

1 Effects of stocking density on reared Siberian sturgeon (*Acipenser baerii*) larval growth,  
2 muscle development and fatty acids composition in a recirculating aquaculture system

3

4 Sturgeon larval density

5

6 Lucia Aidos<sup>1</sup>, Mauro Vasconi<sup>1</sup>, Francesco Abbate <sup>2</sup>, Luisa M. P. Valente <sup>3,4</sup> Marco  
7 Lanfranchi <sup>5</sup> and Alessia Di Giancamillo <sup>1\*</sup>

8

9 <sup>1</sup> Department of Health, Animal Science and Food Safety, Università degli Studi di  
10 Milano Via Celoria, 10 – I-20133 Milano, Italy.

11 <sup>2</sup> Department of Veterinary Sciences, Laboratory of Zebrafish Neuromorphology,  
12 University of Messina, Messina , Italy.

13 <sup>3</sup> ICBAS-Instituto de Ciências Biomédicas de Abel Salazar, University of Porto, Portugal

14 <sup>4</sup> CIMAR/CIIMAR – Centro Interdisciplinar de Investigação Marinha e Ambiental,  
15 Terminal de Cruzeiros do Porto de Leixões, Matosinhos, Portugal.

16 <sup>5</sup> Società Naviglio Agricola SS, Goito, Italy.

17

18 \*Correspondence: Alessia Di Giancamillo, Università degli Studi di Milano, Department  
19 of Veterinary Sciences for Health, Animal Science and Food Safety, Via Celoria 10,  
20 20133 Milan, Italy. Tel: +39 02 50315741; email : alessia.digiancamillo@unimi.it

21

22

23

24 **ABSTRACT**

25 This study evaluated the effects of rearing density on muscle growth and development in  
26 Siberian sturgeon (*Acipenser baerii*) larvae. Three different stocking densities were tested:  
27 low (LD, 30 larvae/l), mid (MD, 80 larvae/l) and high (HD, 150 larvae/l) in a recirculating  
28 aquaculture system. Larvae were sampled at hatching (T0), schooling (T1) and complete  
29 yolk-sac absorption (T2) stage and were weighed and processed for muscle tissue  
30 histometrical analyses and for qualitative morphological study analyses; fatty acid profile  
31 was also determined by Gas Chromatography – Flame Ionization Detector analysis. Low-  
32 density larvae presented a higher weight than MD or HD at T2 ( $P<0.05$ ). Histometrical  
33 analysis revealed that total muscle area was similar at T1 and T2 but higher than T0, while  
34 it was lower at HD at schooling ( $P<0.05$ ). Fatty acids profile revealed no differences  
35 between densities while, during development, there was a selective consumption: sparing  
36 or increasing of essential fatty acids to the detriment of their precursors. Our study  
37 suggests that lower densities appear to be more suitable to rear Siberian sturgeon in this  
38 particular stage of development. Indeed, larvae reared at the lower density were heavier  
39 and longer while larvae reared at the higher density showed lower muscle proliferation  
40 rate. As a consequence, LD larvae may exert an increase of potential growth at a mid-  
41 long term.

42

43

44

45

46

47 **KEYWORDS:** Density, fatty acids, larvae, muscle structure, Siberian sturgeon.

## 48 **1 INTRODUCTION**

49

50 During the past two centuries, the natural stocks of Siberian sturgeon (*Acipenser baerii*)  
51 suffered a sharp decline, due to overfishing, pollution and loss of spawning spots. In 1998,  
52 all sturgeon species were effectively added to the Appendix II of the Convention on  
53 International Trade in Endangered Species of Wild Fauna and Flora (CITES), which  
54 allowed to control the illegal trade of sturgeons and their products and the implementation  
55 of conservation plans.

56 Survival and growth during early stages of development of Siberian sturgeon and  
57 throughout the following life periods is of great importance both for conservation  
58 aquaculture production programs and for commercial purposes. Following an increased  
59 demand of commercial production facilities, there is a growing need for the improvement  
60 of hatchery technologies that allow the production of high quality Siberian sturgeon  
61 larvae. After hatching, only endogenous feeding occurs (Balon, 2001), when larvae  
62 entirely rely on the yolk-sac reserves for energy and growth, until its digestive system is  
63 fully developed. During this stage, larvae may be called either pre-larvae or free-embryo  
64 (Dettlaff et al., 1993). The rate at which the yolk-sac reserves are utilized for tissue  
65 development and for the accomplishment of anaerobic processes, depends both on abiotic  
66 (dissolved oxygen, light, density or temperature) and biotic factors (Heming &  
67 Buddington 1988; Kamler 2008).

68 During the endogenous feeding stage, stress plays an important role and stressing rearing  
69 conditions may cause mortalities and impaired growth (Bates et al. 2014; Boucher et al.  
70 2014). The environmental conditions experienced during early life stages can have an  
71 influence on traits during later ontogenetic stages (Crossman et al. 2011) and may,

72 therefore, have an impact on the performance in aquaculture settings. The major stressors  
73 in aquaculture are stocking density, temperature and low dissolved oxygen. Stocking  
74 density in intensive aquaculture directly influences physiology, welfare and behavior of  
75 reared fish (Schreck et al., 1997; Montero et al., 1999; Ellis et al., 2002; Schram et al.,  
76 2006).

77 In aquaculture systems, the efficiency is maximized by increasing the stocking densities.  
78 Previous studies demonstrated that high densities may lead to lower welfare of some fish  
79 species (Lupatsch et al., 2010; Yvette et al., 2011). However, in species such as wedge  
80 sole (*Dicologlossa cuneata*) and winter flounder (*Pseudopleuronectes americanus*) there  
81 were no negative effects caused by high densities (Fairchild et al., 2001 and Herrera et  
82 al., 2009, respectively).

83 Nevertheless, in sturgeons, high rearing density was shown to be an environmental  
84 stressor; Wuertz et al. (2006) and Jodun et al. (2002) found that the growth of the Atlantic  
85 sturgeon was suppressed if reared in a high stocking density. The cause of the growth  
86 suppression is not fully known, in particular for what regards its impact on muscle  
87 development. In acipenseridae, muscle growth is the result of the fusion of myoblasts  
88 derived from somite, leading to the formation of multinucleated muscle lamellae and later  
89 of polygonal cells (Steinbacher et al., 2006).

90 Fatty acids, in particular polyunsaturated fatty acids of n-3 series, are generally known as  
91 key nutrients in fish larvae (Sargent et al., 1999). Their role has been investigated in larvae  
92 of several sturgeon species, such as white sturgeon (*Acipenser transmontanus*) (Gawlicka  
93 et al., 2002), Russian sturgeon (*Acipenser gueldenstaedtii*) (Sener et al., 2005) and  
94 Persian sturgeon (*Acipenser persicus*) (Hafezieh et al., 2010), where these fatty acids were  
95 identified as an essential element to guarantee an optimal survival rate. In recent studies

96 Luo et al. (2015) and Luo et al. (2017) demonstrated that a correct inclusion of essential  
97 fatty acid, such as EPA and DHA, in broodstock diets showed a positive effect on  
98 reproductive performances and larval survival of Siberian sturgeon, underlining the  
99 importance of fatty acid in broodstock and larval metabolism. It has been demonstrated  
100 that high stocking densities may affect some metabolic pathways, such as those associated  
101 to the lipid metabolism. In gilthead seabream (*Sparus aurata*) it was observed that high  
102 stocking density decreases hepatic oleic acid (18:1n-9), arachidonic acid, and n-3 highly  
103 unsaturated fatty acid contents (Montero et al., 1999).

104 The aim of this study is to provide new insight on larval development and myogenesis in  
105 Sturgeon, which may have positive consequences on the final product quality but also on  
106 the success in farming for re-population purposes. This fact especially considering that  
107 the skeletal muscle constitutes the edible part of the fish. Studies of muscle growth since  
108 precocious ages (yolk sac) are therefore important for an optimal development and  
109 assessment of fish farms for both protective and productive aims. Specifically, we  
110 quantified the effects of rearing conditions on larvae' weight, total length, survival,  
111 muscle development and fatty acid profile in Siberian sturgeon until the complete yolk-  
112 sac absorption, without the "interference" of the exogenous feed.

113

## 114 **2 MATERIALS AND METHODS**

115

### 116 **2.1 Fish larvae rearing and sampling**

117 The experiment was held during March/April 2017 at the Experimental Animal Research  
118 and Application Centre of Lodi, of the University of Milan. Siberian sturgeon fertilized  
119 eggs were transported from the "Società Agricola Naviglio" fish farm to the experimental

120 unit 24 hours after fertilization. Eggs were incubated at 16°C, after hatching temperature  
121 was then increased to 19°C: temperature was chosen taking into account a previous  
122 experimental trial based on three rearing temperature (Aidos et al., 2017). After hatching,  
123 which occurred 5 days after fertilization, larvae were subjected to three different rearing  
124 densities until the yolk-sac was completely absorbed. Rearing density was based on total  
125 volume of the tank: n = 48, 130, or 240 offspring/1.6-L rectangular, glass tank. Chosen  
126 densities are representative of currently utilized protocols in sturgeon production  
127 facilities. Fish were reared in a recirculating aquaculture unit, composed by a sand filter,  
128 a biological submerged filter and a UV lamp sterilization unit. Every density was tested  
129 in triplicate. Water quality parameters as oxygen, temperature and pH were measured  
130 every day by Hach HQ 30d Portable Meter, (Hach Lange, Dussendorf, Germany); O<sub>2</sub>  
131 were constantly close to the saturation value and pH values were within the range  
132 described for this species in this stage of development (Kamler, 2002). Dead larvae were  
133 removed every day, and mortality was estimated by dead larvae daily recording.  
134 Measurements of ammonia, nitrite and nitrate were carried at the beginning, at hatching  
135 and the end of the trial by Hach 2800 Portable Spectrophotometer (Hach Lange,  
136 Dussendorf, Germany and were compliant with values recommended for Siberian  
137 sturgeon. Eggs and larvae were exposed to an artificial photoperiod regime of 12L:12D.  
138 Along the entire experimental period, hatched larvae utilized the nutrients of their yolk  
139 sac and were not fed any exogenous feed. Sampling time points were chosen according  
140 to important steps of Siberian sturgeon larvae behaviour development: hatching (T0),  
141 beginning of the schooling phase (T1) and complete yolk sac absorption phase (T2). For  
142 each sampling time-point, 3 larvae per experimental nursery (total n=9 larvae per  
143 treatment) for histology and for SEM analyses, and a pool of 5 larvae per nursery and per

144 replicate (analysis were performed in duplicate; total n=30 per treatment) for fatty acid  
145 composition were picked up with a wide pipette and killed by over-anaesthesia with Ethyl  
146 3-Aminobenzoate, Methanesulfonic A (Sigma-Aldrich), at a concentration of 100mg/l.  
147 This research was approved by the Ethic Committee of the University of Milan  
148 (OPBA\_22\_2017).

149

## 150 **2.2 Scanning Electron Microscope (SEM)**

151 Samples were immediately fixed in 2.5% glutaraldehyde in Sorensen phosphate buffer  
152 0.1M. After several rinsing in the same phosphate buffer, they were dehydrated in a  
153 graded alcohols series, critical-point dried in a Balzers CPD 030, sputter coated with 3  
154 nm gold in a Balzers BAL-TEC SCD 050 and examined for the correct larval  
155 development and for the detection of morphological abnormalities under a Zeiss EVO LS  
156 10 scanning electron microscope.

157

## 158 **2.3 Histological and immunohistochemical analyses**

159 Whole larvae were immediately fixed in 4% paraformaldehyde in 0.01M phosphate-  
160 buffered saline (PBS) pH 7.4 for 24h at 4°C, then dehydrated in a graded series of ethanol,  
161 cleared with xylene and embedded in paraffin. Serial transverse microtome sections at a  
162 peri-anal level (5 µm-thick) were obtained from each sample. The haematoxylin/eosin  
163 (HE) stain was performed for the evaluation of the structural aspects of the developing  
164 lateral muscle tissues and for histometry (Aidos et al., 2017). Standard histometrical  
165 techniques were applied using an Olympus BX51 light microscope equipped with a DP-  
166 software program (Cell^B, Basic Imaging Software, Olympus, Italy) for determining: i)  
167 total muscle area (TMA), ii) red muscle area (slow muscle cross-sectional area, SMA),

168 iii) white muscle area (fast muscle cross-sectional area, FMA), iv) lamellae fibres area  
169 (LFA), v) polygonal fibres area (PFA), at the three analysed developmental stages:  
170 hatching (T0), schooling (T1) and yolk-sac full absorption (T2). On other transverse  
171 sections, immunostaining was performed to detect proliferating cell nuclear antigen  
172 (PCNA). The applied immunohistochemical procedure has been previously described in  
173 detail (Di Giancamillo et al., 2009). Briefly, endogenous peroxidase activity was blocked  
174 by incubating the sections in 3% H<sub>2</sub>O<sub>2</sub> in PBS. Nonspecific binding sites were blocked  
175 by incubating the sections in normal mouse serum (Dakocytomation, Milan, Italy). Mouse  
176 monoclonal anti-PCNA (dilution 1:200, clone PC10, Sigma-Aldrich, Milan, Italy)  
177 antibodies were applied overnight at room temperature. The used primary antisera were  
178 diluted with a 0.05 M pH 7.4 Tris–HCl saline buffer (TBS: 0.05 M, pH 7.4, 0.55 M NaCl).  
179 After the treatment with the primary antibodies has been completed, the antigen–antibody  
180 complexes were detected with a peroxidase-conjugated polymer that carries secondary  
181 antibody molecules directed against mouse (EnVision™+, DakoCytomation, Glostrup,  
182 Denmark) applied for 120 min at room temperature. Peroxidase activity was then detected  
183 with diaminobenzidine (DAB, DakoCytomation, Glostrup, Denmark) as the substrate.  
184 Appropriate washing with TBS was performed between each step, and all incubations  
185 were carried out in a moist chamber. All sections were finally weakly counterstained with  
186 Mayer’s haematoxylin, dehydrated, and permanently mounted. The specificity tests for  
187 the used antibodies were performed by incubating other sections in parallel with: i) TBS  
188 instead of the specific primary antibodies; ii) TBS instead of the secondary antibodies.  
189 The results of these controls were always negative (i.e. staining was abolished).  
190 Photomicrographs were taken with an Olympus BX51 microscope (Olympus, Milan,  
191 Italy) equipped with a digital camera, and final magnifications were calculated.



192 PCNA-immunopositive cells were for brevity described as “proliferating cells” and their  
193 relative cell number were evaluated by counting the muscle immunopositive nuclei in a  
194 tissue area corresponding to the above mentioned FMA at the three analysed  
195 developmental stages and then converted to number of proliferating cell/mm<sup>2</sup>.

196

#### 197 **2.4 Fatty acids composition**

198 The extraction and determination of total lipids was performed in whole larvae, according  
199 to the Folch (1957) method with chloroform:methanol (2:1). The preparation of fatty acid  
200 methyl esters (FAME) was performed according to Christie (2003). Briefly, the lipid  
201 sample (20 mg) was dissolved 10% methanolic hydrogen chloride (2 mL). A 1 mL  
202 solution of tricosanoic acid (1 mg/ml) in toluene was added as internal standard. After an  
203 incubation at 50 °C overnight, 2 mL of a 1M K<sub>2</sub>CO<sub>3</sub> solution and 5 mL of 5% NaCl were  
204 added to each sample. The FAMEs were extracted with 2×2 mL of hexane and then  
205 evaporated under nitrogen. The sample was dissolved in 1 mL hexane and 1 µL sample  
206 was injected into the gas-chromatograph, in split mode (split ratio 1:100). Fatty acid  
207 analysis was carried out on an Agilent gas-chromatograph (Model 6890 Series GC) fitted  
208 with an automatic sampler (Model 7683) and FID detector. The carrier gas was helium  
209 with a flow rate of 1.0 ml/min and an inlet pressure of 16.9 psi. A HP-Innowax fused  
210 silica capillary column (30m×0.25mm I.D., 0.25 µm film thickness; Agilent  
211 Technologies) was used to separate FAME fatty acid methyl esters. The oven temperature  
212 program for separation was from 100 to 180 °C at 3 °C min<sup>-1</sup>, then from 180 to 250 °C  
213 at 2.5 °C min<sup>-1</sup> and held for 10 min. Carrier gas was helium at 1.0 mL min<sup>-1</sup>, inlet  
214 pressure 16.9 psi. Fatty acids were identified by comparison of retention times with  
215 standard 37 Fatty acids methyl esters (FAME) mixture in dichloromethane and standard

216 Menhaden fish oil, obtained from Supelco (Supelco, Bellafonte, PA, USA), and were  
217 expressed as percentage of total fatty acids.

218

## 219 **2.5 Statistical analysis**

220 Statistical analysis was performed with SAS statistical software (version 9.3, Cary Inc.,  
221 NC). Data from the histometrical analyses (TMA, SMA, FMA, LFA and PFA) and PCNA  
222 cellular counts, were analysed using 2-way ANOVA with densities (LD, MD, HD) and  
223 developmental stages (T0, T1 and T2) as main factors, and co-variated for the total area  
224 corresponding to the TMA (for PCNA, LFA and HFA were used as co-variated factor).  
225 Concerning fatty acid analysis, the normal distribution and homogeneity of variance was  
226 confirmed and comparison between means was performed by analysis of variance. The  
227 Student Newman Keuls was used as post hoc test for comparison of the means among  
228 different rearing density or different sampling point. The data are presented as least-  
229 square means (SEM). Differences between means were considered significant at  $p < 0.05$ .

230

## 231 **3 RESULTS**

232

### 233 **3.1 Development, survival and growth**

234 Throughout the trial,  $O_2$  was constantly close to the saturation value; temperature ranged  
235 from 17 to 19.7°C, and pH values were within the physiological range described for this  
236 species. The duration of the endogenous feeding phase was of 8 days across treatments,  
237 and fish exhibited schooling behaviour in synchrony among density treatments.

238

239 Mortality from schooling to the yolk sac absorption significantly decreased (Figure 1a;  
240  $P < 0.01$ ), and within the schooling stage the LD group showed lower mortality than MD  
241 and HD groups (Figure 1a;  $P < 0.05$ ). At the end of the trial no differences were found  
242 between treatments (Figure 1a).

243 Body weight significantly increased from one stage of development to the other,  
244 regardless of the density (Figure 1b;  $P < 0.001$ ). Higher stocking densities had a  
245 significantly negative effect on the growth of Siberian sturgeon larvae, because at the end  
246 of the experiment, final body weight of the LD group was significantly higher than that  
247 of either the MD group or the HD group ( $P < 0.05$ ; Figure 1b).

248 Total length (TL) significantly increased from one stage of development to the other,  
249 regardless of the density ( $P < 0.001$ ; Figure 1c). At the full yolk-sac absorption stage, TL  
250 decreased as a function of increasing rearing density. Larvae reared at a lower density  
251 were significantly longer than those reared at either MD or HD ( $P < 0.05$ ; Figure 1c). The  
252 interaction between developmental stages and temperature was not significant.

253 **[Figure 1]**

254

### 255 **3.2 SEM**

256 SEM morphological analyses revealed a correct morphological development at all  
257 densities from hatching to larval schooling and yolk-sac absorption, with no  
258 morphological deformities (Figure 2a-c). Briefly at hatching larvae presented an evident  
259 yolk-sac, which was reduced at schooling and completely absent at the end of the trial  
260 (white asterisks, Figure 2a, 2b, 2c respectively). No damaged fins, looped tail or change  
261 in the form of yolk-sac was observed.

262

### 263 3.3 Histological and immunohistochemical analyses

264 Histological analyses revealed an anatomically normal muscle development with an outer  
265 monolayer of slow muscle cells (SM), as well as an inner monolayer of fast muscle cells  
266 (FM) at hatching: changes occurred in both SM and FM at schooling and consequently at  
267 yolk-sac absorption from monolayer to multilayers (Figure 2d-f). Histometrical results  
268 are presented in Figure 2g-i. At the schooling stage, the TMA was significantly higher  
269 for larvae subjected to both LD and MD ( $P<0.05$ ), while at the end of the experiment no  
270 differences were found across treatments (Figure 2g); there were highly significantly  
271 differences from one stage to the other regarding TMA ( $P<0.01$ ; Figure 2g). As for the  
272 SMA, there is a highly significant difference from one stage to the other ( $P<0.05$ ) but no  
273 differences were found at each stage of development between treatments (Figure 2h).  
274 Also regarding the FMA, there was a highly significant increase from hatch to the yolk-  
275 sac absorption stage ( $P<0.01$ ; Figure 2i). However, no differences were found across  
276 treatments. The interaction between developmental stages and density was not significant.

277 **[Figure 2]**

278

279 Regarding the FMA two different types of cells were identified: the inner ones are  
280 lamellae-shaped fibres (LF) and the outer ones are polygonal cells (PC) (Fig, 3a and b).  
281 Regarding the areas occupied by LF and PC, no differences were found between  
282 treatments at the schooling stage (Figure 3c,d respectively). At the end of the trial, though,  
283 the area occupied by the lamellae was significantly higher in larvae subjected to LD or  
284 MD ( $P<0.05$ ; Figure 3c). On the opposite, the area occupied by the polygonal cells, was  
285 significantly higher for larvae subjected to HD ( $P<0.05$ , Figure 3d). The interaction  
286 between developmental stages and density was not significant.

287 [Figure 3]

288

289 Regarding the anti-PCNA of the lateral muscle, immunostaining was observed in FMA  
290 nuclei (arrowheads; Figure 4a-c); quantification of the proliferating cells is reported in  
291 Figure 4d. Proliferating cells of the FMA, revealed that the T0 group was significantly  
292 higher than all the other groups (Figure 4d;  $P < 0.001$ ). At schooling, the LD group showed  
293 a significantly higher number of proliferating cells than larvae subjected to the MD or  
294 HD ( $P < 0.05$ ). At the end of the trial, the LD group still revealed a significantly higher  
295 number of proliferating cells than HD and the MD group presented a significantly higher  
296 number than the HD group (LD vs HD,  $P < 0.01$ ; MD vs HD,  $P < 0.05$ , respectively). At the  
297 end of the trial there were no significant differences between groups LD and MD. The  
298 interaction between developmental stages and density was not significant.

299 [Figure 4]

300

### 301 3.4 FATTY ACIDS

302 The lipid content of Siberian sturgeon larvae was not affected (p-value 0.407) by rearing  
303 treatment. Larvae progressively consumed their lipid reserves: at hatching larvae showed  
304 a lipid content of 2.22 (0.39 SD), while at schooling phase it decreased to 1.49 (0.32 SD)  
305 and to 1.19 (0.26 SD) observed at the end of yolk-sac absorption. Fatty acid composition  
306 of larvae at hatching and reared at three different density are presented in Table 1. Oleic  
307 acid (18:1 n-9, OA) was the fatty acid present in higher amount in all larvae, followed by  
308 palmitic acid (16:0, PA) linoleic acid (18:2 n-6, LA) and docosahexaenoic acid (22:6 n-  
309 3, DHA). The rearing density did not influence the larvae fatty acid composition as there  
310 were no statistically significant differences between treatments ( $P > 0.05$ ). Contrariwise,

311 fatty acid profile changed between different stages of development of Siberian sturgeon  
312 larvae. Some fatty acid decreased their relative amount during larval growing while other  
313 increased. OA is the fatty acid that showed the higher decrease during the trial, passing  
314 from a value of 37.09 g/100g of fatty acids registered in larvae at hatching to a mean value  
315 of 34.8 at the end of yolk absorption. The higher increase was found in DHA, which has  
316 gone from a value of 9.2 to 11.75 g/100g of fatty acids.

317

#### 318 **4 DISCUSSION**

319 In the present study we analysed the effects of three stocking density rearing conditions  
320 on larvae' weight, total length, survival, muscle development and fatty acids profile of  
321 Siberian sturgeon until the complete yolk-sac absorption.

322 In intensive aquaculture, fish are continuously subjected to various environmental  
323 discomfort situations and stocking density is certainly one of the key factors for the  
324 productivity of farms: many studies have concluded that overcrowding is a problem that  
325 can induce a reduction of growth and mortalities of larval and juvenile forms. Although  
326 large quantities of larvae derive from fertilized eggs, mortality or deformities in these  
327 initial phases remain rather high or, in any case, variable. Mohseni et al. (2000) reported  
328 that a higher incidence of deformities may lead to death during the early stages of  
329 ontogeny and development. In our study, SEM morphological analyses have been  
330 performed with the aim of following the correct morphological development: from  
331 hatching, larval morphological development followed the correct steps as described by  
332 Dettlaff et al. (1993) with no abnormalities detected.

333 Regarding the growth parameters, some studies on lake sturgeon (*Acipenser fulvescens*)  
334 and on Atlantic sturgeon (*Acipenser oxyrinchus*), have not shown a significant difference

335 in growth rate in high densities (between 264 and 792 juveniles/litre; Fajfer et al. al 1999,  
336 Mohler et al., 2000). However, many studies indicate that high stocking density increases  
337 stress (Barton 2002; Barton & Iwama 1991; Leatherland & Cho 1985; Pickering &  
338 Duston 1983; Wedemeyer, 1976) and to date, it has been shown that high density is an  
339 environmental stress factor for sturgeons (Wuertz et al., 2006). In our study, in fact, larvae  
340 reared at a low density were significantly heavier and longer than those reared at medium  
341 and high densities. Mortality was significantly lower in the LD group at the schooling  
342 stage, while no differences were observed at the end of the trial. In addition, Li et al.  
343 (2011) in their study on the effects of stock density on growth of the sturgeon Amur  
344 (*Acipenser schrenckii*) found that growth rate decreased significantly by increasing  
345 stocking density, with a significant negative impact. The strong reduction in growth in  
346 high-density reared fish is often related to a decrease in food consumption and a reduction  
347 in the efficiency of conversion (Papoutsoglou et al., 1998; Vijayan & Leatherland 1990).  
348 Even if in our study we evaluated only the endogenous feeding conditions, we still can  
349 compare this data in terms of the efficiency in converting yolk-sac resources. Recent  
350 studies on sturgeon (Falahatkar & Barton 2007, Rafatnezhad et al 2008, Falahatkar et al  
351 2009) suggest that this species is relatively resistant to disturbances caused during  
352 aquaculture practices, but is still relatively sensitive. A reduction in the growth rate in  
353 medium and high density reared sturgeons also corresponds to a different muscular  
354 development. Considering muscle growth, we have to point out that in teleosts, muscle  
355 growth is the result of two processes: hypertrophy and hyperplasia. Muscle fibres grow  
356 by hypertrophy during post-embryonic life to reach a functional maximum (Rowlerson &  
357 Vegetti 2001) and have been described for different species, such as European seabass  
358 (*Dicentrarcus labrax*; Alami-Durante et al., 1997), cod (*Gadus morhua*; Galloway et al,

1999), salmon (*Salmo salar*; Nathanailides et al., 1995) and in other marine species (*Scophthalmus maximus*; *Dicentrarcus labrax*; *Cyprinus carpio*), as reviewed by Valente et al. (2013). A substantial difference between sturgeons and teleosts is that in the latter the white muscle is composed entirely of cylindrical cells from hatching to the adult life. In sturgeons, instead, compared to teleost fishes, there is the fusion of myoblasts derived from somite, leading to the formation of multinucleated muscle lamellae and later on, in polygonal cells (Steinbacher et al., 2006). In our study we observed a change in the three physiological phases considered, a change that occurs in all the densities considered: from hatching to schooling and full yolk-sac absorption stage, the fast fibres undergo a phenomenon of hypertrophy / hyperplasia which increases its number and size. The muscular component observed with HE staining shows only an increase in the size of the myotomes already morphologically observed and then verified with histometry (see below); as for the stages of development, there were no qualitative nor morphological differences among the three tested densities. According to Rowleron & Vegetti (2001), a widely used method for measuring muscle growth involves the cross-sectional areas, which provide an index of hypertrophic or hyperplastic growth. To quantify these qualitative changes, we used histometry. Difference in terms of muscle development has been identified in the definitive polygonal cells and in the primary lamellae: HD has more definitive polygonal cells than LD and MD and, on the contrary, less lamellae, that are primitive fibres. This can indicate an increase in the conversion / differentiation of the primitive lamellae in definitive polygonal cells, which is confirmed by a reduction in both length and weight of the HD group. These preliminary results allow us to suggest that larvae reared at low and medium densities are similar in terms of muscle development, while in the group of larvae raised at high density it is possible to observe an acceleration



383 of muscle development in its final form. According to Rowleron & Vegetti 2001 we  
384 assessed the rate of replication of the cells. PCNA counts revealed to be higher in the fast  
385 fibres at LD at both T1 and T2, suggesting that the turnover was decreased in HD group  
386 and this is consistent with the higher final weight and length reached by larvae subjected  
387 to the lowest density. Up to date the mechanism of polygonal cells formation is still  
388 unclear, but we suggest that in the HD group, a faster differentiation from lamellae-shaped  
389 fibres into polygonal cells was present, thus revealing a correlation with the lowest growth  
390 parameters in the same group.

391 The fatty acid composition has not been influenced by stocking density during our trial.  
392 Density could act as a stressor and induce some alteration of fatty acid metabolism in fish,  
393 as found in gilthead sea bream (Montero et al., 1999). The modification of fatty acid  
394 composition was observed after long time experiments, and it has been linked to the  
395 utilization of polyunsaturated fatty acids (PUFA) in liver in response to a stress situation  
396 and plasma cortisol release. Other authors found similar results in rainbow trout (Bayir &  
397 Bayir, 2017), where the modification of fatty acids profile was also linked with the  
398 increase of  $\Delta$ -6 desaturase in the liver and muscle of trout reared ad high density. All  
399 these studies were performed on fed fish and for longer periods than that used in our  
400 study, so it is possible that the modification of fatty acids profile in response to density  
401 could appear only after a long exposure to longer density-rearing conditions than the one  
402 experimented in our trial. Not taking into account the rearing density but only the  
403 evolution of fatty acids profile in time, during the first days of life of Siberian sturgeon  
404 larvae, we could observe how it changes according to the larval development. Oleic acid  
405 and linoleic acid decreased during the development, probably because larvae rather used  
406 them to satisfy their energy requirements without consuming important and essential fatty

407 acids, which were conversely spared. Arachidonic acid and DHA increased at the end of  
408 the trial when compared with the composition of larvae at hatching; the relative increase  
409 could be due both to a spare effect, as they were not used to obtain energy, and to an ex-  
410 novo synthesis, starting from their precursors. This last hypothesis is supported by the  
411 simultaneous decrease of precursors of ARA and DHA, like linoleic acid (18:2 n-6),  $\gamma$ -  
412 linolenic acid (18:3 n-6),  $\alpha$ -linolenic acid (18:3 n-3) and stearidonic acid (18:4 n-3) and  
413 the increase of ARA and DHA. The sturgeons' ability to elongate and desaturate 18:2n-  
414 6 and 18:3n-3 fatty acids to 20:4n-6, 20:5n-3 and 22:6n-3 is supported also by the findings  
415 of other authors that investigated the effect of substitution of fish oil in sturgeon diet with  
416 vegetable oils, rich on linoleic and linolenic acid (Xu et al., 1993 for white sturgeon, Sener  
417 et al., 2005 for Russian sturgeon and Liu et al., 2018 for hybrid sturgeon (*A. baeri*  $\times$  *A.*  
418 *schrenckii*). The modification of fatty acids profile of Siberian sturgeon larvae before  
419 their first exogenous feeding has been investigated also in a previous trial performed with  
420 three different rearing temperatures (Vasconi et al., 2018). The results of the present trial  
421 are almost comparable with those obtained in our previous experiment; Siberian sturgeon  
422 larvae did not use equally the fatty acids that composed their lipid reserves, as they spare  
423 essential fatty acids at the expense of the others ones.

424

## 425 **5 CONCLUSION**

426

427 It is essential in the success of aquaculture practices to reach a good compromise between  
428 larval quality and economic feasibility. Siberian sturgeon seems to be quite susceptible  
429 to stocking density in early stages, when considering muscle growth and development.  
430 Taking into account the results of the present study for what concerns the higher weight

431 and length achieved by larvae of the LD group, we suggest that lower densities could be  
432 taken in account for the production of high quality sturgeon larvae. Moreover, the lower  
433 muscle fibres proliferation rate showed by larvae subjected to the higher rearing density  
434 may compromise the growth potential of fish reared in these conditions, but this is still to  
435 be confirmed. However, it appears of great importance to assess the stress condition as  
436 well as the gene expression pattern, in order to better understand and characterize the  
437 mechanism of muscle fibres conversion during early development and its impact in future  
438 stages of development.

439

440

#### 441 **ACKNOWLEDGMENTS**

442 This research did not receive any specific grant from funding agencies in the public,  
443 commercial, or not-for-profit sectors. Authors wish to thank Società Agricola Naviglio  
444 for providing the fertilized eggs necessary for this study.

445

#### 446 **REFERENCES**

447 Aidos, L., Valente, L. M. P., Sousa, V., Lanfranchi, M., Domeneghini, C. & Di  
448 giancamillo, A. (2017). Effects of different rearing temperatures on muscle development  
449 and stress response in the early larval stages of *Acipenser baerii*. *European Journal of*  
450 *Histochemistry*, 61, 2850, 287-294. doi:10.4081/ejh.2017.2850.

451

452 Alami-Durante, H., Olive, N. & Rouel, M. (2007). Early thermal history significantly  
453 affects the seasonal hyperplastic process occurring in the myotomal white muscle of

- 454 *Dicentrarchus labrax* juveniles. Cell and Tissue Research, 327, 553–570. doi  
455 10.1007/s00441-006-0321-2
- 456
- 457 Balon, E. K. (2001). Saltatory ontogeny and the life-history model: neglected processes  
458 and patterns of evolution. Journal of Bioeconomics, 3, 1–26.  
459 doi.org/10.1023/A:1016305005962
- 460
- 461 Barton, B.A. (2002) Stress in Fishes: A Diversity of Responses with Particular Reference  
462 to Changes in Circulating Corticosteroids. Integrative and Comparative Biology, 42,  
463 Issue 3, 517–525. doi.org/10.1093/icb/42.3.517
- 464
- 465 Barton, B. A. & Iwama, G. K. (1991). Physiological changes in fish from stress in  
466 aquaculture with emphasis on the response and effects of corticosteroids. Annual Review  
467 of Fish Diseases, 1, 3–26. doi.org/10.1016/0959-8030(91)90019-G
- 468
- 469 Bates, L. C., Boucher, M. A., & Shrimpton, J. M. (2014). Effect of temperature and  
470 substrate on whole body cortisol and size of larval White Sturgeon (*Acipenser*  
471 *transmontanus* Richardson, 1836). Journal of Applied Ichthyology, 30, 1259–1263.  
472 doi.org/10.1111/jai.12570
- 473
- 474 Bayir, M. & Bayir, A. (2017). A comparison of growth, antioxidant levels and fatty acid  
475 metabolism in juvenile rainbow trout (*Oncorhynchus mykiss*) reared in two different  
476 stocking densities: low and high. Marine and Freshwater Behaviour and Physiology, 50  
477 (5-6), 345-357. doi: 10.1080/10236244.2017.1412652

478

479 Boucher, M. A., McAdam, S. O. & Shrimpton, J. M. (2014). The effect of temperature  
480 and substrate on the growth, development and survival of larval White Sturgeon.  
481 *Aquaculture*, 430, 139–148. doi.org/10.1016/j.aquaculture.2014.03.011

482

483 Christie, W. W. (2003). *Lipid Analysis* (3<sup>rd</sup> edition) The Oily Press, Bridgwater, UK

484

485 Crossman, J. A., Forsythe, P. S., Scribner, K. T. & Baker, E. A. (2011). Hatchery rearing  
486 environment and age affect survival and movements of stocked juvenile Lake Sturgeon.  
487 *Fisheries Management and Ecology*, 18, 132–144. doi.org/10.1111/j.1365-  
488 2400.2010.00762.x

489

490 Dettlaff, T. A., Ginzburg, A. S. & Schmalhausen, O. I. (1993). Chapter 3: Development  
491 of Prelarvae. In: *Sturgeon fishes: developmental biology and aquaculture*. Springer-  
492 Verlag Berlin Heidelberg. doi:10.1007/978-3-642-77057-9

493

494 Di Giancamillo A., Rossi R., Vitari F., Pastorelli G., Corino C. & Domeneghini C. (2009).  
495 Dietary conjugated linoleic acids decrease leptin in porcine adipose tissue. *Journal of*  
496 *Nutrition*, 139, 1867 – 1872. doi: 10.3945/jn.109.110627

497

498 Ellis, T., North, B., Scott, A. P., Bromage, N. R., Porter, M. & Gadd, D. (2002). The  
499 relationships between stocking density and welfare in farmed rainbow trout. *Journal of*  
500 *Fish Biology*, 61, 493-531. doi.org/10.1111/j.1095-8649.2002.tb00893.x

501

- 502 Fairchild, E. A. & Howell, W. H. (2001). Optimal stocking density for juvenile winter  
503 flounder *Pseudopleuronectes americanus*. Journal of the World Aquatic Society, 32, 300-  
504 8. doi.org/10.1111/j.1749-7345.2001.tb00453.x  
505
- 506 Fajfer, S. , Meyers, L. , Willman, G. , Carpenter, T. & Hansen, M. J. (1999). Growth of  
507 Juvenile Lake Sturgeon Reared in Tanks at Three Densities. North American Journal of  
508 Aquaculture, 61, 331-335. doi:10.1577/1548-8454(1999)061<0331:GOJLSR>2.0.CO;2  
509
- 510 Falahatkar, B., Poursaeid, S., Shakoorian, M. & Barto, B. (2009). Responses to handling  
511 and confinement stressors in juvenile great sturgeon *Huso huso*. Journal of Fish Biology,  
512 75(4), 784-96. doi: 10.1111/j.1095-8649.2009.02334.x  
513
- 514 Falahatkar, B. & Barton, B. A. (2007). Preliminary observations of physiological  
515 responses to acute handling and confinement in juvenile Beluga *Huso huso*. Aquaculture  
516 Research 38(16), 1786-1789. doi:10.1111/j.1365-2109.2007.01855.x  
517
- 518 Galloway, T. F., Kjorsvik, E. & Kryvi, H. (1999) Muscle growth and development in  
519 Atlantic cod larvae (*Gadus morhua* L.), related to different somatic growth rates. Journal  
520 of Experimental Biology, 202, 2111-2120  
521
- 522 Gawlicka, A., Herold, M. A., Barrows, F. T., De La Noüe, J. & Hung, S. S. O. (2002)  
523 Effects of dietary lipids on growth, fatty acid composition, intestinal absorption and

- 524 hepatic storage in white sturgeon (*Acipenser transmontanus* R.) larvae. Journal of  
525 Applied Ichthyology 18, 673–681. doi10.1046/j.1439-282 0426.2002.00371.x  
526
- 527 Hafezieh, M., Mohd Salah Kamarudin, S., Bin Saad, C.R., Mostafa Kamal, A. S., Agh,  
528 N., Valinassab, T., Sharifian, M. & Hosseinpour, H. (2010). Effect of Enriched *Artemia*  
529 *urmiana* with HUFA on growth, survival and fatty acids composition of Persian sturgeon  
530 larvae (*Acipenser persicus*). Iranian Journal Fisheries Sciences 9, 61-72.  
531 doi.jifro.ir/article-1-23-en.html  
532
- 533 Heming, T. A. & Buddington, R. K. (1988). Yolk absorption in embryonic and larval  
534 fishes. Fish Physiology, XIA,407–446. doi.org/10.1016/S1546-5098(08)60203-4  
535
- 536 Herrera, M., Vargas-Chacoff, L., Hachero, I., Ruíz-Jarabo, I., Rodiles, A.& Navas, J. I.  
537 (2009). Physiological responses of juvenile wedge sole *Dicologlossa 23uneate*  
538 (Moreau) to high stocking density. Aquaculture Research, 40, 790-7.  
539 doi.org/10.1111/j.1365-2109.2008.02162.x  
540
- 541 Jodun, W. A., Millard, M. J.& Mohler, J. (2002) The effect of rearing density on growth,  
542 survival, and feed conversion of juvenile Atlantic sturgeon. North American Journal of  
543 Aquaculture, 64, 10–15. doi.org/10.1577/1548-  
544 8454(2002)064<0010:TEORDO>2.0.CO;2  
545
- 546 Kamler E. (2002). Ontogeny of yolk-feeding fish: an ecological perspective. *Reviews in*  
547 *Fish Biology and Fisheries.*, 12, 79-103. https://doi.org/10.1023/A:1022603204337

548

549 Kamler, E. (2008). Resource allocation in yolk-feeding fish. *Reviews in Fish*  
550 *Biology and Fisheries*, 18, 143–200. doi.org/10.1007/s11160-007-9070-x

551

552 Leatherland, J. F. & Cho, C. Y. (1985). Effect of rearing density on thyroid and interrenal  
553 gland activity and plasma and hepatic metabolite levels in rainbow trout, *Salmo gairdneri*  
554 Richardson. *Journal of Fish Biology*, 27, 583–592. doi.org/10.1111/j.1095-  
555 8649.1985.tb03203.x

556

557 Li, D., Liu, Z. & Xie, C. (2012). Effects of rearing density on juvenile *Acipenser*  
558 *schrenckii* digestibility, feeding rate and growth. *Fish Physiology and Biochemistry*, 38,  
559 511–520. doi 10.1007/s10695-011-9531-y

560

561 Liu, C., Wang, J., Ma, Z., Li, T., Xing, W., Jiang, ....., Luo, L. (2018). Effects of totally  
562 replacing dietary fish oil by linseed oil or soybean oil on juvenile hybrid sturgeon,  
563 *Acipenser baerii* Brandt ♀ × *A. schrenckii* Brandt ♂. *Aquaculture Nutrition*, 24, 184–194.  
564 doi.org/10.1111/anu.12546

565

566 Lupatsch, I., Santos, G. A. & Schrama, J. W. (2010). Effect of stocking density and  
567 feeding level on energy expenditure and stress responsiveness in European sea bass  
568 *Dicentrarchus labrax*. *Aquaculture*, 298, 245–50.  
569 doi.org/10.1016/j.aquaculture.2009.11.007

570



- 571 Mambrini, M., Sanchez, M. P., Chevassus, B., Labbe, L., Quillet, E. & Boujard, T. (2004).  
572 Selection for growth increases feed intake and affects feeding behavior of brown trout.  
573 Livestock Production Science, 88, 85-98. doi.org/10.1016/j.livprodsci.2003.10.005  
574
- 575 Mohler, J. W., King, M. K. & Farrell, P. R. (2000). Growth and survival of first-feeding  
576 and fingerling Atlantic Sturgeon under culture conditions. *North American Journal of*  
577 *Aquaculture*, 62, 174-183. doi.org/10.1577/1548-  
578 8454(2000)062<0174:GASOFF>2.3.CO;2  
579
- 580 Mohseni, M., Pourkazemi, M., Kazemi, R., Norooz Foshkhomi, M. R., Mojazi Amiri, B.,  
581 Kaladkova, L. N. (2000). Study on the effects of stocking density of eggs and larvae on  
582 the survival and frequency of morphological deformities in Persian sturgeon, great  
583 sturgeon and stellate sturgeon. *Iranian Journal of Fisheries Sciences*, Volume 2, issue 1  
584
- 585 Montero, D., Izquierdo, M. S., Tort, L., Robaina, L., & Vergara, J. M. (1999). High  
586 stocking density produces crowding stress altering some physiological and biochemical  
587 parameters in gilthead sea bream, *Sparus aurata*, juveniles. *Fish Physiology and*  
588 *Biochemistry*, 20, 53–60. doi.org/10.1023/A:1007719928905  
589
- 590 Nathanailides, C., Stickland, N. C. & Albors, O. L. (2011). Influence of pre-hatch  
591 temperature on the development of muscle cellularity in post-hatch Atlantic salmon  
592 (*Salmo salar*). *Canadian Journal of Fisheries and Aquatic Sciences*, 52(4), 675-680.  
593 doi:10.1139/f95-068  
594

- 595 Papoutsoglou, S. E., Tziha, G., Vrettos, X. & Athanasiou, A. (1998). Effect of stocking  
596 density on behavior and growth rate of European sea bass (*Dicentrarchus labrax*)  
597 juveniles reared in a closed circulated system. *Aquacultural Engineering* 18(2), 135-144.  
598 doi:10.1016/S0144-8609(98)00027-2  
599
- 600 Pickering, A. D. & Duston, J. (1983). Administration of cortisol to brown trout, *Salmo*  
601 *trutta* L., and its effects on the susceptibility to *Saprolegnia* infection and furunculosis.  
602 *Journal of Fish Biology*, 23, 163-175. doi:10.1111/j.1095-8649.1983.tb02891.x  
603
- 603 Sargent, J., McEvoy, L., Estevez, A., Bell, G., Bell, M., Henderson, J. & Tocher, D.  
604 (1999). Recent developments in the fatty acids nutrition of fish. *Aquaculture* 177. 191-  
605 199 .doi: 10.1016/S0044-307 8486(99)00083-6  
606
- 607 Rafatnezhad, S. , Falahatkar, B. & Tolouei Gilani, M. H. (2008). Effects of stocking  
608 density on haematological parameters, growth and fin erosion of great sturgeon (*Huso*  
609 *huso*) juveniles. *Aquaculture Research*, 39, 1506-1513. doi:10.1111/j.1365-  
610 2109.2008.02020.x  
611
- 612 Rowlerson, A. & Vegetti, A. (2001). Cellular mechanism of post-embryonic muscle  
613 growth in aquaculture species. In: Johnston IA, editor. *Muscle development and growth*  
614 (pp 103-140). Academic Press, San Diego  
615
- 616 Sener, E., Yildiz, M., & Savaş, E. (2005). Effects of Dietary Lipids on Growth and Fatty  
617 Acid Composition in Russian Sturgeon (*Acipenser gueldenstaedtii*) Juveniles. *Turkish*  
618 *Journal of Veterinary and Animal Sciences*. 29, 1101-1107

619

620 Schram, E., Van der Heul, J. W., Kamstra, A. & Verdegem, M. C. J. (2006). Stocking  
621 density dependent growth of Dover sole (*Solea solea*). *Aquaculture*, 252, 339-47.  
622 doi.org/10.1016/j.aquaculture.2005.07.011

623

624 Schreck, C. B., Olla B. L. & Davis M. W. (1997). Behavioral responses to stress. In:  
625 Iwama, G. K., Pickering, A. D., Sumpter, J. P. & Schreck, C. B., editors, Cambridge:  
626 Cambridge University Press. *Fish stress and health in aquaculture* (62, 145-170)

627

628 Steinbacher, P., Haslett, J. R., Sanger, A. M. & Stoiber, W. (2006). Evolution of  
629 myogenesis in fish: a sturgeon view of the mechanisms of muscle development. *Anatomy  
630 and Embryology*, 211, 311-22. doi: 10.1007/s00429-006-0082-4

631

632 Yvette, S., Wunderink, S. E. & Silke, H. (2011). Chronic and acute stress responses in  
633 Senegalese sole (*Solea senegalensis*): the involvement of cortisol, CRH and CRHe BP.  
634 *General and Comparative Endocrinology*, 171, 203-10. doi:10.1016/j.ygcen.2011.01.010

635

636 Valente, L. M. P., Moutou, K. A., Conceicao, L. E. C., Engrola, S., Fernandes, J. M. O.  
637 & Johnston, I. A. (2013). What determines growth potential and juvenile quality of  
638 farmed fish species? *Reviews in Aquaculture*, 5 (Suppl. 1), S168–S193. doi:  
639 10.1111/raq.12020

640

641 Vasconi, M., Aidos, L., Di Giancamillo, A., Bellagamba, F., Domeneghini, C. & Moretti,  
642 V. (2018). Effect of temperature on fatty acid composition and development of unfed

643 Siberian sturgeon (*A. baerii*) larvae. Journal of Applied Ichthyology, 2018;00:1-7. doi:  
644 10.1111/jai.13725

645

646 Vijayan, M. M. & Leatherland, J. F. (1990). High stocking density affects cortisol  
647 secretion and tissue distribution in brook charr, *Salvelinus fontinalis*. Journal of  
648 Endocrinology, 124(2), 311-8

649

650 Wedemeyer, G.A., Meyer, F.P., & Smith, L. (1976). Environmental stress and fish  
651 diseases: Book 5. Snieszko, S. F. & Axelrod, H.R. (Eds) TFH Publications, Inc

652

653 Wuertz, S., Lutz, I., Gessner, J., Loeschau, P., Hogans, B., Kirschbaum, F.& Kloas, W.  
654 (2006). The influence of rearing density as environmental stressor on cortisol response  
655 of Shortnose sturgeon (*Acipenser brevirostrum*). Journal of Applied Ichthyology, 22  
656 (suppl 1), 269-263. doi.org/10.1111/j.1439-0426.2007.00966.x

657

658 Xu, R., Hung, S. S.& German, J. B. (1993). White sturgeon tissue fatty acid compositions  
659 are affected by dietary lipids. Journal of Nutrition, 123(10), 1685-92.  
660 doi:10.1093/jn/123.10.1685

661

662

### 663 **Figure legends**

664 **FIGURE 1** Water parameters from T0 until the end of the trial for each stocking density:

665 a) dissolved oxygen (mg/l); b) temperature (°C); c) pH.

666 **Figure 1** a) mortality (%); b) Larval growth expressed in mg of body weight; c) Larval  
667 length expressed in mm of body length for stocking density,; Error bars indicate the  
668 standard error of the mean for each treatment/stage of development; <sup>A,B</sup>Means with  
669 different superscripts differ significantly between stages of development ( $P < 0.05$ );  
670 <sup>a,b</sup>Means with different superscripts differ significantly between treatments ( $P < 0.05$ ).

671 **Figure 2.** Images of the three stocking densities at different time points – SEM  
672 representative figures at HD; a) at hatching; b) at schooling; c) yolk-sac full absorption.  
673 The figures have same scale bar as located in Figure 3a: 200  $\mu\text{m}$ ; asterisks, yolk-sac; thin  
674 arrows, tail; He, head. d-f) HE staining representative figures for hatching, schooling and  
675 yolk-sac full absorption, respectively, at MD. FM, fast fibres; SM, slow fibres. The  
676 figures have the same scale bar as located in Figure 3d: 200  $\mu\text{m}$ . g) quantitative  
677 representation of TMA: area expressed in  $\mu\text{m}^2$ ; n=9/group; h) quantitative representation  
678 of SMA: area expressed in  $\mu\text{m}^2$ ; n=9/group; i) quantitative representation of FMA: area  
679 expressed in  $\mu\text{m}^2$ ; n=9/group; <sup>A,B</sup>Means with different superscripts differ significantly  
680 between stages of development ( $P < 0.05$ ); <sup>a,b</sup>Means with different superscripts differ  
681 significantly between treatments ( $P < 0.05$ ).

682

683 **Figure 3.** HE-staining with representative images at MD for a,b) lamellae-shaped fibres  
684 (LF) and polygonal cells (PC); c) quantitative representation of lamellae-shaped fibres  
685 area; d) quantitative representation of and polygonal cells area. Area expressed in  $\mu\text{m}^2$ ;  
686 n=9/group; <sup>a,b</sup>Means with different superscripts differ significantly between treatments  
687 ( $P < 0.05$ ).

688 **Figure 4.** a,b,c) Representative images of PCNA-immunolocalization (arrowheads); for  
689 LD at different timepoints; d) quantitative representation of PCNA counts. Area

690 expressed in number/mm<sup>2</sup>; n=9/group; <sup>A,B</sup>Means with different superscripts differ  
691 significantly between stages of development (P< 0.05); <sup>a,b</sup>Means with different  
692 superscripts differ significantly between treatments (P< 0.05).