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## ► To cite this version:

Miquel Perello-Amoros, Cécile Ralliere, Joaquim Gutiérrez, Jean-Charles Gabillard. Myomixer is expressed during embryonic and post-larval hyperplasia, muscle regeneration and differentiation of myoblats in rainbow trout (*Oncorhynchus mykiss*). *Gene*, 2021, 790, pp.145688. 10.1016/j.gene.2021.145688 . hal-03284436

**HAL Id: hal-03284436**

**<https://hal.inrae.fr/hal-03284436v1>**

Submitted on 24 May 2023

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1 **Myomixer is expressed during embryonic and post-larval hyperplasia, muscle regeneration**  
2 **and differentiation of myoblasts in rainbow trout (*Oncorhynchus mykiss*)**

3  
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## 13 **1. Abstract**

14 In contrast to mice or zebrafish, trout exhibits post-larval muscle growth through hypertrophy and  
15 formation of new myofibers (hyperplasia). The muscle fibers are formed by the fusion of  
16 mononucleated cells (myoblasts) regulated by several muscle-specific proteins such as Myomaker  
17 or Myomixer. In this work, we identified a unique gene encoding a Myomixer protein of 77 amino  
18 acids (aa) in the trout genome. Sequence analysis and phylogenetic tree showed moderate  
19 conservation of the overall protein sequence across teleost fish (61% of aa identity between trout  
20 and zebrafish Myomixer sequences). Nevertheless, the functionally essential motif, AxLyCxL is  
21 perfectly conserved in all studied sequences of vertebrates. Using *in situ* hybridization, we observed  
22 that *myomixer* was highly expressed in the embryonic myotome, particularly in the hyperplastic  
23 area. Moreover, *myomixer* remained readily expressed in white muscle of juvenile (1 and 20 g)  
24 although its expression decreased in mature fish. We also showed that *myomixer* is up-regulated  
25 during muscle regeneration and *in vitro* myoblasts differentiation. Together, these data indicate that  
26 *myomixer* expression is consistently associated with the formation of new myofibers during  
27 somitogenesis, post-larval growth and muscle regeneration in trout.

## 28 **2. Introduction**

29 Skeletal muscle consists of myofibers derived from the fusion of progenitor cells called myoblasts.  
30 In mammals, myofibers formation occurs throughout embryogenesis and during muscle  
31 regeneration in adult. Myoblasts proliferate, differentiate into myocytes that fuse to form  
32 multinucleated myotubes, and mature into functional myofibers (Dumont et al., 2015). The fusion  
33 process is highly regulated by numerous key proteins involved in distinct steps, including cell-cell  
34 recognition and adhesion, cytoskeletal reorganization and finally membrane fusion. Among those  
35 proteins, the transmembrane Myomaker protein is expressed only in skeletal muscle and is  
36 absolutely required for myoblast fusion (Millay et al., 2013). Indeed, in *myomaker* knockout mice,  
37 muscle is formed only by mononucleated myoblasts. Similarly, the muscle of *myomaker* knockout  
38 mice fails to regenerate after injury, which shows that *myomaker* is also essential for formation of  
39 new myofibers during muscle regeneration (Millay et al., 2014). Consequently, *myomaker*  
40 expression is upregulated during periods of myofiber formation (embryogenesis and muscle  
41 regeneration), and downregulated thereafter (Millay et al., 2014, 2013). In addition, ectopic  
42 expression of *myomaker* in fibroblasts promotes fusion with C2C12 myoblasts, showing its direct  
43 involvement in the fusion process (Millay et al., 2016, 2014). The mechanism of action of  
44 Myomaker remains poorly understood even though it has been shown that the C-terminal end of the  
45 protein is essential to its function (Millay et al., 2016).

46 Recently, another muscle-specific peptide called Myomixer with fusogenic activity was identified in  
47 mice (Bi et al., 2017; Quinn et al., 2017). The *myomixer* knockout in mice leads to muscle  
48 formation with mononucleated cells, and *in vitro*, the peptide allows the fusion of a fibroblast with a  
49 myoblast. Interestingly, the ectopic expression of *myomixer* and *myomaker* in fibroblasts promotes  
50 fibroblast-fibroblast fusion, suggesting that they should act together (Quinn et al., 2017).  
51 Nevertheless, Leikina et al. (2018) showed that Myomaker and Myomixer are involved in distinct  
52 step of the myoblast fusion process. Whereas Myomaker is essential for hemifusion of the plasma

53 membrane, Myomixer promotes the formation of fusion pores, and the fusogenic activities of these  
54 proteins do not require direct interaction (Leikina et al., 2018).

55 In zebrafish, Myomaker and Myomixer have been characterized and there are also essential for  
56 myoblast fusion (Landemaine et al., 2014; Millay et al., 2016; Shi et al., 2017; Zhang and Roy,  
57 2017). Both proteins are expressed in embryonic myotome and their expression declines before  
58 hatching. Recently, we identified the unique *myomaker* ortholog in rainbow trout and revealed its  
59 unusual sequence. Indeed, the trout Myomaker protein contains 14 minisatellites and two sequence  
60 extensions leading to a protein of 434 aa instead of 221 in zebrafish (Landemaine et al., 2019). *In*  
61 *vitro*, ectopic expression of trout *myomaker* in mouse fibroblasts promotes fusion with C2C12  
62 myoblasts. Given the original structure of trout Myomaker, we wondered whether the sequence and  
63 expression pattern of trout *myomixer* were conserved.

64 In this work, we showed that Myomixer protein sequence was moderately conserved across  
65 evolution and that the unique trout *myomixer* gene was highly expressed in skeletal muscle even  
66 after hatching and was upregulated during muscle regeneration and myotube formation.

### 67 **3. Materials and methods**

#### 68 *3.1. Animals*

69 All the experiments presented in this article were developed under the current legislation that  
70 regulates the ethical handling and care procedures of experimentation animals (décret no. 2001-464,  
71 May 29, 2001) and the muscle regeneration study was approved by the INRAE PEIMA  
72 (Pisciculture Expérimentale INRAE des Monts d'Arrée) Institutional Animal Care and Use  
73 Committee (B29X777-02). The LPGP fish facility was approved by the Ministère de  
74 l'Enseignement Supérieur et de la Recherche (authorization no. C35-238-6).

### 75 *3.2. Muscle regeneration experiment*

76 As described in Landemaine et al., (2019), this experiment was carried out at the INRAE facility  
77 PEIMA (Sizun, Brittany, France). Briefly,  $1530 \pm 279$  g rainbow trout (*O. mykiss*) were anesthetized  
78 with MS-222 (50 mg/l) and using a sterile 1.2-mm needle, the left side of each fish was injured by a  
79 puncture behind to the dorsal fin and above the lateral line. The right side was used as a control for  
80 each fish. White muscle samples from both sides (within the injured region and opposite) were  
81 taken at 0, 1, 2, 4, 8, 16, and 30 days post-injury using a sterile scalpel after proper sacrifice by an  
82 MS-222 overdose. The obtained samples were properly stored in liquid nitrogen until further  
83 processing for gene expression analyses. Along the experiment, no infection was detected and the  
84 survival rate was 100%.

### 85 *3.3. Trout satellite cell culture*

86 Satellite cells from trout white muscle (15-20g body weight) were cultured as previously described  
87 (Froehlich et al., 2013; Gabillard et al., 2010). Briefly, 40 g of tissue were mechanically and  
88 enzymatically (collagenase C9891 and trypsin T4799) digested prior to filtration (100  $\mu$ m and 40  
89  $\mu$ m). The cells were seeded in poly-L-lysine and laminin precoated 6-well treated polystyrene plates  
90 at a density of 80,000 cells/cm<sup>2</sup> and incubated at 18°C. The cells were cultured for 3 days in F10  
91 medium (medium F10, Sigma, N6635) supplemented with 10% fetal bovine serum to stimulate cell  
92 proliferation. Then, the medium was changed to Dulbecco's modified Eagle's medium (Sigma,  
93 D7777) containing 2% fetal bovine serum to stimulate cell differentiation and cultured in this  
94 medium for an additional 3 days. Cells were washed twice with PBS and collected with TRI reagent  
95 solution (Sigma–Aldrich, catalog no. T9424) at 3<sup>rd</sup> (PM) and 4<sup>th</sup> (DM1), 5<sup>th</sup> (DM2) and 6<sup>th</sup> (DM3)  
96 day of culture. Samples were immediately stored at -80°C until further processing for gene  
97 expression analysis.

### 98 3.4. Amplification and sequencing of myomixer sequence

99 The *O. mykiss myomixer* nucleotide sequence containing the full coding region was obtained from  
100 the Trout Genome browser of the French National Sequencing Center (Genoscope). We designed  
101 PCR (Polymerase Chain Reaction) primers in two different exons (forward, 5'-  
102 TTGGCTTTCCTTCCTCTTCAG-3'; and reverse, 5'-TGCGATCTGACTGGTGTCTCC -3'). PCR  
103 reaction was carried out from a rainbow trout muscle cDNA (complementary DNA) and the PCR  
104 product was run in agarose gel, purified and sequenced (Eurofins) and the obtained sequence was  
105 used to design primers for quantitative PCR (qPCR). The validated sequence of *myomixer* cDNA  
106 was deposited in GenBank with the accession number MN230110.

### 107 3.5. Phylogenetic analysis

108 Several Myomixer amino acid sequences obtained from different databases were aligned with the  
109 Mafft server software, version 7 (<https://mafft.cbrc.jp/alignment/server/>) using the default  
110 parameters and the G-INS-i iterative refinement method. The subsequent phylogenetic analysis was  
111 performed using the neighbour-joining method with MEGA X software in a bootstrapped method  
112 (500) to assess the robustness of the tree.

### 113 3.6. RNA extraction, cDNA synthesis, and quantitative PCR analyses

114 For three individual fish (~150g), sample of white muscle, red muscle, skin, heart, brain, adipose  
115 tissue, liver, spleen, pituitary, kidney, ovary, gill, testis and intestine were collected and immediately  
116 stored in liquid nitrogen. Total RNA was extracted from cell cultures or from 100 mg of tissue (or  
117 less in the case of some small organs and tissues for the screening) using TRI reagent (Sigma-  
118 Aldrich, catalog no. T9424) and its concentration was determined using the NanoDrop ND-1000  
119 spectrophotometer. One µg of total RNA was used for reverse transcription (Applied Biosystems  
120 kit, catalog no. 4368813). Trout *myomixer* primers for quantitative PCR (qPCR) (forward, 5'-  
121 AGACTTCCGTGACTCCTACCAG-3'; and reverse, 5'-TGCGATCTGACTGGTGTCTCC-3')  
122 were designed in two exons to avoid genomic DNA amplification. The secondary structure

123 formation in the predicted PCR product were determined with the mFOLD software. Quantitative  
124 PCR analyses were performed with 5 µl of cDNA using SYBR® Green fluorophore (Applied  
125 Biosystems), following the manufacturer's instructions, with a final concentration of 300 nM of  
126 each primer. The PCR program used was as follows: 40 cycles of 95 °C for 3 s and 60 °C for 30 s.  
127 The relative expression of target cDNAs within the sample set was calculated from a serial dilution  
128 (1:4–1:256) (standard curve) of a cDNA pool using StepOne™ software V2.0.2 (Applied Bio-  
129 systems). Subsequently, qPCR data were normalized using *elongation factor-1 alpha (eF1a)* gene  
130 expression as previously detailed.

### 131 3.7. *In situ hybridization*

132 Trout embryos at days 10, 14 and 18 were fixed with 4% paraformaldehyde (PFA 4%) overnight at  
133 4°C and stored in methanol at -20 °C until use. Whole-mount *in situ* hybridization was performed  
134 using RNAscope®, an hybridization amplification-based signal system (Wang et al., 2012)  
135 according to the manufacturer's protocol (Advanced Cell Diagnostics #322360). Embryos were  
136 rehydrated in a decreasing methanol/PBS+0.1% Tween-20 series (75% MetOH/25% PBST; 50%  
137 MetOH/50% PBST; 30% MetOH/70% PBST; 100% PBST) for 10 min each. Once rehydrated,  
138 embryos were transferred to a 2 ml Eppendorf tube. After 15 min treatment of 1x Target Retrieval  
139 (ACD #322000) at 100°C, embryos were treated with Protease Plus solution (ACD #322331), at  
140 40°C for 5-45 min according to the stage. Embryos were incubated with the custom set of probes  
141 designed by ACD Biotechnie (20 pairs of 18-25 nt) overnight at 40°C in sealed Eppendorf tubes.  
142 Detection of specific probe binding sites was performed using RNAscope® 2.5 HD Detection  
143 Reagents-RED kit (ACD #322360), according to the manufacturer. Images of the embryos were  
144 obtained using a Zeiss Stemi 2000-C stereo microscope. For the histological examination of  
145 sections, the samples were embedded in 5% agarose in distilled water. Blocks were sectioned at 35  
146 µm on a Leica vibratome (VT1000S). Images of the sections were obtained using a Nikon 90i  
147 microscope.



148 For the detection of *myomixer* and *myomaker* expression in 1 g and 20 g trout muscle, samples of  
149 white muscle were fixed with 4% paraformaldehyde overnight at 4°C and embedded in paraffin.  
150 Then, cross-sections (7µm) of muscle were cut using a microtome (HM355; Microm Microtech,  
151 Francheville, France) and *in situ* hybridization was performed using RNAscope® 2.5HD detection  
152 reagent RED kit (ACD #322360). Briefly, sections were baked at 60°C for 1 hour, dewaxed and air-  
153 dried. After 10 min in hydrogen peroxyde solution (ACD #322335), sections were treated with 1x  
154 Target Retrieval (ACD #322000) for 15 min at 100°C, following 25 min with Protease Plus solution  
155 (ACD #322331) at 40°C. All steps at 40°C were performed in a ACD HyBEZ II Hybridization  
156 System (#321720). Images of the sections were obtained using a Nikon 90i microscope.  
157 For multiplex RNAscope *in situ* hybridization, trout embryos of 17 dpf (day post fertilisation) were  
158 fixed as previously described in PF4% and embedded in paraffin. Cross-sections (7µm) were then  
159 hybridized using the RNAscope Multiplex Fluorescent Assay v2 (ACDBio #323100) according to  
160 the manufacturer's protocols. This assay allows simultaneous visualization of up to three RNA  
161 targets, with each probe assigned a different channel (C1, C2 or C3). Each channel requires its own  
162 amplification steps. *Pax7* and *myomixer* transcripts were targeted with fluorescent dyes Opal 520  
163 (Akoya Biosciences #FP1487001KT) and Opal 620 (Akoya Biosciences #FP1495001KT)  
164 respectively. Nuclei are counter-stained with DAPI.

### 165 3.8. Statistical analyses

166 The data were analyzed using the nonparametric Kruskal–Wallis rank test followed by the  
167 Wilcoxon-Mann-Whitney test. All analyses were performed using the R statistical package (3.6.3  
168 version).

## 169 **4. Results**

### 170 *4.1. Identification of the trout myomixer gene*

171 We performed a BLAST search in the trout genome (Berthelot et al., 2014) using the sequence of  
172 zebrafish Myomixer protein (Swiss-Prot: P0DP88.1) and we found only one locus with *myomixer*  
173 sequence similarity in the scaffold\_4105 of the trout genome. We also identified two ESTs  
174 (Expressed Sequence Tag; GDKP01024145.1; GDKP01044688.1) corresponding to the *myomixer*  
175 transcript that encoded a protein of 77 aa (deposited in GenBank<sup>TM</sup> with accession number  
176 MN230110). Because both ESTs had little overlap, we performed RT-PCR with a primer on each  
177 ESTs to confirm that both ESTs belonged to the same transcript. The sequence of the PCR product  
178 obtained (599nt), validated that both ESTs belonged to a unique *myomixer* transcript. Sequence  
179 alignment between the genomic sequence and the EST sequences revealed the presence of two  
180 exons, the first containing the full coding sequence. As shown in the figure 1, the trout Myomixer  
181 protein was moderately conserved and shared 61% identity with zebrafish Myomixer and only 25%  
182 with the mouse one. In addition, trout Myomixer sequence shared 95% of identity with other  
183 salmonid Myomixer but only 60-65% of identity with other teleost fish. Despite this overall  
184 moderate sequence conservation, the functionally essential motif, AxLyCxL (x corresponds to  
185 leucine, isoleucine, valine and y corresponds to serine, threonine, alanine or glycine) (Shi et al.,  
186 2017) was conserved in trout Myomixer as well as several charged amino acids in the middle of the  
187 protein (arginine at position 40 and 45; lysine at position 39). The phylogenetic analysis of  
188 Myomixer proteins from several vertebrate species showed a phylogenetic tree consistent with the  
189 vertebrate evolution (figure 2). It was noteworthy that all the Myomixer protein sequences studied  
190 in salmonid were more divergent than the Myomixer sequences in other teleost.

### 191 *4.2. Myomixer is expressed in embryonic and postlarval trout muscle*

192 We performed whole-mount *in situ* hybridization to examine *myomixer* expression during  
193 embryonic myogenesis. *Myomixer* expression was detected as soon as the early stage of

194 somitogenesis (10 dpf) in the deep myotome (figure 3A and D). Then, *myomixer* transcript was  
195 readily detected at 14 (figure 3B and E) and 18 (figure 3C and F) dpf in all somites when  
196 multinucleated fibers begin to form. *Myomixer* expression was also detected in the head muscles  
197 (18dpf) and a transient signal was observed in the otic vesicle (14dpf). In addition, cross-sections  
198 (figure 3F) of 18 dpf embryos have shown that *myomixer* expression was highest in the lateral part  
199 of the myotome. Double in situ hybridization for *pax7* and *myomixer* indicated that *myomixer* was  
200 not expressed in the undifferentiated myogenic dermomyotome-like epithelium surrounding the  
201 primary myotome (figure 3G-I) that was positive for *pax7*. In contrast, the myotome strongly  
202 expressed *myomixer* but contained rare *pax7* positive cells. After hatching, *myomixer* expression  
203 was still readily detected by *in situ* hybridization in the muscle of 1 g and 20 g trout (figure 3J and  
204 K). The signal, consisting of small red dots (1-2/fiber cross-section) adhering to myofibers was  
205 scattered throughout the muscle and was less frequent in muscle of 20 g trout than in 1 g trout. The  
206 patterns of *myomixer* and *myomaker* expression in white muscle of 20 g trout were similar (figure  
207 3K and L).

208 The qPCR quantification of *myomixer* expression in white muscle of 15g, 150g and 1500g trout  
209 (figure 4A) showed that *myomixer* remained clearly expressed after hatching, although its  
210 expression declined as fish weight increased. We also analyzed trout *myomixer* expression in  
211 several tissues by qRT-PCR to determine whether its expression was restricted to skeletal muscle.  
212 As shown in figure 4B, *myomixer* was strongly expressed in white and red skeletal muscle but not  
213 in heart. *Myomixer* expression was also detected at low level in non-muscle tissues such as skin and  
214 brain.

#### 215 *4.3. Myomixer is up-regulated during muscle regeneration and myotube formation in* 216 *vitro*

217 To determine whether *myomixer* is up-regulated during the muscle regeneration, we measured its  
218 expression in muscle following mechanical injury. In our previous study, we observed that the

219 formation of new fibers and the increase of *myogenin* expression occurred 30 days following injury  
220 (Landemaine et al., 2019). Consistently, *myomixer* expression remained stable up to 16 days and  
221 was sharply up-regulated on day 30 with 6-fold higher expression in injured muscle than in the  
222 control one (figure 5).

223 We extracted satellite cells from white muscle of trout, and induced their differentiation and fusion  
224 *in vitro* (Gabillard et al., 2010). Quantitative PCR analysis showed that *myomixer* expression was  
225 significantly up-regulated 3 days after differentiation induction and paralleled *myomaker* expression  
226 (figure 6A and 6B).

227

228

## 229 **5. Discussion**

230 The fusion of myocytes is highly regulated by numerous key membrane-anchored proteins such as  
231 Myomaker and Myomixer (Petrany and Millay, 2019). In the particular context of the persistence of  
232 muscle hyperplasia during post-larval growth of trout and the original structure of trout Myomaker  
233 protein, our work aimed at characterizing the sequence of *myomixer* and its expression during *in*  
234 *vivo* and *in vitro* myogenesis in this species.

235 The *in silico* analysis of the trout genome and the EST databases allowed us to identify a unique  
236 *myomixer* gene. The alignments of Myomixer protein sequences evidenced a moderate conservation  
237 of the overall amino acid sequence across vertebrate lineage. In addition, phylogenetic analysis  
238 showed a greater divergence in salmonid Myomixer sequences. This higher rate of protein sequence  
239 evolution could result from a relaxation of selection pressure or changes of the functional  
240 constraints on Myomixer protein (Zhang and Yang, 2015) although some amino acid residues are  
241 still conserved. For instance, the motif AxLyCxL, essential for Myomixer activity (Shi et al., 2017)  
242 is present in trout Myomixer protein and in all vertebrate species studied. Thus, despite overall  
243 divergence in Myomixer sequences, the key amino acids are conserved in salmonids.

244 Our expression analyses showed that *myomixer* is strongly expressed in the embryonic myotome  
245 during somitogenesis (10 dpf to 18 dpf), when myoblasts fused to form mature myofibers (Barresi  
246 et al., 2001; Steinbacher et al., 2007). Sections of trout embryos of 10 dpf revealed that *myomixer*  
247 was expressed in the fibers of the deep myotome formed during the primary wave of myogenesis.  
248 Then, the highest expression of *myomixer* was observed in the dorsal, ventral and lateral domains of  
249 the myotome, where the secondary wave of myogenesis (stratified hyperplasia) takes place  
250 (Steinbacher et al., 2007). In addition, double *in situ* hybridization for *pax7* and *myomixer* showed  
251 mutually exclusive expression patterns. Indeed, *pax7* is expressed in undifferentiated myogenic  
252 cells present in the dermomyotome-like epithelium surrounding the primary myotome (Dumont et  
253 al., 2008). The *pax7*-positive cells spread into the myotome should correspond to the muscle stem

254 cells (also called satellite cells) that persist in adult muscle. In contrast, *myomixer* is strongly  
255 expressed in differentiated myogenic cells in the area of muscle hyperplasia (Steinbacher et al.,  
256 2007). This expression pattern is in agreement with those obtained in zebrafish that shows a strong  
257 expression of *myomixer* from 14 hpf to 24 hpf (Shi et al., 2017). However, at the end of  
258 somitogenesis (18 dpf) of the trout embryos, *myomixer* expression is maintained in all somites,  
259 whereas in zebrafish its expression is no longer detected in the anterior somites at a comparable  
260 stage (24 dpf). Effectively, in mouse and zebrafish the expression of *myomixer* declines soon after  
261 somitogenesis (Bi et al., 2017; Shi et al., 2017), whereas in trout its expression is maintained  
262 throughout post-larval growth, *i.e.* in fry, juvenile and to a lesser extend in mature fish. Our results  
263 clearly indicate that the expression pattern of *myomixer* is similar to that of the *myomaker* in trout  
264 (Landemaine et al., 2019) during embryonic and post-larval stages. In addition, we did not observe  
265 *myomixer* and *myomaker* expression in myofibers, but only in small cells that should be fusing  
266 muscle precursors. These results are in agreement with those obtained in mouse which show that  
267 muscle overload induces *myomaker* expression in muscle precursors (myocytes) but not in  
268 myofibers, reinforcing the essential role of this protein in muscle hypertrophy and hyperplasia (Goh  
269 and Millay, 2017). Accordingly, in zebrafish, *myomixer* and *myomaker* expression is no longer  
270 detected in white muscle after hatching (Landemaine et al., 2014; Shi et al., 2017) after which post-  
271 larval muscle growth proceeds only by hypertrophy (Johnston et al., 2009). In contrast, in trout,  
272 muscle hyperplasia persists during post-larval growth (Steinbacher et al., 2007) and is accompanied  
273 by a maintenance of *myomixer* and *myomaker* expression indicating that they are markers of muscle  
274 hyperplasia rather than fiber hypertrophy.

275 Our qPCR analyses showed that *myomixer* expression was strongly stimulated in white muscle 30  
276 days after injury, in parallel with the appearance of newly formed myofibers (Landemaine et al.,  
277 2019; Montfort et al., 2016). This kinetic of *myomixer* expression during muscle regeneration, is  
278 comparable to that one of *myomaker* and *myogenin* (Landemaine et al., 2019). Moreover, our results

279 are in agreement with our previous transcriptomic analysis showing that numerous genes essential  
280 for hyperplastic muscle growth (*myod*, *myogenin*, *M-cadherin*, etc.) were up regulated 30 days post  
281 injury (Montfort et al., 2016). Furthermore, we showed that *myomixer* and *myomaker* were up  
282 regulated 3 days after induction of satellite cells differentiation. This latter result is reminiscent to  
283 previous data showing that *myogenin* and *myomaker* expression increase during fusion of trout  
284 myocytes (Landemaine et al., 2019). Thus, these results strongly suggest that *myomixer* is up  
285 regulated during the fusion of myocytes. It is noteworthy that recent studies using loss-of-function  
286 approaches (Zhang et al., 2020) demonstrated in human myoblasts that Myomaker and Myomixer  
287 function is very well conserved among mammals, although the regulation of these genes by other  
288 MRFs could present slight differences. The technical difficulties in performing this kind of  
289 experiments in longer-lived fish species same as the rainbow trout caused that this approach was not  
290 contemplated in the objectives of the present work, although it would be a logical continuation of  
291 the current work to study the interrelationship of Myomaker and Myomixer with other proteins that  
292 regulate the muscle growth in salmonids. Taken together, these results strongly suggest that  
293 Myomixer, like Myomaker, plays a crucial role in myoblast fusion, muscle development and muscle  
294 regeneration.

## 295 **6. Conclusions**

296 In conclusion, our work shows that despite moderate sequence conservation, *myomixer* expression  
297 is consistently associated with the formation of new myofibers during somitogenesis, post-larval  
298 growth and muscle regeneration in trout and can be considered as a good marker of hyperplasia.

## 299 **Acknowledgments**

300 We particularly thank A Patinote and C. Duret for trout rearing and egg production and L Goardon  
301 from the fish facility PEIMA (Pisciculture Expérimentale INRAE des Monts d'Arrée) for muscle  
302 regeneration experiments. This work was supported by INRAE and the "Ministerio de Economía y

303 Competitividad” (MINECO) from the Spanish Government and the fellowship of M Perrello was  
304 supported by MINECO (BES-2016-078697).



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307 **Figure 1. The sequence alignment of vertebrate Myomixer proteins.**

308 The alignment was performed from the complete protein sequences using ClustalW  
309 multiple alignment tool. The amino acid residues present in all sequences are uppercase  
310 and lowercase when present in at least 6 sequences. The AxLyCxL motif was indicated: x  
311 corresponds to leucine, isoleucine, valine and y denotes serine, threonine, alanine or  
312 glycine. Accession numbers are as follows: *O. mykiss*, QII57370; *D. rerio*, P0DP88 ; *S. salar*,  
313 XM-014180492; *S. aurata*, ERR12611\_isotig14560 (<http://sea.ccmarmar.ualg.pt:4567/>); *G.*  
314 *gallus*, CD218366.1; *C. porosus*, XP\_019405207; *M. musculus*, Q2Q5T5 and *H. sapiens*,  
315 A0A1B0GTQ4.

316  
317 **Figure 2. Phylogenetic analysis of Myomixer in tetrapods and teleosts.**

318 The phylogenetic tree was constructed from a multiple alignment of the complete  
319 sequences of the proteins using the neighbour-joining method. The numbers at the tree  
320 nodes represent percentage of bootstrap values after 500 replicates.

321  
322 **Figure 3. Patterns of myomixer expression during embryonic development.**

323 (A-F) Embryos were analyzed by whole mounted *in situ* hybridization at day 10, 14 and 18  
324 post fertilization. The corresponding vibratome section (35µm) was presented for each  
325 stage. Asterisks indicate the dorsal and ventral domains of the myotome and arrowhead  
326 indicates the dermomyotome-like epithelium. (G-I) Double *in situ* hybridization for *pax7*  
327 and *myomixer* of 17 dpf embryo sections. The nuclei are counter-stained with DAPI and  
328 arrowhead indicates the dermomyotome-like epithelium. (J-L) The expression of *myomixer*  
329 and *myomaker* in muscle of 1 g and 20 g trout was also studied using *in situ* hybridization  
330 on cross sections (7µm).

331  
332 **Figure 4. Expression of myomixer in tissues and white muscle of different-weight**  
333 **trout.**

334 The quantification of *myomixer* expression was performed by qPCR analysis in muscle of  
335 different weight trout (A) and in several tissues (150 g; B). The qPCR results are presented  
336 as a ratio of *myomixer* and *eF1a* expression, and the bars represent the standard error.  
337 The letters (a-d) in A indicate the significant differences between means ( $p < 0.05$ ; Kruskal–  
338 Wallis rank test followed by the Wilcoxon-Mann-Whitney test).

339  
340 **Figure 5. Expression of myomixer during muscle regeneration in trout.**

341 Gene expression profile of *myomixer* during muscle regeneration in rainbow trout  
342 normalized with *eF1a* expression. Bars represent the standard error and the letters  
343 indicate the significant differences between means within the same treatment (control or  
344 injured). The asterisk indicates significant differences between treatments at a given time.  
345 Statistical significance ( $p < 0.05$ ) was determined using the Kruskal–Wallis rank test  
346 followed by the Wilcoxon-Mann-Whitney test.

347  
348 **Figure 6. Expression of myomixer and myomaker during trout satellite cell**  
349 **differentiation.**

350 The cells were cultivated in proliferative medium (PM) and then in differentiation medium  
351 for 1, 2, and 3 days (DM1, DM2, and DM3). The qPCR results are normalized with *eF1a*  
352 expression and bars represent the standard error. Different letters indicate significant  
353 differences between means. Statistical significance ( $p < 0.05$ ) was determined using the  
354 Kruskal–Wallis rank test followed by the Wilcoxon-Mann-Whitney test.

355



Figure 2

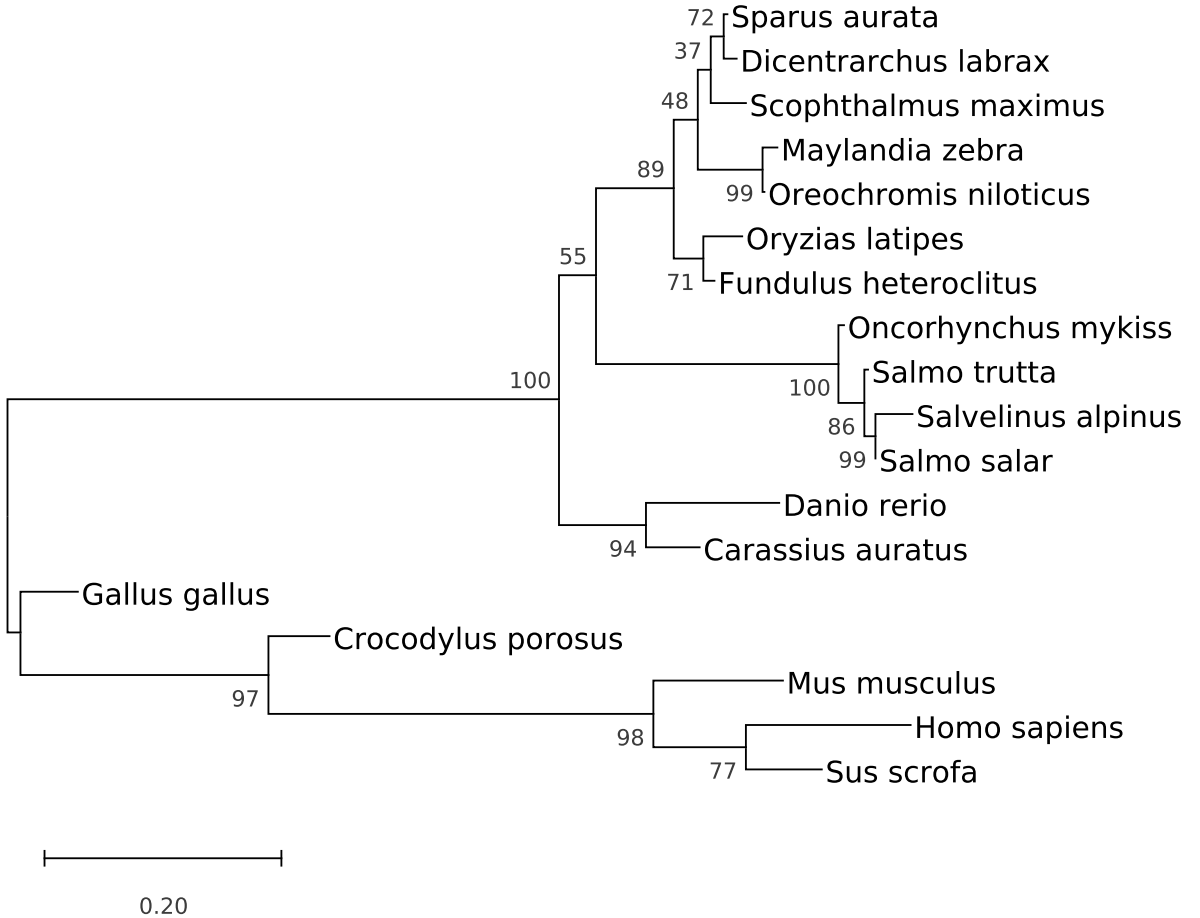


Figure 3

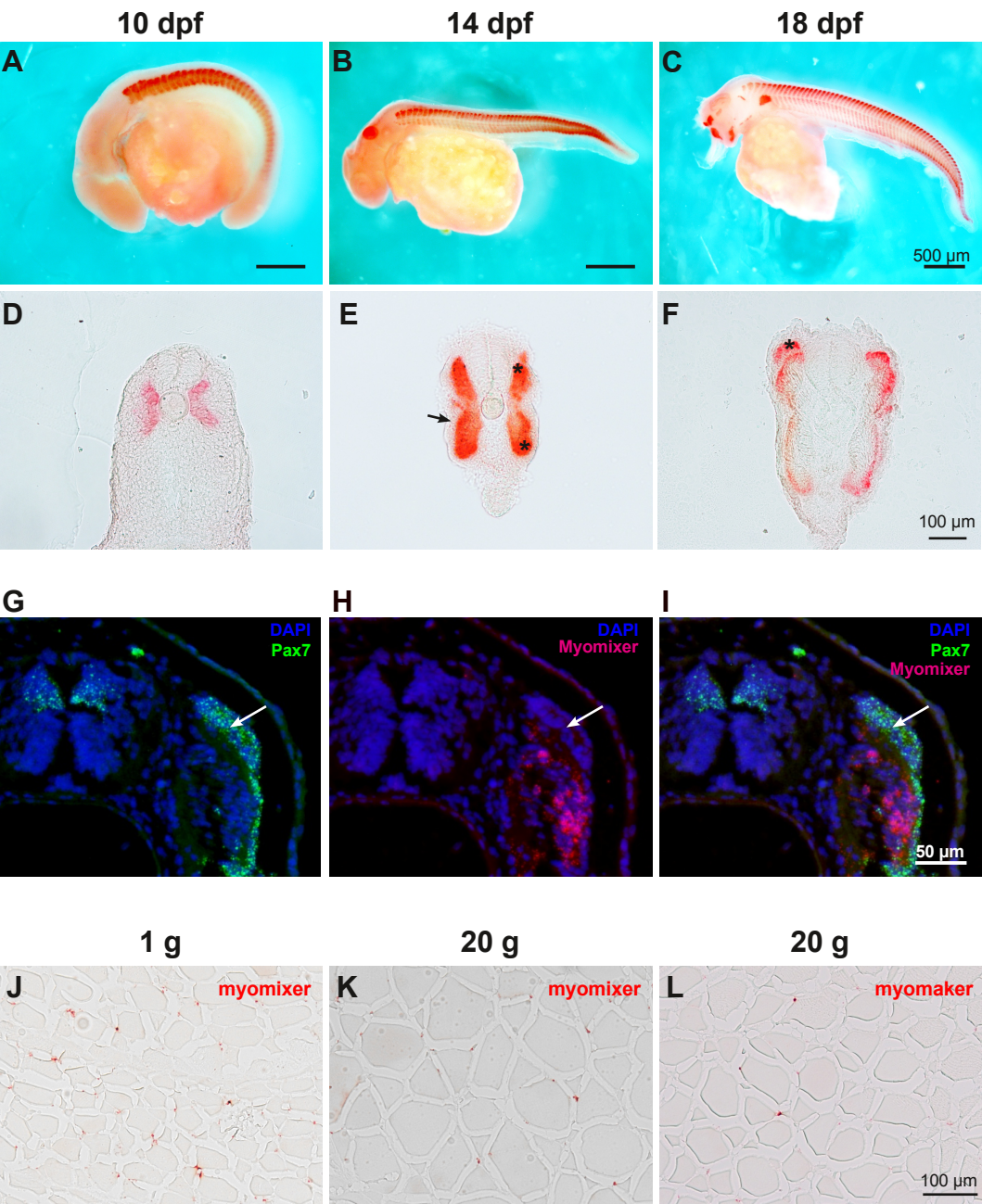
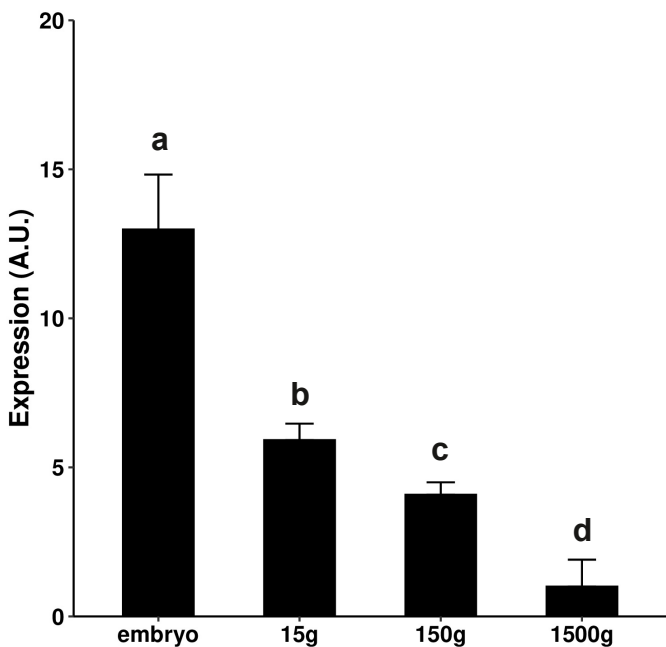


Figure 4

A



B

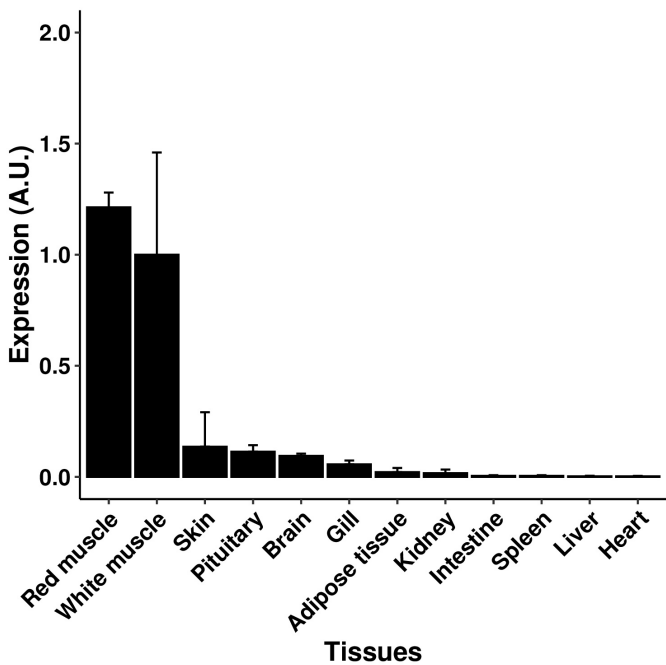


Figure 5

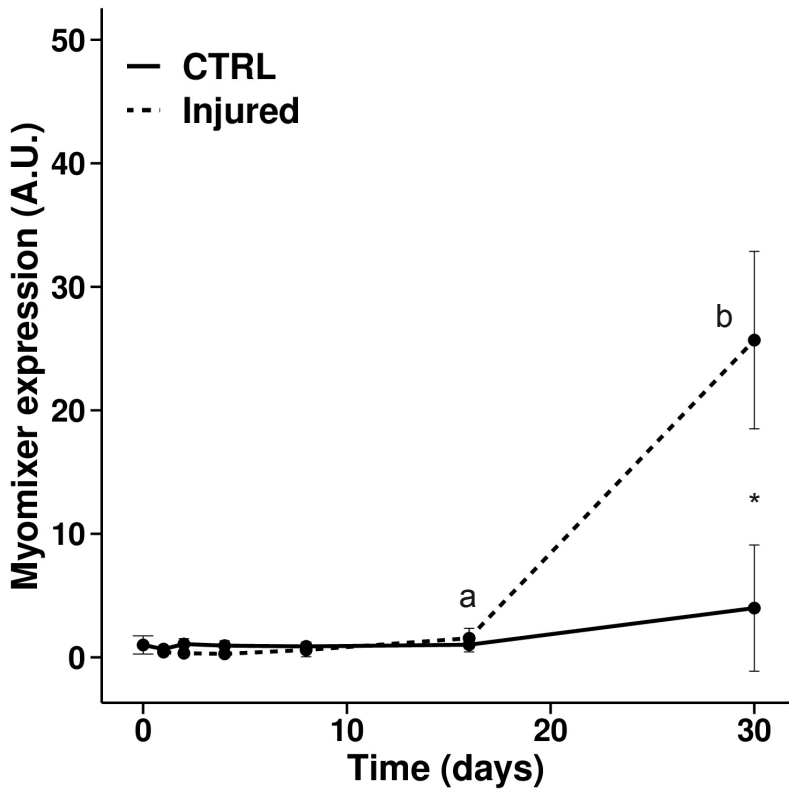




Figure 6

