *S. aurata*. Twenty-four h after the last meal, four groups of fish
507 were intraperitoneally administered with saline (control), 2 g/Kg BW glucose, 150 mg/Kg BW
508 metformin and 2 g/Kg BW glucose + 150 mg/Kg BW metformin, respectively. Five and 24 h
509 following the treatment, the liver was collected. Enzyme activity levels of IDH (A) and OGDH (B)

510 are presented as mean ± SE (n = 6 fish). Statistical significance for independent variables (sampling
511 time and treatment) are indicated as follows: *$P < 0.05$; **$P < 0.01$; *NS* not significant. Homogeneous
512 subsets for the treatment are shown with different letters ($P < 0.05$).

513

514 Fig. 4. Effect of glucose and metformin administration on key enzymes in amino acid metabolism in
515 the liver of *S. aurata*. Twenty-four h after the last meal, four groups of fish were intraperitoneally
516 administered with saline (control), 2 g/Kg BW glucose, 150 mg/Kg BW metformin and 2 g/Kg BW
517 glucose + 150 mg/Kg BW metformin, respectively. Five and 24 h following the treatment, the liver
518 was collected and RNA isolated. Enzyme activity values of ALT (A), AST (B) and mRNA levels as
519 well as enzyme activity of GDH (C, D) are presented as mean ± SE (n = 6 fish). Expression levels for
520 GDH were normalized using ribosomal subunit 18s, β-actin and EF1α as housekeeping genes.
521 Statistical significance for independent variables (sampling time and treatment) are indicated as
522 follows: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; *NS* not significant. Homogeneous subsets for the
523 treatment are shown with different letters ($P < 0.05$).

524

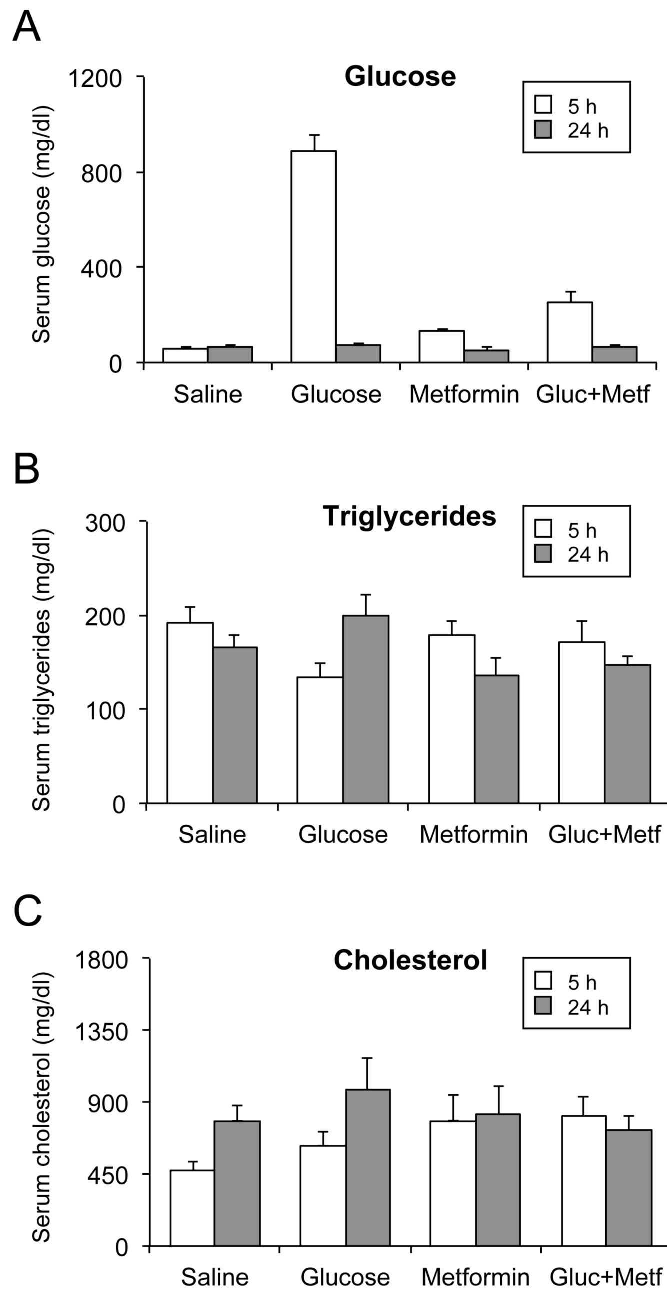
525 Fig. 5. Effect of glucose and signalling metformin administration on the mRNA levels of signalling
526 proteins involved in the control of intermediary metabolism in the liver of *S. aurata*. Twenty-four h
527 after the last meal, four groups of fish were intraperitoneally administered with saline (control), 2
528 g/Kg BW glucose, 150 mg/Kg BW metformin and 2 g/Kg BW glucose + 150 mg/Kg BW metformin,
529 respectively. Five and 24 h following the treatment, the liver was collected and RNA isolated. The
530 mRNA levels of mTOR (A), PGC1β (B), SREBP1 (C) and Lpin1 (D) are presented as mean ± SE (n
531 = 6 fish). Expression levels for each gene were normalized using ribosomal subunit 18s, β-actin and
532 EF1α as housekeeping genes. Statistical significance for independent variables (sampling time and
533 treatment) are indicated as follows: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; *NS* not significant.
534 Homogeneous subsets for the treatment are shown with different letters ($P < 0.05$).

535 Table 1. Oligonucleotides used in the present study.

Primer	Sequence (5’ to 3’)	Gene, GenBank accession no.
JYA01F1	TGTGTCAGCTCTCAACTCGACC	GK, AF169368
JYA02R1	AGGATCTGCTCTACCATGTGGAT	
JYA03F1	TGCTGGGGACAAAACGAACTCTTCC	PFK1, KF857580
JYA04R1	AAACCCTCCGACTACAAGCAGAGCT	
AE1305	CAGATGGTGAGCCGTGTGAGAAGGATG	FBPase1, AF427867
AE1306	GCCGTACAGAGCGTAACCAGCTGCC	
CG1543	GGTATTTCGGGGAGCTGCTGAG	GDH, MF459045
CG1544	CGCATCAGGGACGAGGACA	
AS1601	GGAGACTGTTTTGAGGTCGCC	mTOR, MH594580
AS1602	ACCTCCATCACCGTGTGGCA	
LS1703	ACCTCTTCTACCCCAACCAACAAC	Lpin1, MH594582
LS1704	TCCACCACCTCGCCCAG	
LS1705	GCATGGCTCGCGACGGC	PGC1β, MH594581
LS1706	GTGTTTTCAGTGGGCCATGGCATTG	
JS1406	CAGCAGCCCGAACACCTACA	SREBP1, JQ277709
JS1407	TTGTGGTCAGCCCTTGGAGTTG	
ASEF1Fw	CCCGCCTCTGTTGCCTTCG	EF1α, AF184170
ASEF1Rv	CAGCAGTGTGGTTCCGTTAGC	
JDRT18S	TTACGCCCATGTTGTCCTGAG	18s, AM490061
JDRT18AS	AGGATTCTGCATGATGGTCACC	
QBACTINF	CTGGCATCACACCTTCTACAACGAG	β-actin, X89920
QBACTINR	GCGGGGGTGTTGAAGGTCTC	

536

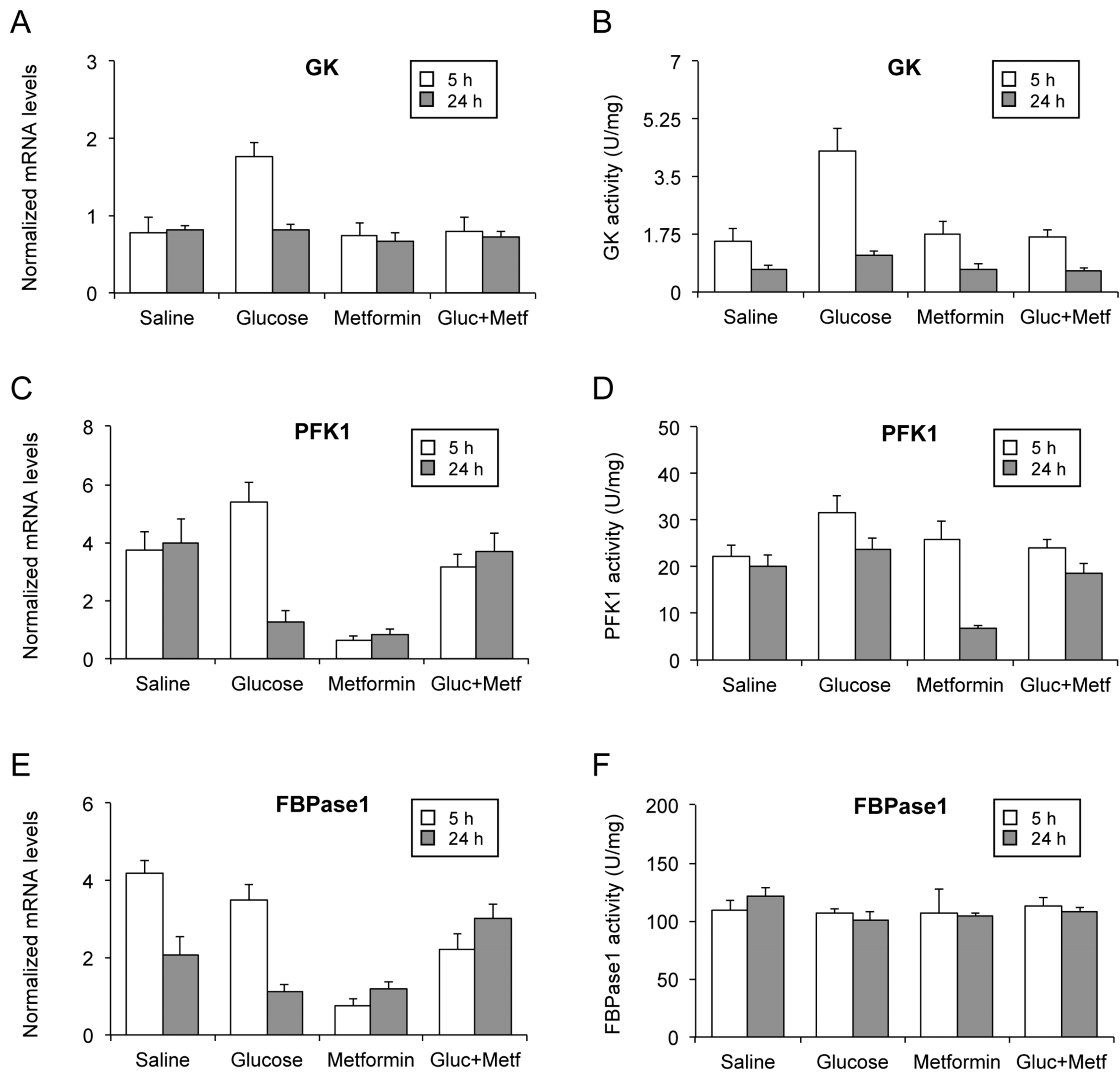
Figure 1



Two-way ANOVA

Dependent variable	Interaction	Time	Treatment	Treatment: Saline	Treatment: Gluc	Treatment: Metf	Treatment: Gluc+Metf
Glucose	***	***	***	a	c	ab	b
Triglycerides	*	NS	NS	-	-	-	-
Cholesterol	NS	NS	NS	-	-	-	-

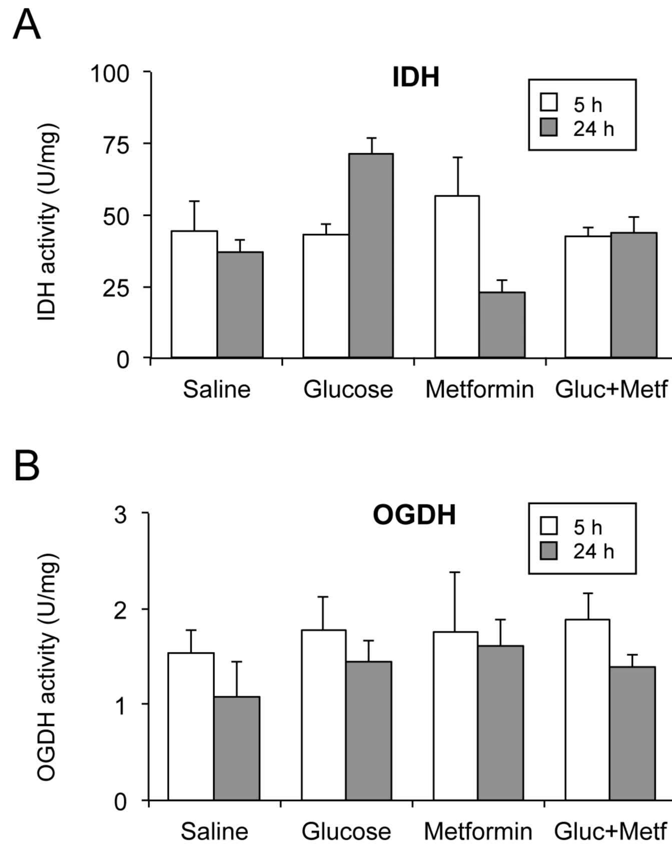
Figure 2



Two-way ANOVA

Dependent variable	Interaction	Time	Treatment	Treatment: Saline	Gluc	Metf	Gluc+Metf
GK mRNA	**	NS	***	a	b	a	a
GK activity	**	***	***	a	b	a	a
PFK1 mRNA	***	*	***	b	b	a	b
PFK1 activity	*	***	**	ab	b	a	ab
FBPase1 mRNA	***	**	***	b	b	a	b
FBPase1 activity	NS	NS	NS	-	-	-	-

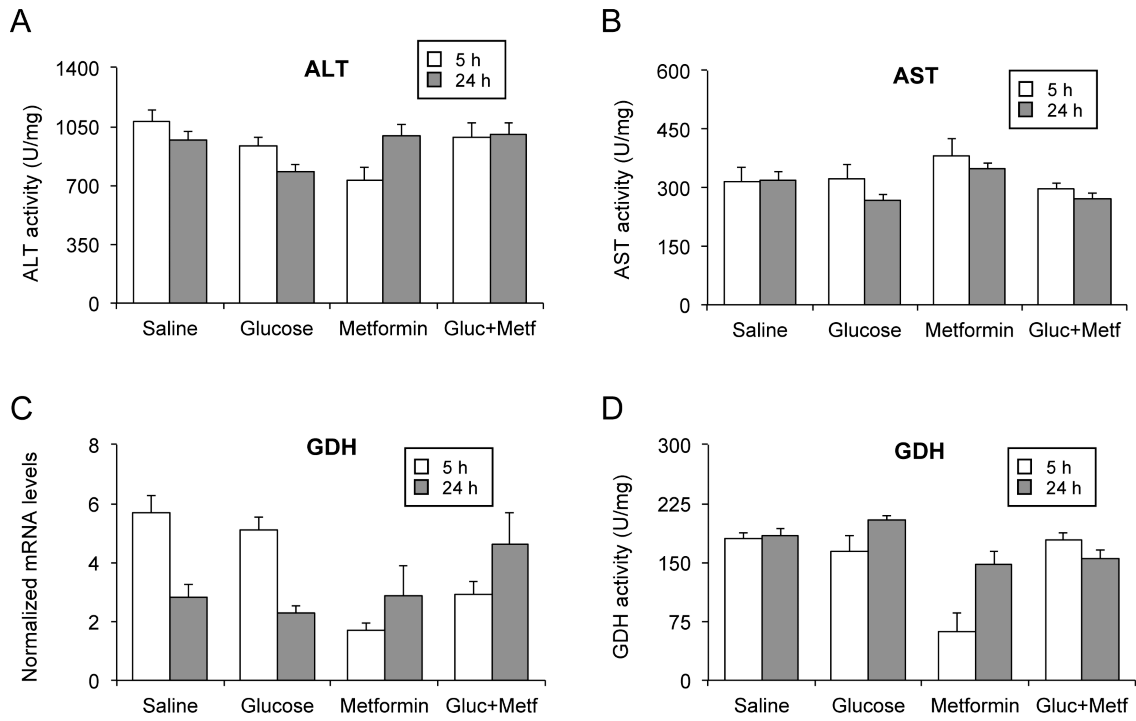
Figure 3



Two-way ANOVA

Dependent variable	Interaction	Time	Treatment	Treatment: Saline	Gluc	Metf	Gluc+Metf
IDH activity	**	NS	*	a	b	a	ab
OGDH activity	NS	*	NS	-	-	-	-

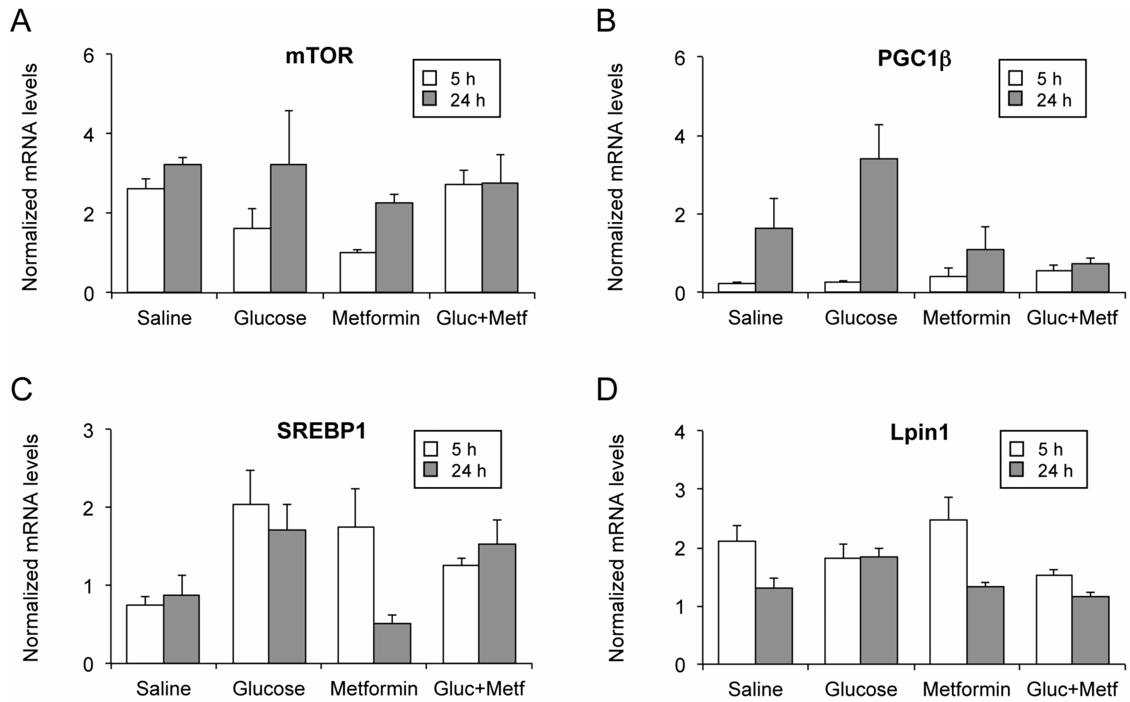
Figure 4



Two-way ANOVA

Dependent variable	Interaction	Time	Treatment	Treatment: Saline	Gluc	Metf	Gluc+Metf
ALT activity	*	NS	*	b	a	ab	ab
AST activity	NS	NS	*	ab	ab	b	a
GDH mRNA	***	NS	*	b	ab	a	ab
GDH activity	**	*	***	b	b	a	b

Figure 5



Two-way ANOVA

Dependent variable	Interaction	Time	Treatment	Treatment: Saline	Gluc	Metf	Gluc+Metf
mTOR mRNA	NS	NS	NS	-	-	-	-
PGC1β mRNA	*	***	*	ab	b	a	a
SREBP1 mRNA	NS	NS	**	a	b	a	ab
Lpin1 mRNA	NS	**	NS	-	-	-	-