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1 **Amino acids down-regulate the expression of several autophagy related genes in**
2 **rainbow trout myoblasts**

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15

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17

18 **Running title: Amino acids control the expression of autophagy genes**

19

20 **Abbreviations used:** AA, Amino acids; AAR, Serum deprived medium containing amino
21 acids plus Rapamycin; AMPK, AMP-activated protein kinase; Atg4b autophagy-related 4b;
22 Atg12l, autophagy-related 12-like; Baf A₁, bafilomycin A₁; CM, Complete Medium; EF1 α ,
23 Elongation Factor-1 α ; eIF2 α , eukaryotic initiation factor-2 α ; 4E-BP1, eukaryotic
24 initiation factor 4E binding protein; FoxO, forkhead box-O transcription factor; Gabarapl1,
25 gamma-aminobutyric acid type A (GABA(A)) receptor-associated protein-like 1; GCN2,

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26 **general control nonderepressible 2**; IGF1, insulin-like growth factor-1; LC3B, microtubule-
27 associated light chain 3B; MM, Minimal Medium; PI3K, phosphatidylinositol 3-kinase; **R**,
28 **Serum and amino acids deprived medium containing Rapamycin**; S6, ribosomal protein S6;
29 TOR, target of rapamycin; **TORC1, TOR complex 1**.
30

31 **ABSTRACT**

32

33 Many fish species experience long periods of fasting often associated with seasonal
34 reductions in water temperature and prey availability or spawning migrations. During periods
35 of nutrient restriction, changes in metabolism occur to provide cellular energy via catabolic
36 processes. Muscle is particularly affected by prolonged fasting as proteins of this tissue act as
37 a major energy source. However, the molecular components involved in muscle protein
38 degradation as well as the regulatory networks that control their function are still incompletely
39 defined in fish. The present work aimed to characterize the response of the autophagy-
40 lysosomal degradative pathway to nutrient and serum availability in primary culture of
41 rainbow trout myoblasts. In this aim, 4-day old cells were incubated in a serum and amino
42 acid-rich medium (complete medium), a serum and amino acid-deprived medium (minimal
43 medium) or a minimal medium plus amino acids, and both the transcription-independent
44 short-term response and the transcription-dependent long-term response of the autophagy-
45 lysosomal degradative pathway were analyzed. We report that serum and amino acids
46 withdrawal is accompanied by a rapid increase of autophagosome formation but also by a
47 slower induction of the expression of several autophagy-related genes (*LC3B*, *gabarp11*,
48 *atg4b*). We also show that this later response is controlled by amino acid (AA) availability
49 and that both TOR-dependent and TOR-independent pathways are involved in this effect.
50 Together these results suggest an important role for AA released by muscle proteolysis during
51 the fasting period in regulating the subtle balance between energetic furniture and muscle
52 protein sparing.

53

54 **Key words:** Fish, myoblast, Amino acids, autophagosome, gene expression, signaling

55

56 INTRODUCTION

57

58 Many fish species migrate long distances to the spawning grounds. Extreme examples
59 are the temperate eel species (2000 – 6000 km)^{1,2} and salmonid species (up to 3000 km)^{3,4} In
60 the course of the migration, fish enter into a long term starving phase and degrade almost all
61 their lipid and about half their white muscle mass, thereby reducing their ability to burst
62 swim.⁵ Not all degradation products are funneled into oxidative pathways, but substantial
63 amounts provide the building blocks for the developing oocytes, some of them mediated
64 through hepatic vitellogenin biosynthesis. Fuel usage changes drastically, with protein
65 increasingly important as migration progresses, and likely the sole energy source for the last
66 half of the migration and the energetically costly spawning process itself. Carbohydrates are
67 used intermittently, and rebuilt from amino acids for spawning.^{5,6} Overall, these data
68 highlight the importance of a tight control of muscle protein degradation in these species not
69 only to fuel migration but also to fuel gonadal maturation and spawning. Individual variations
70 in this control could therefore lead to differences in reproductive success or life span.
71 However, the molecular components involved in muscle protein degradation as well as the
72 regulatory networks that control their function are still incompletely defined.

73

74 Protein degradation in the skeletal muscle of vertebrates is thought to be essentially
75 mediated by the activity of two highly conserved pathways, the ubiquitin-proteasomal
76 pathway and the autophagic/lysosomal pathway. While ubiquitin-proteasome-dependent
77 degradation has been investigated in depth and its contribution to muscle loss has been
78 already well documented in mammals,⁷⁻¹¹ the role of autophagy has only recently begun to be
79 investigated and new evidence has demonstrated that this pathway plays a critical role in
80 controlling muscle mass.^{12,13} In fish, the ubiquitin-proteasome system has been intensively

81 investigated in recent years and significant progresses have been made in understanding the
82 mechanisms that control this system.¹⁴⁻²⁰ In contrast, limited studies have focused on the
83 autophagic/lysosomal pathway which is recognized to be up-regulated during muscle
84 wasting,^{19, 21, 22} and then the regulatory networks that control this pathway remains little
85 understood in fish.

86

87 The autophagic/lysosomal pathway is an evolutionary conserved process that is
88 responsible for the degradation of long-lived proteins and for the elimination of redundant or
89 damaged cellular structures, e.g., mitochondria. During autophagy, portions of cytoplasm and
90 cell organelles are sequestered into vacuoles, called autophagosomes, with subsequent fusion
91 of autophagosomes with lysosomes and digestion of the content of the vacuoles by lysosomal
92 hydrolases.²³ In mammalian models, two main mechanisms have been identified for the
93 induction of the autophagosome formation in response to nutrient and growth factor
94 starvation. The first is a rapid and transient transcription-independent induction mediated by
95 mammalian target of rapamycin (mTOR) and AMP-activated protein kinase (AMPK)
96 complexes, the major nutrient and energy sensors in eukaryotic cells, respectively.²⁴ The
97 second regulatory pathway is a slower mechanism requiring gene expression and thought to
98 allow the persistent generation of autophagosomes during prolonged fasting.^{13, 25} Recent
99 studies in mammals have shown that the transcription factors Forkhead box-O (FoxO3a and
100 FoxO1) are necessary and sufficient to activate autophagy by controlling the expression of
101 some critical autophagy-related genes (*LC3b* and *Gabarapl1* (the mammalian Atg8-related
102 proteins), *Vps34*, *Ulk2*, *atg12*).^{26, 27} FoxO proteins are also known as downstream targets of
103 the insulin-like growth factor-1 (IGF1) / insulin – phosphatidylinositol 3-kinase (PI3K) –
104 protein kinase Akt signalling and are thus considered to be major mediators of growth factor
105 effect on autophagy-related gene expression.^{26, 27}

106

107 Recently, we have shown that fasting rainbow trout (*Oncorhynchus mykiss*) for 14
108 days strongly induces the expression of several major genes involved in autophagy
109 (*gabara11*, *LC3b*, *atg4b*, *atg12l*) in white muscle.²² These results are consistent with the
110 strong activation of the lysosomal degradative system under catabolic situations, as observed
111 by the induction of the expression and activity of cathepsins **L, B, D and S** during salmonid
112 migration, maturation and starvation,^{19, 21} and provide new evidence for the importance of the
113 autophagic/lysosomal pathway for muscle atrophy in rainbow trout. However, **although recent**
114 **studies have shown the well conservation of the above mentioned signalling proteins (Akt,**
115 **TOR, AMPK, FoxO) in rainbow trout,**^{15, 22, 28-32} little is known about the mechanisms
116 involved in the induction of the autophagosome formation in response to nutrient and growth
117 factor starvation in fish. To our knowledge, the only available data suggest that the regulatory
118 networks that control the expression of autophagy-related genes have probably diverged
119 throughout evolution.^{22, 33} Indeed, in contrast to what is described in mammalian models, our
120 recent results failed to demonstrate any insulin or IGF1 effect on the expression of autophagy-
121 related genes.^{22, 27} Therefore, the aim of the present study was to characterize both the
122 transcription-independent short-term response and the transcription-dependent long-term
123 response of the autophagy-lysosomal degradative pathway to nutrient and serum withdrawal
124 in primary culture of rainbow trout muscle cells. A particular attention was paid to the nature
125 of the stimuli that control the latter response, which would be of particular importance in
126 salmonid species that, in the course of their spawning migration, enter into a long term
127 starving phase.

128

129 RESULTS

130

131 Serum and amino acid withdrawal induces autophagosome formation in trout 132 myoblasts.

133 Rapid changes in macroautophagy have long been known to occur in response to changes in
134 nutrient and/or hormonal (Insulin/IGF1) supply.³⁴ To test whether serum and amino acid
135 withdrawal was also accompanied by an increase of autophagosome formation in our cell
136 culture model, we monitored by immunofluorescence the subcellular localisation of the LC3
137 protein in cells incubated in a serum and amino acid-rich (Complete Medium, CM) or -
138 deprived medium (Minimal Medium, MM) for 4 h. As shown in Figure 1A,
139 immunofluorescence of CM incubated cells showed LC3 staining as a weak diffuse
140 cytoplasmic pool. In contrast, MM incubated cells revealed the appearance of punctate LC3
141 staining that represent autophagosomes,³⁵ indicating that serum and amino acid withdrawal
142 induced autophagosome formation in our cell culture model. Furthermore, the addition of
143 bafilomycin A₁, a vacuolar ATPase inhibitor that inhibits autophagosome-lysosome fusion
144 and prevents the degradation of LC3,³⁶ had a weak effect in CM incubated cells but strongly
145 induced the number of punctate LC3 structures in MM incubated cells, further supporting the
146 evidence of a short term induction of autophagosome formation by serum and amino acid
147 withdrawal in our cell culture model.

148 Given that autophagy is a highly dynamic and complex process that is tightly regulated at
149 multiple steps, and in order to strengthen our conclusion, we performed in lysates from CM
150 and MM incubated cells western blots for LC3B, which is cleaved and conjugated to
151 phosphatidylethanolamine during autophagosome formation to generate a faster-migrating
152 form, LC3-II.³⁷ As shown in Figure 1B, the serum and AA withdrawal was associated with an
153 increase of the ratio LC3-II/LC3-I (the non-cleaved form of LC3B) (compare lanes CM and

154 MM) which is a hallmark of autophagy induction. Furthermore, the LC3-II/LC3-I ratio in the
155 presence of bafilomycin A₁ is higher in MM incubated cells (compare lanes CM+Baf A₁ and
156 MM+Baf1A₁), indicating that autophagic flux is increased during serum and AA withdrawal.

157 Finally, in order to better characterize the autophagy flux in our cell culture model, we
158 monitored by western blotting the LC3 lipidation in 4-day old cells cultivated in CM and MM
159 for 1, 2.5, 4, 6, 12 and 24 h. As shown in Figure 1C, the ratio of LC3-II to LC3-I was
160 significantly increased after 2 h in MM ($P<0.05$) and remained significantly elevated until 6 h
161 before declining to the level of the CM incubated cells.

162 Overall, these results demonstrated that serum and amino acid withdrawal was accompanied
163 by a rapid and transient increase of autophagy in our cell culture model, indicating that these
164 serum and AA-deprived cells can serve as a relevant model to characterise the factors
165 involved in the induction of this proteolytic system.

166

167 **Serum and amino acid withdrawal induces the expression of several autophagy related** 168 **genes in trout myoblasts.**

169 To test if a serum and AA withdrawal might also induce the expression of autophagy related
170 genes in our cell culture model, we analysed by qRT-PCR the mRNA levels of several
171 autophagy related genes in CM and MM incubated cells. As shown in Figure 2, among the
172 four genes studied, *atg12l* was the only gene whose expression was not significantly affected
173 by serum and amino acid withdrawal. The mRNA levels of *gabarapl1* and *LC3B* significantly
174 increased after 24 h of serum and AA removal. Those for *atg4b* reached significantly higher
175 levels than those of CM incubated cells as early as 2.5 h after serum and AA removal and
176 remained higher until 24 h. Altogether, these results demonstrate that in addition to the short-
177 term (4 h) effect on the autophagosomal formation demonstrated above, the serum and AA

178 withdrawal allows also a long-term (24 h) induction of the expression of several autophagy
179 related genes in our cell culture model.

180

181 **AA availability controls the expression of several autophagy related genes and the**
182 **activity of several AA and growth factors sensing signalling pathways in trout**
183 **myoblasts.**

184 There is growing evidence that AA play an important role in controlling gene expression.^{28, 29,}

185 ³⁸⁻⁴⁰ In order to test if AA play a role in the above demonstrated induction of autophagy

186 related gene expression in MM incubated cells, we analysed by qRT-PCR the mRNA levels

187 of the autophagy related genes in CM, MM or MM plus AA (AA) incubated cells for 24h (Fig

188 3). As presented above, serum and AA removal induced the expression of *gabap11*, *LC3B*

189 and *atg4b* and had no effect on *atg12l*. The addition of AA pool into the MM medium

190 reduced the levels of transcripts of the first three genes to that observed in CM cells, but had

191 no effect on that of *atg12l*. These results demonstrated for the first time that the expression of

192 autophagy related genes are regulated by the availability of AA in the cells.

193 In order to clarify the mechanisms involved in the observed effect of AA on the autophagy-

194 related gene expression we then monitored the activation of several AA and growth factors

195 sensing signalling pathways in **CM**, MM and MM plus AA incubated cells for 30 min (Fig 4)

196 and 4 h (data not shown). The phosphorylation of the two TOR effectors S6 and the

197 Eukaryotic Initiation Factor 4E Binding Protein (4E-BP1) was strongly induced after AA

198 addition **to reach levels similar (S6) or higher (4E-BP1) that those observed in CM incubated**

199 **cells**. In contrast, the phosphorylation status of eIF2 α , known to play an essential role in the

200 amino acid regulation of a large number of genes, was not affected by the treatments in the

201 times monitored. Finally, we examined the phosphorylation status of FoxO1 and FoxO3a,

202 which are considered to play a key role in the control of autophagy-related gene expression in

203 mammals. As shown in Figure 4, serum and AA removal decreased their phosphorylation
204 (compare CM and MM) but the addition of AA pool into the MM medium had no effect
205 (compare MM and AA). To further exclude FoxO action on the target genes, we then
206 monitored by immunofluorescence the localization of FoxO1 in CM, MM and MM plus AA
207 incubated cells for 4 h. As shown in Figure 4B, immunofluorescence of MM and MM plus
208 AA incubated cells revealed that FoxO1 co-localizes with Hoescht staining, indicating that
209 AA addition do not affect the nuclear localization of the studied protein. In contrast, CM
210 incubated cells showed the loss of nuclear staining for FoxO1, indicating that this treatment
211 prevents nuclear translocation of FoxO1.

212 Overall, these results demonstrated that AA availability controls the expression of several
213 autophagy related genes as well as the activation of the protein TOR in our cell culture model.
214 These results ask the question of the involvement of this protein in the above demonstrated
215 effect of AA on the autophagy-related gene expression.

216

217 **AA control the expression of several autophagy related genes via TOR-dependent and**
218 **-independent mechanisms.**

219 Recent findings demonstrated the involvement of the complex TORC1 in the regulation of the
220 expression of several metabolic- and growth-related genes.⁴¹ In order to determine whether
221 TORC1 mediates the above presented effect of AA addition on the expression of autophagy
222 related genes, MM incubated cells were stimulated with AA in presence or absence of the
223 TOR inhibitor rapamycin for 30 min, 4 h and 24 h. We first checked the specificity of
224 rapamycin treatment in our cell culture model by monitoring by western blot the activation of
225 several TOR-dependent and -independent signalling pathways in cells stimulated for 30 min
226 (Fig 5) and 4 h (data not shown). Whatever the time monitored, the AA mediated induction of
227 phosphorylation of the two TOR effectors S6 and 4EBP1 was strongly abolished in presence

228 of rapamycin. In contrast, the phosphorylation of eIF2 α and FoxO proteins was not affected
229 by rapamycin treatment. Overall, these results demonstrated that rapamycin treatment
230 specifically inhibits the activity of TOR in our cell culture model.

231 We then analysed by qRT-PCR the mRNA levels of autophagy related genes in **CM and** MM
232 incubated cells stimulated with or without AA in presence or absence of rapamycin for 24 h
233 (Fig 6). The expression of *atrogen1* has been previously shown to be downregulated by AA
234 via a TOR-dependent mechanism^{39, 40} and was therefore monitored as control. As expected,
235 *atrogen1* was strongly down regulated (> 2-fold) after 24 hours of stimulation with AA but
236 not with AA plus rapamycin. In contrast, the effect of AA on the expression of *gabapll1* and
237 *LC3B* genes was not abolished in presence of rapamycin, suggesting that AA act on these
238 genes via a TOR-independent way. Finally, the AA mediated down-regulation of the
239 expression of *atg4b* gene was significantly affected by rapamycin treatment supporting an
240 involvement of the TORC1 complex in the observed effect of AA on the expression of this
241 gene. Taken together, these results demonstrate for the first time that AA regulates the
242 expression of several autophagy-related genes via mechanisms involving both TOR-
243 dependent and TOR-independent pathways.

244

245 DISCUSSION

246

247 In mammals, new evidence has demonstrated the important role of the
248 autophagic/lysosomal pathway in regulating muscle mass and identified the transcription
249 factors FoxO as a key factor of the control of this proteolytic system in response to nutrient
250 and growth factor starvation by inducing several autophagy-related genes.^{26, 27} In rainbow
251 trout, our previous findings showed that fasting fish for 14 days or serum depletion of trout
252 myoblasts strongly induces the expression of several autophagy-related genes.²² However, we
253 failed to link the phosphorylation status of FoxOs with the expression of autophagy-related
254 genes, suggesting a moderate role for this transcription factor on the autophagic/lysosomal
255 pathway in this species. The aim of the present study was therefore to clarify the signal
256 transduction pathway involved in the regulation of the expression of autophagy-related genes
257 in rainbow trout and monitor the potential role of amino acids in this control by exploring the
258 regulation of the expression of *gabrap11*, *LC3B*, *atg12l* and *atg4b* genes in culture of
259 rainbow trout myoblasts.

260

261 ***Short- and long-term induction of the autophagy-lysosomal degradative pathway by***
262 ***serum and AA withdrawal in trout myoblasts*** – In mammals, rapid changes in the autophagy-
263 lysosomal degradative pathway have long been known to occur in response to changes in
264 nutrient and/or hormonal (Insulin/IGF1) supply.^{34, 42} In the present study we demonstrated
265 that serum and amino acid withdrawal was also accompanied by a short term increase of
266 autophagosome formation in our cell culture model, as evidenced by the appearance of
267 punctate LC3 structures as well as the increase of LC3 processing (a reliable marker of
268 autophagosome formation⁴³) in MM incubated cells. This rapid induction of autophagosome
269 formation in starved cells could be explained by its critical role in counteracting nutrient

270 deprivation by delivering damaged or long-lived proteins, macromolecules and organelles
271 within the lysosomal degradative machinery to generate nutrients and energy levels
272 compatible with cell survival.^{44, 45}

273 In addition to this rapid transcription-independent induction of autophagosome
274 formation in response to nutrient and growth factor starvation, a slower mechanism requiring
275 gene expression has been described.^{26, 27} Here, we demonstrated that several autophagy-
276 related genes are induced in MM incubated cells well after (24h) the short term (4h) increase
277 of autophagosome formation. These results are consistent with previous findings in mammals
278 showing that during fasting, skeletal muscle shows a persistent generation of autophagosomes
279 that continues for days.²⁵ Such prolonged autophagic induction requires transcriptional control
280 in order to replenish LC3 and Gabarap, critical proteins that are destroyed during
281 autophagosome fusion and lysosome. Altogether, these results demonstrated an evolutionary
282 conservation of the mechanisms involved in the induction of the autophagy-lysosomal system
283 between lower and higher vertebrates and indicated that these serum and AA-deprived trout
284 myoblasts may serve as a relevant model to characterise the factors involved in the
285 transcriptional regulation of autophagy genes.

286

287 ***Role of AA in the control of the expression of autophagy related genes*** – Amino
288 acids are not only substrates for various metabolic pathways, but can also serve as signaling
289 molecules controlling signal transduction pathways.^{46, 47} In addition, there is growing
290 evidence indicating that amino acids play an important role in controlling gene expression
291 through different pathways including at least GCN2 and TORC1.^{28, 29, 38-40, 48, 49} In a previous
292 study, we reported that a pool of AA was able to regulate the expression of several
293 carbohydrate and lipid metabolism related genes in rainbow trout hepatocytes.²⁹ Here, we
294 demonstrated that AA availability controls also the mRNA levels of several autophagy-related

295 genes in trout myocytes. These results indicate that in addition to the short-term inhibitory
296 effect previously described,^{44, 50} AA exert also a long-term effect on the autophagy proteolytic
297 system. Consequently, they suggest that the amount of factors controlling the formation of the
298 autophagosome is tightly tuned to and by environmental conditions. Whether this long-term
299 effect of AA on autophagy-related genes is specific to the studied species, which displays
300 very unusual features of its protein metabolism compared to mammals (i.e. a high dietary
301 amino acid requirement and a continuous growth of the skeletal muscle throughout their life⁵¹,
302 ⁵²) is worth investigating.

303

304 ***Mechanisms underlying the action of amino acids on the expression of autophagy-***
305 ***related genes*** – To our knowledge, no findings have been published to date on the effects of
306 AA on the regulation of the expression of autophagy-related genes and the mechanisms
307 involved remained to be determined. In this aim, we monitored the effect of AA availability
308 on several key proteins of the main AA and growth factors sensing signalling pathways in our
309 cell culture model. Recent studies in mammals have shown that FoxO1 and FoxO3a are
310 necessary and sufficient to activate autophagy by controlling the expression of some critical
311 autophagy-related genes.^{26, 27} We therefore monitored their phosphorylation status in our cell
312 cultures and did not observe any effect of AA supplementation, excluding a possible
313 involvement of these proteins in the observed inhibition of expression of studied genes by
314 AA. Similarly, our results show no effect of AA availability on the phosphorylation status of
315 eIF2 α at the monitored time, also rejecting a possible contribution of the GCN2 pathway in
316 the AA mediated down regulation of autophagy-related genes. In contrast, we demonstrate
317 here that AA addition leads to induce the phosphorylation of the two TOR effectors S6 and
318 4E-BP1, suggesting that the TOR protein is activated in these conditions and making possible
319 its involvement in the observed effect of AA on the expression of autophagy-related genes.

320 Indeed, numerous previous studies revealed the importance of the TOR protein in mediating
321 the effect of AA on the expression of several metabolic and growth-related genes^{28, 29, 39, 40, 49}
322 Here, we demonstrated that the treatment of trout myocytes with the TOR inhibitor rapamycin
323 did not prevent the inhibition of the expression of *gabarapl1* and *LC3B* genes by AA but
324 significantly affected that of *atg4b*. This indicates that AA act on the expression of
325 autophagy-related genes via both TOR-dependent and –independent ways. In this regard, new
326 findings have provided direct evidence on the role of several other factors in mediating the
327 induction of the expression of autophagy related genes in muscle wasting.⁵³ Collectively, our
328 results and those previously published highlight the complexity of mechanisms involved in
329 the control of the expression of autophagy-related genes. Further studies are warranted to
330 follow these different mechanisms as affected by AA availability in trout myocytes.

331

332 ***Conclusions and Physiological significance*** – In conclusion, in the present study we
333 demonstrated the existence of both short- and long-term control of the autophagy-lysosomal
334 degradative system **in rainbow trout myoblasts**. From an evolutionary adaptative point of
335 view, this is of particular importance in salmonids that, in the course of their spawning
336 migration, enter into a long starving phase and degrade about half of their white muscle mass.
337 Such a prolonged degradation of muscle proteins requires transcriptional control in order to
338 replenish critical proteins that are destroyed during autophagosome fusion with lysosome. We
339 also demonstrated that AA availability controls the mRNA levels of several autophagy-related
340 genes in this species. To our knowledge, such effects of AA on the regulation of the
341 expression of autophagy-related genes have not been demonstrated so far and whether this
342 effect is specific to the studied species is worth investigating. However, such a regulation
343 system could originate from an evolutionary adaptation of migrating salmonids that have to
344 deal between energy supply (mainly coming from muscle protein bound AA) and muscle

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345 protein sparing to achieve their spawning migration. Amino acids released by muscle
346 proteolysis would thus regulate themselves this subtle balance that allows salmonids to finally
347 arrive to spawning grounds. **Studies along these lines either with migrating salmonids or those**
348 **under experimentally induced starvation are necessary to verify our hypotheses.**
349

350 MATERIALS AND METHODS

351

352 *Animals*

353 Juvenile immature rainbow trout (~ 5 g) were maintained in our own experimental facilities
354 (INRA, Donzacq, France) at 18°C under natural photoperiods (12 h/12 h). All experiments
355 were carried out in accordance with legislation governing the ethical treatment of animals
356 (Decret N° 2001-464, May 29, 2001), and investigators were certified by the French
357 Government to carry out animal experiments (N° agrément 35-47). All animal work was
358 approved by the Ministère de l'Enseignement Supérieur et de la Recherche (Autorisation
359 N°A352386).

360

361 *Myosatellite cell isolation and culture*

362 Myoblasts were carried out as follows: for each culture, 30 to 60 animals, each weighing
363 approximately 5 g, were killed by a blow to the head and then immersed for 30 s in 70%
364 ethanol to sterilize external surfaces. Cells were isolated, pooled, and cultured following
365 previously described protocols.^{22, 54} Briefly, after removal of the skin, dorsal white muscle was
366 isolated under sterile conditions and collected in Dulbecco's modified Eagle's medium
367 (DMEM) containing 9 mM NaHCO₃, 20 mM HEPES, 15% horse serum, and antibiotic-
368 antimycotic cocktail (100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 g/ml fungizone) at
369 pH 7.4. After mechanical dissociation of the muscle in small pieces, the tissue was
370 enzymatically digested with a 0.2% collagenase solution in DMEM for 1 h at 18°C and gentle
371 shaking. The suspension was centrifuged (300 g for 5 min at 15°C) and the resulting pellet
372 was subjected to two rounds of enzymatic digestion with a 0.1% trypsin solution in DMEM
373 for 20 min at 18°C with gentle agitation. After each round of trypsinization the suspension
374 was centrifuged and the supernatant was diluted in 2 volumes of cold DMEM supplemented

375 with 15% horse serum and the same antibiotic-antimycotic cocktail mentioned above. After
376 two washes with DMEM, the cellular suspension was filtered through 100- and 40- μ m nylon
377 filters. All experiments were conducted three times with cells seeded at a density of 1.5 to 2 x
378 10^6 per well, in six-well plastic plates (Nunc, 140675). Plates and coverslips were previously
379 treated with poly-L-lysine and laminin to facilitate satellite cell adhesion. Cells were incubated
380 at 18°C, the optimal temperature for culture of trout origin, with a complete medium (CM)
381 containing Earle's Balanced Salt (EBSS) culture medium (Sigma, E7510) supplemented with
382 10% fetal bovine serum (Sigma, F7524), MEM vitamins solution (Invitrogen, 11120-037),
383 MEM essential amino acid mixture (Invitrogen, 11130-036) and MEM non-essential amino
384 acid mixture (Invitrogen, 11140-035) and antibiotic-antimycotic cocktail under an air
385 atmosphere. The medium was renewed every 2 days and observations of morphology were
386 regularly made to control the state of the cells.

387

388 *Treatment conditions*

389 In the first experiment, 4-day old cells (myoblasts, as verified by visual microscopy) were
390 incubated in a serum and amino acid-rich (Complete Medium) or -deprived medium (Minimal
391 Medium) with or without bafilomycin A₁ (a vacuolar ATPase inhibitor that inhibits
392 autophagosome-lysosome fusion and prevents the degradation of LC3), **for different times**
393 **specified in figure legends**. In the following experiments, after 4 days of culture, the cells
394 were subjected or not to serum and amino acid deprivation in a minimal medium (MM) for
395 1h. Then, according to each experimental design, cells were incubated in either amino-acids
396 free (MM) or amino acids (AA) containing medium (MEM essential and non-essential AA
397 mixtures) for different times specified in figure legends. In experiments involving rapamycin
398 (specific TOR-inhibitor), the inhibitor was added 30 min prior stimulation with AA. At the
399 end of the stimulation periods, the mediums were removed, the wells were washed with ice-

400 cold PBS and the cells were used for immunofluorescence, gene expression or western blot
401 analysis. Each experiment was performed three times.

402

403 *Immunofluorescence analysis*

404 Cells on glass coverslips were briefly washed two times by PBS and fixed 10 min with
405 methanol. For permeabilization cells were incubated 3 min in 0.1% TritonX100/PBS. After
406 three washes, cells were saturated for 1 h with 3% BSA, 0,1% Tween20 in PBS (PBST). Cells
407 were incubated 3 h with the **primary** antibody anti- LC3B (Cell Signaling Technologies,
408 2775) diluted in blocking buffer. The secondary antibody anti-Rabbit Alexa488 (Invitrogen,
409 A-21441) was diluted in PBST and applied for 1 h. Cells were mounted with Mowiol 4-88
410 (Calbiochem, 475904) containing Hoescht (0.5 µg/ml). Cells were photographed using a
411 Canon digital camera coupled to a Canon 90i microscope.

412

413 *Gene expression analysis*

414 Total RNA samples were extracted from muscles using Trizol reagent (Invitrogen, 15596018)
415 according to the manufacturer's recommendations. One microgram of the resulting total RNA
416 was reverse transcribed into cDNA using the SuperScript III RNaseH- Reverse Transcriptase
417 kit (Invitrogen, 18080085) and oligo dT (Promega, C1101) or Random primers (Promega,
418 C1181) according to the manufacturer's instructions.

419 Target gene expression levels were determined by real-time quantitative RT-PCR (qRT-PCR)
420 with 300 nM of each primer. Primers were designed to overlap an intron if possible (Primer3
421 software) using known sequences in trout nucleotide databases (<http://www.sigenae.org/>) as
422 previously described.²² Real-time RT-PCR was carried out on an iCycler iQ TM real time
423 PCR detection system using iQ TM SYBR® Green Supermix (BIO-RAD, 172-5006).
424 Relative quantification of the target gene transcripts with a chosen reference gene transcript

425 (EF1 α and Rps29) was made following the Pfaffl method with the Relative Expression
426 Software tool (REST C)^{55, 56} In experiments involving rapamycin Rps29 was used as
427 reference transcripts because EF1 α RNA was not stable during the experimental conditions.
428 PCR was performed using 10 μ l of the diluted cDNA mixed with five pmoles of each primer
429 in a final volume of 25 μ l. The PCR protocol was initiated at 95 $^{\circ}$ C for 3 min for initial
430 denaturation of the cDNA and hot-start iTaq TM DNA polymerase activation and continued
431 with a two-step amplification program (20 s at 95 $^{\circ}$ C followed by 30 s at specific primer
432 hybridization temperature) repeated 40 times. Melting curves were systematically monitored
433 (temperature gradient at 0.5 $^{\circ}$ C/10 s from 55 to 94 $^{\circ}$ C) at the end of the last amplification cycle
434 to confirm the specificity of the amplification reaction. The different PCR products were
435 initially checked by sequencing to confirm the nature of the amplicon. Each PCR run included
436 replicate samples (duplicate of reverse transcription and duplicate of PCR amplification) and
437 negative controls (reverse transcriptase-free samples, RNA-free samples).

438

439 *Protein extraction and Western blotting*

440 Protein homogenates from cells were prepared as previously described³¹ Protein
441 concentrations were determined with Bradford reagent method⁵⁷ Cell lysates (10 μ g of
442 protein) were subjected to SDS-PAGE and Western blotting using the appropriate antibody:
443 Anti-phospho FoxO1 (Thr24)/FoxO3a (Thr32) (Cell Signaling Technologies, 9464), **anti-**
444 **FoxO1 (Epitomics, 1874-1)**, anti-anti-phospho S6 (Ser235/Ser236) (Cell Signaling
445 Technologies, 4856), anti-carboxyl terminal S6 (Cell Signaling Technologies, 2217), anti-
446 phospho 4E-BP1 (Thr37/Thr46) (Cell Signaling Technologies, 9459), **anti-4E-BP1 (Cell**
447 **Signaling Technologies, 9452)**, anti-phospho eiF2 α (Ser51) (Cell Signaling Technologies,
448 3398), anti-carboxyl terminal eiF2 α (Cell Signaling Technologies, 9722), anti-LC3B (Cell
449 Signaling Technologies, 2775), **anti- β -actin (Santa-Cruz Biotechnology, sc-47778)**, **anti- β -**

450 Tubulin (Cell Signaling Technologies, 2146). Anti-phospho FoxO1 (Thr24)/FoxO3a (Thr32),
451 anti-FoxO1, anti-phospho S6 (Ser235/Ser236), anti-carboxyl terminal S6, anti-phospho 4E-
452 BP1 (Thr37/Thr46), anti-4E-BP1, anti- β -tubulin and anti- β -actin have been previously
453 validated in trout.^{15, 20, 22, 28, 29, 31-33} For anti-phospho eIF2 α , anti-carboxyl terminal eIF2 α and
454 anti-LC3B the amino acid sequences of the corresponding proteins were monitored in the
455 SIGENAE database⁵⁸ to check for well-conservation of the antigen sequences with the
456 corresponding sequences from mammals, ensuring a good specificity of the used mammalian
457 antibodies in our samples. Preliminary experiments were also performed with murine C2C12
458 cell line as control. After washing, membranes were incubated with an IRDye infrared
459 secondary antibody (LI-COR Inc, 956-32221). Bands were visualized by Infrared
460 Fluorescence using the Odyssey® Imaging System and quantified by Odyssey infrared
461 imaging system software (Application software, version 1.2).

462

463 *Statistical analysis*

464

465 The results of gene expression and protein phosphorylation analyses were expressed as means
466 \pm SEM ($n = 3$ independent observations, each the mean of 6 and 3 replications for gene
467 expression and protein phosphorylation, respectively) and analyzed by one-way ANOVA
468 followed by Student-Newman-Keuls test. For all statistical analyses, the level of significance
469 was set at $P < 0.05$.

470

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474

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477 Low utilisation of dietary carbohydrates in carnivorous rainbow trout: role of amino acids,
478 glucose and insulin interactions?).

479

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629 **FIGURE LEGENDS**

630

631 **Figure 1. Effect of serum and AA withdrawal on autophagosome formation in trout**

632 **myoblasts.** 4-day old cells were incubated in a serum and amino acid-rich (Complete
633 Medium, CM) or -deprived medium (Minimal Medium, MM) with or without bafilomycin A₁

634 (Baf A₁). (A), Immunolocalization of LC3B (green) was performed after 4 h incubation as
635 described in MATERIALS & METHODS. Nuclei were stained with Hoescht (blue). **Graph**

636 **represents the quantification of the number of LC3 puncta per cell. Results are expressed as**
637 **means ± SEM, n=4-5 (mean of 4-5 replications) and were analysed by one-way ANOVA**

638 **followed by Student Newman Keuls test for multiple comparison (P<0.05). Different letters**
639 **represent significantly different values.** (B), After 4 h incubation, cell lysates (10 µg) were

640 analysed by Western blot with an anti-LC3B antibody. β-actin was used as loading control.
641 **The ratio of LC3-II to LC3-I is shown quantitatively as a graph.** (C), Cells were incubated in a

642 **serum and amino acid-rich (Complete Medium, CM) or -deprived medium (Minimal Medium,**
643 **MM) for 0.5, 2, 4, 6, 12, 24 h. Cell lysates (10µg) were analyzed by Western blot with the**

644 **indicated antibodies. A representative blot is shown. Graphs represent the ratio of LC3-II to**
645 **LC3-I. Results are means ± SEM, n=3 (mean of 3 replications) and were analyzed using one**

646 **way ANOVA followed by the Student-Newman-Keuls test for multiple comparisons**
647 **(p<0.05). Different letters represent significantly different values.**

648

649 **Figure 2. Effect of serum and AA withdrawal on autophagy-related gene expression in**

650 **trout myoblasts.** 4-day old cells were incubated in a serum and amino acid-rich (Complete
651 Medium, CM) or -deprived medium (Minimal Medium, MM) for 1, 2.5, 4, 8 or 24 h.

652 *Gabarapl1*, *LC3B*, *atg4b* and *atg12l* mRNA levels were estimated using real-time RT-PCR.

653 Expression values were normalised with elongation factor 1α (EF1α)-expressed transcripts.

654 Results are expressed as means \pm SEM, ($n = 3$ independent experiments, each the mean of 6
655 replications) and were analysed by one-way ANOVA followed by Student Newman Keuls
656 test for multiple comparison ($P < 0.05$). Different letters represent significantly different
657 values.

658

659 **Figure 3. Effect of AA on autophagy-related gene expression in trout myoblasts.** 4-day
660 old cells were incubated in a serum and amino acid-rich (Complete Medium, CM) or -
661 deprived medium (Minimal Medium, MM) for 1 h. The Minimal Medium was then replaced
662 for 24 h with an amino acid-free (MM) or amino acids (AA) containing medium (MEM
663 essential and non-essential AA mixtures). *LC3B*, *gabara11*, *atg12l* and *atg4b* mRNA levels
664 were estimated using real-time RT-PCR. Expression values were normalised with elongation
665 factor 1 α (EF1 α)-expressed transcripts. Results are expressed as means \pm SEM, ($n = 3$
666 independent experiments, each the mean of 6 replications) and were analysed by one-way
667 ANOVA followed by Student Newman Keuls test for multiple comparison ($P < 0.05$).
668 Different letters represent significantly different values.

669

670 **Figure 4. Effect of AA on the phosphorylation of S6, 4E-BP1, eIF2 α , FoxO1 and FoxO3a**
671 **proteins in trout myoblasts.** 4 days cultivated cells were incubated in a serum and AA
672 deprived medium for 1 h. Afterwards, the culture medium was replaced with a serum and AA
673 rich medium (Complete Medium group CM), a serum and AA-free medium (Minimal
674 Medium group MM) or a medium containing amino acids (Amino acids group AA) for 30
675 min before harvest. Cell lysates (10 μ g) were analyzed by Western blot with the indicated
676 antibodies. A representative blot is shown. Graphs represent the ratio between the
677 phosphorylated protein and the total amount of the targeted protein. For FoxO proteins, filled
678 and dashed columns represent FoxO1 and FoxO3a, respectively. Results are means \pm SEM,

679 n=3 (mean of 3 replications) and were analyzed using one way ANOVA followed by the
680 Student-Newman-Keuls test for multiple comparisons ($p<0.05$). Different letters represent
681 significantly different values. (B), Immunolocalization of FoxO1 (red) was performed as
682 described in MATERIALS & METHODS. Nuclei were stained with Hoescht (blue).

683

684 **Figure 5. Effect of AA with or without rapamycin on the phosphorylation of S6, 4E-BP1,**
685 **eIF2 α , FoxO1 and FoxO3a proteins in trout myoblasts.** 4 days cultivated cells were
686 incubated in a serum and AA deprived medium for 30 min and then preincubated for 30 min
687 with or without 100 nM rapamycin. Afterwards, the culture mediums were replaced for 30
688 min with a serum and AA rich medium (Complete Medium group CM), a serum and AA-free
689 medium (Minimal Medium group MM and Rapamycin group R) or a medium containing
690 amino acids (Amino acids group AA and Amino acids plus Rapamycin group AAR). Cell
691 lysates (10 μ g) were analyzed by Western blot with the indicated antibodies. A representative
692 blot is shown. Graphs represent the ratio between the phosphorylated protein and the total
693 amount of the targeted protein. For FoxO proteins, filled and dashed columns represent
694 FoxO1 and FoxO3a, respectively. Results are means \pm SEM, n=3 (mean of 3 replications) and
695 were analyzed using one way ANOVA followed by the Student-Newman-Keuls test for
696 multiple comparisons ($p<0.05$). Different letters represent significantly different values.

697

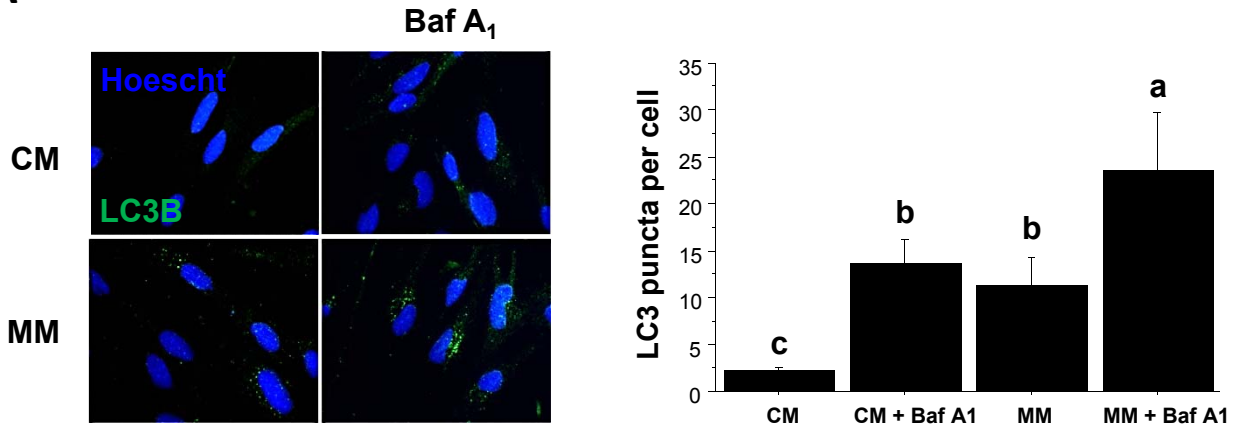
698 **Figure 6. Effect of AA with or without rapamycin on the expression of several**
699 **autophagy-related genes in trout myoblasts.** 4 days cultivated cells were incubated in a
700 serum and AA deprived medium for 30 min and then preincubated for 30 min with or without
701 100 nM rapamycin. Afterwards, the culture mediums were replaced for 24 h with a serum and
702 AA rich medium (Complete Medium group CM), a serum and AA-free medium (Minimal
703 Medium group MM and Rapamycin group R) or a medium containing amino acids (Amino

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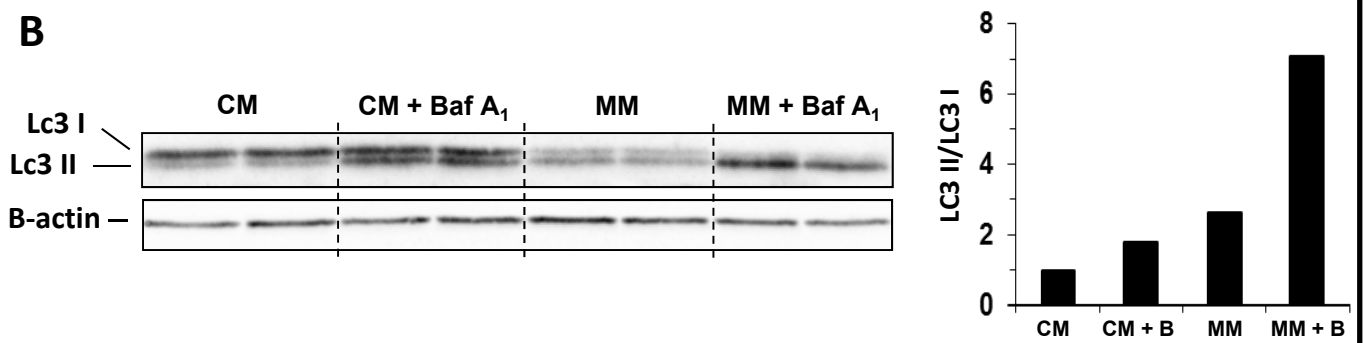
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704 acids group AA and Amino acids plus Rapamycin group AAR). *Atrogin1*, *LC3B*, *gabarapl1*,
705 *atg12l* and *atg4b* mRNA levels were estimated using real-time RT-PCR. For each treatment,
706 six replicate were performed. Expression values were normalised with *Rps29* expressed
707 transcripts. Results are expressed as fold of the CM group and presented as means \pm SEM,
708 n=6 (mean of 6 replications). They were analysed by one-way ANOVA followed by Student
709 Newman Keuls test for multiple comparison ($P < 0.05$). Different letters represent significantly
710 different values.

A



B



C

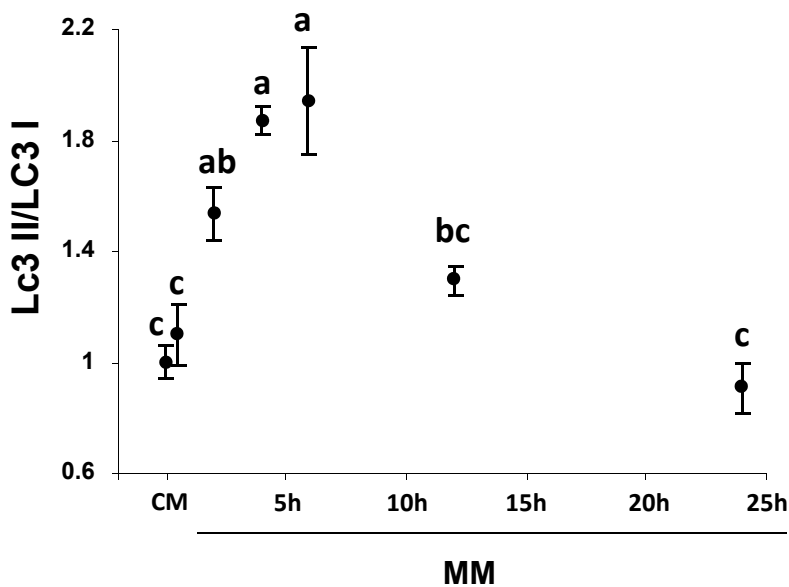
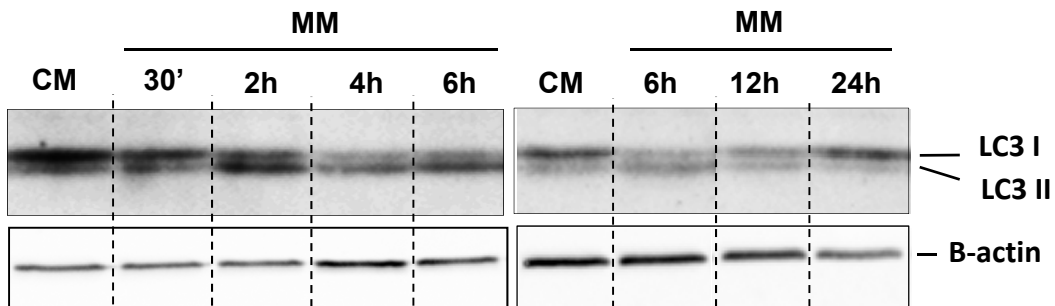


Fig. 1

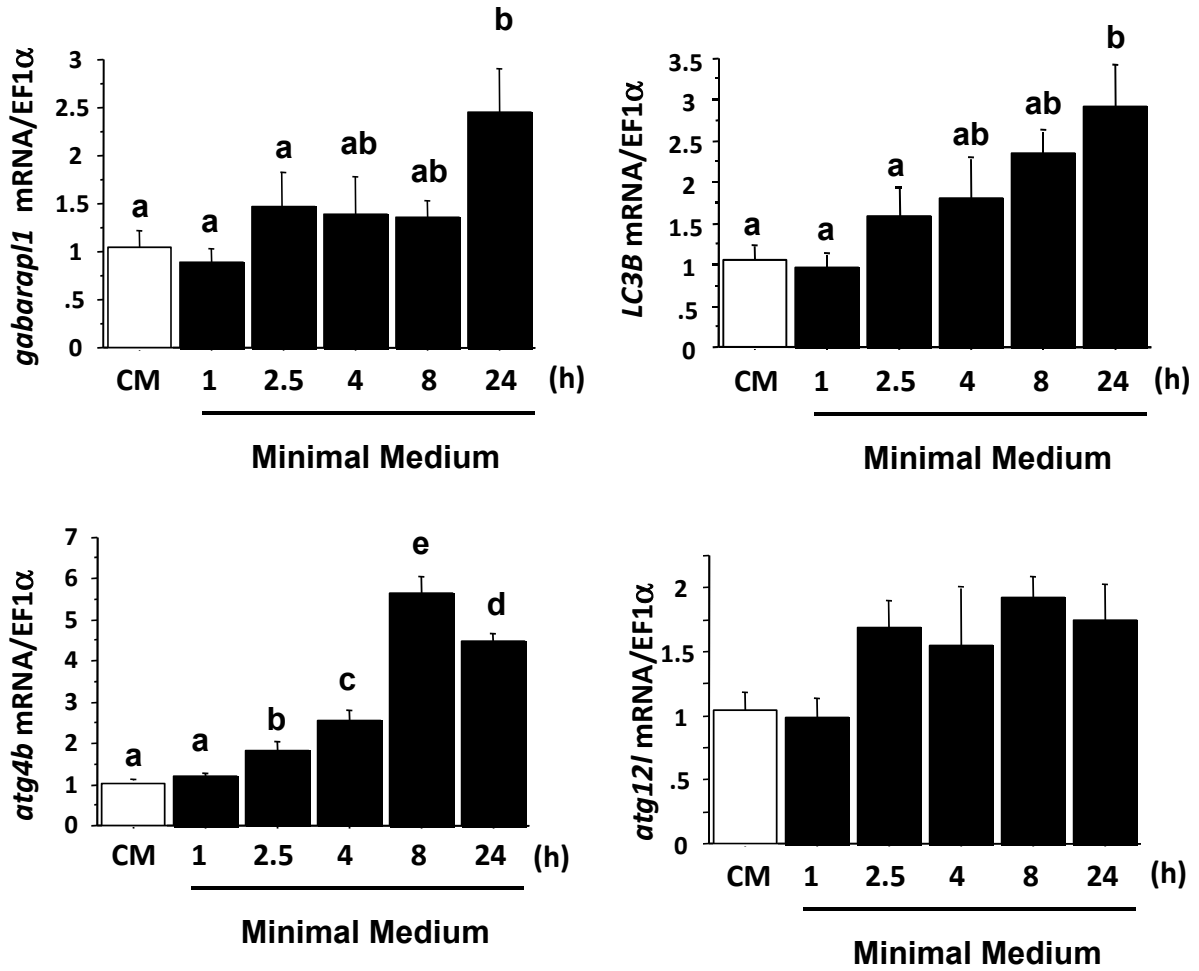


Fig. 2

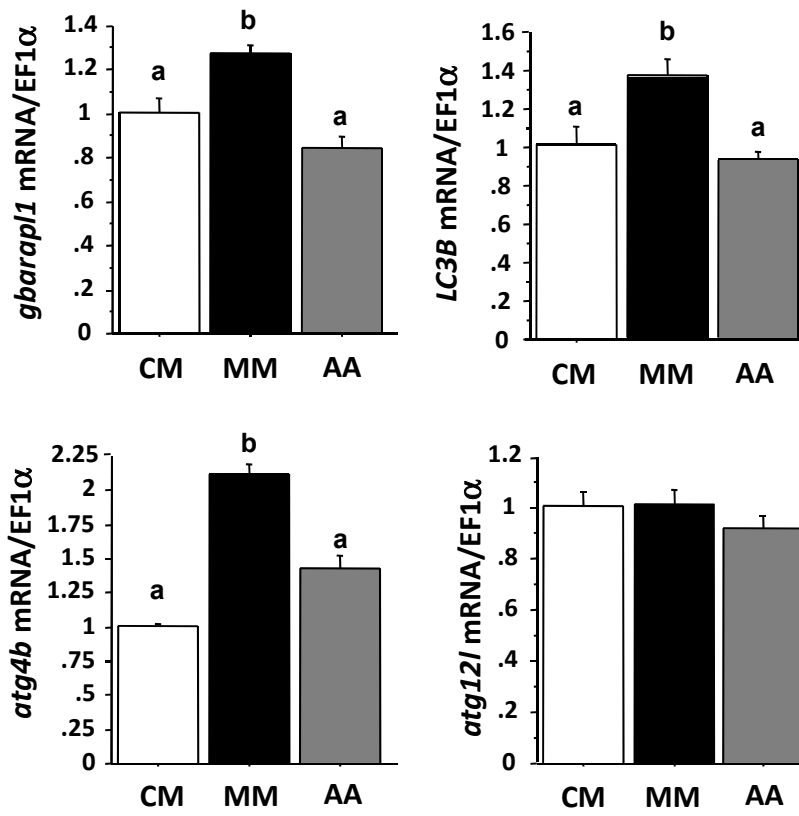


Fig. 3

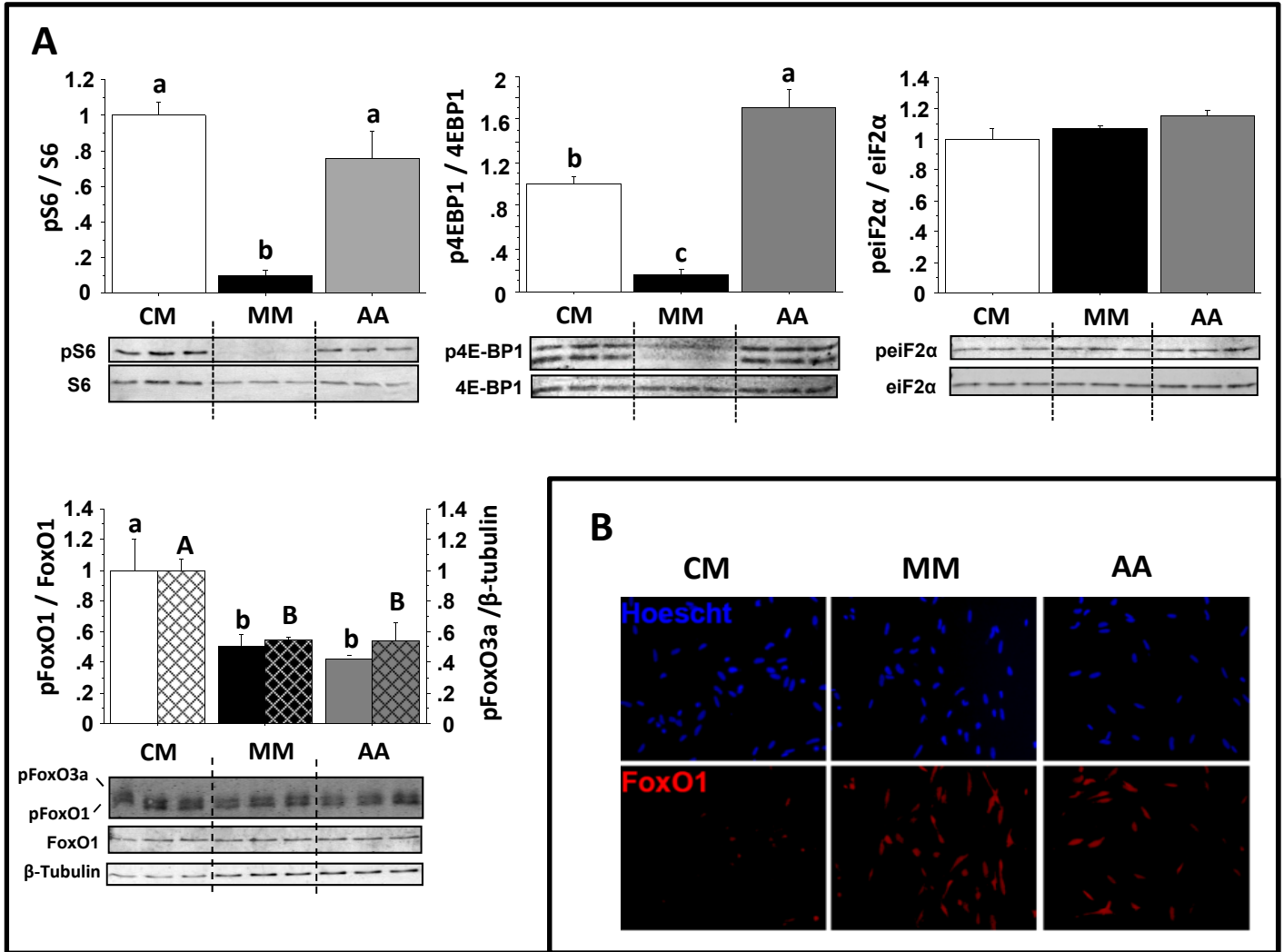


Fig. 4

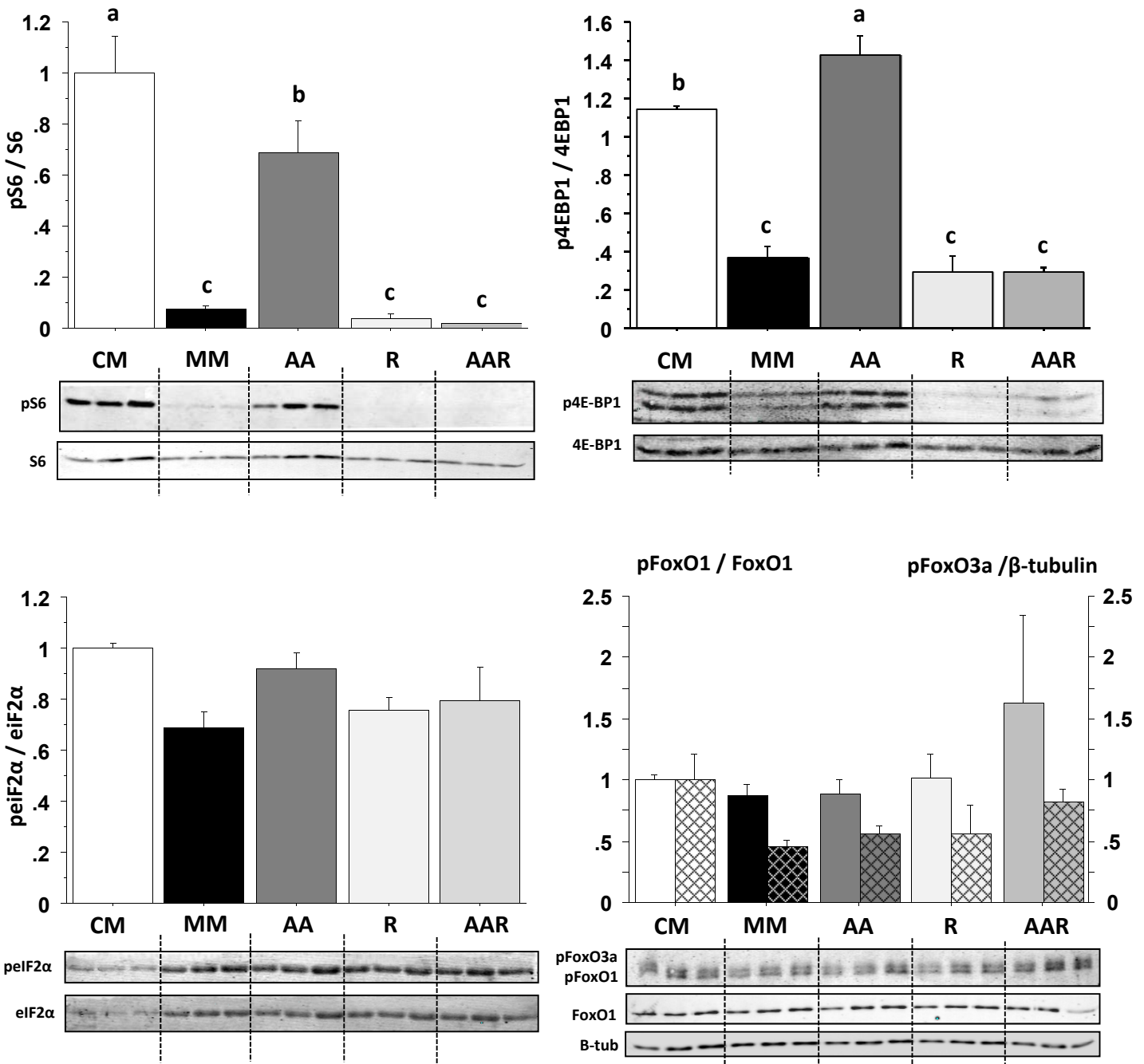


Fig. 5

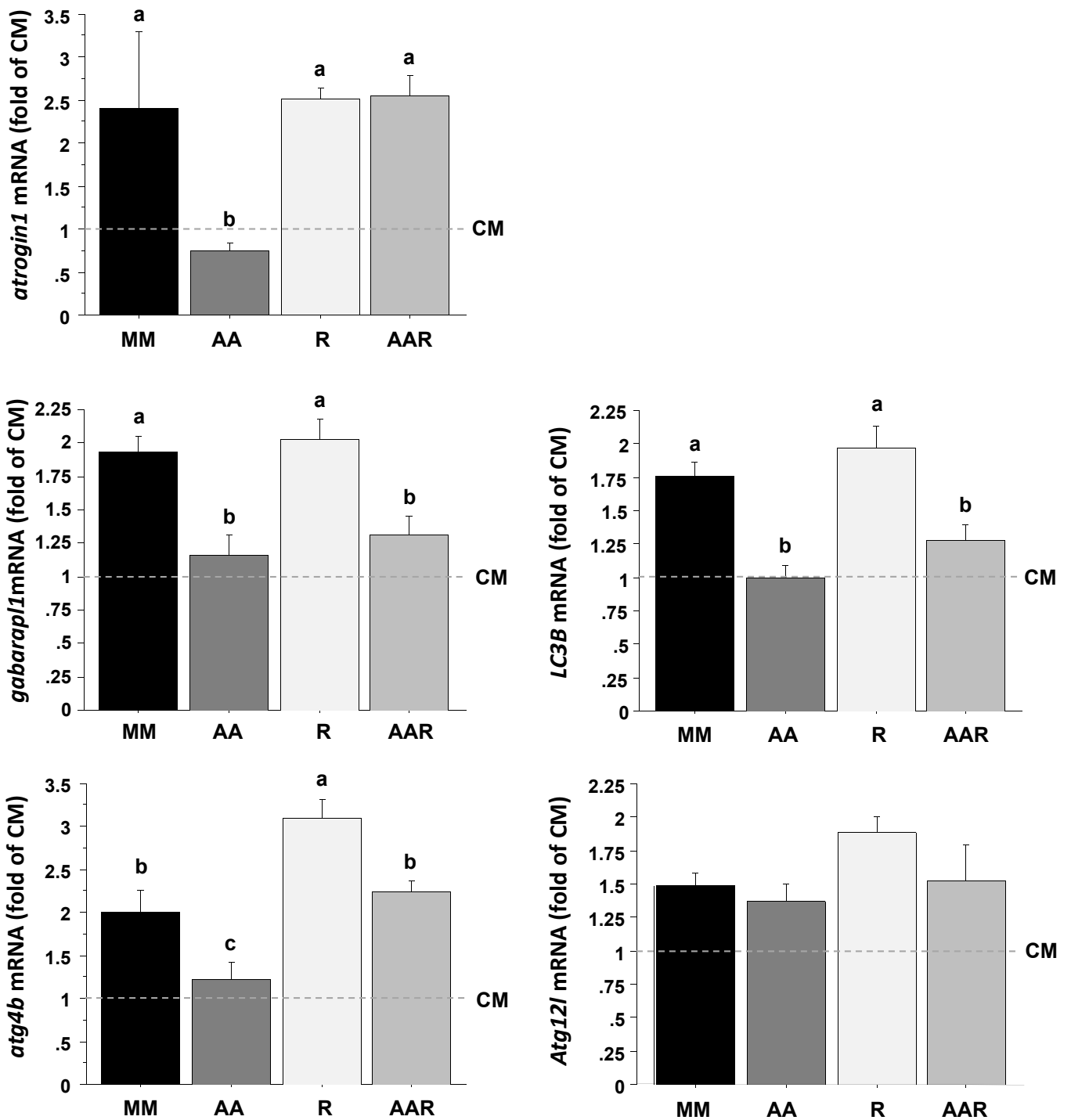


Fig. 6