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Lactate signaling and fuel selection in rainbow trout:

Mobilization of energy reserves

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Running head: Lactate signaling and fuel selection in fish

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1 **Abstract**

2 Lactate is now recognized as a regulator of fuel selection in mammals because it
3 inhibits lipolysis by binding to the hydroxycarboxylic acid receptor 1 (HCAR1). The goals
4 of this study were to quantify the effects of exogenous lactate on: (i) lipolytic rate or rate
5 of appearance of glycerol in the circulation (R_a glycerol) and hepatic glucose production
6 (R_a glucose), and (ii) key tissue proteins involved in lactate signaling, glucose transport,
7 glycolysis, gluconeogenesis, lipolysis and β -oxidation in rainbow trout. Measurements of
8 fuel mobilization kinetics show that lactate does not affect lipolysis as it does in
9 mammals (R_a glycerol remains at $7.3 \pm 0.5 \mu\text{mol kg}^{-1} \text{min}^{-1}$), but strongly reduces
10 hepatic glucose production (16.4 ± 2.0 to $8.9 \pm 1.2 \mu\text{mol kg}^{-1} \text{min}^{-1}$). This reduction is
11 likely induced by decreasing gluconeogenic flux through the inhibition of cytosolic
12 phosphoenolpyruvate carboxykinase (Pck1, alternatively called Pepck1; 60% and 24%
13 declines in gene expression and protein level, respectively). It is also caused by lactate
14 substituting for glucose as a fuel in all tissues except white muscle that increases *glut4a*
15 expression and has limited capacity for monocarboxylate transporter (Mct)-mediated
16 lactate import. We conclude that lipolysis is not affected by hyperlactatemia because
17 trout show no activation of autocrine Hcar1 signaling (gene expression of the receptor is
18 unchanged or even repressed in red muscle). Lactate regulates fuel mobilization via
19 Pck1-mediated suppression of gluconeogenesis and by replacing glucose as a fuel.
20 This study highlights important functional differences in the Hcar1 signaling system
21 between fish and mammals for the regulation of fuel selection.

22 **Keywords:** fish metabolism, lactate, lipolysis, hepatic glucose production,
23 carbohydrates, lipids, Hcar1.

24 INTRODUCTION

25 In addition to its traditional roles as glycolytic end-product, oxidative fuel and
26 gluconeogenic precursor, lactate is now recognized as an essential metabolic signal (1-
27 5). The hydroxycarboxylic acid receptor (HCAR1: a G-protein coupled receptor
28 previously called GPR81) was originally identified as a key binding target for lactate in
29 mammalian adipocytes (6), but has since been detected in muscle (7-9) and liver (10),
30 among others. It has now also been found in fish (11, 12). Upon lactate binding, the
31 HCAR1 receptor acts via the G_i protein to inhibit the cAMP/PKA pathway (8). Functional
32 studies have linked this lactate-induced HCAR1 signaling pathway to lipid metabolism in
33 several tissues, notably the retention of neutral lipids through the inhibition of lipolysis in
34 adipose tissue (13), the inhibition of lipolysis in muscle (7-9), and conversely, enhanced
35 lipid oxidation in the (fasted) liver (10). Studies probing the metabolic roles of HCAR1
36 lactate signaling in muscle have also reported stimulation of mitochondrial biogenesis,
37 both via cAMP/PKA dependent and independent pathways (7-9). In muscle, lactate has
38 been found to mediate these effects via a positive autocrine feedback loop because
39 acute and chronic lactate administration resulted in dose-dependent increases in
40 HCAR1 mRNA and protein abundance *in vitro* and *in vivo* (7-9). Importantly, this
41 feedback loop appears to be dependent on the suppression of cAMP signaling (8),
42 suggesting that the (auto)stimulation of HCAR1 expression and translation is an
43 indicator of HCAR1 signaling activity.

44 Multiple factors affecting metabolic fuel selection have been characterized in fish,
45 particularly rainbow trout (14). Glucose mobilization (the rate of hepatic glucose
46 production or R_a glucose) is reduced by exogenous glucose or alanine (15, 16), insulin

47 (17), propranolol (18), prolonged swimming (19) and low temperature (20), but it is
48 stimulated by epinephrine (18). The mobilization of lipid reserves (lipolysis or rate of
49 appearance of glycerol in the circulation: R_a glycerol) is also increased by epinephrine,
50 but contrary to its effect in mammals, it is inhibited by norepinephrine in fish (21).
51 Surprisingly, fish lipolytic rate remains unaffected by endurance swimming (22) or
52 graded exercise up to critical swimming speed (U_{crit}) (23). Whether lactate signaling or
53 Hcar1 are involved in modulating fuel selection in fish is unknown. Therefore, this study
54 aims to determine how exogenous lactate modulates fuel selection *in vivo* by measuring
55 its effects on hepatic glucose production and lipolytic rate of rainbow trout. Exogenous
56 supply rather than exercise-induced hyperlactatemia was selected here to focus on
57 lactate as a signal and to avoid the confounding impact of the metabolic, hormonal, and
58 neural changes caused by swimming. Our goals were to quantify the effects of elevated
59 lactate levels on: (i) R_a glucose and R_a glycerol, and (ii) key tissue proteins involved in
60 lactate signaling or carbohydrate and lipid metabolism that could potentially explain the
61 observed behavior of glucose and glycerol kinetics. We anticipated that lactate would
62 cause the Hcar1-mediated inhibition of lipolysis as it does in mammals (24, 25) and a
63 decrease in hepatic glucose production (24). It was predicted that these trout responses
64 would be reflected by corresponding changes in key regulatory proteins of lipid and
65 carbohydrate metabolism.

66

67 **METHODS**

68 **Animals**

69 Hormonally feminized rainbow trout (*Oncorhynchus mykiss*) with a Fulton's
70 condition factor K of 1.12 ± 0.05 (N=26) [$K=(10^5 \times M_b) \div L^3$]; where M_b is body mass in
71 grams and L is total length in millimeters (26)] were purchased from Linwood Acres
72 Trout Farm (Campbellcroft, Ontario, Canada). They were divided into 3 treatment
73 groups: for *in vivo* glucose kinetics (series 1 experiments), *in vivo* glycerol kinetics
74 (series 2 experiments), and tissue measurements of gene expression (qPCR), protein
75 expression (Western blots) and enzyme activities (series 3 experiments). The physical
76 characteristics of the 3 groups are in Table 1. All fish were held in a 1,200 L flow-
77 through tank supplied with dechloraminated Ottawa tap water at 13°C, on a 12 h: 12 h
78 light: dark photoperiod and they were fed Profishnet floating fish pellets (Martin Mills,
79 Elmira, ON, Canada) 5 days a week. They were acclimated to these conditions for at
80 least 3 weeks before experiments. All the procedures were approved by the Animal
81 Care Committee of the University of Ottawa (protocol BL-1625) and adhered to the
82 guidelines established by the Canadian Council on Animal Care (CCAC).

83

84 **Catheterizations**

85 Fish were anesthetized with ethyl 3-aminobenzoate methanesulfonate (MS-222;
86 60 mg l^{-1} buffered with 0.2 g l^{-1} sodium bicarbonate) and surgically fitted with 2
87 catheters (for the glucose and glycerol kinetics experiments) or a single catheter (for
88 gene expression, protein expression and enzyme activity experiments) (BTPE-50
89 catheters, Instech Laboratories, Plymouth Meeting, PA, USA) in the dorsal aorta (27).
90 The catheters were kept patent by flushing with Cortland's saline containing 50 U ml^{-1}
91 heparin (H3393, Sigma-Aldrich, St. Louis, MO, USA). After surgery, the fish were left to

92 recover overnight in a 90 L swim-tunnel (Loligo Systems, Tjele, Denmark) where they
93 were kept in the resting state at a water velocity of 0.5 body length per second (BL s⁻¹).
94 This weak current reduces stress and helps the flow of water over the gills, but it does
95 not require swimming to maintain body position because the animals are sitting at the
96 bottom of the chamber (15). The swim tunnel was supplied with the same quality water
97 as the holding tank and kept at 13°C.

98

99 **Glucose and glycerol kinetics (series 1 and 2)**

100 The rates of hepatic glucose production (or rate of appearance of glucose: R_a
101 glucose) and glycerol production (or lipolytic rate: R_a glycerol) were measured by
102 continuous infusion of [6-³H] glucose (American Radiolabeled Chemicals, St. Louis,
103 MO, USA; specific activity 2220 GBq mmol⁻¹) and [2-³H] glycerol (American
104 Radiolabeled Chemicals; 925 GBq mmol⁻¹). These tracer methods have been
105 thoroughly validated in fish (28) and specifically tested in rainbow trout to assess the
106 effects of a variety of physiological stresses on R_a glucose (14, 19, 20, 29). The method
107 to quantify R_a glycerol is also well-established for rainbow trout (21-23). All infusates
108 were freshly prepared immediately before each experiment by drying an aliquot of the
109 tracer obtained from the supplier under N₂ and resuspending in Cortland's saline. For
110 glucose kinetics, a priming dose of tracer equivalent to 3 h of infusion was injected as a
111 bolus at the start of each infusion to reach isotopic steady state in <45 min (28, 30). A
112 priming dose was not required to measure glycerol kinetics because the total glycerol
113 pool is small and isotopic steady state is reached in <45 min without it (21, 23, 30). For
114 each flux measurement, the infusate was administered continuously at ~1 ml h⁻¹ (the

115 exact infusion rate was determined for each fish to adjust for differences in body mass)
116 using a calibrated syringe pump (Harvard Apparatus, South Natick, MA). Infusion rates
117 averaged $1523 \pm 36 \text{ Bq kg}^{-1} \text{ min}^{-1}$ (N=8) for labeled glucose and $9227 \pm 297 \text{ Bq kg}^{-1}$
118 min^{-1} (N=10) for labeled glycerol. These trace amounts (labeled + unlabeled
119 metabolites) had no effect on glucose or glycerol metabolism because they accounted
120 for $<0.0000001\%$ of baseline endogenous hepatic glucose production (glucose kinetics)
121 and $<0.00001\%$ of baseline endogenous rate of appearance of glycerol in the circulation
122 (glycerol kinetics). Blood samples ($\sim 100 \mu\text{l}$ each) were drawn 50, 55 and 60 min after
123 starting the tracer infusion to quantify baseline glucose or glycerol kinetics, and every 20
124 min thereafter during a 3 h administration of exogenous lactate at a rate of $30 \mu\text{mol kg}^{-1}$
125 min^{-1} . Lactate was infused at $\sim 1 \text{ ml h}^{-1}$, but the exact rate was adjusted individually for
126 each fish to correct for differences in body mass. This rate is equivalent to twice the
127 baseline endogenous rate of lactate release in the circulation (31) and caused a gradual
128 increase in blood lactate concentration that reached $\sim 13 \text{ mM}$ after 3 h in previous
129 experiments (32). The total amount of blood sampled from each fish accounted for
130 $<10\%$ of the total blood volume. Blood samples were immediately centrifuged to
131 separate plasma (5 min; 13,000 RPM) and stored at -20°C until analyses.

132

133 **Blood sample analyses for metabolite kinetics (series 1 and 2)**

134 Plasma lactate, glucose and glycerol concentrations were measured
135 spectrophotometrically (Spectra Max Plus 384 Microplate reader, Molecular Devices,
136 Sunnyvale, CA, USA) using standard NAD^+/NADH -coupled enzymatic assays at 340
137 nm (16, 33). Radioactivity was measured by scintillation counting (Perkin-Elmer Tricarb

138 2910TR, Waltham, MA, USA) in Bio-Safe II™ scintillation cocktail (Research Products
139 International, Mt Prospect, IL, USA). [6-³H] glucose activity was measured after drying
140 each plasma sample under N₂ to eliminate tritiated H₂O. [2-³H] glycerol activity was
141 measured by incubating samples with ATP and hexokinase to phosphorylate the
142 labeled free glucose that was then removed by ion-exchange column chromatography
143 [DOWEX-1 (formate form) and DOWEX-50 (HCl form)] before drying under N₂ (33). A
144 previous study had shown that 10.6 ± 0.9% of labelled glycerol is lost during this
145 separation procedure (23) and glycerol activities were corrected accordingly.

146

147 **Tissue measurements (series 3)**

148 To avoid working with radioactive tissues, these experiments were carried out on
149 a different group of fish than series 1 and 2. Fish saline (control group) or lactate
150 (treatment group; 30 μmol lactate kg⁻¹ min⁻¹) was administered at ~1 ml h⁻¹ for 3 h. Blood
151 samples were taken before starting saline or lactate administration (0 h) and after 3 h of
152 saline or lactate infusion. The animals were then euthanized by a sharp blow on the
153 head and tissues were collected. The whole liver, red muscle (along the lateral line) and
154 white muscle (perpendicular to the dorsal fin) were collected and stored at -80°C until
155 analyses.

156

157 *RNA extraction and gene expression*

158 Total RNA from liver, red and white muscle was extracted and quantified as
159 previously (16, 34). QuantiTect reverse transcriptase kit (Qiagen, Toronto, ON, Canada)
160 was used to generate cDNA using total RNA and following the manufacturer's protocol.

161 Two-step real-time RT-PCR assays were performed to quantify fold-changes in relative
162 hepatic mRNA abundances of the lactate-sensitive hydroxycarboxylic acid receptor 1
163 (*hcar1*), key gluconeogenic transcripts (phosphoenolpyruvate carboxykinase, *pck1*,
164 *pck2a*, *pck2b* and glucose-6-phosphatase, *g6pca*, *g6pcb1a*, *g6pcb1b*) and glucose
165 transporters (*glut4a* and *glut4b*, alternatively called *slc2a4*). Additionally, multiple
166 transcripts involved in lipid metabolism were measured: fatty acid oxidation [carnitine
167 palmitoyltransferase 1; *cpt1* (NM_001124735.1 which is equivalent to Trout_B1b as
168 described in Morash et al., 2010) , hydroxyacyl CoA dehydrogenase; *hoad*), transport
169 (fatty acid translocase; *cd36*) and synthesis (fatty acid synthase; *fasn*). Standard curves
170 from pooled cDNA serial dilution were generated to optimize reaction conditions and
171 diluted samples were run in duplicates. Negative controls (no-reverse transcriptase and
172 no-template) were included in each assay to control for DNA contamination. All primer
173 sequences and amplification efficiencies are presented in Tables S2 (carbohydrate
174 metabolism targets) and S3 (lipid metabolism targets). Amplification efficiencies were
175 calculated from serially diluted 7-point standard curves and ranged between 92.8-
176 108.7%, with R^2 values >0.93 . Relative transcript abundance derived from standard
177 curves was normalized using the NORMA-Gene procedure (35). Finally, mRNA fold
178 changes were calculated relative to the control group.

179

180 *Western blotting*

181 Total protein from liver, red and white muscle was extracted, quantified and
182 prepared as previously (34) using a protease inhibitor, (88665, Thermo Fisher Scientific,
183 Waltham, MA, USA), kinase inhibitor (S1504, Sigma-Aldrich) and phosphatase inhibitor

184 (S6508, Sigma-Aldrich). Proteins were migrated for 2 h at 100 V and blotted at 100 V for
185 2 h on nitrocellulose membrane (pore size 0.2 μm , Bio-Rad Laboratories, Hercules, CA,
186 USA) in 20% Western transfer buffer. The membranes were incubated in Odyssey
187 blocking buffer (LI-COR Biosciences, Lincoln, NE, USA) for 1 h at room temperature.
188 Membranes were incubated overnight at 4°C in rabbit-raised Pck1 (A2036) and Glut4
189 (A7637) primary antibodies (ABclonal Technology, Woburn, MA, USA). Membranes
190 were washed and incubated with IRDye 800 CW goat-anti-rabbit secondary antibody
191 (925-32211, LI-COR) for 1.5 h at room temperature, protected from light. The primary
192 and secondary antibodies were diluted 1:500 and 1:20,000, respectively, as advised by
193 the manufacturers. Following incubation, the membranes were washed and visualized
194 by infrared fluorescence using the Odyssey Imaging System (LI-COR Biosciences)(16,
195 34). Band intensity was quantified by Odyssey Infrared Imaging System software (v.3.0;
196 LI-COR Biosciences), normalized to total protein stain intensity and expressed as a
197 relative-fold change compared with control groups for all tissue.

198

199 *Enzyme activities*

200 Tissue homogenates were prepared as previously (36). Enzyme activity was
201 determined kinetically at 21.0°C by measuring the oxidation of NADH or reduction of
202 NADP^+ at 340 nm and expressed in $\mu\text{mol min}^{-1} \text{g}^{-1}$ protein. The activity was assayed in
203 triplicate. Control reactions were run for 10 min for each enzyme assay after adding
204 reagents. Background activity was subtracted from enzyme activity. The conditions
205 of the individual enzyme assay were as detailed below (values given are final
206 concentrations in the well). Hexokinase (Hk; EC 2.7.1.1) and glucokinase (Gck; EC

207 2.7.1.2) assay mixture contained 300 mM imidazole, 100 mM KCl, 10 mM MgCl₂, 1 mM
208 NADP⁺, 5 U ml⁻¹ G6PDH, 5 mM ATP and glucose (omitted for control), 5 mM for Hk and
209 50 mM for Gck, reflecting different affinities for the substrate. Phosphoenolpyruvate
210 carboxykinase (Pck; EC 4.1.1.32) reaction mixture contained 50 mM imidazole, 20 mM
211 NaHCO₃, 1 mM MnCl₂, 0.5 mM PEP (Na₃), 0.12 mM NADH, 6 U ml⁻¹ malate
212 dehydrogenase and 0.2 mM deoxyguanosine diphosphate (dGDP; omitted for control).
213 Hydroxyacyl CoA dehydrogenase (Hoad; EC 1.1.1.35) reaction mixture contained 50
214 mM imidazole, 5 mM EDTA, 1 mM KCN, 0.05 mM NADH and 0.1 mM acetoacetyl CoA
215 (omitted for control).

216

217 **Calculations and statistics**

218 R_a glucose and R_a glycerol were calculated using the steady-state equation of
219 Steele (37), where R_a (in $\mu\text{mol kg}^{-1} \text{min}^{-1}$) is the infusion rate of labeled glucose or
220 glycerol (in $\text{Bq kg}^{-1} \text{min}^{-1}$) divided by the measured specific activity of glucose or glycerol
221 (in $\text{Bq } \mu\text{mol}^{-1}$). Statistical comparisons for metabolite kinetics (series 1 and 2) were
222 performed using one-way repeated-measures analysis of variance (RM-ANOVA) with
223 the Dunnett's post hoc test to determine which time points were statistically different
224 from baseline. Glucose, glycerol and lactate concentrations were analyzed by two-way
225 RM ANOVA (series 3; table 3), where the treatment and (sampling) time were the
226 effects. Based on rearing conditions, body mass between experimental series were
227 different. Gene expression, protein expression and enzyme activity results were
228 analyzed using a two-tailed unpaired t-test to assess the effects of lactate. Before all
229 tests, normality and homoscedasticity were assessed using the Shapiro-Wilk and

230 Levene's test, respectively. When the assumptions of normality or equality of variances
231 were not met, the data were normalized by natural logarithm or square root
232 transformation. When transformation failed to normalize the distribution, Kruskal-Wallis
233 non-parametric one-way ANOVA on ranks (series 1 and 2) or non-parametric Mann-
234 Whitney U test (series 3) were performed. Values presented are means \pm s.e.m. and a
235 level of significance of $P < 0.05$ was used in all tests.

236

237 **RESULTS**

238 **Series 1: Glucose kinetics**

239 During the measurement of glucose kinetics, exogenous lactate infusion had no
240 effect on plasma glucose concentration ($p > 0.05$; Fig. 1A), but progressively increased
241 plasma lactate concentration to 10.9 ± 0.4 mM over 3 hours ($p < 0.001$; Fig. 1B and
242 Table 2). Elevating plasma lactate caused an increase in glucose specific activity
243 ($p < 0.001$ at 1 h and $p < 0.05$ at 2.33 h; Fig. 2A) and a decrease in hepatic glucose
244 production from a baseline rate of 16.4 ± 2.0 $\mu\text{mol kg}^{-1} \text{min}^{-1}$ to a final value of $8.9 \pm$
245 1.2 $\mu\text{mol kg}^{-1} \text{min}^{-1}$. ($p < 0.001$; Fig. 2B and Table 2).

246

247 **Series 2: Glycerol kinetics**

248 During the measurement of glycerol kinetics, the infusion of exogenous lactate
249 caused large increases in plasma glycerol concentration ($p < 0.001$; Fig. 3A) and plasma
250 lactate concentration ($p < 0.001$; Fig. 3B). Glycerol concentration doubled and lactate
251 concentration reached 10.5 ± 1.4 mM, a level similar to the glucose kinetics
252 experiments (Table 2). Increasing the availability of plasma lactate caused minor

253 fluctuations in glycerol specific activity (Fig. 4A; $p<0.05$) and glycerol production or rate
254 of lipolysis ($p<0.05$; Fig. 4B). For these 2 parameters, however, post-hoc tests could not
255 identify any particular means that were significantly different from baseline (Fig. 4 and
256 Table 2).

257

258 **Series 3: Gene expression, protein signaling and enzyme activities**

259 *Metabolite concentrations*

260 Plasma metabolite concentrations of the fish used for tissue measurements
261 (series 3) and their statistical analyses are in Table 3. Control animals that received a
262 saline infusion showed no changes in glucose, glycerol or lactate levels. Those given an
263 infusion of exogenous lactate also maintained steady glucose concentration, but more
264 than doubled glycerol concentration. In addition, they showed a large increase in
265 plasma lactate concentration like the animals used to measure *in vivo* metabolite fluxes
266 (series 1 and 2). Interactions between time and treatment effects were significant for
267 glycerol and lactate concentrations.

268

269 *Gene expression*

270 Exogenous lactate reduced the relative mRNA expression of the
271 hydroxycarboxylic acid 1 (lactate) receptor *hcar1* in red muscle ($p<0.01$), showed a non-
272 significant trend to decrease it in white muscle ($p=0.057$), and had no effect in liver
273 ($p>0.05$; Fig. 5). The impacts of lactate on target genes that play key roles in the
274 regulation of carbohydrate and lipid metabolism are shown in Fig. 6. For carbohydrate
275 metabolism (left panels of Fig. 6), exogenous lactate decreased the relative mRNA

276 abundances of the glucose transporters *glut4a* and *glut4b* in red muscle ($p < 0.01$ and
277 $p < 0.001$ respectively), but increased them in white muscle ($p < 0.05$; Fig. 6). In liver,
278 lactate reduced the relative expression of the *pck1* paralogue of phosphoenolpyruvate
279 carboxykinase ($p < 0.05$), but had no effect on other paralogues of this gluconeogenic
280 enzyme (*pck2a* and *pck2b*; $p > 0.05$; Fig. 6). None of the glucose-6-phosphatase
281 paralogues (*g6pca*, *g6pcb1a* and *g6pcb1b*) were affected by lactate ($p > 0.05$; Fig. 6).
282 For proteins involved in lipid metabolism (right panels of Fig. 6), the relative transcript
283 abundance of two β -oxidation enzymes were quantified. Lactate did not affect *cpt1* in
284 liver, red muscle or white muscle ($p > 0.05$). The relative expression of *hoad* was
285 stimulated in white muscle ($p < 0.01$), but remained constant in liver and red muscle
286 ($p > 0.05$; Fig. 6). The relative mRNA expression of *cd36* was decreased in liver ($p < 0.05$),
287 but remained unaffected by lactate in red and white muscle ($p > 0.05$; Fig. 6). Similarly,
288 exogenous lactate caused no measurable change in the transcript abundance of *fasn* in
289 liver ($p > 0.05$; Fig. 6).

290

291 *Protein expression*

292 The relative protein abundance of Pck1 and Glut4 was quantified by Western
293 blotting (Fig. 7). Exogenous lactate decreased the protein expression of Pck1 in liver
294 ($p < 0.05$), echoing what had been observed for the mRNA abundance of this
295 gluconeogenic enzyme (Fig. 6). The expression of Glut4 in white or red muscle was not
296 affected by lactate ($p > 0.05$).

297

298 *Enzyme activities*

299 The effects of exogenous lactate on the activities of target enzymes playing key
300 roles in the regulation of carbohydrate and lipid metabolism are presented in Fig. 8. For
301 carbohydrate metabolism, lactate caused the activation of Hk in white muscle ($p < 0.05$),
302 but had no effect on liver Pck, Hk, or Gck ($p > 0.05$). For lipid metabolism, Hoad activity
303 was reduced by lactate in liver ($p < 0.01$), but remained constant in white and red muscle
304 ($p > 0.05$; left panels of Fig. 8).

305

306 **DISCUSSION**

307 This study shows that high lactate does not inhibit lipolysis in fish as it does in
308 mammals, but strongly decreases hepatic glucose production. This lack of effect on
309 lipolytic rate is due to weak putative Hcar1-mediated signaling because the expression
310 of the trout receptor is not modified by elevated lactate except in red muscle where it
311 actually declines. Therefore, the known HCAR1 autocrine feedback mechanism that
312 slows lipolysis in mammals does not seem to operate in fish. Trout R_a glucose is greatly
313 reduced by high lactate, likely through a decrease in gluconeogenesis caused by the
314 inhibition of Pck1. The overall need for glucose may be reduced because it is replaced
315 by lactate as an oxidative fuel in tissues that readily use carbohydrates, such as brain,
316 heart, and liver. Indeed, previous work suggests that the majority of lactate produced is
317 oxidized at rest and during recovery from exercise in rainbow trout (38). The liver (38),
318 heart (39) and brain (40) can all readily oxidize lactate in the resting state. Opposite
319 responses in *glut4* expression of red vs white muscle suggest that glucose import is
320 reduced in red muscle where it is replaced by lactate, but increased in white muscle: a
321 tissue with a very limited capacity for Mct-mediated transmembrane lactate transport

322 (41). Results emphasize the importance of measuring metabolite fluxes directly rather
323 than to rely on changes in concentration when investigating metabolic responses.
324 Without directly quantifying glycerol and glucose kinetics, it would have been impossible
325 to demonstrate that lactate does not affect lipolysis (because glycerol concentration
326 increases dramatically) and strongly inhibits hepatic glucose production (because
327 glycemia remains steady).

328

329 **Lactate infusion protocol mimics strenuous exercise**

330 We have administered exogenous lactate in the circulation rather than exercise
331 the animals. Before starting exogenous supply, the fish showed no signs of stress and
332 had circulating lactate (~1 mM), glucose (~7 mM) and glycerol levels (~0.2 mM) (Figs. 1
333 and 3) well within normal resting values (23, 42, 43). Lactate was then infused at twice
334 the baseline endogenous rate of release in the circulation (31) to reach plasma
335 concentrations of 10.9 ± 0.4 mM and 10.5 ± 1.4 mM after 3 h in series 1 and 2
336 experiments, respectively (Figs. 1 and 3, Table 2). The rate of exogenous supply was
337 equal to the natural endogenous release of trout when swimming at ~3 body lengths/s
338 (42). Final plasma lactate concentrations were similar to those measured in previous
339 exogenous supply experiments (32) and closely mimicked a normal increase in blood
340 lactate induced by strenuous exercise (38, 41).

341

342 **High lactate does not inhibit fish lipolysis**

343 The rate of appearance of glycerol in the circulation is the best index of whole-
344 organism lipolytic rate (30) and it has been widely used to assess lipid mobilization in

345 mammals (33, 44-47) as well as fish (21-23). This study is the first to investigate the
346 effects of exogenously increased circulating lactate on lipolytic rate *in vivo* for any
347 species. How lactate modulates glycerol and fatty acid fluxes has never been
348 measured, and this type of manipulation provides a novel research avenue to improve
349 the understanding of lactate signaling.

350

351 Elevated lactate levels do not affect trout R_a glycerol that remains at an average
352 baseline value of $7.3 \pm 0.5 \mu\text{mol kg}^{-1} \text{min}^{-1}$ (Fig. 4). This contrasts greatly with the well-
353 known anti-lipolytic effect of hyperlactatemia reported in mammals (5, 48) and suggests
354 that other reported responses of mammalian lipid metabolism to lactate, like tissue
355 triacylglycerol accumulation and lowering of plasma fatty acid levels (7-9) are also
356 absent in trout. This idea is supported by the observation that lactate has no effect on
357 the gene expression of *fasn* (Fig. 6), that encodes for a protein essential for lipogenesis
358 (49, 50). Similarly, the expression of *lpl* does not respond to lactate and *hs1* mRNA
359 shows opposite responses in red muscle (activation that would oppose lipid storage)
360 and white muscle (inhibition that would promote storage) (Fig. S1). What could then be
361 the underlying physiological reasons why lipid metabolism of fish remains mostly
362 insensitive to lactate? In mammals, lipolytic suppression is mediated by lactate binding
363 to HCAR1 in adipose tissue and muscle (25). Even though fish possess this lactate
364 receptor (11, 12), the signaling pathway does not seem to operate in trout because
365 Hcar1 is not activated by lactate and may therefore have a different function than in
366 mammals. Mammalian HCAR1 is regulated via an autocrine feedback loop (3) whereby
367 lactate increases the expression of the receptor (7, 51). In trout, lactate has no

368 stimulating effect on *hcar1* expression in any tissue, and even suppresses it by 78% in
369 red muscle (Fig. 5). Rainbow trout always maintain very high rates of lipolysis and
370 triacylglycerol/fatty acid substrate cycling under a wide range of physiological conditions
371 that include rising plasma lactate concentration up to ~10 mM in the resting state (Fig.
372 4), endurance exercise lasting several days (22), and graded exercise up to critical
373 swimming speed (23). In mammals, lactate inhibits lipolysis (5) and similar stresses of
374 endurance and strenuous exercise cause major changes in fat mobilization (45, 52).
375 The lack of Hcar1 signaling observed here may be responsible for the high lipolytic
376 activity and the “lipolytic inertia” commonly observed in fish. Therefore, this study
377 highlights major functional differences between fish and mammals for the role of lactate
378 and Hcar1 signaling in metabolic fuel selection.

379

380 **High lactate reduces hepatic glucose production**

381 Elevated lactate causes a decline in R_a glucose from 16.4 ± 2.0 to 8.9 ± 1.2 μmol
382 $\text{kg}^{-1} \text{min}^{-1}$ (Fig. 2B), and this 46% reduction occurs within one hour of exogenous
383 supply. A similar decrease in resting glucose production was reported in endurance-
384 trained humans (24), but not in sedentary subjects (53) when circulating lactate was
385 clamped at 4 mM. R_a glucose represents the combined contributions of
386 gluconeogenesis and glycogenolysis, but does not provide information on the relative
387 importance of each pathway. However, tissue measurements show that a reduction in
388 gluconeogenic flux is most likely responsible for the observed decrease in total glucose
389 production because lactate infusion reduces both *pck1* transcript abundance (-60%; Fig.
390 6) and Pck1 protein levels (-24%; Fig. 7). In mammals, PCK1 is the most significant

391 regulating step of gluconeogenesis. It catalyzes the conversion of oxaloacetate to
392 phosphoenolpyruvate that serves as a substrate for downstream gluconeogenic
393 enzymes, among them fructose 1,6-bisphosphatase and glucose-6-phosphatase which
394 ultimately cause the release of free glucose in the circulation. The Pck1 transcript has a
395 very short half-life (~30 min) and there is no evidence that Pck is subject to covalent
396 modifications or allosteric regulation. Its activity depends on transcription and/or *pck1*
397 degradation (54), and, therefore, the observed inhibition of trout R_a glucose is caused,
398 at least in part, by a decrease in gluconeogenesis mediated by the downregulation of
399 Pck1.

400

401 The role of Pck2, the mitochondrial isozyme, has recently been the subject of
402 renewed interest, especially in the context of substrate preference for gluconeogenesis
403 (54-56). Because Pck2 silencing reduces the ability of mammals to use lactate as a
404 gluconeogenic substrate (56) and hypoxia induces the expression of *pck2b* in rainbow
405 trout alevins (55), we have quantified the hepatic expression of *pck2* transcripts, but
406 found no effect of lactate (Fig. 6). The relative expression of cytosolic and mitochondrial
407 Pck appears to be species, tissue, and physiological state-dependent (54, 57). Overall,
408 hepatic Pck activity does not change following lactate infusion (Fig. 8) and previous
409 reports show strong compartmentalization of Pck activity to mitochondria in rainbow
410 trout (57, 58). This suggests that other factors than changes in *pck1* transcript and
411 Pck1 protein abundance are involved in reducing R_a glucose in trout. Therefore, further
412 work is needed to establish the exact localization of Pck isozymes and to characterize
413 the relative influence of each on the regulation of trout gluconeogenesis.

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We have also measured the hepatic expression of several paralogues of glucose-6-phosphatase: the enzyme that liberates glucose from both hepatic *de novo* gluconeogenesis as well as glycogenolysis (58). Lactate has no effect on the expression of *g6pc* paralogues (Fig. 6) and this supports the idea that the decrease in R_a glucose is mainly caused by upstream gluconeogenic suppression of Pck1. It would be useful to know the phosphorylation status and the activities of glycogen synthase and glycogen phosphorylase, as well as the size of liver glycogen stores to assess potential contributions of glycogen metabolism to the reduction in R_a glucose. We cannot discard the possibility that changes in R_a glucose arise from changes in glycogenolysis, but a previous study using labeled lactate shows that, under similar conditions, tracer incorporation into the glucose or glycogen pools is extremely limited (38). Overall, therefore, rainbow trout reduce hepatic glucose production mainly through the inhibition of gluconeogenesis and probably not by modulating glycogen breakdown.

429 **Effects of high lactate on fuel selection**

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Like other animals, trout maintain high rates of lactate production and utilization, even at rest (31, 32, 42). The most important pathway for lactate disposal is oxidation (38) and the end product is a preferred oxidative fuel over other substrates like glucose and fatty acids (14). In trout, lactate oxidation increases strongly with exogenous supply (32) and the contribution of other fuels to energy metabolism decreases accordingly in the liver (38), heart (39) and brain (40) under resting conditions. Here in trout, the high availability of lactate causes the same decrease in circulating glucose utilization as for

437 R_a glucose (Fig. 2B) because glycemia remains constant (Fig. 1A). This decrease in
438 glucose disposal is driven by a reduction in glucose oxidation potentially supported by
439 the lowered expression of *gckb* (Fig. 6), although no change in Gck activity could be
440 detected (Fig. 8). Fatty acid oxidation is another pathway that may be reduced,
441 particularly in the liver where both transport (*cd36* expression; Fig. 6) and β -oxidation
442 (Hoad activity; Fig. 8) are inhibited. Overall, therefore, exogenous supply causes the
443 partial replacement of glucose and fatty acids by lactate as an oxidative fuel for energy
444 metabolism.

445

446 **Red and white muscles metabolize carbohydrates differently**

447 Hyperlactatemia causes opposite responses in *glut4* paralogue expression of red
448 (-42% and -43% for *glut4a* and *glut4b*, respectively) versus white muscle (+166% and
449 +178% for *glut4a* and *glut4b*, respectively) (Fig. 6), suggesting that glucose utilization is
450 reduced in red muscle and increased in white muscle. Tissue glucose and lactate
451 metabolism are constrained by transmembrane transport through Glut (59) and Mct
452 isoforms (41). Substitution of glucose by lactate appears to take place in red muscle,
453 but may not be possible in white muscle that has an extremely low capacity for lactate
454 transport. White muscle expresses Mct isoforms very poorly, especially the Mct1
455 paralogues that specialize in lactate import for oxidation (41). The overall switch from
456 glucose to lactate seen at the whole organism level means that relatively more glucose
457 becomes available for white muscle that, unlike other tissues, may take advantage of
458 this opportunity and increase glucose utilization. This idea is supported by the fact that
459 white muscle is the only tissue showing a rise in Hk activity (Fig. 8) and further

460 emphasizes that important differences in carbohydrate metabolism exist between the
461 two muscle types. Conversely, the expression of both *glut4* paralogues decreased
462 significantly in the red muscle of lactate infused fish. This finding is in line with an
463 observed, but non-significant, decrease in Hk activity in red muscle, suggesting that
464 increasingly available glucose is not used as a fuel by this tissue. Similarly, the
465 observed (non-significant) tendency of hepatic Gck activity to decrease suggests that
466 the liver does not increase its use of glucose either.

467

468 **Perspectives and significance**

469 Higher lactate availability does not reduce lipolytic rate because rainbow trout
470 only show weak Hcar1 signaling that may have a different metabolic function than in
471 mammals. The trout receptor responds differently because its expression remains
472 unaffected by lactate in all tissues except red muscle where it is even strongly
473 suppressed. This lack of Hcar1 activation may contribute to the high lipolytic rate and
474 the “lipolytic inertia” observed quasi permanently in fish. Rainbow trout maintain high R_a
475 glycerol during hyperlactatemia, and elevated rates of triacylglycerol/fatty acid cycling
476 during all types of exercise even when strenuous. Results show that trout R_a glucose is
477 greatly diminished because high lactate decreases gluconeogenic flux by inhibiting
478 Pck1 via reductions in *pck1* transcript abundance and Pck1 protein levels.
479 Hyperlactatemia also reduces overall tissue need for glucose by substituting it for
480 lactate as an oxidative fuel. However, this substitution does not occur in white muscle
481 that activates *glut4* expression and can probably not stimulate lactate import because of
482 its limited capacity for Mct-mediated transmembrane lactate transport. This study

483 highlights clear functional differences of the Hcar1-mediated lactate signaling system
484 between fish and mammals in the regulation of fuel selection for energy metabolism.

485

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494

495 **Author Contributions**

496 GGMT, JAM, LT and JMW: conception and design of research; GGMT, ET, and
497 EF performed experiments; all authors analyzed data; all authors interpreted results of
498 experiments; GGMT and JMW prepared figures; GGMT drafted manuscript; all authors
499 edited and revised manuscript, and all authors approved the final version of the
500 manuscript.

501

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Table 1: Physical characteristics of the animals

	<i>In vivo</i> Glucose kinetics (series 1)	<i>In vivo</i> Glycerol kinetics (series 2)	Tissue measurements (series 3)
Sample size	8	10	8
Body mass (g)	478 ± 25	661 ± 39	217 ± 7
Body length (cm)	34.8 ± 0.6	38.0 ± 0.8	27.6 ± 0.3
Fulton's condition factor (K)	1.13 ± 0.03	1.20 ± 0.03	1.04 ± 0.02
Hematocrit (%)	19.2 ± 1.6	19.6 ± 1.2	24.4 ± 0.8

Three types of experiments were carried out on adult rainbow trout to measure rates of glucose mobilization (series 1), rates of fatty acid mobilization (series 2) or gene expression, protein expression, and enzyme activities (series 3). Values are means ± s.e.m.

Table 2: Initial (baseline) and final values after 3 h of exogenous lactate infusion for plasma metabolite concentrations and fluxes measured in the glucose kinetics experiments (series 1) and glycerol kinetics experiments (series 2).

	Baseline (0 h)	Final (3 h)
<i>Glucose kinetics (series 1)</i>		
Plasma glucose (mM)	7.1 ± 0.5	7.9 ± 0.7
R_a glucose ($\mu\text{mol kg}^{-1} \text{min}^{-1}$)	16.4 ± 2.0	8.9 ± 1.2 ***
Plasma lactate (mM)	0.8 ± 0.2	10.9 ± 0.4 ***
<i>Glycerol kinetics (series 2)</i>		
Plasma glycerol (mM)	0.21 ± 0.03	0.48 ± 0.04 ***
R_a glycerol ($\mu\text{mol kg}^{-1} \text{min}^{-1}$)	8.2 ± 1.4	5.7 ± 0.7
Plasma lactate (mM)	1.1 ± 0.4	10.5 ± 1.4 ***

R_a glucose: rate of glucose mobilization or hepatic glucose production.

R_a glycerol: rate of mobilization of fatty acids (glycerol production or lipolytic rate).

Values are means ± s.e.m (N=8-10). Significant effects of exogenous lactate infusion are indicated by *** (P<0.001).

Table 3: Initial and final values with statistical analyses for plasma glucose, glycerol and lactate concentrations measured in fish used for tissue measurements of gene expression, protein expression and enzyme activities (series 3). The animals were divided into 2 groups that received either an infusion of Cortland's saline (controls) or an infusion of lactate (treatment), both for 3 h.

	Group	Baseline (0 h)	Final (3 h)	Time effect	Treatment effect	Time×treatment interaction
Glucose (mM)	Control	4.52 ± 0.37	4.57 ± 0.64	ns	ns	ns
	Lactate	4.57 ± 0.43	4.12 ± 0.48			
Glycerol (mM)	Control	0.21 ± 0.02	0.24 ± 0.03	**	*	**
	Lactate	0.20 ± 0.02	0.51 ± 0.09			
Lactate (mM)	Control	0.67 ± 0.05	0.67 ± 0.06	****	****	****
	Lactate	0.64 ± 0.04	14.9 ± 1.16			

Values are means ± s.e.m.

ns (not significant) $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$.

510 **Figure captions**

511 **Fig. 1.** Circulating glucose (A) and lactate concentrations (B) of rainbow trout while
512 hepatic glucose production was quantified during exogenous lactate infusion that
513 started at time 0 (series 1). Values are means \pm s.e.m. (N=8). *** Indicates significant
514 differences from mean resting values (P<0.001).

515
516 **Fig. 2.** Effects of exogenous lactate on glucose specific activity (A) and hepatic glucose
517 production (B) in rainbow trout (series 1). Values are means \pm s.e.m. (N=7-8).
518 Significant differences from mean baseline values are indicated by * (P<0.05), **
519 (P<0.01) and *** (P<0.001).

520
521 **Fig. 3.** Circulating glycerol (A) and lactate concentrations (B) of rainbow trout while
522 lipolytic rate (= rate of appearance of glycerol) was quantified during exogenous lactate
523 infusion that starts at time 0 (series 2). Values are means \pm s.e.m. (N=10). Significant
524 differences from mean baseline values are indicated by ** (P<0.01) and *** (P<0.001).

525
526 **Fig. 4.** Effects of exogenous lactate on glycerol specific activity (A) and rate of
527 production of glycerol or lipolytic rate (B) in rainbow trout (series 2). Values are means \pm
528 s.e.m. (N=10).

529
530 **Fig. 5.** Effects of exogenous lactate on the relative mRNA expression of the
531 hydroxycarboxylic acid receptor 1 (*hcar1*) in rainbow trout tissues. Values are means \pm
532 s.e.m. (N=6-8). Significant effect of lactate is indicated by ** (P<0.01). Relative mRNA
533 abundance was normalized using the NORMA-Gene procedure (35) and fold changes
534 were calculated relative to the control group.

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539 **Fig. 6.** Effects of exogenous lactate on the relative mRNA expression of genes
540 associated with carbohydrate (left panels) and lipid metabolism (right panels) in rainbow
541 trout tissues. Glucose transporters 4a and 4b (*glut4a* and *glut4b*), phosphoenolpyruvate
542 carboxykinase (*pck1*, *pck2a* and *pck2b*), glucose 6 phosphatase (*g6pca*, *g6pcb1a* and
543 *g6pcb1b*), carnitine palmitoyltransferase 1 (*cpt1*), hydroxyacyl CoA dehydrogenase
544 (*hoad*), fatty acid translocase (*cd36*), and fatty acid synthase (*fasn*). Values are means
545 \pm s.e.m. (N=5-8). Significant effects of lactate are indicated by * (P<0.05), ** (P<0.01)
546 and *** (P<0.001). Relative mRNA abundance was normalized using the NORMA-Gene
547 procedure (35) and fold changes were calculated relative to the control group.

548

549 **Fig. 7.** Effects of exogenous lactate on the protein expression of liver
550 phosphoenolpyruvate carboxykinase (Pck1) and muscle glucose transporter 4 (Glut4) in
551 rainbow trout. Values are means \pm s.e.m. (N=6). Significant effect of lactate is indicated
552 by * (P<0.05).

553

554 **Fig. 8.** Effects of exogenous lactate on the activity of enzymes involved in carbohydrate
555 (left panels) and lipid metabolism (right panels) in rainbow trout tissues.
556 Phosphoenolpyruvate carboxykinase (Pck) hexokinase (Hk), glucokinase (Gck)
557 hydroxyacyl CoA dehydrogenase (Hoad). Values are means \pm s.e.m. (N=7-8).
558 Significant effects of lactate are indicated by * (P<0.05), and ** (P<0.01).

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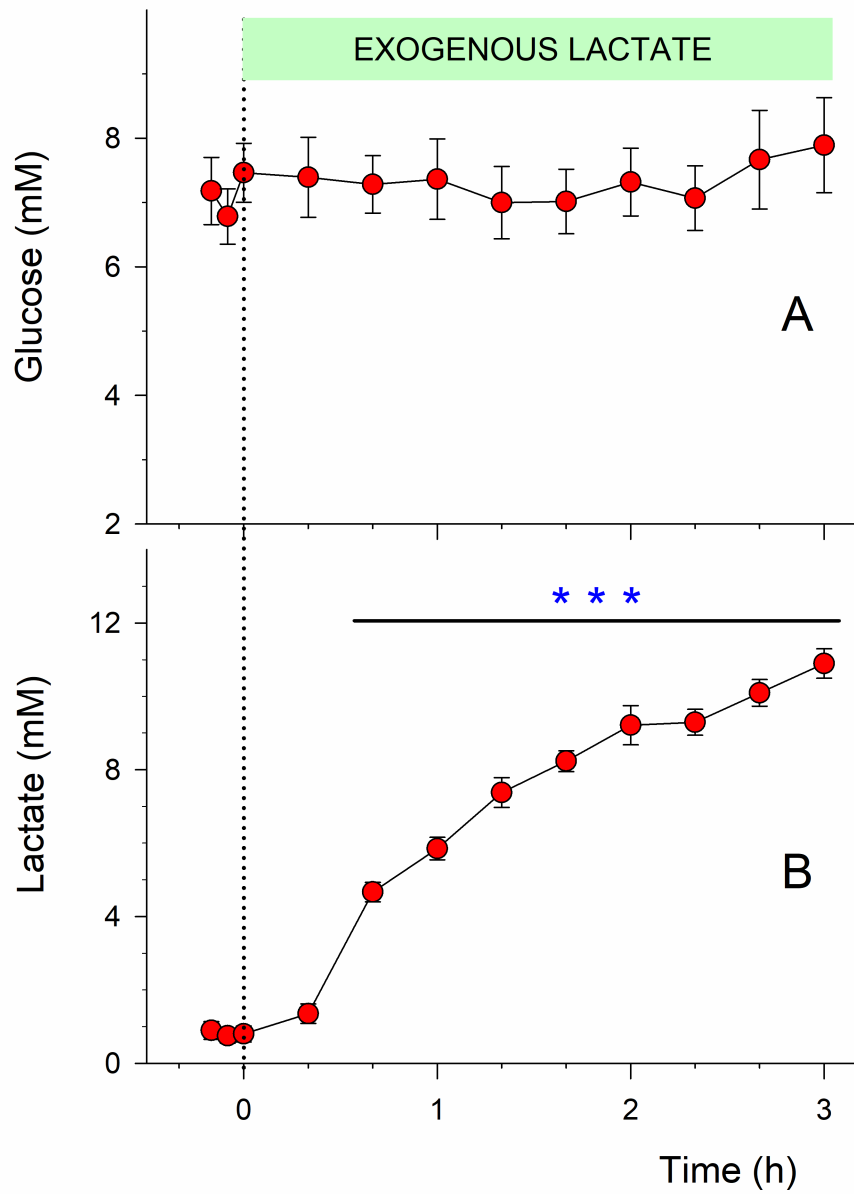
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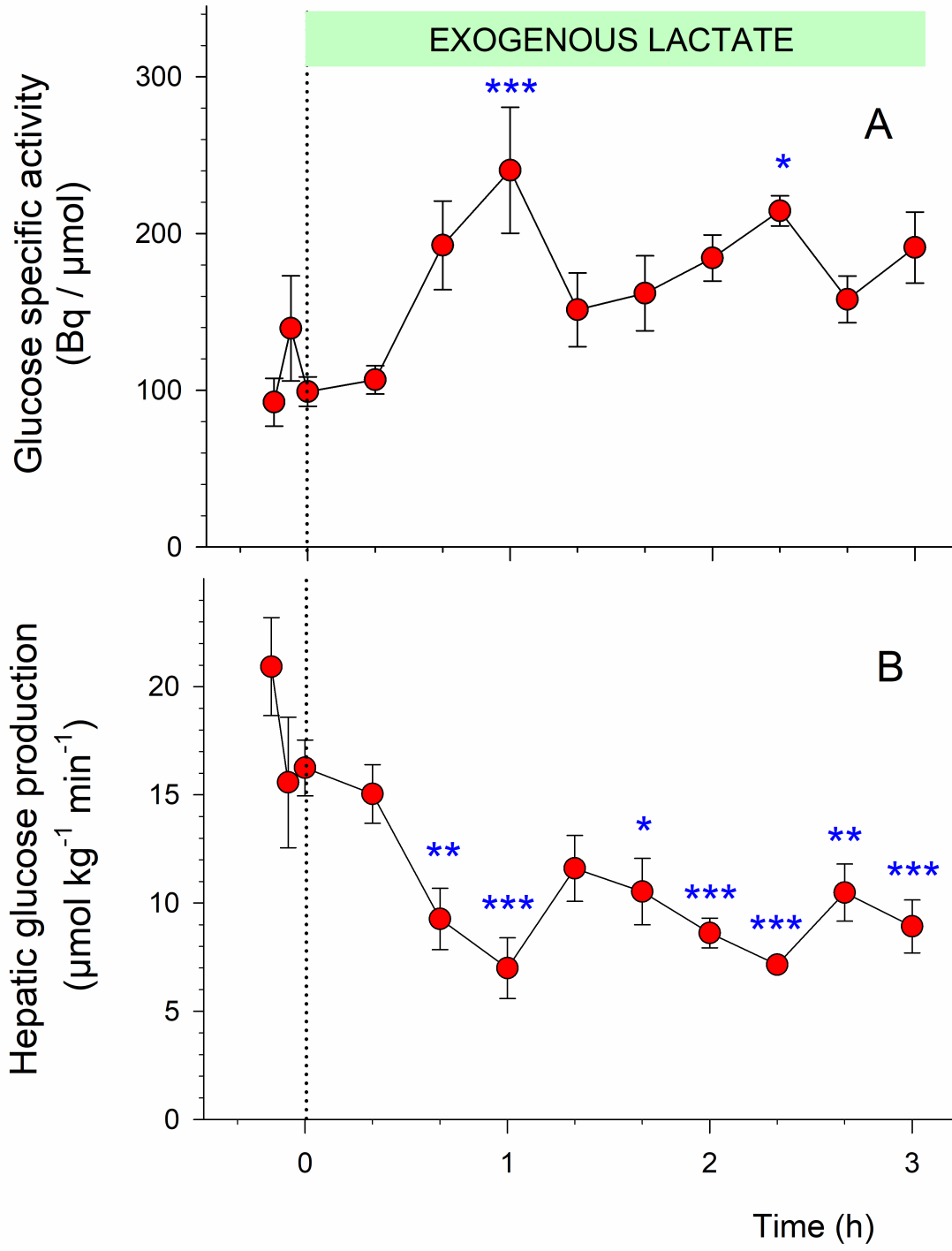
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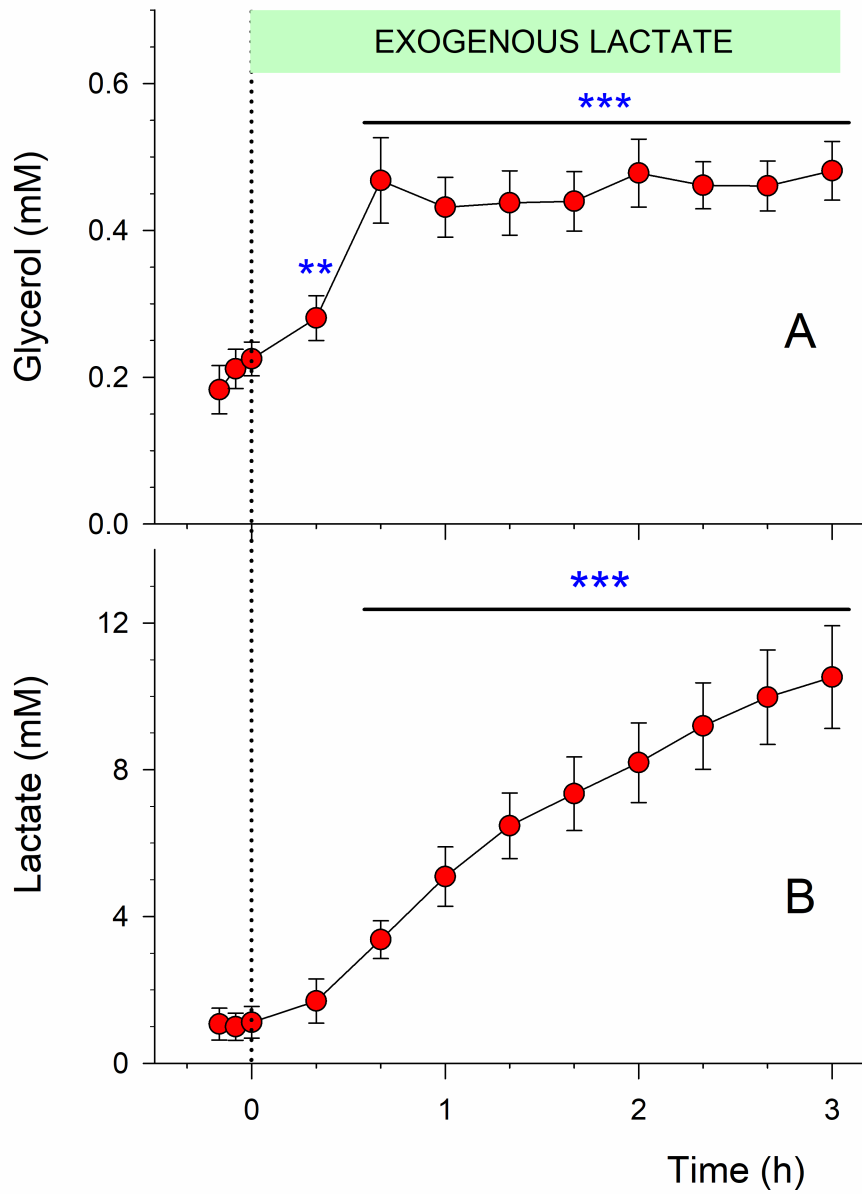
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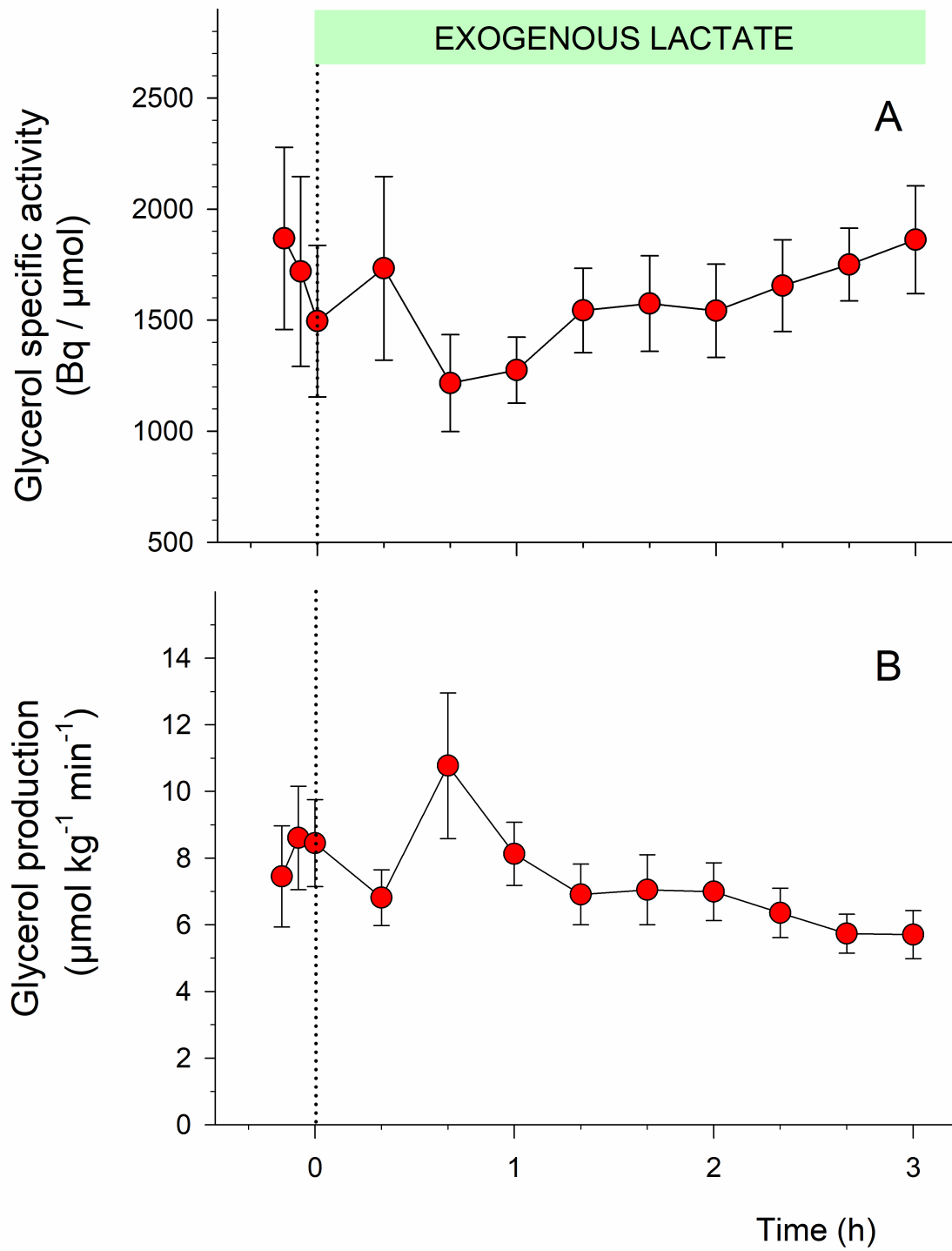
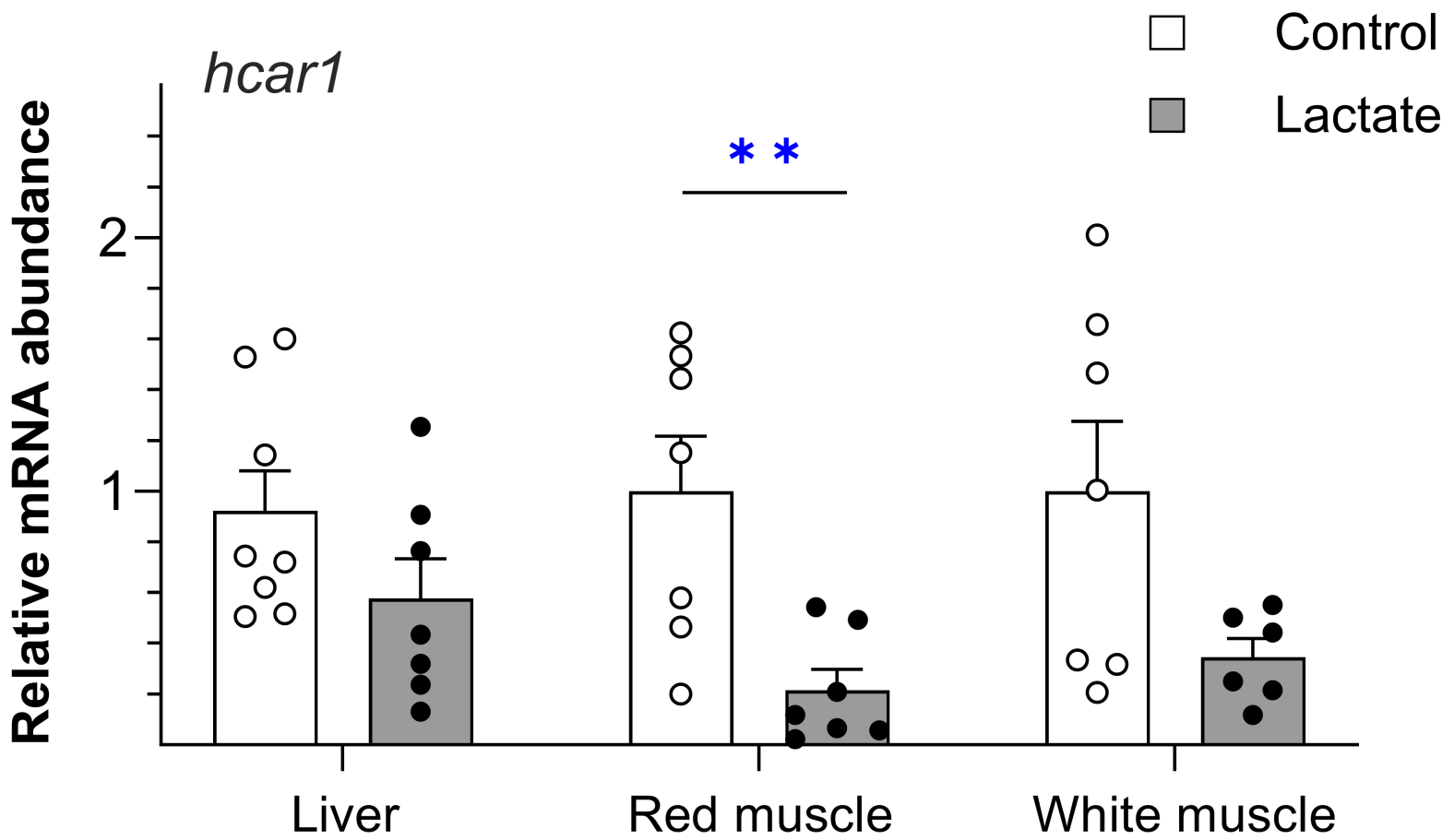


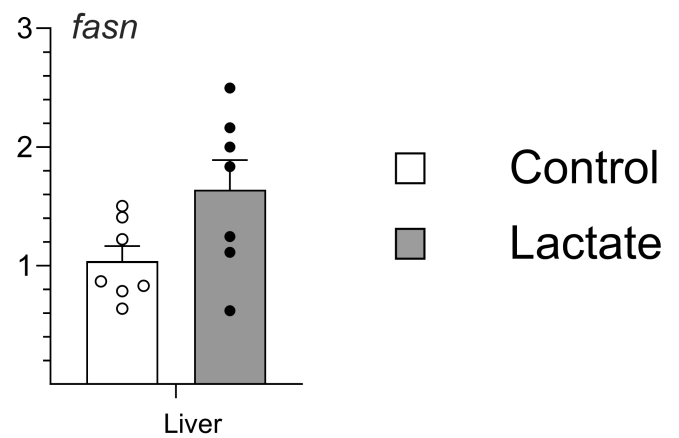
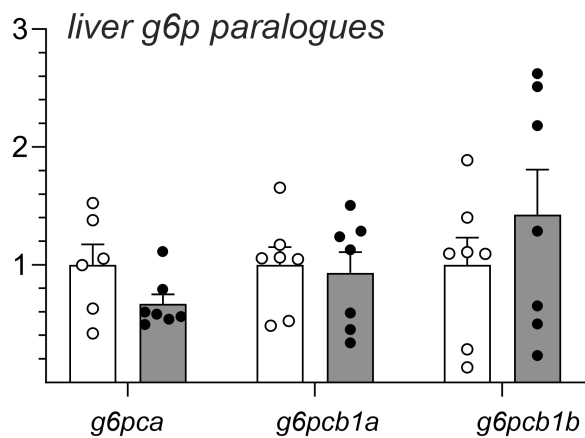
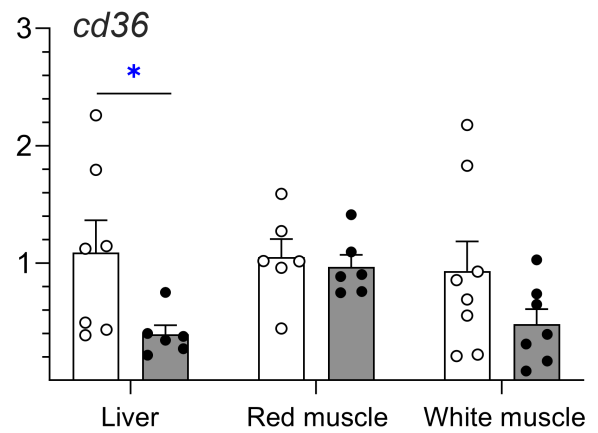
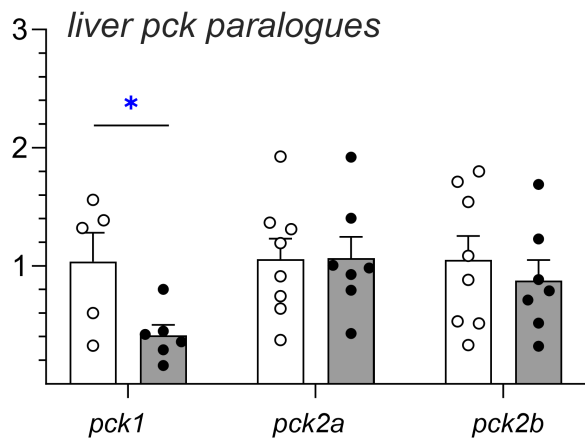
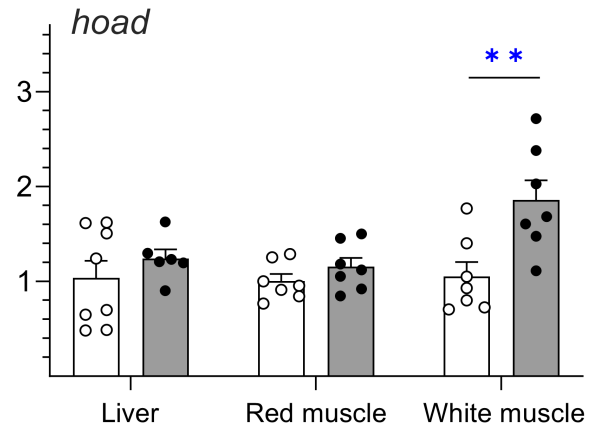
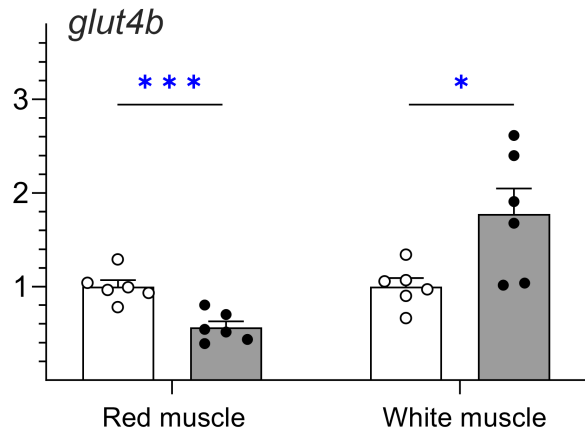
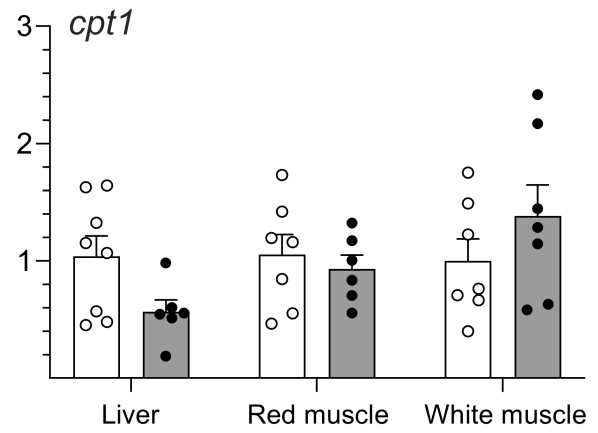
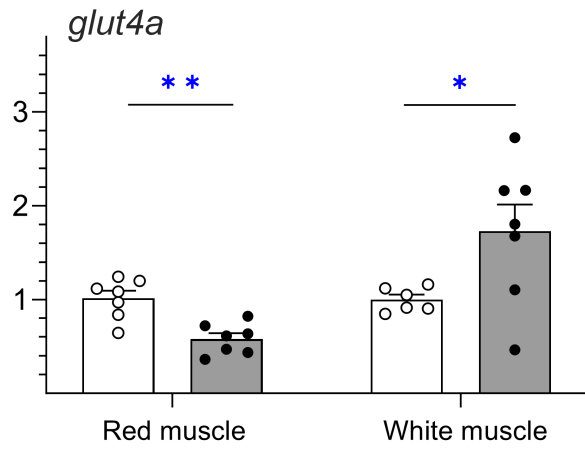
Fig 4



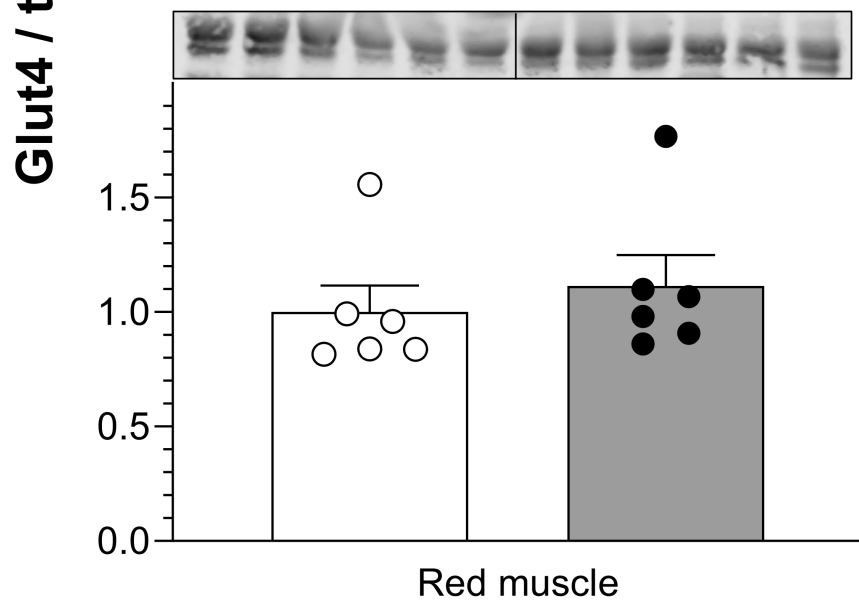
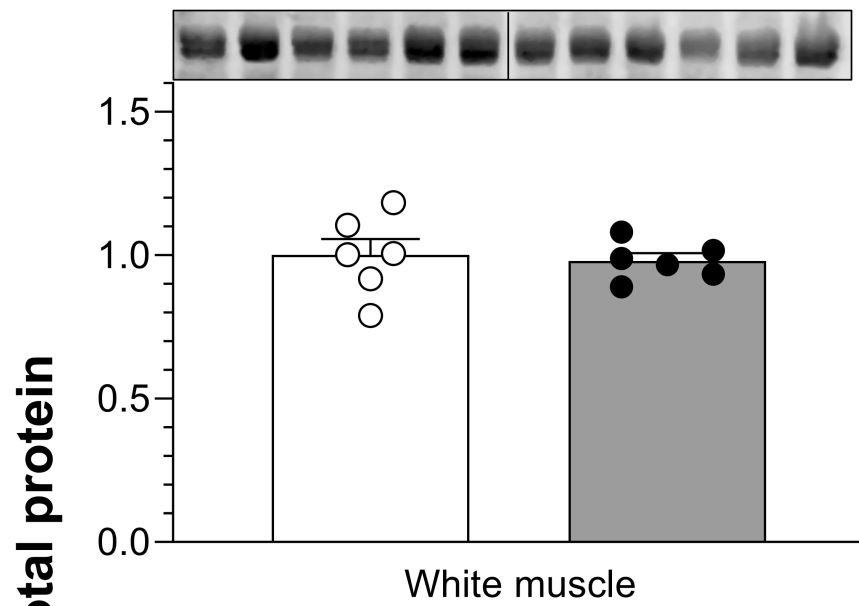
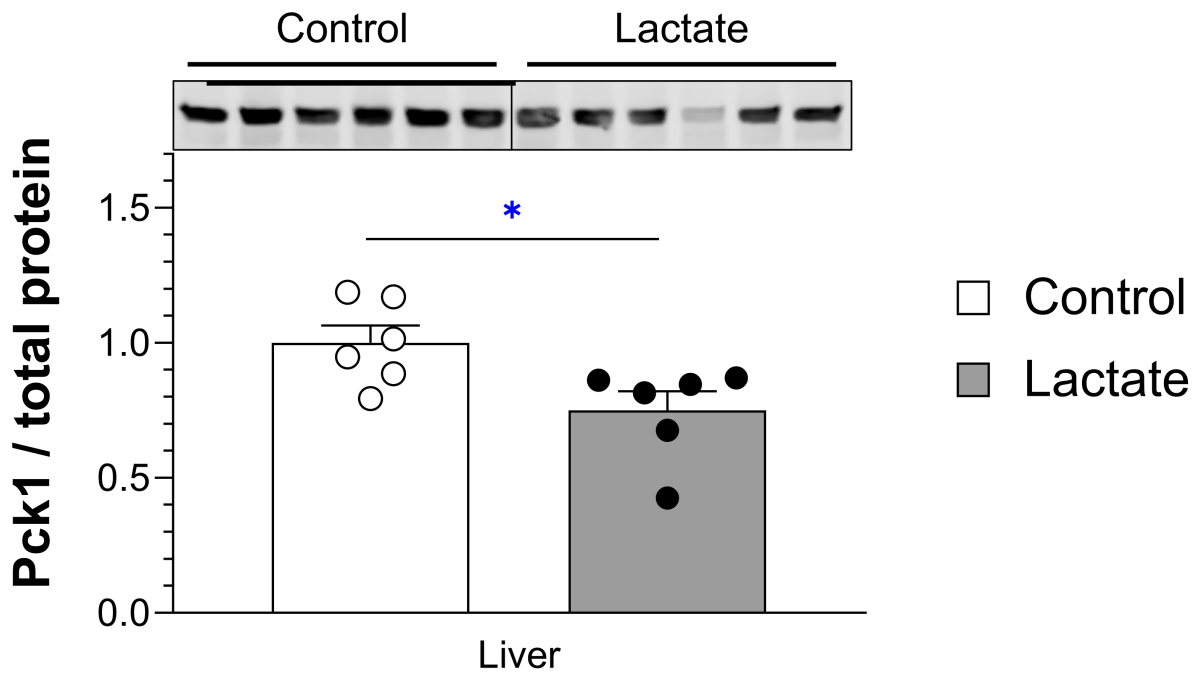
Carbohydrates

Lipids

Relative mRNA abundance

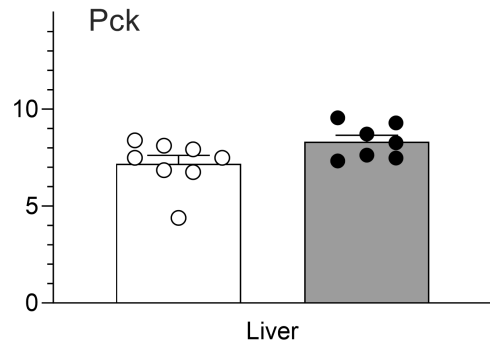


Control
Lactate



Carbohydrates

Lipids



□ Control
■ Lactate

Enzyme activity ($\mu\text{mol min}^{-1} \text{g protein}^{-1}$)

