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

Sturgeon larval density

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ABSTRACT

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

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

1 INTRODUCTION

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

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

During the endogenous feeding stage, stress plays an important role and stressing rearing conditions may cause mortalities and impaired growth (Bates et al. 2014; Boucher et al. 2014). The environmental conditions experienced during early life stages can have an 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

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

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

2 MATERIALS AND METHODS

2.1 Fish larvae rearing and sampling

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

unit 24 hours after fertilization. Eggs were incubated at 16°C, after hatching temperature was then increased to 19°C: temperature was chosen taking into account a previous experimental trial based on three rearing temperature (Aidos et al., 2017). After hatching, which occurred 5 days after fertilization, larvae were subjected to three different rearing densities until the yolk-sac was completely absorbed. Rearing density was based on total volume of the tank: n = 48, 130, or 240 offspring/1.6-L rectangular, glass tank. Chosen densities are representative of currently utilized protocols in sturgeon production facilities. Fish were reared in a recirculating aquaculture unit, composed by a sand filter, a biological submerged filter and a UV lamp sterilization unit. Every density was tested in triplicate. Water quality parameters as oxygen, temperature and pH were measured every day by Hach HQ 30d Portable Meter, (Hach Lange, Dussendolf, Germany); O_2 were constantly close to the saturation value and pH values were within the range described for this species in this stage of development (Kamler, 2002). Dead larvae were removed every day, and mortality was estimated by dead larvae daily recording. Measurements of ammonia, nitrite and nitrate were carried at the beginning, at hatching and the end of the trial by Hach 2800 Portable Spectophotometer (Hach Lange, Dussendolf, Germany and were compliant with values recommended for Siberian sturgeon. Eggs and larvae were exposed to an artificial photoperiod regime of 12L:12D. Along the entire experimental period, hatched larvae utilized the nutrients of their yolk sac and were not fed any exogenous feed. Sampling time points were chosen according to important steps of Siberian sturgeon larvae behaviour development: hatching (T0), beginning of the schooling phase (T1) and complete yolk sac absorption phase (T2). For each sampling time-point, 3 larvae per experimental nursery (total n=9 larvae per 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),

iii) white muscle area (fast muscle cross-sectional area, FMA), iv) lamellae fibres area (LFA), v) polygonal fibres area (PFA), at the three analysed developmental stages: hatching (T0), schooling (T1) and yolk-sac full absorption (T2). On other transverse sections, immunostaining was performed to detect proliferating cell nuclear antigen (PCNA). The applied immunohistochemical procedure has been previously described in detail (Di Giancamillo et al., 2009). Briefly, endogenous peroxidase activity was blocked by incubating the sections in 3% H_2O_2 in PBS. Nonspecific binding sites were blocked by incubating the sections in normal mouse serum (Dakocytomation, Milan, Italy). Mouse monoclonal anti-PCNA (dilution 1:200, clone PC10, Sigma-Aldrich, Milan, Italy) antibodies were applied overnight at room temperature. The used primary antisera were diluted with a 0.05 M pH 7.4 Tris–HCl saline buffer (TBS: 0.05 M, pH 7.4, 0.55 M NaCl). After the treatment with the primary antibodies has been completed, the antigen–antibody complexes were detected with a peroxidase-conjugated polymer that carries secondary antibody molecules directed against mouse (EnVisionTM+, DakoCytomation, Glostrup, Denmark) applied for 120 min at room temperature. Peroxidase activity was then detected with diaminobenzidine (DAB, DakoCytomation, Glostrup, Denmark) as the substrate. Appropriate washing with TBS was performed between each step, and all incubations were carried out in a moist chamber. All sections were finally weakly counterstained with Mayer's haematoxylin, dehydrated, and permanently mounted. The specificity tests for the used antibodies were performed by incubating other sections in parallel with: i) TBS instead of the specific primary antibodies; ii) TBS instead of the secondary antibodies. The results of these controls were always negative (i.e. staining was abolished). Photomicrographs were taken with an Olympus BX51 microscope (Olympus, Milan, Italy) equipped with a digital camera, and final magnifications were calculated.

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

2.4 Fatty acids composition

The extraction and determination of total lipids was performed in whole larvae, according to the Folch (1957) method with chloroform:methanol (2:1). The preparation of fatty acid methyl esters (FAME) was performed according to Christie (2003). Briefly, the lipid sample (20 mg) was dissolved 10% methanolic hydrogen chloride (2 mL). A 1 mL solution of tricosanoic acid (1 mg/ml) in toluene was added as internal standard. After an incubation at 50 °C overnight, 2 mL of a 1M K_2CO_3 solution and 5 mL of 5% NaCl were added to each sample. The FAMEs were extracted with 2×2 mL of hexane and then evaporated under nitrogen. The sample was dissolved in 1 mL hexane and 1 µL sample was injected into the gas-chromatograph, in split mode (split ratio 1:100). Fatty acid analysis was carried out on an Agilent gas-chromatograph (Model 6890 Series GC) fitted with an automatic sampler (Model 7683) and FID detector. The carrier gas was helium with a flow rate of 1.0 ml/min and an inlet pressure of 16.9 psi. A HP-Innowax fused silica capillary column (30m×0.25mm I.D., 0.25 µm film thickness; Agilent Technologies) was used to separate FAME fatty acid methyl esters. The oven temperature program for separation was from 100 to 180 °C at 3 °C min-1, then from 180 to 250 °C at 2.5 °C min-1 and held for 10 min. Carrier gas was helium at 1.0 mL min−1, inlet pressure 16.9 psi. Fatty acids were identified by comparison of retention times with standard 37 Fatty acids methyl esters (FAME) mixture in dichloromethane and standard

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

2.5 Statistical analysis

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

3 RESULTS

3.1 Development, survival and growth

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

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

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

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

[Figure 1]

3.2 SEM

SEM morphological analyses revealed a correct morphological development at all densities from hatching to larval schooling and yolk-sac absorption, with no morphological deformities (Figure2a-c). Briefly at hatching larvae presented an evident yolk-sac, which was reduced at schooling and completely absent at the end of the trial (white asterisks, Figure 2a, 2b, 2c respectively). No damaged fins, looped tail or change in the form of yolk-sac was observed.

3.3 Histological and immunohistochemical analyses

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

[Figure 2]

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

[Figure 3]

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

[Figure 4]

3.4 FATTY ACIDS

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

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

4 DISCUSSION

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

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

Regarding the growth parameters, some studies on lake sturgeon (*Acipenser fulvescens*) 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,

359 1999), salmon (*Salmo salar*; Nathanailides et al., 1995) and in other marine species
360 (*Scophthalmus maximus*; *Dicentrarcus labrax; Cyprinus carpio*), as reviewed by Valente
361 et al. (2013). A substantial difference between sturgeons and teleosts is that in the latter
362 the white muscle is composed entirely of cylindrical cells from hatching to the adult life.
363 In sturgeons, instead, compared to teleost fishes, there is the fusion of myoblasts derived
364 from somite, leading to the formation of multinucleated muscle lamellae and later on, in
365 polygonal cells (Steinbacher et al., 2006). In our study we observed a change in the three
366 physiological phases considered, a change that occurs in all the densities considered: from
367 hatching to schooling and full yolk-sac absorption stage, the fast fibres undergo a
368 phenomenon of hypertrophy / hyperplasia which increases its number and size. The
369 muscular component observed with HE staining shows only an increase in the size of the
370 myotomes already morphologically observed and then verified with histometry (see
371 below); as for the stages of development, there were no qualitative nor morphological
372 differences among the three tested densities. According to Rowlerson & Vegetti (2001),
373 a widely used method for measuring muscle growth involves the cross-sectional areas,
374 which provide an index of hypertrophic or hyperplastic growth. To quantify these
375 qualitative changes, we used histometry. Difference in terms of muscle development has
376 been identified in the definitive polygonal cells and in the primary lamellae: HD has more
377 definitive polygonal cells than LD and MD and, on the contrary, less lamellae, that are
378 primitive fibres. This can indicate an increase in the conversion / differentiation of the
379 primitive lamellae in definitive polygonal cells, which is confirmed by a reduction in both
380 length and weight of the HD group. These preliminary results allow us to suggest that
381 larvae reared at low and medium densities are similar in terms of muscle development,
382 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 Rowlerson & 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 Δ-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

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

5 CONCLUSION

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

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

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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 (ºC); c) pH.

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

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

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

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

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