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Advances in the early larval stages of Siberian sturgeon: muscle development and structure under different rearing conditions

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I dedicate this Thesis to Giuseppe, Lori, Fede and Bea

Abstract

Fish larval stages are particularly sensitive to environmental conditions, as these can severely affect survival and potential growth. In fish species destined for production and commercialisation, a suitable early environment is of great importance, for what concerns growth efficiency and quality at harvest. Temperature and stocking density are among the most important factors affecting the early stages of development, regarding somatic and muscle growth of fish. Moreover, environmental enrichment is considered to improve biological functioning of captive animals, including fish, by improving their psychological and behavioral needs.

Siberian sturgeon (*Acipenser baerii*) is a species at risk of extinction and, therefore, production of this species in aquaculture is of vital importance, not only to provide the market with caviar and meat, but also for repopulation purposes. Taking into account that little is known regarding the impact of environmental conditions in early life stages of the Siberian sturgeon, the aim of this Thesis is to provide new knowledge on the influence on muscle growth and development during the endogenous feeding phase. This was performed by addressing morphological, physiological and molecular aspects of muscle growth structure and development. This Thesis may contribute to identify and select the most suitable environmental conditions to improve Siberian sturgeon larval rearing in aquaculture production.

Three trials have been performed, one in each year of my PhD, where 1) rearing temperature, 2) stocking density and 3) environmental enrichment were examined in important timepoints of Siberian sturgeon larvae development: hatching, schooling and complete yolk-sac absorption stage.

In **Chapter 1,** is presented a general overview of Siberian sturgeon biology and development, a brief description of the muscle morphology and of the methods used to study muscle growth. Following, the types of sturgeon muscle growth are described, as well as the mechanisms involved in muscle growth regulation. Finally, the environmental effects on growth are presented along with a short overview of the plasticity of the myogenic phenotype.

In **Chapters 2**, **3** and **4**, the effects of three different rearing temperatures (16, 19 or 21 °C) applied from hatching until the yolk-sac full absorption were assessed. Siberian sturgeon fertilized eggs were all incubated at 16°C and newly-hatched larvae were then subjected to three different temperatures throughout the endogenous feeding phase. All the three experimental

rearing temperatures were congruent with a correct development of farmed *A. baerii* (**Chapter 2**, **3** and **4**), but a rearing temperature of 22°C lead to a higher developmental rate, which could be advantageous in commercial hatcheries, as it shortens the endogenous feeding phase and allows to feed the larvae sooner with exogenous feed. In **Chapter 2**, histometrical, histochemical and immunohistochemical analyses were performed in order to characterize muscle growth (total muscle area, TMA; slow muscle area, SMA; fast muscle area, FMA), development (anti proliferating cell nuclear antigen –PCNA) as well as stress status by specific stress biomarkers (heat shock protein 70 or 90; HSP70 or HSP90). Histometry revealed that both TMA and FMA were larger in the schooling stage at 19°C while no differences were observed in the SMA at any of the tested rearing temperatures. PCNA quantification revealed a significantly higher number of proliferating cells in the yolk-sac absorption phase at 22°C than at 16°C. HSP90 immunopositivity seems to be particularly evident at 19°C. HPS70-immunopositivity was never observed in the developing lateral muscle. It was possible to conclude from this study that a temperature of 19°C could be taken into account by commercial hatcheries, as it supported larger size in developing larvae and suggesting a higher growth potential. The aim of **Chapter 3** was to investigate the fatty acid composition of yolk-stage Siberian sturgeon larvae reared at three different temperatures. The fatty acid composition of larvae was affected by temperature. The larvae reared at lower temperatures (16°C) showed adaptive behavior compared to the larvae reared at two higher temperatures (19°C and 22°C), conserving a greater quantity of polyunsaturated fatty acids and consuming saturated fatty acids for energetic purposes. **Chapter 3** study suggests that at a lower temperature sturgeon shows an adaptation to the variation of the temperature, carried out with the purpose to conserve the fatty acids that guarantee a greater fluidity to the cell membranes at the lower temperatures.

The aim of **Chapter 4** was to assess growth, muscle development and stress status in Siberian sturgeon larvae at different rearing temperatures. Immunofluorescence localization of myogenin and Igf1, the expression of genes involved in muscle development and growth (*myog* and *Igf-1*) and in the stress status (*Hsp70*, *Hsp90α*, *Hsp90β*) and iv) were performed as well as whole body cortisol. Both at the schooling stage and at the end of the trial, there were no significant differences regarding larval weight among temperatures. Both at schooling and at the end of the trial, larvae reared at 16°C showed a lower level of cortisol than those reared at 19°C or 22°C. Igf-1 immunopositivity was particularly evident in red muscle at the schooling stage at all temperatures and it was similarly expressed in white muscle at both schooling and yolk-sac absorption stage at all tested temperatures. Myogenin immunopositive cells were detected in

the cytoplasm of undifferentiated cells at all stages and at all temperatures considered. At schooling, all of the analysed genes resulted significantly more expressed in larvae reared at 16°C compared to the larvae reared in the other two rearing temperatures (*Hsp70*, *hsp90α*, *hsp90β, myog*, *Igf1).* No differences were found in the expression of the analysed genes between larvae reared at 19°C and 22°C. Conversely, at the end of the trial, no significant differences were found in the expression of all genes among rearing temperatures. From **Chapters 2**, **3** and **4**, it would appear that, taking into account all analysed variables, it would seem that a temperature of ranging from 16 to 19°C would be a good compromise between growth potential and stress status.

In **Chapters 5** and **6**, the effects of different rearing stocking densities were examined during the pre-larval phase. After hatching, Siberian sturgeon newly-hatched larvae were subjected to three different stocking densities (30, 80 or 150 larvae/litre) until the yolk-sac was fully absorbed. In **Chapter 5,** muscle growth and development were evaluated by assessing body weight and length, muscle histometrical analyses, qualitative morphological study analyses and fatty acid profile. At the end of the trial, larvae reared at the lower density were heavier and longer and presented a higher proliferation rate of the muscle fibres. Total muscle area was lower for larvae reared at the highest density at schooling, which, from a morphological point of view, showed an acceleration in muscle development that may be detrimental at a medium-long term. 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. Results of **Chapter 5** suggest that lower densities appear to be more suitable to rear Siberian sturgeon in this particular stage of development. In **Chapter 6**, growth (Specific Growth Rate, SGR and Condition Factor, K), muscle development and stress status were evaluated, through immunofluorescence, whole body cortisol and Real-Time PCR. SGR was significantly improved at the yolk-sac absorption stage for larvae reared at the lower density, while no differences were found regarding K. Alpha skeletal muscle actin (ACTA1) immunofluorescence was detected in the cytoplasm of muscle cells in all developmental stages and no morphological differences among densities were detected regarding muscle structure at all stages. The levels of relative expression of different genes involved in the growth process (*igf1* and *igf2*), in the myogenesis process (*myog*) and in the regulation of cellular stress (*glut1*, *glut2* and *hsp70*) were analysed. All the genes examined have shown an up-regulation in both development stages at all the rearing densities considered, with the exception of the *myog* gene which was, instead, always down-regulated. This down-regulation is significantly greater in larvae reared in high-density in

the phase of the complete absorption of the yolk sac. Cortisol levels did not differ significantly, both in time and across densities. To conclude, taking into account the SGR and the gene expression results of **Chapter 6**, we suggest that lower densities are used in these stages of development, as these showed a higher growth potential and lower stress levels.

From **Chapter 5** and **6**, it is evident that the lowest density tested was the most favourable one but, it is not applicable in a commercial hatchery as it is space consuming and, thus, not economically feasible. Therefore, we would suggest that an intermediate density could be a good compromise from a commercial hatchery point of view.

In **Chapter 7** the morpho-functional and behavioural responses of early life phases in Siberian sturgeon (*Acipenser baerii*) towards two types of substrate (Bioballs type 1: ϕ35mm; Bioballs type 2: ϕ38mm BB1 and BB2 respectively) vs. no substrate (CTR), were examined, from hatching and during the endogenous feeding period. Number of larvae swimming in the water column were analysed, larval growth in terms of weight and length and histometrical analyses were also performed in order to evaluate muscle development. Larvae reared with BB1 substrate were present in a lower number in the water column, were heavier and longer at the end of the trial, and showed larger areas of total muscle area, slow muscle area and fast muscle area. Moreover, larvae reared with no substrate showed an acceleration of muscle differentiation, which can have negative consequences on its growth potential. It would seem more favourable to provide a substrate rather than a bare bottom for Siberian sturgeon in these early phases of development, in particular a substrate with characteristics similar to those of BB1.

This Thesis ends with a general discussion in **Chapter 8**. The effects of temperature, stocking density and environmental enrichment during larval phases in relation to muscle growth and development are discussed and the main conclusions of the present work and future research direction are also presented. Overall, the present Thesis shows that Siberian sturgeon, in the early stages of development, does not appear to be particularly sensitive to temperature variations, even if the results may suggest a range temperature of 16-19°C. The discourse concerning density and environmental enrichment is different: the obtained results suggest a medium rearing density and the presence of a substrate in the tanks.

Riassunto

Gli stadi larvali dei pesci sono particolarmente sensibili alle condizioni ambientali, poiché possono influenzarne la sopravvivenza e il potenziale di crescita. In particolare, nelle specie ittiche destinate alla produzione e alla commercializzazione, le condizioni ambientali adeguate sono di notevole rilevanza per quanto riguarda l'efficienza della crescita e la qualità del prodotto finale. La temperatura e la densità di allevamento sono tra i fattori più importanti per quello che riguarda le prime fasi dello sviluppo, la crescita somatica e muscolare dei pesci. Inoltre, l'arricchimento ambientale, in molto casi, è in grado di migliorare il funzionamento biologico degli animali in cattività, compresi i pesci, ottimizzando i loro bisogni psicologici e comportamentali.

Lo storione siberiano (*Acipenser baerii*) è una specie a rischio di estinzione e, pertanto, la sua produzione in acquacoltura è di importanza vitale, non solo per fornire caviale e carne, ma anche per scopi di ripopolamento. Tenendo presente che si sa poco sull'impatto delle condizioni ambientali nelle fasi iniziali della vita dello storione siberiano, lo scopo di questa tesi è quello di fornire nuove conoscenze sull'influenza dell'ambiente circostante sulla crescita e sullo sviluppo muscolare durante la fase di alimentazione endogena. Ciò è stato effettuato affrontando in maniera multidisciplinare valutando aspetti morfologici, fisiologici e molecolari dello sviluppo e della crescita muscolare. Questa tesi può contribuire a identificare e selezionare le condizioni ambientali più idonee per migliorare l'allevamento larvale dello storione siberiano nella produzione in acquacoltura.

Sono stati effettuati tre studi, uno in ogni anno del mio dottorato di ricerca, in cui 1) temperatura di allevamento, 2) densità di allevamento e 3) arricchimento ambientale sono stati esaminati considerando delle tempistiche precise dello sviluppo delle larve di storione siberiano: schiusa, raggruppamento in schools (schooling) e completo riassorbimento del sacco vitellino.

Nel **Capitolo 1** viene presentata una panoramica generale della biologia e dello sviluppo dello storione siberiano, una breve descrizione della morfologia muscolare e dei metodi utilizzati per studiarne la crescita. Successivamente, vengono descritti i tipi differenti di crescita muscolare dello storione ed i meccanismi coinvolti nella regolazione della crescita stessa. Infine, gli effetti ambientali sulla crescita sono presentati insieme a una breve panoramica della plasticità del fenotipo miogenico.

Nei **Capitoli 2**, **3** e **4**, sono stati valutati gli effetti di tre diverse temperature di allevamento (16, 19 o 21°C) applicate partendo dalla schiusa fino completo riassorbimento del sacco vitellino. Le uova fecondate di storione siberiano sono state incubate a 16°C e le larve, appena schiuse, sono state sottoposte a tre diverse temperature durante tutta la fase di alimentazione endogena. Tutte e tre le temperature di allevamento sperimentali sono risultate congruenti con un corretto sviluppo di *A. baerii* (**Capitolo 2**, **3** e **4**), ma la temperatura di 22°C porta ad un tasso di sviluppo più veloce, che potrebbe essere vantaggioso nelle hatchery commerciali, dal momento che riduce i tempi di alimentazione endogena e permette di nutrire prima le larve con alimento artificiale. Nel **Capitolo 2** sono state eseguite analisi istometriche, istochimiche e immunoistochimiche per caratterizzare la crescita muscolare (area muscolare totale, TMA; area muscolare lenta, SMA; area muscolare veloce, FMA), sviluppo (anti-proliferating nuclear antigen, anti-PCNA) e lo stato di stress con specifici biomarcatori (heat shock protein 70 o 90; HSP70 o HSP90). L'istometria ha rivelato che sia TMA che FMA erano più grandi nella fase di schooling a 19°C, mentre non si sono osservate differenze nella SMA in nessuna delle temperature di allevamento testate. La quantificazione del PCNA ha rivelato un numero significativamente più elevato di cellule proliferanti nella fase di riassorbimento del sacco vitellino a 22°C rispetto a 16°C. L'immunopositività di HSP90 sembra essere particolarmente evidente a 19°C. L'immunopositività dell'HPS70 non è mai stata osservata nel muscolo laterale in sviluppo. È possibile concludere, da questo studio, che una temperatura di 19°C potrebbe essere presa in considerazione dalle hatcheries commerciali, in quanto ha portato a dimensioni maggiori nelle larve in via di sviluppo e ad un maggiore potenziale di crescita. Lo scopo del **Capitolo 3** era quello di studiare la composizione degli acidi grassi delle larve di storione siberiano allevato a tre diverse temperature, fino al riassorbimento del sacco vitellino.

Le larve allevate alle temperature minori (16°C) hanno mostrato un comportamento adattivo rispetto alle larve allevate alle due temperature maggiori (19°C e 22°C), conservando una maggiore quantità di acidi grassi polinsaturi e consumando a scopo energetico acidi grassi saturi.

Lo studio del **Capitolo 3** suggerisce che ad una temperatura inferiore lo storione presenta un adattamento legato alla variazione della temperatura, effettuato con lo scopo di conservare gli acidi grassi che garantiscono una maggiore fluidità alle membrane cellulari alle temperature minori.

Lo scopo del **Capitolo 4** è stato quello di valutare la crescita, lo sviluppo muscolare e lo stato di stress nelle larve di storione siberiano a diverse temeprature di allevamento. La localizzazione in immunofluorescenza della miogenina e Igf1, l'espressione dei geni coinvolti nello sviluppo e

nella crescita dei muscoli (myog e Igf-1) e nello stato di stress (Hsp70, Hsp90α, Hsp90β) sono stati eseguiti così come il livello di cortisolo di tutta la larva. Sia allo schooling che alla fine della prova, non ci sono state differenze significative riguardo il peso larvale tra le temperature testate e le larve allevate a 16°C hanno mostrato un livello inferiore di cortisolo rispetto a quelle allevate a 19°C o a 22°C. L'immunopositività di Igf-1 era particolarmente evidente nel muscolo rosso nella fase di schooling a tutte le temperature ed è stata espressa in modo simile nel muscolo bianco sia allo schooling che alla fase di completo assorbimento del sacco vitellino a tutte le temperature testate. Le cellule immunopositive alla miogenina sono state osservate nel citoplasma delle cellule indifferenziate in tutte le fasi e a tutte le temperature testate. Allo schooling, tutti i geni analizzati sono risultati significativamente più espressi nelle larve allevate a 16°C rispetto alle larve allevate nelle altre due temperature di allevamento (*hsp70, hsp90α, hsp90β, myog, Igf1*). Non sono state riscontrate differenze nell'espressione dei geni analizzati tra larve allevate a 19°C e 22°C. Al contrario, alla fine dello studio, non sono state riscontrate differenze significative nell'espressione di tutti i geni tra le temperature di allevamento. Dai **Capitoli 2**, **3** e **4**, sembrerebbe che, prendendo in considerazione tutte le variabili analizzate, una temperatura di 19°C sia un buon compromesso tra potenziale di crescita e stato di stress.

Nei **Capitoli 5** e **6**, gli effetti di diverse densità di allevamento sono stati esaminati durante la fase pre-larvale. Dopo la schiusa, le larve di storione siberiano sono state sottoposte a tre diverse densità di allevamento (30, 80 o 150 larve / litro) fino al completo riassorbimento del sacco vitellino. Nel **Capitolo 5**, la crescita e lo sviluppo del muscolo sono stati valutati, tramite peso corporeo e lunghezza, analisi istometriche del muscolo, analisi qualitative dello studio morfologico e profilo degli acidi grassi. Alla fine dello studio, le larve allevate alla densità inferiore erano più pesanti e più lunghe e presentavano un più alto tasso di proliferazione delle fibre muscolari. L'area muscolare totale era più bassa nelle larve allevate alla massima densità allo schooling e, da un punto di vista morfologico, mostrava un'accelerazione nello sviluppo muscolare che può essere sfavorevole a medio-lungo termine. Il profilo degli acidi grassi non ha rivelato differenze tra le densità mentre, durante lo sviluppo, c'era un consumo selettivo: risparmio o aumento di acidi grassi essenziali a scapito dei loro precursori. I risultati del **Capitolo 5** suggeriscono che densità più basse sembrano essere più adatte a sostenere lo storione siberiano in questa particolare fase di sviluppo. Nel **Capitolo 6** sono stati valutati la crescita (Specific Growth Rate, SGR e Condition Factor, K), lo sviluppo muscolare e lo stato di stress, attraverso immunofluorescenza, cortisolo corporeo e Real-Time PCR. Il SGR è stata significativamente migliorato allo stadio di riassorbimento del sacco vitellino per le larve

allevate alla densità più bassa, mentre non sono state riscontrate differenze per quanto riguarda l'actina del muscolo scheletrico (ACTA1). L'immunofluorescenza è stata rilevata nel citoplasma delle cellule muscolari in tutte le fasi dello sviluppo e non ci sono state le differenze tra le densità per quanto riguarda la struttura muscolare in tutte le fasi di sviluppo. Sono stati analizzati i livelli di espressione di diversi geni coinvolti nel processo di crescita (*igf1* e *igf2*), nel processo di miogenesi (*myog*) e nella regolazione dello stress cellulare (*glut1, glut2* e *hsp70*). Tutti i geni esaminati hanno mostrato una up-regulation in entrambi gli stadi di sviluppo rispetto alla schiusa a tutte le densità di allevamento considerate, ad eccezione del gene *myog* che era, invece, sempre sotto-regolato. Questa sotto-regolazione è significativamente maggiore nelle larve allevate ad alta densità nella fase di riassorbimento completo del sacco vitellino. I livelli di cortisolo non hanno mostrato differenze significative, sia nel tempo che tra densità. Per concludere, tenendo conto del SGR e dei risultati dell'espressione genica del **Capitolo 6**, suggeriamo che in queste fasi di sviluppo si possano utilizzare basse densità, poiché queste hanno mostrato un maggiore potenziale di crescita e livelli di stress inferiori.

Dai **Capitoli 5 e 6**, si evidenzia come la densità più bassa testata è la più favorevole, ma non è applicabile in una hatchery commerciale per una questione di spazio e, di conseguenza, non è economicamente concretizzabile. Pertanto, suggeriamo che una densità intermedia possa essere un buon compromesso dal punto di vista dell'allevatore.

Nel **Capitolo 7** sono stati studiate le risposte morfo-funzionali e comportamentali delle prime fasi della vita nello storione siberiano verso due tipi di substrato (Bioballs tipo 1: ϕ35mm; Bioballs tipo 2: ϕ38mm BB1 e BB2 rispettivamente) *versus* nessun tipo di substrato (CTR), a partire dalla schiusa fino alla fine del periodo di alimentazione endogena. Sono stati analizzati il numero di larve che nuotavano nella colonna d'acqua durante tutta la prova, sono state eseguite anche analisi di crescita in termini di peso e lunghezza e analisi istologiche per valutare lo sviluppo muscolare. Le larve allevate con substrato BB1 erano presenti in un numero inferiore nella colonna d'acqua, erano più pesanti e più lunghe alla fine della prova e mostravano anche misure istometriche più ampie (area muscolare totale, area muscolare lenta e area muscolare). Inoltre, le larve allevate senza substrato hanno mostrato un'accelerazione del differenziamento muscolare, che può avere conseguenze negative sul suo potenziale di crescita. Questi dati preliminari suggeriscono un effetto positivo della presenza di substrato piuttosto che un fondo vuoto per lo storione siberiano nelle prime fasi di sviluppo, in particolare un substrato con caratteristiche simili a quelle di BB1.

Questa Tesi termina con una conclusione generale nel **Capitolo 8**. Vengono discussi gli effetti della temperatura, della densità e dell'arricchimento ambientale durante le fasi larvali in relazione alla crescita e allo sviluppo muscolare e vengono presentate le principali conclusioni del presente lavoro e le considerazioni per il futuro della ricerca. Complessivamente, la presente Tesi mostra che per lo storione siberiano nelle prime fasi di sviluppo, non risulta essere particolarmente sensibile a variazioni di temperatura, anche se i risultati possono suggerire una temperatura di 19 ° C. Diverso il discorso relativo a densità e arricchimento ambientale: i risultati suggeriscono una densità di allevamento media e la presenza di un substrato nelle vasche.

Chapter 1

General Introduction

1. General Introduction

1.1. General aspects of Siberian sturgeon biology and production

Sturgeons are frequently named "living fossils" as they belong to an ancient order of bony fishes, existing since the Jurassic Period, 201-145 million years ago. The value of sturgeon products increased since the middle ages from a commercial point of view in many regions. There are several historical facts that refer to sturgeons, such as its representation on coins in Karthago (actual Tunisia), in 600 b.c.; Aristotle mentioning the use of sturgeon swim bladders for wine clearing and Ovid naming the sturgeons to be “the noble fish” in the “Halieuticon”.

Siberian sturgeon (*Acipenser baerii* Brandt, 1869) is one of the 25 species of the Acipenseridae family and its first description was based on specimens caught in the Ob and Lena rivers (Brand, 1869). The Siberian sturgeon has the following taxonomic classification:

- Super-class: Osteychthyes
- Class: Actinopterygii
- Sub-class: Chondrostei
- Order: Acipenseriformes
- Family: Acipenseridae
- Species: Acipenser baerii

Living sturgeons show several distinctive primitive morphological characteristics that are also evident in fossil specimens: most of the endocranium is cartilaginous, the notochord is present in the adult stage, heterocercal tail and body provided with five rows of bony scutes with small star-like scutes between the main ones. *A. baerii* shows extended snout and four barbels in front of the mouth (Jones et al. 1978). The back is light grey to dark brown and the belly colour varies from white to light yellow. Characteristics such as ventral mouth, rostral chemosensory barbels and flattened body, are related to their benthic behaviour (Figure 1.1)

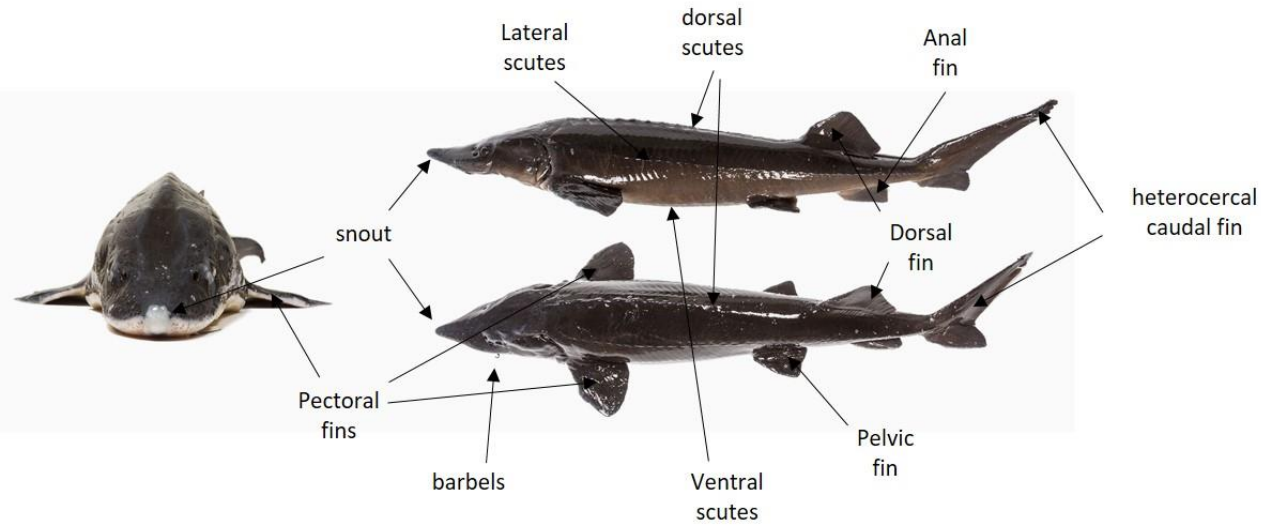

Figure 1.1. External anatomy of *Acipenser baerii* (Brandt). Adapted from original picture hosted at: https://www.acipensersrl.com/acipenser-species-sturgeon.html

Siberian sturgeon is present in all hydrographic basins from the Ob-Irtych basin to the Kolyma; it can be, therefore, found in the Ienisseï, Khatanga, Lena and Indigirka basins. Populations are also present in the hydrographic system of Lake Baïkal (FAO). This benthic species can be found in deep and shallow parts of rivers, with moderate to swift current, usually at depths of 1 to 8 m (Ruban, 2005). Adults live essentially in freshwater although it is frequent that some individuals occur in estuaries.

Sturgeons can live in a wide range of temperatures, from 1°C to 26°C and is fairly resistant to low O_2 content. The maximum size reached in nature is 2m of total length and 210 kg, but they usually do not exceed 65 kg (Sokolov and Vasilev, 1989). In a natural environment, males reach sexual maturity at 9-15 years old and females at 16-20 years. However, in a water recirculation system, sexual maturity can be anticipated to 5 years (Ruban, 2005). Spawning happens in the summer and its periodicity is of 3-5 years in females and 2-3 years in males. Spawning occurs in strong-current habitats in the main stream of large and deep rivers with stone or gravel substrate (Kottelat and Freyhof, 2007). Caviar (not fertilized ovocites) can be more than 10% of the body weight of a mature female. Incubation lasts about 16 days (at 10-15°) and larval development lasts about 20 days (at 18°) (www.fishbase.de).

Siberian sturgeon is carnivorous and, in the wild, feeds on crustaceans, insect larvae, molluscs and worms (Ruban, 2005).

Over the past 30 years the over-exploitation of natural sturgeon stocks for caviar production, as well as a serious river fragmentation and a habitat deterioration due to pollution, have led to a

severe population falloff. Therefore, in 1997 the International Union for Conservation of Nature (IUCN) added all of the world-wide commercially utilized sturgeon species to the Annex II of the CITES regulations. As a consequence, trading quotas have been internationally agreed in order to protect these highly endangered species. The Acipenseriformes have the highest ratio of Critically Endangered species amid all of the most threatened animal orders (17 CR – critically endangered; 2 EN – endangered; 4 VU – vulnerable; 2 NT – near threatened; 2 LC – least concern). Following the continuing high demand from the caviar markets, there was a big interest on sturgeon farming, so that the number of farms increased considerably during the past 30 years (Bronzi and Rosenthal, 2014). In the past ten years the production of caviar from fished sturgeons is almost zero (also according to CITES quotas), while the caviar produced from farmed sturgeons has increased, contributing significantly to the global markets.

The farming of *A. baerii* began in the former USSR in the 1970s. At the same time, the first individuals (from parents that had originated in the Lena River) arrived in France, to be used as a biological model. Since then, the diffusion of this species had a sharp increase and, together with the Russian Federation, Siberian sturgeon is known to be present in Europe (Belgium, France, Italy, Germany, Hungary, Poland, and Spain), America (United States, Uruguay), and Asia (China). In Europe, Siberian sturgeon is the most widespread and common species utilized in sturgeon aquaculture. The global aquaculture production of *A. baerii* in 2016 was of 218t with a value of 1934 USD, and the main producer countries were Belarus, followed by Bulgaria and United Arab Emirates (FAO Yearbook of Fishery and Aquaculture, 2016).

The growing market demand for sturgeon products, have increased the need for the development of advanced hatchery technology from commercial farms, for the rearing of this species larvae. Larvae rearing is considered one of the most difficult stages in the intensive farming of sturgeons. Once the larva hatches, its survival depends both on the culture system and management but also on the nutritional input from artificial feeds (Conte et al., 1988).

At a rearing temperature of 18◦C, newly hatched Siberian sturgeon larvae (also called free-embryos or pre-larvae) range from 10.4 to 12.6mm total length (TL) (Sokolov and Vasil'ev 1989; Gisbert et al. 2000), and show non- mature digestive system and gill clefts and sense organs are poorly differentiated (Dettlaff et al., 1993). At this point, larvae are not able to feed and consume the reserves contained in its yolk-sac. Until 3 days post hatching (dph), free embryos show a positive phototaxis, swim-up and drift behaviour, inability to swim for a long time in a certain direction and prefer white substrates. (Gisbert and Ruban, 2003).

At 4 dph larvae measure 14.8–15.6mmTL and there is a significant change in behaviour: free-embryos pass from pelagic to benthic. This is a consequence of the development of the pectoral fins, eye differentiation, presence of rudimentary sensorial structures and the substantial reduction of the yolk sac volume (Gisbert, 1999). At 7-8dph larvae reach 17.9–19.5mmTL and exhibit schooling behaviour: they look for covered bottoms and aggregate in shoals. (Gisbert et al., 1999). From this moment on, the further proliferation of taste buds on barbels and the development of extra-oral taste, allows free embryos to sense feed chemical stimuli. Free-embryos are now ready to assume exogenous feed (Devitsina and Kazhlayev, 1993).

1.2. Muscle morphology and fibre types in fish

The swimming muscle of fish accounts for about 40 – 60% of the total body mass (Bone, 1978). The muscle tissue is organized in myotomes located on either side of the body, which are separated from each other by connective tissue sheets, called myosepta. These myosepta consist on attachment sites for the muscle fibres and have an important role in force transmission: the force of contraction of myotomal muscle fibres is transmitted, via tendons, to the axial skeleton and caudal fin, resulting in body waving and forward propulsion (Webb, 1984). The myosepta form a sequence of overlaid cones that are arranged along axes parallel to the midline. There is a phylogenetic increase in complexity in shape, from the sole v-shape in amphioxus through the narrow w-shape of the agnatha, to the deep w-shape of gnathostomes (Bone, 1978) (Figure 1.2). Each myotome is formed by a superficial, wedge-shaped region that lies directly underneath the lateral line, where the muscle fibres are parallel to the body axis, and by a deeper part where the muscle fibres are organized in a helical manner, forming angles of up to 40° (Sänger and Stoiber, 2001). This kind of arrangement of the muscle fibres is thought to allow analogous degrees of sarcomere shortening at different body flexures (van Raamsdonk et al., 1980; Rome et al., 1988).

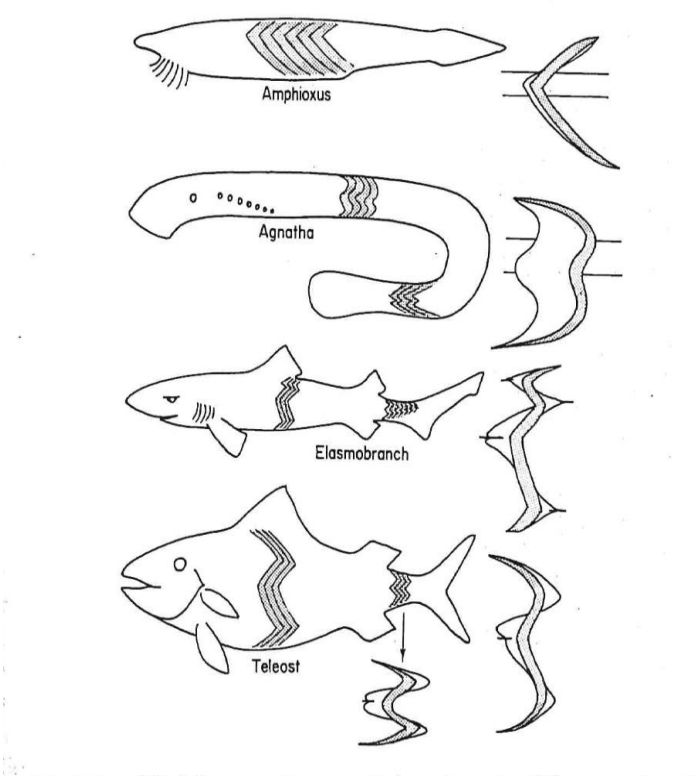

Figure 1.2. Schematic simplified structure of myotomal shape in several fish groups. At the right, an enlarged view of the respective midtrunk myotome. From Bone, 1978.

In teleosts a collagenous horizontal septum divides the axial muscle into dorsal (epaxial) and ventral (hypaxial) (Bone, 1978). However, in sturgeons, this horizontal septum is absent in the early larval stages and arises much later in development at the mid-notochord dorso-ventral separation. The horizontal septum is made of connective tissue and plays an important role in the biomechanics of adult teleost movement (Westneat and Wainwright, 2001). It is commonly considered as an evolutionary conserved characteristic of gnathostome fish (Liem et al. 2001). Only in 2003 Gemballa and collaborators confirmed the presence of a biomechanically functional horizontal septum in adult sturgeon, which is a unifying system of a force-transmitting system including red muscles.

A single muscle fibre is surrounded by a cell membrane or sarcolemma. There is a network of several proteins, physically connected to the internal myofilament structure, that are associated with the sarcolemma. Single muscle fibres are composed mostly (about 80 %) of protein (contractile, regulatory, cytoskeletal) and sarcoplasm (about 8 %), if not taking into account the water content. When together, the myofilaments form sarcomeres, which are the elementary contractile units of the skeletal muscle and that are connected end-to-end. Approximately 70–80 % of the total protein content of a single fibre is made of the myofilaments (proteins) actin and myosin. When a nerve impulse reaches a skeletal muscle fibre, it origins a change in the electric

potential across the sarcolemma. Skeletal muscle fibres are able to convert this electric signal into depolarization, through an increase of cytosolic Ca2+ from the sarcoplasmic reticulum, which allows contraction. Myosin is the main molecular motor that interacts with the filaments of actin and couples the hydrolysis of ATP. It consists of two heavy chains and a couple of light chains. Each heavy chain is composed of a structural α-helical rod and a globular head. In the globular head region is where the light chain and actin bind and is where ATPase activity takes place in many vertebrates as reviewed by Sellers (2000).

Mammalian muscle is characterised by a mosaic outline of fibre types. On the contrary, the axial muscle of fish shows zones of fibre types, which are anatomically different. The axial muscles are arranged in layers of slow-red and fast-white fibres. The majority of the muscle mass consists on fast-white fibres covered by a thin layer of slow-red fibres (Figure 1.3) (Blaxter, 1987). Between these two, there is a pink or intermediate layer of muscle fibres (Dal Pai-Silva et al., 1996), also present in adult sturgeons (Radaelli et al., 1999). Muscle colour is related with the degree of vascularization of each type of muscle. Slow-red muscle is dark because of its high myoglobin contents, the mitochondrial density, and the degree of capillarization; fast-white muscle presents the opposite features of the slow-red fibres, while intermediate-pink muscle possesses intermediate characteristics. Muscle fibres can also be classified and named according to the type of metabolism: aerobic for slow-red fibres (oxidative) and glycolytic for fast-white fibres (anaerobic). Intermediate-pink fibres are characterized as fast contracting with intermediate resistance to fatigue and intermediate speed of shortening (Johnston et al., 1975; Bone, 1978).

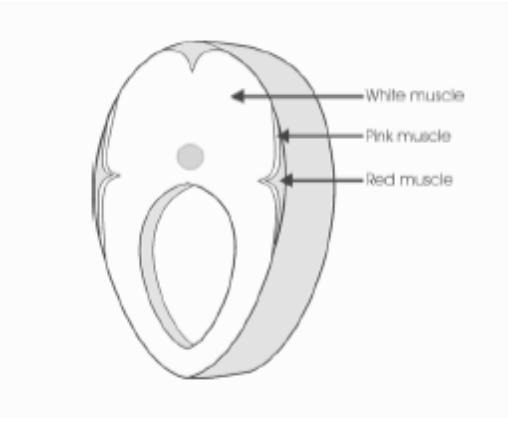

Figure 1.3. A cross-section of a fish muscle showing the location of red (high aerobic, slow twitch), pink (high aerobic, high glycolytic and fast twitch) and white (anaerobic, high glycolytic and fast twitch) muscle fibres From Kiessling et al., 2006

1.2.1. *Slow-red muscle fibres*

Among all teleost species investigated, up to now only the stickleback (*Gasterosteus aculeatus* L.) seems to lack true slow-red fibres (Kilarski and Kozlowska, 1983). Like in teleosts, also in adult sturgeons it is possible to observe a superficial layer of well-vascularised slow fibres with a wedge-like thickening inserted at the level of the lateral line: Stellate sturgeon, *Acipenser stellatus* (Flood et al., 1987), White sturgeon, *Acipenser transmontanus* (Radaelli et al., 1999), Russian sturgeon, *Acipenser gueldenstaedtii* (Nikol'skaya et al., 2004), Siberian sturgeon (Daczewska and Saczko, 2005), Sterlet sturgeon, *Acipenser ruthenus* and *A. ruthenus* x *A. gueldenstaedti* hybrids (Steinbacher et al., 2006a). Slow muscle fibres generally present a parallel alignment to the body axis suited to slow speed body movements (Rome, 1994).

Slow-red muscle fibres are small in diameter (ca. 25-45 μm) and usually account for less than 10% and never more than 30% of the myotomal musculature, as observed in several marine fish species (Greer-Walke and Pull, 1975).

Continued swimming speeds are sustained by the contraction of the slow-red muscle fibres, using aerobic pathways and mainly carbohydrates and/or lipids as fuels in species like Striped Bass (*Morone saratilis*), Goldfish, (*Carassius auratus)* and Common carp *(Cyprinus carpio)* (reviewed by Sänger and Stoiber, 2001). The constant use of the superficial slow-red muscle action requires a reasonable supply of oxygen-carrying blood. This is provided by broad vascularization and a high concentration of myoglobin in the muscle cells, which confers its red colour (Bone, 1978).

1.2.2. *Fast-white muscle fibres*

A fish is composed mainly of white muscle, which represents the bulk of myotomal muscle, accounting for at least 70%. The fibre diameters range between 50 and 100 μm or even more (Sänger and Stoiber, 2001). In a cross-sectional area of the skeletal muscle, the total area occupied by the fast-white muscle varies along the length of the fish: it reaches a peak in the anterior part of the animal and declines caudally, as observed in scup, *Stenotomus chrysops* (Zhang et al., 1996).

Muscle fibres in teleosts present a cylindrical shape in fish species such as zebrafish (*Danio rerio;* Van Raamsdonk et al. 1978), grayling, *Thymallus thymallus* (Merkel, 1995) and rainbow trout, *Oncorhynchus mykiss* (Stoiber and Sänger, 1996), and in other vertebrates, such as, amphibians (Pool frog, *Rana lessonae;* Zaire dwarf clawed frog, *Hymenochirus boettgeri*; Daczewska and

Paluka 1999; Daczewska, 2001) and birds (Przybylski and Blumberg 1966). In sturgeons, however, at hatch and in early larval stages, fast fibres consist of mononucleated lamellae, a feature that is also present in the larval and adult musculature of amphioxus, *Branchiostoma lanceolatum* (Peachy, 1961; Flood, 1968). Later on in the development, Steinbacher and collaborators (2006a) observed that these lamellae-shaped fibres in *Acipenser ruthenus* and *A. ruthenus* x A*. gueldenstaedti* hybrids give place to polygonal fibres, through a process that is not completely clear yet (Figure 1.4).

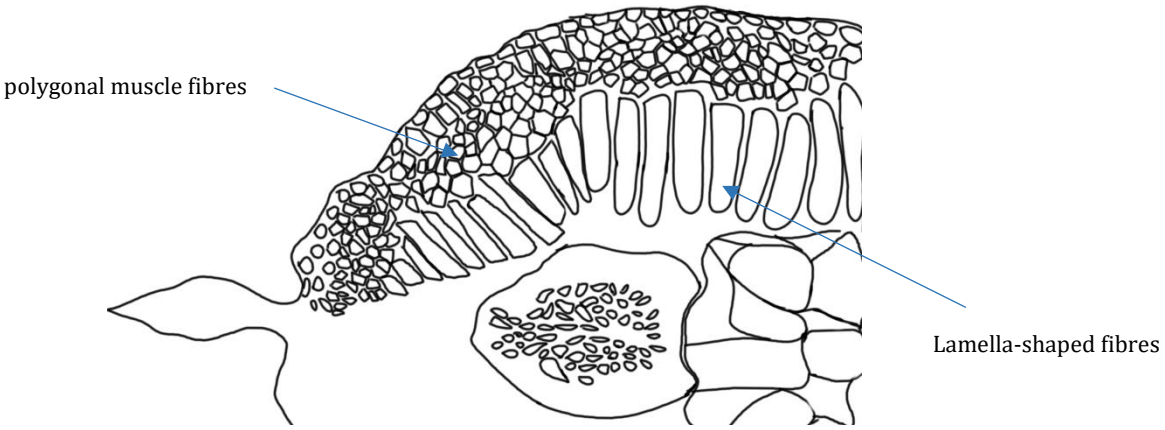

Figure 1.4. schematic representation of a cross section of a sturgeon pre-larva, where it is possible to observe the lamella-shaped fibres in a central position, close to the notochord and the polygonal muscle fibres, located externally (designed by Aidos L. and Polito, U.)

Concerning innervation, in the majority of bony fish fast-white fibres are poly-neuronally innervated (Bone, 1978), while in elasmobranches and primitive teleosts, as well as in sturgeons (Radaelli et al., 1999), these are focally innervated.

The white fibres are fast, exhaust quickly and are generally used at high swimming speeds, e.g., in fast-start burst swimming for prey capture and escape response (Videler, 1993).

The fast swimming is driven by phosphagen hydrolysis and subsequent activation of anaerobic glycogenolysis, resulting in the accumulation of lactic acid in the fast-white muscle (Sänger and Stoiber, 2001), which takes up to 24 h to remove after a burst of all out activity.

The white colour of this type of muscle fibres is due to poor vascularisation and to the lower concentrations of myoglobin, when compared to the slow-red fibres. Lipid droplets concentrations are also lower, together with the glycogen content, with granules mainly located

amongst myofibrils (Sänger and Stoiber, 2001). The myofibrils show a clear radial orientation and present a relatively even width (around 1 μm diameter).

Fast-white muscle fibres may be recognized by a lack of Succinate dehydrogenase (SDH) activity (Sänger and Stoiber, 2001). These fibres mATPase activity shows a trend to acid lability and alkaline stability. These reactions, though, are species specific (Johnston and Lucking, 1978; Kilarski and Kozlowska, 1985; Rowlerson et al., 1985; Assis et al., 2004; Aguiar et al., 2005).

1.2.3. *Intermediate-pink muscle fibres*

An intermediate or pink fibres zone is inserted between slow-red and fast-white fibre areas in most juvenile and adult teleost species. In sturgeon juveniles and adults, pink fibres appeared in an intermediate layer, as well as scattered throughout the fast-white muscle; in adults, the pink layer, though, is much more developed surrounding the lateral line (Radaelli et al., 1999). The exact function of the intermediate-pink muscle fibres is not clear, even if, from initial biochemical studies it would seem that pink muscle has an inherent speed of contraction intermediate between that of the slow-red and fast-white muscle in rainbow trout (Johnston et al., 1977).

Pink muscle is intermediate also regarding metabolism (Johnston et al., 1977; rainbow trout), contraction kinetics (Coughlin et al., 1996, scup), arrangement of myofibrils in cross-section, content of lipid and glycogen granules, amounts of sub-sarcolemmal and inter-myofibrillar mitochondria (tubular and lamellar) and relative length of transverse tubules and sarcoplasmic reticulum (T-SR) junction and sarcoplasm (Johnston et al., 1975, 1977, rainbow trout; mirror carp, *Cyprinus carpio* var. *specularis*; Sänger, 1992, several cyprinids). Pink muscle is recruited at intermediate swimming speeds in rainbow trout (Johnston et al., 1977) and at the maximum stable swimming speed both red and pink muscle are enrolled in scup (Coughlin et al., 1996).

In scup intermediate-pink muscle has lower cross-sectional area than slow-red regardless its position in the fish body (Zhang et al., 1996).In the anterior and middle regions, it forms a diverse band that is medial to the slow-red muscle and has an area of 70% of the red muscle. On the opposite, always in scup, in posterior locations, intermediate-pink muscle has very low cross-sectional area, and the small amount of pink fibres are scattered throughout the red muscle layer (Zhang et al., 1996). Therefore, intermediate-pink muscle shows the highest cross-sectional area and are more organized in the region of lowest strain during swimming.

1.2.4. *Methods used to study muscle growth*

Research on fish muscle growth is important for the increasing developing global aquaculture industry, mostly regarding production and quality. Most of the fish species show continuous growth throughout their lives. Growth in fish has been thoroughly studied since it is a good indicator of the animal's health: rapid growth indicates abundancy of food and other favourable external conditions, while slow growth likely indicates the opposite. Growth in fish is generally determined as changes in body weight, length or condition factor (i.e. weight/length relationship) over time.

Muscle tissue post-embryonic growth implies an increase in the number and in the diameter of the fibres, as well as a contemporary adjustment of the associated connective tissue, nerve and blood supply. Muscle growth can therefore be studied in terms of the contribution of hyperplasia (proliferation of muscle fibres) and hypertrophy (increase in fibre size) (Rowlerson and Vegetti, 2001).

A long established method to measure growth in fish, consists on measuring fibre morphometric variables, such as diameter or cross-sectional area and number of muscle fibres measured within a representative area of the musculature (lateral/trunk) of different ages, sizes, or conditions. Muscle morphometry has been performed in several fish species, such as rainbow trout (Weatherley et al., 1979; 1980; Kiessling et al., 1991; Fauconneau et al., 1997; Valente et al., 1999), European seabass, *Dicentrarchus labrax* (Veggetti et al., 1990; Periago et al., 2005), gilthead seabream, *Sparus aurata* (Rowlerson et al., 1995), Common carp and European seabass (Alami-Durante et al., 2000; 2006; 2007), Atlantic herring,*Clupea harengus*, and Atlantic salmon, *Salmo salar* (Johnston et al., 1998), Atlantic cod, *Gadus morhua* (Galloway et al., 1999a), Pacu, *Piaractus mesopotamicus* (Assis et al., 2004), Blackspot seabream, *Pagellus bogaraveo* (Silva, 2009), Atlantic salmon (Johnston et al., 2002; Nathanallides et al., 2011), Senegalense sole, *Solea senegalensis* (Campos et al, 2013a; 2013b), Wuchang bream, *Megalobrama amblycephala* (Zhu et al, 2014). In practice, cross-sections are taken at the anal opening level, including the area surrounding the lateral line nerve in order to have all the three main muscle layers (slow-red, intermediate-pink and fast-white) represented. At this point, it is possible to calculate the surface occupied by each muscle layer, as well as the number and size of muscle fibres within each layer. An increase in fibre size indicates hypertrophic growth and the increase in number of small fibres represents hyperplastic growth (recruitment of new fibres). The outline of fibre numbers and size distributions are frequently referred to as the 'muscle cellularity'

(Nathanailides et al., 2011; Alami-Durante et al., 2000; 2006; 2007; Stoiber et al., 2002; Periago et al., 2005).

Various biochemical measures of muscle growth have been investigated but the most commonly used is to measure the relative contents or rates of synthesis of protein and nucleic acids in the muscle. For instance, protein growth is well correlated with the RNA concentration and with the RNA:DNA ratio, as observed in brown trout (*Salmo trutta*; Grant, 1996), European seabass (Alami-Durante et al., 2007) and Pacific bluefin tuna, *Thunnus orientalis* (Tanaka et al., 2007). The protein:DNA ratio has been used to determine hypertrophic growth in Atlantic cod (Pelletier et al., 1995) and in Pacific Bluefin tuna (Tanaka et al., 2007); on the opposite, the DNA concentration gives an indication on a hyperplastic growth, as shown in rainbow trout (Valente et al., 1998).

Changes in the phenotype of muscle fibres, mainly in contractile protein expression, may be indicators of muscle growth. Myosin isoforms constitute reliable markers of muscle development in fish, as they show development transitions of different muscle fibre types and can be identified by immunostaining (Veggetti et al., 1993, guppy, Johnston and Horne, 1994, Atlantic herring; Mascarello et al., 1995, gilthead seabream).

Cell proliferation gives an indication of hyperplastic growth in fish, by revealing the sites and intensity of the mitotic activity in cells (Johnston et al., 1995, Atlantic herring; Rowlerson et al., 1995, gilthead seabream; Veggetti et al., 1999, common sole, *Solea solea*). Cell proliferation may be accessed through *in vivo* labelling with incorporation of tritiated thymidine or 5BrdU (5-bromo-deoxy-uridine) or by staining tissue sections with proliferating cell nuclear antigen (PCNA). However, both these methods may be limited to phases of rapid hyperplastic growth because of the relatively low frequency of mitotic events in muscle at other times.

1.3. Fish muscle development and growth

Myogenesis is common to all vertebrates and involves sequential complex events including the specification, proliferation, differentiation, migration and fusion of precursor cells to form multinucleated muscle fibres. In several teleost species, three distinct phases of fibre production are acknowledged (Steinbacher et al. 2006b; Rescan, 2008). The first phase is fully embryonic in which adaxial and posterior somitic cells lead to the formation of two morphologically and functionally distinctive muscle types that form the primary myotome. A second phase takes place during the late embryo and early larval stages and involves the production of new muscle fibres

in specific zones (stratified hyperplasia). The third phase starts in larvae and continues into adult stages, and involves new fibre production all over the myotome giving rise to a mosaic of fibre diameters (mosaic hyperplasia). To conclude, muscle formation in fish comprises myogenesis, the production (hyperplasia, or recruitment) and successive expansion of muscle fibres (hypertrophy and elongation).

1.3.1. *First phase: embryonic myogenesis*

Myogenesis initiates in an earlier stage of development in fish embryos when compared with amniotes, such as birds and mammals. Embryonic muscle develops in cellular sections called somites, which are paired and located along the body axis, separated by the notochord and the neural tube.

Somite cells differentiate into a myotome composed of four types of muscle fibres: muscle pioneers (MPs), red-slow muscle, white-fast muscle and intermediate-pink muscle, as observed in zebrafish (reviewed by Johnston et al., 2011).

The specification of cells in each of these lineages depends on the members of a family of master transcription factors for vertebrates myogenesis that are called myogenic regulatory factors (MRFs) (myoD, Myf5, mrf4, myogenin). The sequence identity of the MRFs is conserved among vertebrates including teleosts and play a crucial role in specification and determination of the muscle cell lineage (Holterman and Rudnicki, 2005). During myogenesis, each MRF is expressed in a consecutive manner (Cornelison et al., 2000). Myod and Myf5 have a role in the initial specification of the myogenic lineage, while Myogenin and Mrf4 are activated during myoblast differentiation and cell fusion (Cornelison et al., 2000). Myogenin also works together with other transcription factors including myoD, in order to induce and maintain the differentiation and the beginning of the specific muscle gene expression (Hinits and Hughes, 2007). In zebrafish, myogenic precursor cells (MPCs) for slow and fast fibres, before the somatic form, are spatially separated already in the gastrulation stage (Hirsinger et al., 2004). There is a population of mononuclear MPCs called the adiaxial cells, that can be found near the notochord and are precursors of both slow muscles and the pioneer cells of the muscles, which lie near the horizontal septum, in zebrafish (Devoto et al., 1996).

As in the teleosts, the first muscle cells of the axial musculature to appear in sturgeons are adjacent to the axial organs. These adaxial cells present a flattened shape and a radial orientation respect to the notochord and show a myogenic identity. Before somitogenesis, these cells start

to differentiate by myofibrillar myosin synthesis. During subsequent differentiation, the adiaxial cells undergo lateral transmyotomic migration and eventually form the slow fibre layer on the lateral surface of the myotome. Similar to teleosts (Devoto et al., 1996, zebrafish; Stoiber et al. 1998, European pearlfish, *Rutilus frisii meidingeri*; Stickney et al. 2000, zebrafish), the area of the adiaxial cells of the sturgeon is first extended dorsally and ventrally along the medial surface of the myotomes. Afterwards, the adiaxial cells are gradually relocated laterally through the rest of the myotome until they form the slow superficial layer of muscle fibres as observed in adult sturgeons (Radaelli et al. 1999). In contrast to the adiaxial cells, other cells positioned laterally in the first somites are not clearly distinguishable from the dermatome and the sclerotome. It remains unknown weather this type of myogenesis of the slow muscles also occurs in the chondrichthyan or sarcopterygian fish. Sturgeons have a population of adaxial cells that give rise to a regular original layer of slow fibres and a mid-lateral insertion of the slow muscle layer at the position of the horizontal septum equivalent to that of the teleosts (Steinbacher et al., 2006a). Sturgeons, however, require more than 14 dph before the formation of the horizontal septum (Steinbacher et al., 2006a). The sturgeon pattern is analogous to that of birds and anurans, in which does not exist a dorso-ventral separation of the myotome by specific cells (Grimaldi et al. 2004). This delayed formation of the collageneous horizontal septum in sturgeons, when compared to the teleosts, may be associated with the absence of teleost-type MPs in these fishes. MPs are a population of cells showing high levels of gene expression and early differentiation in zebrafish (Felsenfeld et al. 1991; Hatta et al. 1991; Ekker et al. 1992) and may have a role in establishing cellular separation between the dorsal and ventral myotome at the site of the future horizontal septum.

The sturgeon myotome is characterized by the presence of exceptionally long cellular processes of both slow and fast muscles cells. The longer slower fibre processes end at the medial surface of the myotome between the fast fibres. Most of the shorter fast fibre processes end at the lateral surface of the myotome between the slow fibres. These processes are maintained until the hatching stage and it is, therefore, possible to hypothesize that it represents an ancient characteristic, as small cellular processes are also found in slow fibres within the teleost myotome (Stoiber, 1996). In both sturgeons and teleost, it is probable that the primary function of these processes is to facilitate the migratory movements of the precursors of slow and fast fibres. Steinbacher et al. (2006a) suggest that in sturgeons, these processes may have an additional function as mechanical "dividers" that drive the fusions of precursor cells of the fast muscles to form a highly ordered lamellar structure perpendicular to the notochord. In the most

derived teleosts, this mechanism seems to have been bypassed in favour of their simpler development system, so that the preservation of the long processes is not required. Following, the stratified growth of the sturgeon's fast muscle takes place according to the teleost scheme.

1.3.2. *Second phase: stratified hyperplasia*

Stratified hyperplasia is the major source of new fibres during late embryonic and early larval growth. It consists on an extension of the embryonic myogenesis, which leads to the completion of the definitive muscle layers (slow-red, intermediate-pink and fast-white), through the apposition along a growth zone.

Fast-white new fibres are added at the dorsal and ventral apices of the myotome and between the slow-red and already existing fast-white fibres in gilthead seabream (Rowlerson et al., 1995) and several European cyprinid species and rainbow trout (Stoiber and Sanger, 1996). Also in *A. baerii*, the presence of mononucleated proliferative active cells was observed in the intermyotomal space and subsequently between slow-red and fast-white muscles (Daczewska and Saczko, 2005). It has been demonstrated that myogenic precursors detach from the dermomyotome and constitute a major source of myogenic cells that are responsible for stratified growth, as observed in species like zebrafish (Stellabotte *et al.* 2007), pearlfish (Marschallinger et al. 2009) and rainbow trout (Steinbacher et al., 2007).

Phenotypically and morphologically new red-slow fibres appear during larval life in some species, like European seabass (Scapolo et al., 1988), Atlantic herring (Johnston and Horne, 1994), gilthead seabream, (Mascarello et al., 1995), catfish, *Clurias gariepinus* (Koumans and Akster, 1995) in a position close to the lateral line nerve. In many fish species, the appearance of intermediate fibres takes place close to the horizontal septum during the stratified hyperplastic growth phase (reviewed by Rowlerson and Veggetti 2001).

During the early larval period, much more fast-white than slow-red fibres are added, but it is not known if the dermomytome also gives rise to slow fibres during stratified hyperplasia in zebrafish (Stellabotte et al., 2007). The timing and length of stratified hyperplastic growth is species specific, as reviewed by Valente et al. (2013) and there is no relation between the timing of stratified hyperplasia and the start of exogenous feeding, even if the first attempts of the larvae at cruise swimming in search of food are extremely important for their survival at that age.

1.3.3. *Third phase: mosaic hyperplasia*

In fish that reach a final large size like sturgeons, a second and quite different hyperplastic process takes place, resulting in a large increase in the total number of fibres in all muscle layers, especially in the fast-white muscle layer of teleosts (Rowlerson and Vegetti, 2001). It was believed that in fish species that remain small, such as guppy, *Poecilia reticulata* (Veggetti *et al.*, 1993), bluntnose minnow, *Pimephales notatus* (Weatherley and Gill, 1984) and zebrafish (Biga and Goetz, 2006), there was not a second hyperplastic growth phase. However, in a study with zebrafish Patterson et al. (2008) showed that, during late larval growth, mosaic hyperplasia contributes to trunk muscle growth. Further research seems necessary to clarify this topic.

New fibres are a result of the activation and proliferation of myogenic precursor cells that are widely scattered throughout the myotome in gilthead seabream (Rowlerson et al., 1995). This process results in a transverse section of muscle cut with a typical mosaic appearance that is an alternation of fibres of different ages and diameters (Figure 1.5). This type of hyperplastic growth is, therefore, called mosaic hyperplasia and, in farmed fish, has an economical importance since it is the main phase contributing to muscle growth (Johnston, 2006).

It is not known the origin of the myogenic precursor cells involved in this growth phase in fish. It has been suggested by Stellabotte et al. (2007) that in zebrafish the cells responsible for the mosaic hyperplasia are either dermomyotome cells or a population of cells other than the dermomyotome.

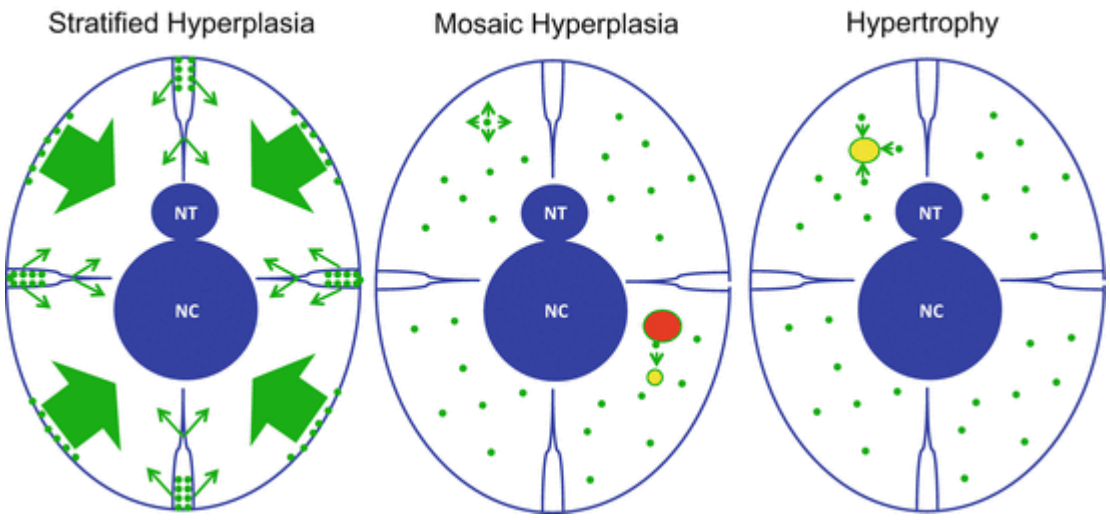

Figure 1.5. Schematic representation of the two types of hyperplastic growth (stratified and mosaic) and of hypertrophic growth. From Gurevich et al., 2015.

The relative timing and importance of each phase of hyperplasia may be related to the evolutionary history, growth potential and final body size achieved by each single species. In some species like gilthead seabream, mosaic hyperplasia only starts after the stratified phase has ceased, but in other species it may initially overrun with stratified hyperplasia (Stoiber and Sänger, 1996; Johnston et al., 1998).

Muscle growth by mosaic hyperplasia is more intense during the juvenile stage and decreases gradually until the fish reaches a certain fraction of body size after which growth occurs by hypertrophy only in species of freshwater teleosts from five families (Cyprinidae, Centrarchidae, Percidae, Salmonidae, Esocidae) (Weatherley et al., 1988), rainbow trout (Stickland, 1983) and gilthead seabream (Rowlerson et al., 1995).

In fish, growth rate and maximum size is highly regulated by the intensity and length of the mosaic hyperplastic growth. Largest and fastest growing fish (like smallmouth bass, *Micropterus dolomieu*; lake whitefish, *Coregonus clupeaformis;* rainbow trout, muskellunge, *Esox masquinongy*), usually show greater hyperplasia than slow growing fish (like bluntnose minnow, longnose dace, *Rhinichthys cataractae*; Weatherley et al., 1988). Furthermore, the capacity of teleosts to grow rapidly and to achieve a large final size is dependent on the body length at which the recruitment of new muscle fibres into the growing axial muscle finishes.

The slow-red and the intermediate-pink muscle layers undergo, as well, additional growth in juveniles by hyperplasia in Atlantic salmon (Higgins and Thorpe, 1990), Southern smelt, *Retropinna retropinna* (Meyer-Rochow and Ingram, 1993) and Atlantic herring (Johnston et al., 1998) and at least in gilthead seabream this appears to be a mosaic hyperplasia (Mascarello et al., 1995).

1.3.4. *Hypertrophy*

During the course of post-embryonic life muscle growth occurs by hypertrophy until the fibres reach a maximum diameter of 100-300 μm for fast-white fibres in most fish; slow-red fibres are smaller because they are more dependent on the oxygen supply from adjacent capillaries (e.g., Johnston, 1982; Sänger, 1993). Body mass, activity patterns and metabolic demand have an important role on the setting of the maximum fibre diameter in notothenioid fishes (Johnston et al., 2003) and in Artic charr, *Salvelinus alpinus* (Johnston et al., 2004).

As muscle fibres increase in size, they are stuffed with myofibrils and obtain additional nuclei (Johnston, 1993). Adding nuclei during growth (both in diameter and in length), allows to

maintain a constant ratio of nuclear to cytoplasmic volume. A population of satellite cells already present in the muscle fuse with existing muscle fibres to provide the additional nuclei (Johnston, 2001). In order to supply the number of nuclei required during growth, this population must be capable of proliferation. Hypertrophic growth continues even after hyperplastic growth has stopped (Stickland, 1983; Weatherley et al., 1988; Kiessling et al. 1991; review by Rowlerson and Vegetti, 2001).

1.4. Muscle growth regulation

Growth is centrally regulated by the hypothalamic-pituitary axis, through the growth hormone (GH)/prolactin/somatolactin hormone families (Kawauchi and Sower, 2006). The growth-hormone, directly or indirectly, participates in the most important physiological processes in fish, comprehending the regulation of osmotic balance, in the metabolism of lipid, protein and carbohydrate, skeletal and muscle growth, reproduction and immune function, and also aspects related with behaviour, such as appetite or aggression (Reinecke et al. 2005; Wood et al. 2005; De-Santis and Jerry 2007).

Individual growth reflects the processes of catabolism and anabolism and is influenced by several physiological processes such as food intake, digestion, absorption, assimilation and excretion. These physiological processes are, on their turn, influenced by the size of the body and a series of biotic factors including day length, temperature and availability of oxygen. An important characteristic of individual growth in teleosts is its indeterminate nature, with body length and mass that increase constantly, though at a slow rhythm until mortality o senescence take place.

In vertebrates, GH directly acts through the receptors located on the muscle sarcolemma and indirectly by inducing the production and release of insulin-like growth factor (Igf) in the liver and peripheral tissues (Wood et al., 2005). The Igf system comprehends Igf-I, Igf-II, different receptors and six binding proteins (Igf BP) and is one of the central pathways that regulates protein synthesis in skeletal muscle. The Igfs also have roles in the development of many tissues and functions in the responses of hypoxia, osmoregulation and reproduction in adult teleosts (Wood et al., 2005). During the synthesis of protein in the muscle, Igf-I and -II activate the phosphatidylinositol 3'-kinase (PI3K)– protein kinase B–Akt/mTOR (mammalian target of rapamycin) signalling pathway, by binding to the Igf1R in the sarcolemma and triggering a phosphorylation cascade that leads to an increase of the Myogenic Regulatory Factor (MRF)

myoD (myoblast determination protein) translation and protein synthesis (Bodine et al. 2001; Wilson and Rotwein 2006). The Igf-I structure, regulation and function in fish, are similar to those in mammals (Reinecke et al. 2005), in which Igf-I stimulates muscle growth through myoblast proliferation (Engert et al. 1996). Also in farmed rainbow trout muscle cells, Igf1R increases during differentiation (Castillo et al. 2006). In fish, Igf-I may contribute more for the regulation of muscle growth than insulin. zebrafish and salmonids have at least two forms of the Igf1R (Igf1Ra and Igf1Rb), which display diverse expression during development (Maures et al. 2002) and that are differently regulated according to the nutritional status (Montserrat et al. 2007; Valente et al. 2012).

Several studies have shown that GH differentially regulates the expression of MRFs in fish muscle, including genes involved in the activation and engagement of myogenic cells, genes involved in differentiating muscle cells and genes encoding for structural muscle proteins (reviewed by Fuentes, et al. 2013).

1.5. Environmental effects on growth

In nature, fish eggs usually are exposed to all types of constrictions like variations of temperature, availability of oxygen, salinity, pH, photoperiod or ammonia (Moyle and Cech, 1982), which can cause considerable embryonic mortality. Such factors interact with each other and may influence growth rates, and with others such as the degree of competition, the amount and quality of food ingested, and the age and state of the maturity of the fish.

Moreover, since their development is not complete, fish larvae are also usually more sensitive to surrounding environment variations than juveniles or adults

In farmed fish species, great effort has been put over the years in order to offer the best rearing conditions for successful development of embryos and larvae. In teleosts, environmental conditions during early stages, which are often short and specific developmental time windows, can have an impact later, frequently in an irreversible manner (Johnston, 2006). Rearing environment during these early stages of development can, therefore, influence the following muscle growth potential, animal well-being and the quality of the final flesh.

1.6. Plasticity of the myogenic phenotype

Phenotypic plasticity may be defined as the capacity of an organism to react to an environmental input through a change of form, state, movement or rate of activity. Growth plasticity in fish species involves an adaptive reaction in the myotomal muscle dynamics (in favour of hyperplasia vs hypertrophy of existing fibres) facing a change in environmental factors such as temperature and food supply, and has significant intra- and interspecific variation. Muscle plasticity in teleosts frequently comprehends structural changes in cellular organelles or supporting structures such as capillaries and usually needs several weeks to reach a new steady state (reviewed by Johnston, 2006).

It is possible to establish two wide categories of muscle plasticity, based on the reversibility of the response. During embryonic and larval stages, muscle plasticity to the environment is generally irreversible because of the rapid rhythm of the ontogenetic changes. After the adult body plan has been established, seasonal acclimatization to environmental changes leads to fully reversible alterations in muscle phenotype.

As previously mentioned, muscle cellularity presents significant plasticity regarding exercise, feeding and environmental factors. Exercise is acknowledged as a powerful stimulus for muscle hypertrophy even if its effects on fibre recruitment have not been measured yet. Little is known about the effects of diet composition on muscle growth characteristics, but rather more is known about the environmental temperature effects on muscle cellularity (Johnston, 2006), in several fish species, such as, Atlantic herring (Johnston et al., 1995), Atlantic halibut, *Hippoglossus hippoglossus* (Galloway et al., 1999b), Danube bleak, *Alburnus chalcoides* (Stoiber et al., 2002), European seabass (Alami-Durante et al., 2006), Atlantic salmon (Nathanallides et al., 2011), Senegalese sole (Campos et al., 2013a, 2013b), Wuchang bream (Zhu et al., 2014), among others.

However, in sturgeons, the influence of environmental conditions on growth plasticity was not assessed yet and few studies have been performed on these species muscle structure (Kryvi et al., 1980; Flood et al., 1987; Radaelli et al., 1999; Nikol'skaya et al., 2004; Daczewska and Saczko, 2005; Steinbacher et al., 2006a; Adamek et al., 2017)

There are many obstacles in understanding the mechanisms of muscle plasticity induced by environmental change. These include the complexity of environmental interactions present in nature, which are frequently difficult to replicate in an experimental set.

1.7. Aims of the Thesis

This Thesis aims to contribute to a better knowledge on the effects of rearing conditions in the early life stages of Siberian sturgeon, in particular during the endogenous feeding phase (until the full yolk-sac absorption). Muscle growth and development will be evaluated during the free-embryo stage regarding three different rearing environmental factors: temperature, stocking density and bottom substrate, in order to: i) evaluate muscle structure and development; and ii) study the influence of the selected external factors on the growth of this fish species. This Thesis addresses morphological (in particular, the histology of the muscle fibre types from the axial musculature), molecular and physiological aspects of the growth plasticity of muscle in Siberian sturgeon larvae. This knowledge may contribute to identify and select the most favourable conditions to improve Siberian sturgeon larvae growth in aquaculture.

Three experiments were performed:

The first one comprises the manipulation of the larval rearing temperature from hatching until the complete yolk-sac absorption (**Chapters 2, 3** and **4**).

In **Chapter 2**, the morphology of the muscle fibres was assessed as well as the stress status of free-embryos. In **Chapter 3**, growth and muscle development were assessed, as well as the larvae fatty acid profile. **Chapter 4** regards growth and stress related gene expression in Siberian sturgeon free-embryos.

In the second experiment, newly-hatched Siberian sturgeon larvae were subjected to three rearing densities (**Chapter 5** and **6**).

In **Chapter 5,** growth and muscle development were assessed, as well as the larvae fatty acid profile. **Chapter 6** regards the larval stress evaluation along with growth related gene expression.

In the third and last experiment (**Chapter 7**), the environmental enrichment was evaluated by subjecting newly hatched larvae to two different rearing substrates, respect to no substrate at all. Chapter 7 assesses larval behavioural, growth and muscle development of Siberian sturgeon free-embryos.

1.8. References

Adamek, D., Rzepkowska, M., Panagiotopoulou, H., Ostaszewska, T., Fajkowska, M., Kamaszewski, M. & Kolman, R. (2017). Morphological Differences of White Muscle Fibres and Genetic Diversity of Fast and Slow Growing Atlantic Sturgeons (*Acipenser oxyrinchus*). *Turkish Journal of Fisheries and Aquatic Sciences*, **17**(5), 959-966. http://doi.org/10.4194/1303-2712-v17_5_11

Aguiar, D.H., Barros, M.M., Padovani, C.R., Pezzato, L.E. & Dal Pai-Silva, M. (2005). Growth characteristics of skeletal muscle tissue in *Oreochromis niloticus* larvae fed on a lysine supplemented diet. *Journal of Fish Biology*, **67**(5), 1287-1298. https://doi.org/10.1111/j.1095-8649.2005.00823.x

Alami-Durante, H., Bergot, P., Rouel, M. & Goldspink, G. (2000). Effects of environmental temperature on the development of the myotomal white muscle in larval carp (*Cyprinus carpio* L.). *Journal of Experimental Biology*, **203**(24), 3675-3688. http://jeb.biologists.org/content/203/24/3675.abstract

Alami-Durante, H., Rouel, M. & Kentouri, M. (2006). New insights into temperature induced white muscle growth plasticity during *Dicentrarchus labrax* life: a developmental and allometric study. *Marine Biology* **149**(6), 1551-1565. https://doi.org/10.1007/s00227-006-0304-6

Alami-Durante, H., Olive, N. & Rouel, M. (2007). Early thermal history significantly affects the seasonal hyperplastic process occurring in the myotomal white muscle of *Dicentrarchus labrax* juveniles. *Cell and Tissue Research,* **327**(3), 553-570. doi 10.1007/s00441-006-0321-2

Assis, J. M. F., Carvalho, R. F., Barbosa, L., Agostinho, C. A. & Dal Pai-Silva, M. (2004). Effects of incubation temperature on muscle morphology and growth in the pacu (*Piaractus mesopotamicus*). *Aquaculture*, **237**(1-4), 251-267. https://doi.org/10.1016/j.aquaculture.2004.04.022

Biga, P.R. & Goetz, F.W. (2006). Zebrafish and giant danio as models for muscle growth: determine vs. indeterminate growth as determined by morphometric analysis*. American Journal of Physiology-regulatory Integrative and Comparative Physiology*, **291**(5), R1327-R1337. https://doi.org/10.1152/ajpregu.00905.2005

Blaxter, J.H.S. (1987). Structure and development of the lateral line. *Biological Reviews,* **62**, 471-514. https://doi.org/10.1111/j.1469-185X.1987.tb01638.x

Bodine, S.C., Stitt,, T.N., Gonzalez, M., Kline, W.O., Stover, G.L., Bauerlein, R., Zlotchenko, E., Scrimgeour, A., Lawrence, J.C., Glass, D.J. and Yancopoulos, G.D. (2001). Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. *Nature Cell Biology*, **3,** 1014-1019. https://doi.org/10.1038/ncb1101-1014

Bone, Q. (1978). Locomotor muscle. In: Hoar, W. S. & Randall, D. J. ed., *Fish physiology* Volume VII. Academic Press. pp. 361-424DOI:10.1016/S1546-5098(08)60168-5

Brandt, J. G. 1869/1870. Einige Worte ber die curopisch-asiatischen Strarten (Sturionides). *Bulletin of Academic Imperial Sciences St.-Petersbourg,* **14**, 171–175.

Bronzi, P. & Rosenthal, H. (2014). Present and future sturgeon and caviar production and marketing: A global market Overview. *Journal of Applied Ichthyology,* **30**, 1536–1546

Campos, C., Valente, L.M., Conceição, L.E.,Engrola, S.,Sousa, V., Rocha, E. & Fernandes, J.M. (2013a) Incubation temperature induces changes in muscle cellularity and gene expression in Senegalese sole (*Solea senegalensis*).*Gene,* **516**(2):209-217. doi: 10.1016/j.gene.2012.12.074

Campos, C.,Valente, L.M., Conceição, L.E., Engrola, S. & Fernandes, J.M. (2013b). Temperature affects methylation of the myogenin putative promoter, its expression and muscle cellularity in Senegalese sole larvae. *Epigenetics*, **8**(4):389-97. doi: 10.4161/epi.24178

Castillo, J., Ammendrup-Johnsen, I., Codina, M., Navarro, I. & Gutierrez, J. (2006). IGF-I and insulin receptor signal transduction in trout muscle cells. *American Journal Physiology - Regulatory, Integrative and Comparative Physiology,* **290**(6), R1683-1690

Conte, F. S., Doroshov, S. I., Lutes, P. B. & Strange, E. M. (1988). In: *Hatchery manual for white sturgeon, Acipenser transmontanus R., with application to other North American Acipenseridae*. Oakland: Division of Agriculture and Nature Resources, University of California

Cornelison, D.D.W., Olwin, B.B., Rudnicki, M.A. & Wold, B.J. (2000). MyoD(−/−) satellite cells in single-fiber culture are differentiation defective and MRF4 deficient. *Developmental Biology,* **224**(2), 122–137. https://doi.org/10.1006/dbio.2000.9682.

Coughlin, D. J., L. Valdes, & L. C. Rome. (1996). Contraction dynamics and power production of pink muscle of scup (*Stenotomus chrysops*). *Journal of Experimental Biology,* **199**(12), 2703-2712. http://jeb.biologists.org/content/199/12/2703.abstract

Daczewska, M. (2001). Mechanism of multinucleate myotomal muscle fibre formation in *Hymenochirus boettgeri* (Anura, Pipidae). *Zoomorphology* **121**, 27-36.

Daczewska, M. & Palucka, M. (1999). Development of primary and secondary muscle fibres in the myotomes of *Rana lessonae* (Anura: Ranidae). *Zoologica Poloniae* **44**(1-4), 59-69

Daczewska, M. & Saczko, J. (2005). Myotomal Myogenesis of Axial Muscle in the Sturgeon *Acipenser baeri* (Chondrostei, Acipenseriformes). *Folia biologica*, **53**(1-2), 29-38 DOI:10.3409/1734916054663537

Dal Pai-Silva, M., Dal Pai, V., da Mota, D.L. & de Castro Rodrigues, A. (1996). Histochemical study of muscle fiber types in *Synbranchus marmoratus* Bloch, 1795. *Annals of* Anatomy - Anatomischer Anzeiger, **178**(1), 65-70. https://doi.org/10.1016/S0940-9602(96)80014-8

De-Santis, C. & Jerry, D.R. (2007). Candidate growth genes in finfish - Where should we be looking? *Aquaculture*, **272**(1-4): 22-38. https://doi.org/10.1016/j.aquaculture.2007.08.036

Dettlaff, T. A., Ginzburg, A. S. and Schmalhausen, O. I. (1993). Chapter 3: Development of Prelarvae. In: *Sturgeon fishes: developmental biology and aquaculture*.1st ed., Springer-Verlag Berlin and Heidelberg. http://dx.doi.org/10.1007/978-3-642-77057-9

Devitsina, G.V. & Kazhlayev, A.A. (1993). Development of chemosensory organs in Siberian sturgeon, *Acipenser baeri*, and stellate sturgeon, *A. stellatus*. *Journal of Ichthyology* **33,** 9–19.

Devoto, S.H., Melançon, E., Eisen, J.S. & Westerfield, M. (1996). Identification of separate slow and fast muscle precursor cells in vivo, prior to somite formation. *Development,* **122**(11), 3371–3380. http://dev.biologists.org/content/122/11/3371.abstract

Ekker, M., Wegner, J., Akimenko, M.A. & Westerfield, M. (1992). Coordinate embryonic expression of three zebrafish engrailed genes. *Development*, **116**(4),1001–1010. http://dev.biologists.org/content/116/4/1001.abstract

Engert, J. C., Berglund, E. B. & Rosenthal, N. (1996). Proliferation proceeds differentiation in IGF stimulated myogenesis. *Journal of Cell Biology,* **135**(2), 431-440. http://jcb.rupress.org/content/135/2/431.abstract

Fauconneau, B., André, S., Chmaitilly, J., LeBail, P.Y., Krieg, F. & Kaushik, S.J. (1997). Control of skeletal muscle fibres and adipose cells size in the flesh of rainbow trout. *Journal of Fish Biology,* **50**(2), 296-314. https://doi.org/10.1111/j.1095-8649.1997.tb01360.x

http://www.fao.org/fishery/culturedspecies/Acipenser_baerii/en

FAO Yearbook of Fishery and Aquaculture, 2016; http://www.fao.org/fishery/statistics/yearbook/en

Felsenfeld, A.L., Curry, M. & Kimmel, C.B. (1991). The fub-1 mutation blocks initial myofibril formation in zebrafish muscle pioneer cells. *Developmental Biology,* **148**(1), 23–30. https://doi.org/10.1016/0012-1606(91)90314-S.

https://www.fishbase.de/summary/4683

Flood, P.R. 1968. Structure of the segmental trunk muscle in amphioxus. *Zeitschrift für Zellforschung und Mikroskopische Anatomie,* **84**(3), 389-416. https://doi.org/10.1007/BF00334754

Flood, R, Gulyaev, D & Kryvi, H. (1987). Origin and differentiation of muscle fibre types in the trunk of the sturgeon, A*cipenser stellatus pallas*. *Sarsia,* **72**(3-4)343-346. DOI: 10.1080/00364827.1987.10419733

Fuentes, EN, Antonio Valdés, JA, Molina, A & Björnsson, BT (2013). Regulation of skeletal muscle growth in fish by the growth hormone – Insulin-like growth factor system. *General and Comparative Endocrinology*, **192**, 136-148. http://dx.doi.org/10.1016/j.ygcen.2013.06.009

Galloway, T.F., Korsvik, E. & Kryvi, H. (1999a). Muscle growth and development in Atlantic cod larvae (*Gadus morhua* L.) related to different somatic growth rates. *Journal of Experimental Biology,* **202**(Pt15), 2111-2120

Galloway, T.F , Korsvik, E. & Kryvi, H. (1999b). Muscle growth in yolk-sac larvae of the Atlantic halibut as influenced by temperature in the egg and yolk-sac stage. *Journal of Fish Biology*, **55**(SA), 26–43. https://doi.org/10.1111/j.1095-8649.1999.tb01044.x

Gemballa, S., Hagen, K., Röder, K., Rolf, M. & Treiber, K. (2003). Structure and evolution of the horizontal septum in vertebrates. *Journal of Evolutionary Biology,* **16**(5), 966–975. https://doi.org/10.1046/j.1420-9101.2003.00588.x

Gisbert, E. (1999). Early development and allometric growth patterns in Siberian sturgeon and their ecological significance. *Journal of Fish Biology,* **54**(4), 852–862. https://doi.org/10.1111/j.1095-8649.1999.tb02037.x

Gisbert, E. & Ruban, G.I. (2003). Ontogenetic behavior of Siberian sturgeon, *Acipenser baerii*: A synthesis between laboratory tests and field data. *Environmental Biology of Fishes,* 67(3), 311–319. https://doi.org/10.1023/A:1025851502232

Gisbert, E., Williot, P. & Castellò-Orvay, F. (2000). Influence of egg size on growth and survival of early stages of Siberian sturgeon (*Acipenser baeri*) under small scale hatchery conditions. *Aquaculture,* **183**(1-2), 83–94. https://doi.org/10.1016/S0044-8486(99)00287-2

Grant, G.C. (1996). RNA-DNA ratios in white muscle tissue biopsies reflect recent growth rates of adult brown trout. *Journal of Fish Biology,* **48**(6), 1223-1230. https://doi.org/10.1111/j.1095-8649.1996.tb01816.x

Greer-Walker, M. & Pull, G.A. (1975). A survey of red and white muscle in marine fish. *Journal of Fish Biology*, **7**(3), 295 -300. https://doi.org/10.1111/j.1095-8649.1975.tb04602.x

Grimaldi, A., Tettamanti, G., Martin, B.L., Gaffield, W., Pownall, M.E. & Hughes, S.M. (2004). Hedgehog regulation of superficial slow muscle fibres in Xenopus and the evolution of tetrapod trunk myogenesis. *Development,* **131**(14), 3249–3262. http://dev.biologists.org/content/131/14/3249

Gurevich, D., Siegel, A. & Currie, P.D. (2015). Skeletal Myogenesis in the Zebrafish and Its Implications for Muscle Disease Modelling. In: Brand-Saberi B. (eds) *Vertebrate Myogenesis. Results and Problems in Cell Differentiation*, vol 56. Springer, Berlin: Heidelberg. https://doi.org/10.1007/978-3-662-44608-9_3

Hatta, K., Bremiller, R., Westerfield, M. & Kimmel, C.B. (1991). Diversity of expression of engrailed-like antigens in zebrafish. *Development,* **112**(3), 821–832. http://dev.biologists.org/content/112/3/821.abstract

Higgins, P.J. & Thorpe, J.E. (1990). Hyperplasia and hypertrophy in the growth of skeletal muscle in juvenile Atlantic salmon, *Salmo salar* L. *Journal of Fish Biology,* **37**(4), 505-519. https://doi.org/10.1111/j.1095-8649.1990.tb05884.x

Hinits, Y. & Hughes, S. M. (2007). Mef2s are required for thick filament formation in nascent muscle fibres. *Development,* **134**(13), 2511-2519. http://dev.biologists.org/content/134/13/2511.abstract

Hirsinger, E., Stellabotte, F., Devoto, S. H. & Westerfield, M. (2004). Hedgehog signaling is required for commitment but not initial induction of slow muscle precursors. *Developmental Biology,* **275**(1), 142-157. https://doi.org/10.1016/j.ydbio.2004.07.030

Holterman, C.E. & Rudnicki, M.A., 2005. Molecular regulation of satellite cell function. *Seminars in Cell and Developmental Biology*, **16**(4-5), 575–584. https://doi.org/10.1016/j.semcdb.2005.07.004

Johnston, I.A. (1993a). Temperature influences muscle differentiation and the relative timing of organogenesis in herring (*Clupea harengus*) larvae. *Marine Biology,* **116**(3), 363-379. https://doi.org/10.1007/BF00350053

Johnston, I.A. (2001). Muscle structure and muscle growth patterns are key determinants of product quality in Atlantic salmon. In: *Cultivation of salmon II.* Bergen, Norway 7-10 May 2001

Johnston, I.A. (2006). Environment and plasticity of myogenesis in teleost fish. *The Journal of Experimental Biology*, **209**(12), 2249-2264. http://jeb.biologists.org/cgi/doi/10.1242/jeb.02153

Johnston, I.A. & Lucking, M. (1978). Temperature induced variation in the distribution of different types of muscle fibres in the goldfish (*Carassius carassius*). *Journal of Comparative Physiology*, **124**(2), 111-116. https://doi.org/10.1007/BF00689170

Johnston, I.A. & Horne, Z. (1994). Immunocytochemical investigations of muscle differentiation in the Atlantic herring (*Clupea harengus*: teleostei). J*ournal of the Marine Biological Association of the United Kingdom,* **74**(1), 79-91. https://doi.org/10.1017/S002531540003568

Johnston, I.A., Ward, P.S. & Goldspink, G. (1975). Studies on the swimming musculature of the rainbow trout I. Fibre Types. *Journal of Fish Biology,* **7**(4), 451-458. https://doi.org/10.1111/j.1095-8649.1975.tb04620.x

Johnston, I.A., Davison,, W. & Goldspink, G. (1977). Energy metabolism of carp swimming muscles. *Journal of Comparative Physiology,* **114**(2):203-216. https://doi.org/10.1007/BF00688970

Johnston, I.A., Vieira, V.L.A. & Abercromby, M. (1995). Temperature and myogenesis embryos of the Atlantic herring (*Clupea harengus*). *Journal of Experimental Biology,* **198**(Pt6), 1389-1403. http://www.ncbi.nlm.nih.gov/pubmed/9319285

Johnston, I.A., Cole, N.J., Abercromby, M. & Vieira, V.L.A. (1998). Embryonic temperature modulates muscle growth characteristics in larval and juvenile herring. *Journal of Experimental Biology,* **201**(Pt12), 623-646. http://www.ncbi.nlm.nih.gov/pubmed/9450973

Johnston, I.A., Manthri, S., Alderson, R., Campbell, P., Mitchell, D., Whyte, D., Dingwall, A., Nickell, D., Selkirk, C. & Robertson, B. (2002). Effects of dietary protein level on muscle cellularity and flesh quality in Atlantic salmon with particular reference to gaping. *Aquaculture,* 210(1-4), 259-283. https://doi.org/10.1016/S0044-8486(01)00862-6

Johnston, I. A., D. A. Fernandez, J. Calvo, V. L. Vieira, A. W. North, M. Abercromby & T. Garland, Jr. (2003). Reduction in muscle fibre number during the adaptive radiation of notothenioid fishes: a phylogenetic perspective. *The Journal of Experimental Biology,* **206**(15): 2595-2609. http://jeb.biologists.org/content/206/15/2595.abstract

Johnston, I. A., M. Abercromby, V. L. Vieira, R. J. Sigursteindottir, B. K. Kristjansson, D. Sibthorpe & S. Skulason (2004). Rapid evolution of muscle fibre number in postglacial populations of Arctic charr *Salvelinus alpinus*. *The Journal of Experimental Biology,* **207**(Pt25): 4343-4360

Johnston, I.A., Bower, N.I. &. Macqueen, D.J. (2011). Growth and the regulation of myotomal muscle mass in teleost fish. *The Journal of Experimental Biology*, **214**(10), 1617-1628. http://jeb.biologists.org/cgi/doi/10.1242/jeb.038620

Jones, P.W., F.D. Martin & J.D. Hardy Jr. (1978). Development of fishes of the Mid-Atlantic Bight. An atlas of eggs, larval and juvenile stages. Vol. 1. Acipenseridae through Ictaluridae. U.S. Fish and Wildlife Service, Office of Biological Services, Program FWS/OBS-78/12. pp336 http://pubs.er.usgs.gov/publication/fwsobs78_12

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

Kawauchi, H. & Sower, S.A. (2006). The dawn and evolution of hormones in the adenohypophysis. *General and Comparative Endocrinology*, **148**(1), 3-14. https://doi.org/10.1016/j.ygcen.2005.10.011

Kiessling, A., Storebakken, T., asgtid, T., & Kiessling, K.-H. (1991). Changes in the structure and function of the epaxial muscle of rainbow trout (*Oncorhynchus mykiss*) in relation to ration and age. I. Growth dynamics. *Aquaculture,* **93**(4), 335-356. https://doi.org/10.1016/0044-8486(91)90226-W

Kiessling, A., Ruohonen, K. & Bjørnevik, M. (2006). Muscle fibre growth and quality in fish. *Archives of Animal Breeding*, **49**(SI1), 137-146. http://www.archanimbreed.com/pdf/2006/at06si1p137.pdf

Kilarski, W. & Kozlowska, M. (1983). Ultrastructural characteristic of the teleostean muscle fibres and their nerve endings. The Stickleback (*Gusrerosteus aculeatus* L.). *Zeitschrift fur mikroskopisch-anatomische Forschung*, **97**(6),1022-1036

Kottelat, M. & Freyhof, J. (2007). In: Kottelat (ed), *Handbook of European freshwater fishes*. Berlin: 646 pp.

Koumans, J.T.M. & Akster, H.A. (1995). Myogenic cells in development and growth of fish. *Comparative Biochemistry and Physiology Part A: Physiology*, **110**(1), 3-20, https://doi.org/10.1016/0300-9629(94)00150-R

Kryvi, H., Flood. P.R. & Gulyaev D. (1980). The ultrastructure and vascular supply of the different fibre types in the axial muscle of the sturgeon *Acipenser stellatus*, Pallas. *Cell and Tissue Research*, **212**(1), 117–126. https://doi.org/10.1007/BF00234038

Liem, K.F., Bemis, W.E., Walker, W.F. & Grande, L. (2001). In: Harcourt College Publishers (eds), *Functional anatomy of the vertebrates: An Evolutionary Perspective,* 3rd ed., Philadelphia: Brookes/Cole

Marschallinger, J., Obermayer, A., Sänger, A.M., Stoiber, W., Steinbacher, P. (2009). Postembryonic fast muscle growth of teleost fish depends upon a nonuniformly distributed population of mitotically active Pax7+ precursor cells. *Developmental Dynamics,* **238**(9), 2442–2448. https://doi.org/10.1002/dvdy.22049

Mascarello, F., Rowlerson, A., Radaelli, P. & Veggetti, A. (1995). Differentiation and growth of muscle in the fish *Sparus aurata* (L): I. Myosin expression and organization of fibre types in lateral muscle from hatching to adult. *Journal of Muscle Research and Cell Motility,* **16**(3), 213-222. https://doi.org/10.1007/BF00121130

Maures, T., Chan, S.J., Xu, B., Sun, H., Ding, J. & Duan, C. (2002). Structural, biochemical, and expression analysis of two distinct insulin-like growth factor I receptors and their ligands in zebrafish. *Endocrinology,* **143**(5), 1858-1871. https://doi.org/10.1210/endo.143.5.8768

Merkel, M. (1995). Somitogenesis and myotomal myogenesis in European grayling *Thymallus thymallus* (L.) (Teleostei). *Zoologica Poloniae*, **40**(1-4), 119-130

Meyer-Rochow, V.B. & Ingram, J.R. (1993). Red-white muscle distribution and fibre growth dynamics: a comparison between lacustrine and riverine populations of the Southern smelt *Retropinna retropinna* Richardson. *Proceedings of the Royal Society of London. Series B: Biological*

Sciences, **252**(1334), 85-92. http://rspb.royalsocietypublishing.org/content/252/1334/85.abstract

Montserrat, N., Gabillard, J.C., Capilla, E., Navarro, M.I. & Gutierrez, J. (2007). Role of insulin, insulin-like growth factors, and muscle regulatory factors in the compensatory growth of the trout *(Oncorhynchus mykiss)*. *General and Comparative Endocrinology*, **150**(3), 462-472. https://doi.org/10.1016/j.ygcen.2006.11.009

Moyle, P.B. & Cech, J.J. (1982). In: Pearson Prentice Hall (ed), *Fishes: An introduction to Icthyology*, London

Nathanallides, C., Lopez-Albors, O. & Stickland, N.C. (2011). Influence of prehatch temperature on the development of muscle cellularity in posthatch Atlantic salmon (*Salmo salar*). *Canadian Journal of Fisheries and Aquatic Sciences*, **52**(4), 675-680. https://doi.org/10.1139/f95-068

Nikol'skaya, M.P., Evgen'eva, T.P. & Shagaeva, V.G. (2004). State of Skeletal Muscles in Larvae of Sturgeons of the Volga Basin in Artificial Reproduction. *Doklady Biological Sciences*, **396**(1-6), 230–232. https://doi.org/10.1023/B:DOBS.0000033285.36385.2d

Patterson, S.E., Mook, L.B. & Devoto, S.H. (2008). Growth in the larval zebrafish pectoral fin and trunk musculature. *Developmental Dynamics*, **237**(2), 307-315. https://doi.org/10.1002/dvdy.21400

Peachy, L.D. (1961). Structure of the longitudinal body muscles of Amphioxus .*The Journal of biophysical and biochemical cytology*, **10**(4), 159LP-176. http://jcb.rupress.org/content/10/4/159.abstract

Pelletier, D., Blier, P.U., Lambert, Y. & Dutil, J.D. (1995). Deviation from the general relationship between RNA concentration and growth rate in fish. *Journal of Fish Biology,* **47**(5),920-922. https://doi.org/10.1111/j.1095-8649.1995.tb06015.x

Periago, M.J., Ayala, M.D., López-Albors, O., Abdel, I., Martínez, C., Garcia-Alcázar, A., Ros, G. & Gil, F. (2005). Muscle cellularity and flesh quality of wild and farmed sea bass, *Dicentrarchus labrax* L. *Aquaculture,* **249**(1-4), 175-188. https://doi.org/10.1016/j.aquaculture.2005.02.047

Przybylski. J. & Blumberg, J.M. (1966). Ultrastructural aspect of myogenesis in the chick. *Laboratory Investigation,* **15**(5), 836-863

Radaelli, G., Domeneghini, C., Arrighi, S., Vaini, F. & Mascarello, F. (1999). Histochemical and immunohistochemical investigation of muscle fibres in the sturgeon (Chondrostei; *Acipenser*).

Journal of Applied Ichthyology, **15**(4-5), 87-91. https://doi.org/10.1111/j.1439-0426.1999.tb00213.x

Reinecke, M., Bjornsson, B.T., Dickhoff, W.W., McCormick, S.D., Navarro, I, Power, D.M. & Gutierrez, J. (2005). Growth hormone and insulin-like growth factors in fish: where we are and where to go. *General and Comparative Endocrinology*, **142**(1-2), 20-24. https://doi.org/10.1016/j.ygcen.2005.01.016.

Rescan, P.Y. (2008). New insights into skeletal muscle development and growth in teleost fishes. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution,* **310B**(7), 541–548. https://doi.org/10.1002/jez.b.21230

Rome, L.C., Funke, R.P., Alexander, R. M., Lutz, G.J., Aldridge, H., Scott, F. & Freadman, M. (1988). Why animals have different muscle fibre types. *Nature,* **335**, 824-827. http://dx.doi.org/10.1038/335824a0

Rome, L.C. (1994). The mechanical design of the fish muscular system. In: Maddock, L., Bone, Q., & Rayner, J. M. V. (eds), *Mechanics and Physiology of Animal Swimming,* pp. 75-97. Cambridge: Cambridge University Press. https://doi.org/10.1017/CBO9780511983641

Rowlerson, A. & Vegetti, A (2001). Cellular mechanism of post-embryonic muscle growth in aquaculture species. In: Ian A. Johnston (ed), *Muscle development and growth*, pp 103-140. London: Academic Press

Rowlerson, A., Scapolo, P.A., Mascarello, F., Carpene, E., & Veggetti, A. (1985). Comparative study of myosins present in the lateral muscle of some fish, species variations in myosin isoforms and their distribution in red, pink and white muscle. *Journal of Muscle Research and Cell Motility,* **6**(5), 601-640. https://doi.org/10.1007/BF00711917

Rowlerson, A., Mascarello, F., Radaelli, G., & Veggetti, A. (1995). Differentiation and growth of muscle in the fish *Sparus aurata* (L): II. Hyperplastic and hypertrophic growth of lateral muscle from hatching to adult. *Journal of Muscle Research and Cell Motility*, **16**(3), 223-236. https://doi.org/10.1007/BF00121131

Ruban, G.I. (2005). In: World Sturgeon Conservation Society, Special Publication No1. *The Siberian sturgeon, Acipenser baerii Brandt: species structure and ecology*, p. 203

Sänger, A.M. (1992). Quantitative fine structural diversification of red and white muscle fibres in cyprinids. *Environomental Biology of Fishes,* **33**(1-2), 97-104. https://doi.org/10.1007/BF00002557

Sänger, A.M. (1993). Limits to the acclimation of fish muscle. *Reviews in Fish Biology and Fisheries*, **3**(1), 1-15. https://doi.org/10.1007/BF00043295

Sänger, A.M. & Stoiber, W. (2001). Muscle fiber diversity and plasticity. In: Ian A. Johnston (ed), *Muscle development and growth*, pp. 187-250. London: Academic Press

Scapolo, P.A., Veggetti, A., Mascarello, F., & Romanello, M.G. (1988). Developmental transitions of myosin isoforms and organisation of the lateral muscle in the teleost *Dicentrarchus labrax* (L.). *Anatomy and Embryology*, **178**(4), 287-295. https://doi.org/10.1007/BF00698660

Sellers, J.R. (2000). Myosins: a diverse superfamily (Review). *Biochimica et Biophysica Acta,* **1496**(1), 3-22. https://doi.org/10.1016/S0167-4889(00)00005-7

Silva, P., Valente, L.M., Olmedo, M., Galante, M.H., Monteiro, R.A. and Rocha, E. (2009). Hyperplastic and hypertrophic growth of lateral muscle in blackspot seabream *Pagellus bogaraveo* from hatching to juvenile. *Journal of Fish Biology*, **74**(1), 37-53. doi: 10.1111/j.1095-8649.2008.02122.x

Sokolov, L.I. and Vasil'ev, V.P. (1989). *Acipenser baeri* Brandt, 1869. pp. 263–284. In: Peter J Miller (ed.), *The Freshwater Fishes of Europe. Vol. I/II*, Aula Verlag, Wiesbaden.

Steinbacher, P., Haslett, J.R., Sänger, A.M. and Stoiber, W. (2006). Evolution of myogenesis in fish: a sturgeon view of the mechanisms of muscle development. *Anatomy and Embryology*, **211**(4), 311–322. https://doi.org/10.1007/s00429-006-0082-4

Steinbacher, P., Haslett, J.R., Six, M., Gollmann, H.P., Sänger, A.M. and Stoiber, W. (2006b). Phases of myogenic cell activation and possible role of dermomyotome cells in teleost muscle formation. *Development Dynamics,* **235**(11), 3132-3143. https://doi.org/10.1002/dvdy.20950

Steinbacher, P., Haslett, A., Obermayer, A., Marschallinger, H.C., Bauer, H.C., Sänger, A.M. and Stoiber, W. (2007). MyoD and Myogenin Expression during myogenic phases in brown trout: a precocious onset of mosaic hyperplasia is a prerequisite for fast somatic growth. *Development Dynamics,* **236**(4), 1106-1114. https://doi.org/10.1002/dvdy.21103

Stellabotte, F., Dobbs-McAuliffe, B., Fernandez, D.A., Feng, X. and Devoto, S.H. (2007). Dynamic somite cell rearrangements lead to distinct waves of myotome growth. *Development,* **134**(7), 1253-1257. http://dev.biologists.org/content/134/7/1253.abstract

Stickland, N.C. (1983). Growth and development of muscle fibres in the rainbow trout (*Salmo gairdneri*). Journal of Anatomy 137, 323-333

Stickney, H.L., Barresi, M.J.F. and Devoto, S.H. (2000). Somite development in zebrafish. *Developmental Dynamics*, **219**(3), 287–303. https://doi.org/10.1002/1097-0177(2000)9999:9999<::AID-DVDY1065>3.0.CO;2-A

Stoiber, W. and Sänger, A.M. (1996). An electron microscopic investigation into possible source of new muscle fibres in teleost fish. *Anatomy and Embryology,* **194**, 569-579. DOI:10.1007/BF00187470

Stoiber, W., Haslett, J.R., Goldschmid, A. and Sänger, A.M. (1998). Patterns of superficial fibre formation in the European pearlfish (*Rutilus frisii meidingeri*) provide a general template for slow muscle development in teleost fish. *Anatomy and Embryololy,* **197**(6),485–496. https://doi.org/10.1007/s004290050159

Stoiber, W., Haslett, J.R., Wenk, R., Steinbacher, P., Gollmann, H.P. and Sänger, A. M. (2002). Cellularity changes in developing red and white fish muscle at different temperatures: simulating natural environmental conditions for a temperature freshwater cyprinid. *Journal of Experimental Biology,* **205**(Pt16), 2349–2364. http://dev.biologists.org/content/134/7/1253.abstract

Tanaka, Y., Gwak, W.S., Tanaka, M., Sawada, Y., Okada, T., Misyashita, S. and Kumai, H. (2007). Ontgenic changes in RNA, DNA and protein contents of laboratory-reared Pacific bluefin tuna *Thunnus orientalis. Fisheries Science,* **73**(2), 378-384. https://doi.org/10.1111/j.1444-2906.2007.01345.x

Valente, L.M.P., Gomes, E.F.S. and Fauconneau, B. (1998). Biochemical growth characterization of fast and slow-growing rainbow trout strains: effect of cell proliferation and size. *Fish Physiology and Biochemistry*, **18**(3), 213-224. https://doi.org/10.1023/A:1007774929535

Valente, L.M.P, Rocha, E., Gomes, E.F.S., Silva, M.W., Oliveira, M.H., Monteiro, R.A.F. and Fauconneau, B. (1999). Growth dynamics of white and red muscle fibers in fast and slow growing strains of rainbow trout. *Journal of Fish Biology,* **55**(4), 675-691. https://doi.org/10.1111/j.1095-8649.1999.tb00710.x

Valente, L.M., Moutou, K.A., Conceição, L.E., Engrola, S., Fernandes, J.M. and Johnston, I.A. (2013). What determines growth potential and juvenile quality of farmed fish species? *Reviews in Aquaculture*, **5**(s1), 1753-5123. https://doi.org/10.1111/raq.12020

Van Leeuwen, J.L. (1999). A mechanichal analysis of myomere shape in fish. *Journal of Experimental Biology,* **202**(23), 3405-3414. http://jeb.biologists.org/content/202/23/3405.abstract

Van Raamsdonk, W., Pool, C.W., Kronnie, G. (1978). Differentiation of muscle fibre types in the Teleost *Brachydanio rerio*. *Anatomy and Embryology,* **153**(2), 137-155. https://doi.org/10.1007/BF00343370

Van Raamsdonk, W., te Kronnie, G., Pool, C.W., and Van de Laarse, W. (1980). An immune histochemical and enzymic characterization of the muscle fibres in myotomal muscle of the teleost *Brachydanio rerio*, Hamilton-Buchanan. *Acta histochemical,* **67**(2), 200-216. https://doi.org/10.1016/S0065-1281(80)80024-9.

Videler, J. J. (1993). In: *Fish Swimming,* London: Chapman and Hall

Veggetti, A., Mascarello, F., Scapolo, P.A. and Rowlerson, A. (1990). Hyperplastic and hypertrophic growth of lateral muscle in *Dicentrarchus labrax* (L.). An ultraestrutral and morphometric study. *Anatomy and Embryology,* **182**(1), 1-10. https://doi.org/10.1007/BF00187522

Veggetti, A., Mascarello, F., Scapolo, P.A., Rowlerson, A. and Carnevali, C.M.D. (1993). Muscle growth and myosin isoform transitions during development of a small teleost fish, *Poecilia reticulata* (Peters) (Atheriniformes, Poeciliidae): a histochemical, immunohistochemical, ultrastructural and morphometric study. *Anatomy and Embryology,* **187**(4), 353-361. DOI:10.1007/BF001858

Veggetti, A., Rowlerson, A., Radaelli, G., Arrighi, S. and Domeneghini, C. (1999). Posthatching development of the gut and lateral muscle in the sole. *Journal of Fish Biology,* 55(SA), 44-65. https://doi.org/10.1111/j.1095-8649.1999.tb01045.x

Webb, P.W. (1984b). Form and function in fish swimming. *Scientific American*, **251**, 72-83-68. https://www.jstor.org/stable/24969414

Weatherley, A.H. and Gill, H.S. (1984). Growth dynamics of white myotomal muscle fibres in the bluntnose minnow, *Pimephales notatus* Rafinesque, and comparison with rainbow trout, *Salmo*

gairdneri Richardson. *Journal of Fish Biology,* **25**(1), 13-24. https://doi.org/10.1111/j.1095-8649.1984.tb04846.x

Weatherley, A.H., Gill, H.S. and Rogers, S.C. (1979). Growth dynamics of muscle fibers, dry weight, and condition in relation to somatic growth rate in yearling rainbow trout (*Salmo gairdneri*). *Canadian Journal of Zoology,* **57**(12), 2385-2392. https://doi.org/10.1139/z79-310

Weatherley, A.H., Gill, H.S. and Rogers, S.C. (1980). The relationship between mosaic muscle fibers and size in rainbow trout (*Salmo gairdneri*). *Journal of Fish Biology,* **17**(5), 603-610. https://doi.org/10.1111/j.1095-8649.1980.tb02792.x

Weatherley, A.H.; Gill, H.S.; Lobo, A.F. (1988). Recruitment and maximal diameter of axial muscle fibres in teleosts and their relationship to somatic growth and ultimate size. *Journal of Fish Biology,* **33**(6), 851-859. https://doi.org/10.1111/j.1095-8649.1988.tb05532.x

Westneat, M.W. and Wainwright S.A. (2001). Mechanical design for swimming: muscle, tendon, and bone. *Fish physiology*, **19**, 271–311

Wilson, E.M. and Rotwein, P. (2006). Control of MyoD function during initiation of muscle differentiation by an autocrine signaling pathway activated by insulin-like growth factor-II. *The Journal of Biological Chemistry*, **281**(40), 29962-29971. doi:10.1074/jbc.M605445200

Wood, A.W., Duan, C. and Bern, H.A. (2005). Insulin-like growth factor signalling in fish. *International Reviews in Cytology*, **243**, 215-285. DOI:10.1016/S0074-7696(05)43004-1

Zhang, G., Swank, D.M. and Rome, L.C. (1996). Quantitative distribution of muscle fiber types in the scup *Stenotomus chrysops*. *Journal of Morphology,* **229**(1), 71-81. https://doi.org/10.1002/(SICI)1097-4687(199607)229:1<71::AID-JMOR4>3.0.CO;2-S

Zhu, K., Wanga, H., Wanga, H., Gulb, Y., Yanga, M., Zenga, C. and WangaaKey, W. (2014). Characterization of muscle morphology and satellite cells, and expression of muscle-related genes in skeletal muscle of juvenile and adult *Megalobrama amblycephala*. *Micron,* **64**, 66–75. https://doi.org/10.1016/j.micron.2014.03.009

Chapter 2

Effects of different rearing temperatures on muscle development and stress response in the early larval stages of *Acipenser baerii*

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Effects of different rearing temperatures on muscle development and stress response in the early larval stages of *Acipenser baerii*

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Introduction

Sturgeons (Acipenseriformes) constitute one of the most ancient groups of the Osteichthyes, and it comprises 25 species that are distributed throughout the Northern Hemisphere, Eurasia and North America.[1] During the latest two hundred years, there was a severe worldwide decrease of the sturgeon stocks and, currently, most of the sturgeon species are in the Apendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). Sturgeon farming is, therefore, important not only for the production of caviar and meat but also because it may contribute to the natural stocks protection. Among the sturgeon species, one of the most commonly used in aquaculture is the Siberian sturgeon (*Acipenser baerii*) which is currently reared in 22 countries presenting a total production of about 8800 tonnes

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Key words: Sturgeon larvae; muscle development; temperature; heat shock protein; proliferating cells; histometry.

Contributions: LA, ADG, CD designed the project; LA, ADG, ML performed the *in vivo* experimental procedures; LA, ADG, performed the laboratory analyses; LA, VS, LMPV, performed the histometrical analyses; LA, ADG, CD, performed the statistical analyses and wrote the paper. All authors contributed to the different draft versions of the manuscript and approved the final manuscript.

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2. Effects of different rearing temperatures on muscle development and stress response in the early larval stages of *Acipenser baerii*

Abstract

The present study aims at investigating muscle development and stress response in early stages of Siberian sturgeon when subjected to different rearing temperatures, by analysing growth and development of the muscle and by assessing the stress response of yolk-sac larvae. Siberian sturgeon larvae were reared at 16°C, 19°C and 22°C until the yolk-sac was completely absorbed. Sampling timepoints were: hatching, schooling and complete yolk-sac absorption stage. Histometrical, histochemical and immunohistochemical analyses were performed in order to characterize muscle growth (total muscle area, TMA; slow muscle area, SMA; fast muscle area, FMA), development (anti proliferating cell nuclear antigen –PCNA or anti-caspase) as well as stress conditions by specific stress biomarkers (heat shock protein 70 or 90; HSP70 or HSP90). Larvae subjected to the highest water temperature showed a faster yolk-sac absorption. Histometry revealed that both TMA and FMA were larger in the schooling stage at 19°C while no differences were observed in the SMA at any of the tested rearing temperatures. PCNA quantification revealed a significantly higher number of proliferating cells in the yolk-sac absorption phase at 22°C than at 16°C. HSP90 immunopositivity seems to be particularly evident at 19°C. HPS70-immunopositivity was never observed in the developing lateral muscle.

Key words: sturgeon larvae, muscle development, temperature, heat shock protein, proliferating cells, histometry

2.1. Introduction

Sturgeons (Acipenseriformes) constitute one of the most ancient groups of the Osteichthyes, and it comprises 25 species that are distributed throughout the Northern Hemisphere, Eurasia and North America (Birnstein et al. 1993). During the latest two hundred years, there was a severe worldwide decrease of the sturgeon stocks and, currently, most of the sturgeon species are in the Apendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). Sturgeon farming is, therefore, important not only for the production of caviar and meat but also because it may contribute to the natural stocks protection. Among the sturgeon species, one of the most commonly used in aquaculture is the Siberian sturgeon

(*Acipenser baerii*) which is currently reared in 22 countries presenting a total production of about 8.800 tonnes per year.

Production of high quality fish larvae is of great importance in aquaculture: even if large amounts of larvae hatch from artificially fertilized eggs, mortality in the early stages still remains quite high or present a high, not expected variability, or frequent rates of deformities occur (Leprevost et al., 2017). For the aquaculture industry it is, hence, of vital importance to produce high quality and healthy juveniles.

Embryonic and larval development in bony fish is strongly influenced by environmental factors, mainly by temperature and dissolved oxygen, as it is in adults. In teleost species larvae, the environmental factors determine the myogenesis rate, the subcellular structure of the muscular fibres, the gene expression models and the number and dimension of muscle fibres. In studies performed in seawater larval species, Conceicao et al. (2010) found that environment has also an influence on the efficiency of the contractile proteins deposition. Larval development of sturgeon still represents an extremely difficult stage, often characterised by high mortality rates (Mohler et al., 2000).

In intensive fish farming, aquatic species are continuously subjected to several environmental discomfort conditions such as handling, feed composition, confinement, density, hierarchical relations, as well as water quality variations in its both abiotic (pH, dissolved oxygen, temperature, pollutants, etc.) and biotic (plankton, bacteria, etc.) components that, as a whole, may lead to environmental stress occurrence (Lushchak, 2011). Farmed sturgeon is, as well, subjected to many environmental stressors, like crowding, handling procedures that precede transportation and/or medical treatments, temperature variations, and may be of great interest for the fish farmers to identify the stress effects of the environmental conditions.

A better understanding of the effects of stress could give additional information regarding the environmental causes of the observed discrepancies (Domeneghini et al, 2002; Di Giancamillo et al., 2012) in growth rates among different stocks, with important positive consequences again on sturgeon farming and production. 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.

Heat shock proteins (HSP) are a family of highly conserved proteins that are expressed in response to several biotic and abiotic stressors, in such a way to be potentially identified as damage biomarkers. An intense stress, not only thermal in its nature, induces a long-time

expression of HSPs that are also known to play an important role in fish health (Feder and Hofmann, 1999; Roberts et al., 2010). HSP70 assists the folding of emerging polypeptide chains, act as a molecular chaperone, and intervenes on the repair and degradation of altered or denatured proteins. HSP90 has an active role in supporting various components of cell signalling, including the cytoskeleton, enzymes, and steroid hormone receptors. Heat shock protein 70 expression has been studied in fish in relation to the exposure to pesticides, virus, metals and other toxic compounds (Roy and Bhattacharya, 2006; Eder et al., 2007; Maradonna et al., 2007).

In fish larvae the environmental stressors appear to affect a greater number of developing tissues than in adults (Pederzoli and Mola, 2016). In addition, fish larvae may appear less tolerant than the adults to temperature variations (Stefanovich et al., 2016), especially in critical moments of larvae development. According to Gisbert and Williot (Gisbert and Williot, 2002), after hatching, Siberian sturgeon shows benthic behaviour, then aggregates into schools. Normal schooling behaviour in this species can be used as a quality condition similarly in coregonid species (Zitov and Millard, 1988). When the yolk-sac absorption is complete, schooling behaviour ceases, and most of the larvae is distributed on the bottom of the tank, or swimming along the tank walls and along the surface of water (Gisbert et al., 1999).

There is increasing indication that early events may imprint an individual physiological memory leading to long-term effects on postnatal growth and physiological function, both in animals and humans (Rehfeldt et al., 2011). Environmental factors during the early stages may have a serious impact in an irreversible way in later development (Rehfeldt et al., 2011). It is, therefore, very important to optimize early rearing conditions to promote maximum growth.

The aim of the present study was, therefore, to monitor environmental stress occurrence and lateral muscle development in the early (until yolk sac absorption) larval stages in Siberian sturgeon (*Acipenser baerii*), when subjected to one of the main stress factors occurring in fish farms: rearing temperature. The effects of the three different rearing temperatures as a possible stressor on muscle development in Siberian sturgeon larvae until complete yolk-sac absorption have been studied in this paper concerning: i) lateral muscle structural organization, and cell replication, in order to characterize lateral muscle growth and ii) stress biomarkers.

2.2. Material and Methods

2.2.1. *Fish larvae rearing and sampling*

The experiment was held during April 2016 at the Experimental Animal Research and Application Centre of Lodi, of the University of Milan. Siberian sturgeon fertilized (artificially inseminated) eggs were transported at 14°C from the "Società Agricola Naviglio" fish farm to the experimental unit 24 hours after fertilization. Embryonic incubation was performed at the same temperature (16.2 ± 0.2°C) and hatching occurred 5 days after fertilization. Larvae were maintained in incubators (three per treatment); in one group temperature was maintained at 16°C (16.4 ± 0.2 °C) and, in the two other groups, temperatures were gradually shifted (one degree per hour) to either 19°C (19.3 ± 0.2°C) or 22°C (21.9 ± 0.2 °C). Water temperature was daily monitored and kept under the fixed values for each incubator. Water was saturated with O_2 throughout the trial (>8mg/l) in all incubators pH values were within the range described for this species in this stage of development (25). Eggs and larvae were exposed to an artificial photoperiod regime of 12L:12D. Along the entire experimental period after hatching the larvae utilized the nutrients of their yolk sac and were not fed any exogenous food.

Moreover, sampling temporal points were chosen considering the important steps of Siberian sturgeon larvae behaviour development already described in the introduction: hatching (T0), beginning of the schooling phase (T1) and complete yolk sac absorption phase (T2). Larval development period was calculated as days post-hatch (dph) until the yolk sac full absorption (Figure 2.1).

Survival rate was estimated by dead larvae daily recording.

This research was approved by the Ethic Committee of the University of Milan (OPBA_20_2016).

2.2.2. *Histometry, histochemistry and immunohistochemistry*

For each sampling time point 3 larvae per incubator were collected (total n=9 larvae per treatment) with a wide pipette and killed by over-anaesthesia with Ethyl 3-Aminobenzoate, Methanesulfonic A (Sigma-Aldrich). Whole larvae were immediately fixed in 4% para-formaldehyde in 0.01M phosphate-buffered saline (PBS) pH 7.4 for 24h at 4°C, then dehydrated in a graded series of ethanol, cleared with xylene and embedded in paraffin. Serial transverse microtome sections (5 µm-thick) were obtained from each sample.

The sequential Haematoxylin/eosin (HE) stain was performed for the evaluation of the structural aspects of the developing lateral muscle tissues and for histometry. The Trichrome Masson histochemical stain was applied according Goldner (Goldner, 1938) in order to clearly distinguish developing connective and striated muscle tissues.

Briefly, the histometric variables as above detailed were measured on HE-stained sections of transversal body sections of individual fish larvae, at a peri-anal localization with the transverse sections conducted in a cranio-caudal direction with the first section rostral to the anus. Standard histometrical techniques were applied using an Olympus BX51 light microscope equipped with a DP-software program (Cell^B, Basic Imaging Software, Olympus, Italy) for describing: i) total muscle cross-sectional area (TMA), ii) red muscle area (slow muscle cross-sectional area, SMA), and iii) white muscle area (fast muscle cross-sectional area, FMA) at the three analysed developmental stages: hatching (T0), schooling (T1) and yolk-sac full absorption (T2).

Immunostaining on other transverse sections was performed to detect proliferating cell nuclear antigen (PCNA), caspase, Heat shock protein 70 (Hsp70) and Heat shock protein 90 (Hsp90), both the latter ones as in situ markers of the presence of environmental stressors. The applied immunohistochemical procedure has been previously described in detail (Di Giancamillo et al., 2009 for PCNA; Di Giancamillo et al., 2015 for HSP70 and HSP90). 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), HSP70 (1:100; N27F3-4/Enzo LifeSciences, Farmingdale, NY) and HSP90 (1:100, AC88/Enzo LifeSciences, Farmingdale, NY), as well as anti-caspase-3 (dilution 1:100, ab4051, ABCAM, Italy) antibodies were applied overnight at room temperature. Preliminary antigen retrieval for HSP70, HSP90 and caspase was performed by heat, with a microwave treatment (for 5 min at 450 W in citrate buffer, pH 6). 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 immunoglobulins for HSP70 and HSP 90 or against rabbit immunoglobulins for caspase (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 fibres 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.

2.2.3. *Statistical analysis*

Statistical analysis was performed with SAS statistical software (version 9.3, Cary Inc., NC). Data from the histometrical analyses (TMA, SMA and FMA) and PCNA cellular counts, were analysed using 2-way ANOVA with temperatures (16°C, 19°C, 22°C) and developmental stages (T0, T1 and T2) as main factors, and co-variated for the total area corresponding to the TMA. The data are presented as least-square means (SEM). Differences between means were considered significant at $P < 0.05$.

2.3. Results

2.3.1. *Larval Development*

The higher the temperature, the shorter was larvae development (Figure 2.1). Larvae subjected to 22°C, presented the schooling behaviour at 3dph and at 7dph the yolk-sac was fully absorbed; larvae subjected to both 16°C and 19°C reached the schooling stage 5dph but larvae subjected to 19°C showed a faster absorption of the yolk-sac when compared with larvae subjected to 16°C.

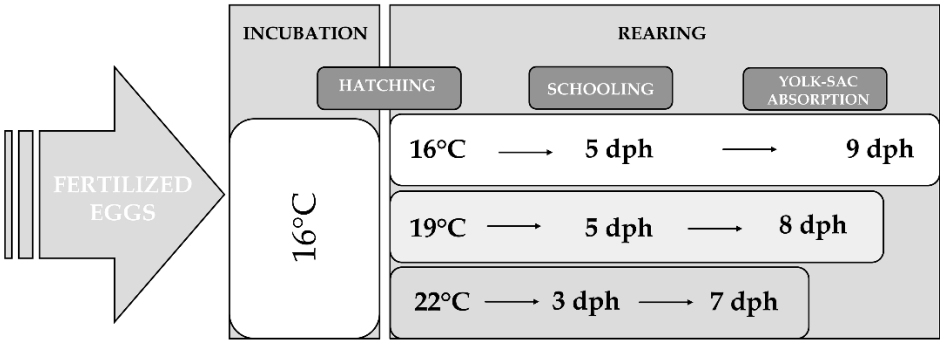

Figure 2.1. Experimental set-up. dph, days post-hatch.

2.3.2. *Histometry and Histochemistry*

HE staining results are presented in Figure 2.2. At hatching (T0; Figure 2.2a, Figure 2.2h), the lateral muscle was already organised in an internal (Figure 2h, bold arrow) as well a peripheral layer (Figure 2.2h, thin arrow). At T1 (Figure 2.2b-d and i), the transversally sectioned myotomes appeared composed by a layer of internal dorso-ventrally flattened lamella-shaped multinucleated muscle cells (future fast fibres; Fig 2.2i, asterisk). The nuclei were present in a central position in muscle cells. At this stage, there was a relevant expansion of the myotome at medium-lateral and dorsal-ventral levels, in comparison with previously, as a result of the addition of new developing fibres (Figure 2.2b-d; bold arrows).

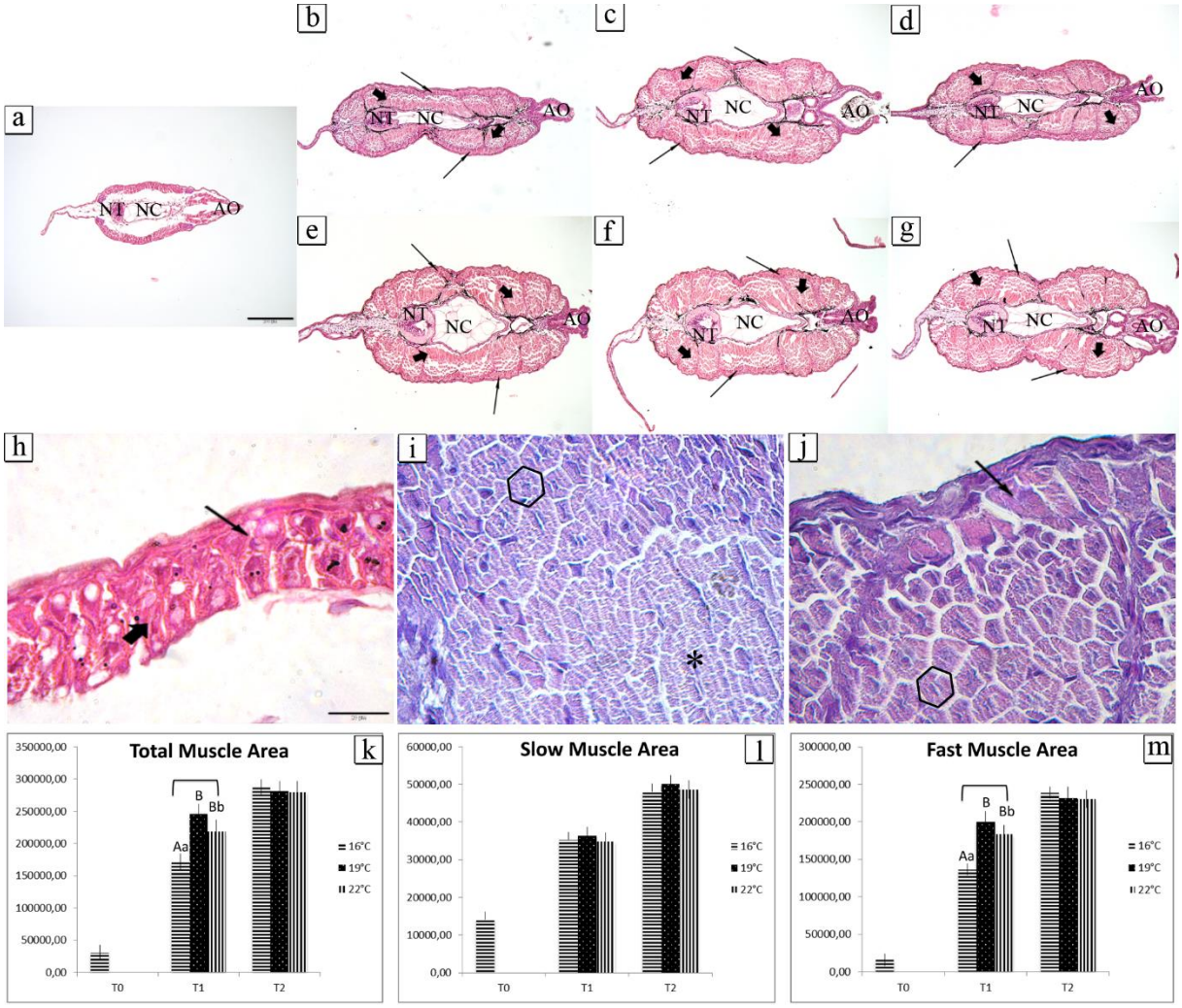

Figure 2.2. Images of the three temperatures at different timepoints - HE staining; a) at hatching, 16°C; b-d) at schooling, at 16°, 19° and 22°C respectively; e-g) yolk-sac full absorption, at 16°, 19°C and 22°C, respectively The figures have the same scale bar as the one located in Figure 1a: 200 µm; thin arrows, slow fibres; bold arrows, fast fibres; NC, notochord; NT, neural tube; AO, anal opening. h-i) HE staining representative figures for hatching, schooling and yolk-sac full absorption, respectively. Bold arrows, fast fibres; thin arrows, slow fibres. The figures have the same scale bar as the one located in Figure 1h: 20 µm. k) quantitative representation of TMA: area expressed in μm^2; n=9/group; Stage P<0.001; Temp. P<0.001; Stage*Temp P<0.001. l) quantitative representation of SMA: area expressed in μm^2; n=9/group; Stage P<0.001; Temp. P= 0.669; Stage*Temp P= 0.954. m)) quantitative representation of FMA: area expressed in μm^2; n=9/group; stage P <0.001; Temp. P<0.001; Stage*Temp P <0.001.

Most of the new generated fibres of the myotome were observed within the prospective fast muscle area, in such a way that the medial row of lamella-shaped fibres appeared overlaid laterally by a 3–4 layer bulk of smaller polygonal cells (Figure 2.2i, hexagon), which extended towards both dorsal and ventral parts. The prospective slow fibres at the periphery of the myotome have been shown to maintain a monolayer arrangement (Figure 2.2b-d, thin arrow) with only a few additional MPCs (MPCs = muscle precursor cells), mostly located at the medial edge of the layer. The myotomes did not show at this stage any anatomical separation between

their dorsal and ventral domains. There was still no evidence of any vascularisation of the myotome area (Figure 2.2b-d). The increase of the total muscle cross sectional area from hatching to the schooling phase was due mainly to an increase of the fast muscle cross sectional area: in only 3 to 5 days the fast muscle cross sectional area increased 11 times while the slow fibres area increased only 2,5 times (Figure 2.2e-g; thin arrows).

When the yolk-sac was fully absorbed (T2), the myotomes did not show any relevant difference in their structure compared to the schooling phase (T1), except for the further enlargement of the prospective fast muscle cross sectional area (Figure 2.2 e-g; bold arrows; Figure2.2j). Histometry revealed that both TMA and FMA were larger in the schooling phase at 19°C (Figure 2.2k and m; 19°C *vs* 16°C, $P<0.01$; 19°C *vs* 22°C, $P<0.05$), while no differences were observed in SMA at any of the tested rearing temperatures ($P>0.05$ all comparisons, Figure 2.2l).

Masson's Goldner Trichrome histochemistry confirmed the histological observations concerning the different areas occupied by the prospective slow and fast muscle fibres and the absence of a horizontal septum (Figure 2.3a-g, asterisk).

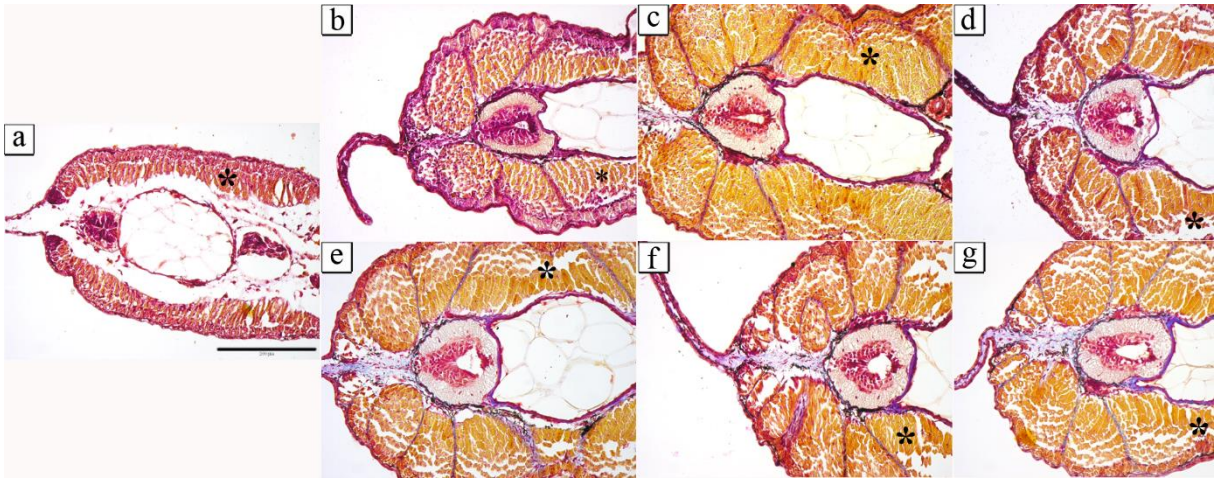

Figure 2.3. Images of the three temperatures at different timepoints - Masson's Trichrome Goldner staining: a) hatching at 16°C; b-d) schooling, at 16°, 19°C and 22°C respectively; e-g) yolk-sac full absorption at 16°, 19° and 22°C, respectively. Asterisks, lack of horizontal septum. Scale bar: 200 µm.

2.3.3. *Immunohistochemistry and cell counts*

Anti-PCNA immunohistochemistry showed at T0 a consistent number of immunopositive nuclei in both layers of the developing lateral muscle area, but mainly in the inner layer (Figure 2.4a). At both T1 and T2 stages (Figs. 2.4b-d and Figs. 2.4f-h respectively) several nuclei of muscle prospective fast fibres in all the experimental groups were shown to be immunoreactive (thin arrows), while the prospective slow fibres nuclei revealed to be always negative (asterisks). An

additional and representative record was the presence of immunopositivity in some neuroblast nuclei of the neural tube (Figure 2.4f, bold arrows). Quantitative evaluation of the proliferating prospective fast muscle cells (FMA) revealed that in the T0 group was significantly higher than all the other groups (Figure 2.4e; $P<0.001$). In the T2 group a significantly higher number of proliferating cells was detected at 22°C reared larvae than in those ones reared at 16°C (Figure 2.4e, $P<0.05$). The anti-caspase immunohistochemistry was never detected in the larvae irrespective of the temperatures, except for the presence of small groups of immunoreactive cells in the epithelial layer of the yolk sac and of the developing alimentary canal (data not shown).

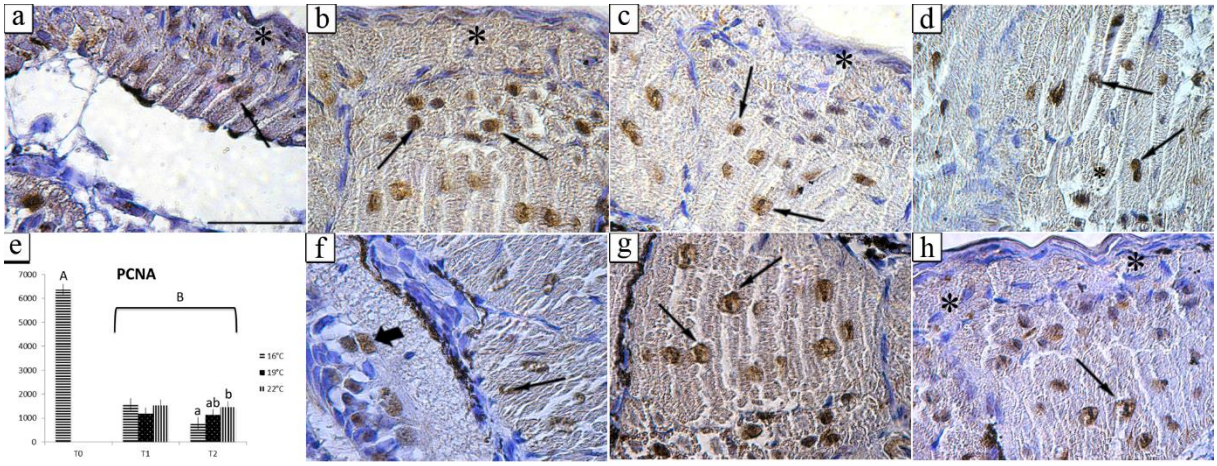

Figure 2.4. Images of the three temperatures at different timepoints - PCNA-immunolocalization. a) hatching at 16°C; b-d) schooling at 16°, 19° and 22°C, respectively; f-h) yolk-sac full absorption at 16°, 19° and 22°C, respectively. Immunopositive neuroblasts, bold arrow; immunopositive nuclei in the fast fibres, thin arrows; immunonegative slow fibres, asterisks; Scale bar: 20 µm. e) quantitative representation of PCNA counts. Area expressed in number/mm^2

A HPS70-immunopositivity was never observed in the developing lateral muscle, irrespective of the different analysed rearing temperatures (data not shown). On the contrary, a HSP90 immunopositivity was clearly detected in a large number of nuclei of the prospective muscle fast fibres, mainly in larvae subjected to 19°C (Figure 2.5a-g; asterisks) and in the neuroblasts (Figure 2.5a-g, thin arrows).

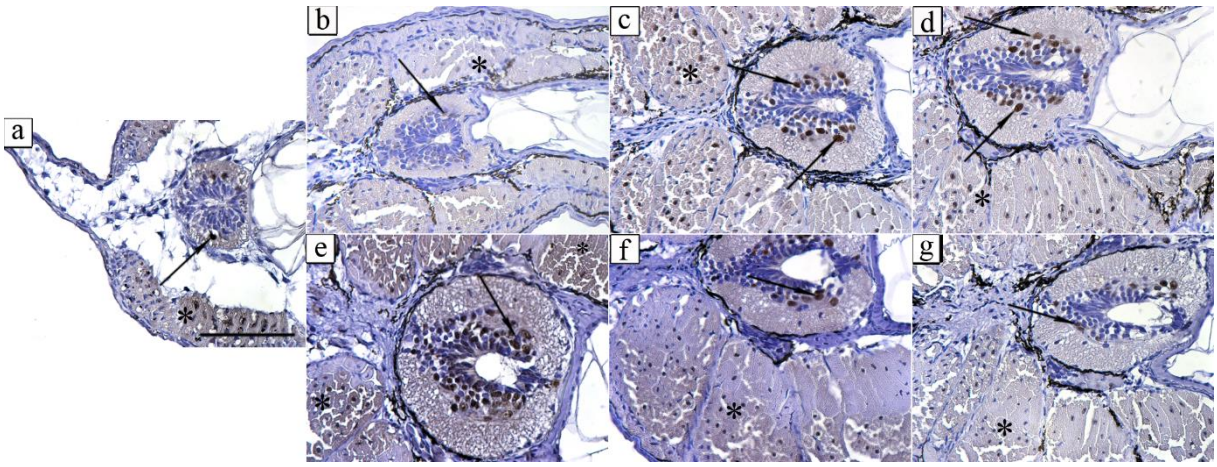

Figure 2.5. Images of the three temperatures at different timepoints - HSP90-immunolocalization. a) hatching at 16°C; b-d) schooling, at 16°, 19°C and 22°C respectively; e-g) yolk-sac full absorption at 16°, 19° and 22°C, respectively. Immunopositive neuroblasts, thin arrows immunopositive in fast fibres, asterisks. Scale bar: 100 µm.

2.4. Discussion

The present work investigated the impact of different rearing temperatures on muscle development of precocious (yolk sac) Siberian sturgeon larvae. Three rearing temperatures (16, 19 or 22 °C) were tested during Siberian sturgeon endogenous feeding larval phase, in order to analyse the short-term effect of temperature on the developing lateral muscle.

2.4.1. *Temperature effects on developmental rate and survival*

Temperature is one of the most important environmental factors that affect development and growth in teleost fishes (Moyle and Cech, 1982; Donaldson et al., 2008), and the larval phase is particularly susceptible to temperature changes (Johnston et al., 1995; Kamler, 2002). In the present study the time interval, during which larvae subjected to the highest rearing temperature (22°C) reached the complete yolk-sac absorption stage was numerically 20% less than the time required by larvae subjected to the lowest temperatures. Similarly, an increase of rearing temperatures (but within the physiological range) lead to an increase in the developmental rate in several marine species like Atlantic cod (Pepin et al., 1997), Senegalese sole (Campos et al., 2013) and gilthead seabream (Garcia de la Serrana et al., 2012), as well as in freshwater species like brown trout (Réalis-Doyelle et al., 2016), Atlantic salmon (Ojanguren et al., 1999) and in several species of Sturgeon (Hardy and Litvak, 2004). Moreover, it is also known that temperature can be associated with fish survival rates (Boucher et al., 2014). In fact, in studies conducted upon different sturgeon species a clear effect of temperature on survival to

hatch was shown (Wang et al., 1987; Van Eenennaam et al., 2005), whereas during the endogenous feeding stage temperature did not influence survival rates until the complete yolk-sac absorption (Gisbert et al., 2000, Boucher et al., 2014). In our study, survival rates from hatch to the complete yolk-sac absorption varied between 88 and 90% but there were no significant differences among tested rearing temperatures, which is in accordance with the above mentioned studies.

2.4.2. *Different temperatures and early events in myogenesis*

Our results showed that since the hatching phase it was already possible to identify in *A. baerii* a superficial monolayer of prospective slow fibres and a deep layer of prospective fast fibres. In addition, with the development going on until the yolk sac full absorption stage, the inner layer of prospective fast fibres becomes larger and larger likely through the recruitment of MPCs. The tested rearing temperatures did not show effects upon the described arrangement.

The horizontal septum is a specialized structure of the myotome, present in all gnathostome fishes, that divide the differentiating myotomes into dorsal and ventral muscle masses (Bone, 1989). In our study we found that, until the end of the trial, yolk-sac larvae showed no clear separation between the dorsal and ventral components of the myotomes. This is in accordance with a study on sterlet, where the formation of such septum occurred only 14 days after hatching, suggesting that physical factors such as mechanical strain from initial swimming movements are necessary for the development of this structure (Steinbacher et al., 2006). In comparison, in teleost fish the horizontal septum appears well defined already in the embryo (Currie et al., 2001; Chauvigné et al., 2006; Steinbacher et al., 2006). This aspect constitutes a major difference between teleosts and sturgeons.

2.4.3. *Different temperatures and lateral muscle histometry*

Muscle growth in fish has been extensively studied mainly in teleosts, mostly in intensively farmed species. In our study we measured in serial transverse sections the total cross sectional muscle area, the prospective slow muscle cross sectional area and the prospective fast muscle cross sectional area at three specific stages of development: hatching, schooling, and full yolk sac absorption. We found that total muscle cross sectional area and fast muscle cross sectional area were significantly larger at schooling stage for larvae subjected to the rearing temperature of 19°C. This difference was not significant at the yolk-sac absorption stage, where larvae subjected

to 16°C as the applied incubation temperature seemed to have recovered both in terms of total muscle cross sectional area and in fast muscle cross sectional area. On the contrary, the slow muscle area was not affected by temperature at any stage of development. These data remain unclear and should be further investigated.

2.4.4. *Proliferating cells*

In teleosts, skeletal muscle growth is the result of two processes: hypertrophy (increase in fibre size) combined with hyperplasia (formation of new fibres), both stratified and mosaic in types, often in temporal succession (Rowlerson and Vegetti, 2001). Taking into account the lamellar shape of the prospective muscle fast fibres, we applied, according to Veggetti et al. (1999), PCNA-immunohistochemistry in order to detect the possibly occurring hyperplastic growth in this muscle area. PCNA-immunoreative nuclei were detected in cells of the deep layer of the developing muscle layer since hatching, but in both schooling and yolk sac absorption stages PCNA-immunoreactive nuclei were only detected in prospective fast fibres, with a significant increment of proliferating cells between 16°C- and 22°C-reared larvae in T2 group. The PCNA-immunoreactive nuclei of prospective slow fibres layer were scarce, in all the applied temperatures and stages of development, which is in accordance with the findings of Daczewska and Saczko (2005). Similarly to what observed by Daczewska and Saczko (2005) again, we can at this moment conclude that, limited to the observed precocious development stages, the growth of the developing lateral muscle of *A. baerii* is based upon both hypertrophic and hyperplastic mechanisms that however concerns the prospective fast muscle cross sectional area only. Anti-PCNA cell counts showed higher proliferation in larvae subjected to 22°C than 16°C, thus indicating a hyperplastic mechanism at 22°C even if limited to the yolk sac absorption phase, whereas the hypertrophic one appeared influenced by the temperature of 19°C at schooling stage, an aspect which deserves further studies.

2.4.5. *Apoptotic cells*

A cellular turnover cannot at present be identified because apoptotic nuclei, identifiable applying anti-caspase immunoreactivity, have never been detected in the developing lateral muscle, likely due to the precocity of the studied developmental stages, in which proliferating cells only have been observed. Apoptosis is reputed a fundamental process during fish development and may be influenced by altered environmental factors such as salinity, pH, oxygen consumption, UV radiation, as well as temperature, in such a way to be eventually responsible of deformities and

abnormalities (AnvariFar et al., 2016). It is in addition to underline that the absence of apoptotic cells in the developing lateral muscle is a demonstration that the experimentally applied rearing temperatures are not detrimental for the development of *A. baerii*.

2.4.6. *Heat-shock proteins 70 and 90*

In this study we have investigated the presence and cellular localization, as immunohistochemically expressed, of both HSP70 and HSP90, considering that they constitute the most frequently described HSPs families in vertebrates including fish species (Deane and Woo, 2010; Rupik et al., 2011; Lewis et al., 2016; Peng et al., 2016). HSP70 was never identified by us in the examined precocious sturgeon larvae: this observation appears in full accordance with the part of the study by Rupik et al. (2011) referring to zebrafish embryos, in which the absence of HSP70 expression has been described in concomitance with physiological temperatures in the environment. In comparison, in studies conducted in various species, including *Acipenseridae,* there was always evidence of an increase of HSPs synthesis as a consequence of a thermal stress (Allen et al., 20016; Bertotto et al., 2011; Han et al., 2012; Linares-Casenave et al., 2013; Zheng et al., 2015; He et al., 2016; Peng et al., 2016; Simide et al., 2016). In addition, studies conducted in other fish species showed the expression of HSP70 linked to the occurrence of transport stress (Simide et al., 2007; Poltronieri et al., 2008). As a preliminary consequence, we can suggest that all the experimentally applied temperatures, not accompanied by a HSP70 expression, are to be considered within the physiological range for this species and for the studied stages.

On the contrary, a HSP90 immunoreactivity was clearly identified in nuclei of the prospective fast muscle fibres as well as in nuclei of the developing grey matter of the neural tube, particularly evident at 19°C. These observations are to be related to a regulative function in myofibrillogenesis attributed to HSP90 during the development of zebrafish, as well to the synthesis of regulatory proteins such as steroid hormone receptors (Rupik et al., 2011). This part of our study enabled us to demonstrate that all the three applied experimental temperatures are accompanied by the expression of HSP90 in the developing lateral muscle, where the protein can likely display its multiple chaperon molecule functions. The immunoreactivity was especially intense at 19°C. Interestingly, this latter rearing temperature is the same, in which we have here showed larger dimensions of both total muscle area and prospective fast muscle area via the hypertrophy mechanism.

In conclusion, even considering that all the three experimental rearing temperatures are congruent with a correct development of cultured *A. baerii* and that a rearing temperature of 22°C leads to a higher developmental rate of *A. baerii* larvae (which could be advantageous in commercial hatcheries), we can at present conclude that a temperature of 19°C can support larger size in developing larvae and likely a speedy acquisition of swimming capacities. Further studies are however necessary in order to investigate the expression of genes involved in the lateral muscle development in Siberian sturgeon yolk-sac larvae, with the additional aim to investigate the possible disjunction between HSP70 mRNA and measurable HSP70 tissue levels.

Acknowledgments

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2.5. References

Allen, P.J., Hodge, B., Werner, I. & Cech, J.J. Jr. (2006). Effects of ontogeny, season, and temperature on the swimming performance of juvenile green sturgeon (*Acipenser medirostris*). Canadian *Journal of Fish Aquatic Science*, **63**(6), 1360–1369. https://doi.org/10.1139/f06-031

AnvariFar, H., Amirkolaie, A.K., Miandare, H.G., Ouraji, H., Jalali, M.J. & Ucuncu, S.I. (2016) Apoptosis in fish: environmental factors and programmed cell death. *Cell and Tissue Research*, **68**(3), 425–439. https://doi.org/10.1007/s00441-016-2548-x

Bertotto, D., Poltronieri, C., Negrato, E., Richard, J., Pascoli, F., Simontacchi, C. & Radaelli, G. (2011). Whole body cortisol and expression of HSP70, IGF-I and MSTN in early development of sea bass subjected to heat shock. *General and Comparative Endocrinology*, 174(1), 44-50. https://doi.org/10.1016/j.ygcen.2011.08.003

Birnstein, V.J. (1993). Sturgeon and paddlefishes: threatened fishes in need of conservation. *Conservation Biology*, **7**(4), 773–787. https://doi.org/10.1046/j.1523-1739.1993.740773.x

Bone, Q. (1978). Locomotor muscle. In: Hoar, W. S. & Randall, D. J. ed., *Fish physiology* Volume VII. Academic Press. pp. 361-424DOI:10.1016/S1546-5098(08)60168-5

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

Campos, C., Fernandes, J.M.O., Conceição, L.E.C., Engrola, S., Sousa, V. & Valente LMP. (2013). Thermal conditions during larval pelagic phase influence subsequent somatic growth of Senegalese sole by modulating gene expression and muscle growth dynamics. *Aquaculture,* 414–415, 46–55. https://doi.org/10.1016/j.aquaculture.2013.07.039

Chauvigné, F., Ralliere, C., Cauty, C. & Rescan, P.Y. (2006) *In situ* hybridisation of a large repertoire of muscle-specific transcripts in fish larvae: the new superficial slow-twitch fibres exhibit characteristics of fast-twitch differentiation. *Journal of Experimental Biology*, **209**(2), 372-379. http://jeb.biologists.org/content/209/2/372

Conceicao, L., Aragao, C., Richard, N., Engrola, S., Gavaia, P. & Mira, S. (2010). Novel methodologies in marine fish larval nutrition. *Fish Physiology and Biochemistry*, 36(1), 1-16. https://doi.org/10.1007/s10695-009-9373-z

Currie, P.D. & Ingham, P.W. (2001). Induction and Patterning of Embryonic Skeletal Muscle Cells in Zebrafish. In Johnston IA (ed), *Muscle development and growth,* p. 1-17. San Diego: Academic Press

Daczewska, M. & Saczko, J. (2005). Myotomal Myogenesis of Axial Muscle in the Sturgeon *Acipenser baeri* (Chondrostei, Acipenseriformes). *Folia biologica*, **53**(1-2), 29-38 DOI:10.3409/1734916054663537

Deane, E.E. & Woo, N.Y.S. (2010). Advances and perspectives on the regulation and expression of piscine heat shock proteins. *Reviews in Fish Biology and Fisheries*, 21(2), 153-185. https://doi.org/10.1007/s11160-010-9164-8

Di Giancamillo, A., Martino, P.A., Arrighi, S. & Domeneghini, C. (2012). Gut Peculiarities of Feed Deprived White Sturgeons (*Acipenser transmontanus*, Richardson 1836). *Open Journal of Veterinarian Medicine*, **2**(2), 52-59. DOI:10.4236/ojvm.2012.22009

Di Giancamillo, A., Rossi, R., Pastorelli, G., Deponti, D., Carollo, V., Casamassima, et al. The effects of dietary verbascoside on blood and liver oxidative stress status induced by a high n-6 polyunsaturated fatty acids diet in piglets. *Journal of Animal Science*, **93**(6):2849-59. https://doi.org/10.2527/jas.2014-8607

Di Giancamillo, A., Rossi, R., Vitari, F., Pastorelli, G., Corino, C. & Domeneghini, C. (2009). Dietary conjugated linoleic acids decrease leptin in porcine adipose tissue. *Journal of Nutrition*, **139**(10), 1867-1872. https://doi.org/10.3945/jn.109.110627

Domeneghini, C., Radaelli, G., Bosi, G., Arrighi, S., Di Giancamillo, A., Pazzaglia, M., et al. (2002). Morphological and histochemical differences in the structure of the alimentary canal in feeding and runt (feed deprived) white sturgeons (*Acipenser transmontanus*). *Journal of Applied Ichthyology*, **18**(4-6), 341-346. https://doi.org/10.1046/j.1439-0426.2002.00384.x

Donaldson, M.R., Cooke, S.J., Patterson, D.A. & MacDonald, J.S. (2008). Cold shock and fish. *Journal of Fish Biology*, **73**(7), 1491-1530. https://doi.org/10.1111/j.1095-8649.2008.02061.x

Eder, K.J., Köhler, H.R. & Werner, J. (2007). Pesticide and pathogen: heat shock protein expression and acetylcholinesterase inhibition in juvenile Chinook salmon in response to multiple stressors. *Environmental Toxicology and Chemistry*, **26**(6), 1233-42. https://doi.org/10.1897/05-462R2.1

Feder, M.E. & Hofmann, G.E. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annual Review Physiology*, 61:243–282.

Garcia de la Serrana, D., Vieira, V.L.A., Andree, K.B., Darias, M., Estévez, A., Gisbert, E. et al. (2012). Development Temperature Has Persistent Effects on Muscle Growth Responses in Gilthead Sea Bream. *PLoS ONE*, **7**(12), e51884. http://doi.org/10.1371/journal.pone.0051884

Gisbert, E., Williot, P. & Castellò-Orvay, F. (2000). Influence of egg size on growth and survival of early stages of Siberian sturgeon (*Acipenser baeri*) under small scale hatchery conditions. *Aquaculture,* **183**(1-2), 83–94. https://doi.org/10.1016/S0044-8486(99)00287-2

Gisbert, E. & Williot, P. (2002). Duration of synchronous egg cleavage cycles at different temperatures in Siberian sturgeon (*Acipenser baerii*). *Journal of Applied Ichthyology*, 18:271–274.

Gisbert, E., Williot, P. & Castello´-Orvay, F. (1999). Behavioural modifications in the early life stages of Siberian sturgeon (*Acipenser baeri*, Brandt). *Journal of Applied Ichthyology,* **15**, 237–242.

Goldner, J. (1938). A modification of the Masson trichrome technique. American Journal of Pathology, **14**, 247-249.

Han, D., Huang, S.S.Y., Wang, W.F., Deng, D.F. & Hung, S.S.O. (2012). Starvation reduces the heat shock protein responses in white sturgeon larvae. *Environmental Biology of Fishes*, 93:333–342.

Hardy, R.S. & Litvak, M.K. (2004). Effects of Temperature on the Early Development, Growth, and Survival of Shortnose Sturgeon, *Acipenser brevirostrum*, and Atlantic Sturgeon, *Acipenser oxyrhynchus*, Yolk-Sac Larvae. *Environmental Biology of Fishes*, **70**(2), 145-154. https://doi.org/10.1023/B:EBFI.0000029345.97187.5b

He, J., Wang, J., Xu, M., Wu, C. & Liu, H. (2016). The cooperative expression of Heat Shock Protein 70KD and 9o KD gene in juveniles *Larimichthys crocea* under *Vibrio alginolyticus* stress. *Fish and Shellfish Immunology*, **58**, 359-369. https://doi.org/10.1016/j.fsi.2016.09.049

Johnston, I., Vieira, V. & Abercromby, M. (1995). Temperature and myogenesis in embryos of the Atlantic herring *Clupea harengus*. *Journal of Experimental Biology*, 198(Pt6), 1389-1403. http://www.ncbi.nlm.nih.gov/pubmed/9319285

Kamler, E. (2002). Ontogeny of yolk-feeding fish: an ecological perspective. *Reviews in Fish Biology and Fisheries*, 12(1), 79-103. https://doi.org/10.1023/A:1022603204337

Leprevost, A., Azais, T., Trichet, M. & Sire, J.I (2017). Vertebral development and ossification in the Siberian sturgeon (*Acipenser baerii*), with new insights on bone histology and ultrastructure of vertebral elements and scutes. *The Anatomical Record*, 300(3), 437-449. https://doi.org/10.1002/ar.23515

Lewis, M., Gotting, M., Anttila, K., Kanerva, M., Prokkola, J.M., Seppanen, E., Kolari, I. & Nikinmaa, M. (2016). Different relationship between hsp70 mRNA and hsp70 levels in the heat shock response of two Salmonids with dissimilar temperature preference. *Frontiers in Physiology,* **7**, 12. https://doi.org/10.3389/fphys.2016.00511

Linares-Casenave, J., Werner, I., Van Eenennaam, J.P. & Doroshov, S.I (2013). Temperature stress induces notochord abnormalities and heat shock proteins expression in larval green sturgeon (*Acipenser medirostris* Ayres 1854). *Journal of Applied Ichthyology*, **29**(5), 958–967. https://doi.org/10.1111/jai.12220

Lushchak, V.I. (2011). Environmentally induced oxidative stress in aquatic animals. *Aquatic Toxicology*, **101**(1), 13-30. https://doi.org/10.1016/j.aquatox.2010.10.006

Maradonna, F. & Carnevali, O. (2007). Vitellogenin, zona radiate protein, cathepsin D and heat shock protein 70 as biomarkers of response to xenobiotics. *Biomarkers,* **12**(3), 240-55. DOI: 10.1080/13547500601070859

Mohler, J.W., King, M.K. & Farrel, P.R. (2000). Growth and survival of first-feeding and fingerling Atlantic sturgeon under culture conditions. *North American Journal of Aquaculture*, **62**(3), 174-183. https://doi.org/10.1577/1548-8454(2000)062<0174:GASOFF>2.3.CO;2

Moyle, P.B. & Cech, J.J. (1982). In: Pearson Prentice Hall (ed), *Fishes: An introduction to Icthyology*, London

Ojanguren, A.F., Reyes-Gavilán, F.G. & Muñoz, R.R. (1999). Effects of Temperature on Growth and Efficiency of Yolk Utilisation in Eggs and Pre-feeding Larval Stages of Atlantic Salmon. *Aquaculture International*, **7**(2), 81-87. https://doi.org/10.1023/A:1009214804949

Pederzoli, A. & Mola, L. (2016). The early stress responses in fish larvae. *Acta Histochemica*, **118**(4), 443-449. https://doi.org/10.1016/j.acthis.2016.03.001

Peng, G., Zhao, W., Shi, Z., Chen, H., Liu, Y., Wei, J. & Gao, F. (2016). Cloning HSP70 and HSP90 genes of kaluga (*Huso dauricus*) and the effects of temperature and salinity stress on their gene expression. *Cell Stress Chaperones*, **21**(2), 349-359. https://doi.org/10.1007/s12192-015-0665-1

Pepin, P., Orr, D.C. & Anderson, J.T. (1997). Time to hatch and larval size in relation to temperature and egg size in Atlantic cod (*Gadus morhua). Canadian Journal of Fisheries and Aquatic Sciences*, 54(s1), 2-10. https://doi.org/10.1139/f96-154

Poltronieri, C., Maccatrozzo, L., Simontacchi, C., Bertotto, D., Funkenstein, B. & Radaelli, G. (2007). Quantitative RT-PCR analysis and immunohistochemical localization of HSP70 in sea bass *Dicentrarchus labrax* exposed to transport stress. *European Journal of Histochemistry*, **51**(2), 125-136. https://doi.org/10.4081/1134

Poltronieri, C., Negrato, E., Bertotto, D., Majolini, D., Simontacchi, C. & Radaelli, G. (2008). Immunohistochemical localization of constitutive and inducible Heat Shock Protein 70 in carp (*Cyprinus carpio*) and trout (*Oncorhynchus mykiss*) exposed to transport stress. *European Journal of Histochemistry,* **52**(3), 191-198. https://doi.org/10.4081/1211

Réalis-Doyelle, E., Pasquet, A., De Charleroy, D., Fontaine, P. & Teletchea, F. (2016). Strong effects of temperature on the early life stages of a cold stenothermal fish species, brown trout (*Salmo trutta* L.). *PLoS One*, **11**(5) e0155487. https://doi.org/10.1371/journal.pone.0155487

Rehfeldt, C., Te Pas, M.F.W., Wimmers, K., Brameld, J.M., Nissen, P.M. & Berri, C. (2011). Advances in research on the prenatal development of skeletal muscle in animals in relation to the quality of muscle-based food. I. Regulation of myogenesis and environmental impact. *Animal*, **5**(5), 718–730. https://doi.org/10.1017/S1751731110002089

Roberts, R.J., Agius, C., Saliba, C., Bossier, P. & Sung, Y.Y. (2010). Heat shock proteins (chaperones) in fish and shellfish and their potential role in relation to fish health: a review. *Journal of Fish Diseases*, **33**(10), 789–801. https://doi.org/10.1111/j.1365-2761.2010.01183.x

Rowlerson, A. & Vegetti, A (2001). Cellular mechanism of post-embryonic muscle growth in aquaculture species. In: Ian A. Johnston (ed), *Muscle development and growth*, pp 103-140. London: Academic Press

Roy, S. & Bhattacharya, S. (2006). Arsenic-induced histopathology and synthesis of stress proteins in liver and kidney of Channa punctatus. *Journal of Fish Diseases*, **65**(2), 218-29. https://doi.org/10.1016/j.ecoenv.2005.07.005.

Rupik, W., Jasik, K., Bembenk, J. & Widlak, W. (2011). The expression patterns of heat shock genes and proteins and their role during vertebrate's development. *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology*, **159**(4), 349-366. https://doi.org/10.1016/j.cbpa.2011.04.002

Simide, R., Richard, S., Prévot-D'Alvise, N., Miard, T. & Gaillard, S. (2016). Assessment of the accuracy of physiological blood indicators for the evaluation of stress, health status and welfare in Siberian sturgeon (*Acipenser baerii*) subject to chronic heat stress and dietary supplementation. *International Aquatic Research*, **8**(2), 121–135. https://doi.org/10.1007/s40071-016-0128-z

Stefanovich, D.I., Manzon, L.A., McDougall, C.S., Boreham, D.R., Somers, C.M., Wilson, J.Y., Manzon, R.G. (2016). Thermal stress and the heat shock response in embryonic and young adult of the year juvenile lake whitefish. *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology*, **193**, 1-10. https://doi.org/10.1016/j.cbpa.2015.12.001

Steinbacher, P., Haslett, J.R., Sänger, A.M. and Stoiber, W. (2006). Evolution of myogenesis in fish: a sturgeon view of the mechanisms of muscle development. *Anatomy and Embryology*, **211**(4), 311–322. https://doi.org/10.1007/s00429-006-0082-4

Van Eenennaam, J.P., Linares-Casenave, J., Deng, X. & Doroshov, S.I. (2005). Effect of incubation temperature on green sturgeon embryos, *Acipenser medirostris*. *Environmental Biology of Fishes*, **72**(2), 145-154. https://doi.org/10.1007/s10641-004-8758-1

Veggetti, A., Rowlerson, A., Radaelli, G., Arrighi, S. & Domeneghini, C. (1999). Post-hatching development of the gut and lateral muscle in the sole. *Journal of Fish Biology*, **55**(SA):44–65. https://doi.org/10.1111/j.1095-8649.1999.tb01045.x

Wang, Y.L., Buddington, R.K. & Doroshov, S.I (1987). Influence of temperature on yolk utilization by the white sturgeon, *Acipenser transmontanus. Journal of Fish Biology,* **30**(3), 263–271. https://doi.org/10.1111/j.1095-8649.1987.tb05751.x

Zheng, K., Wang, W., Hung, S.S.O., & Deng, D.F. (2015). Feeding rates affect expression of heat-shock protein 70 in green sturgeon fry. *North American Journal of Aquaculture*, **77**(2), 206–210. https://doi.org/10.1080/15222055.2014.987931

Zitov, R.E. & Millard, J.L. (1988). Survival and growth of lake whitefish (*Coregonus clupeaformis*) larvae fed only formulated dry diets. *Aquaculture,* **69**(1-2), 105–113. https://doi.org/10.1016/0044-8486(88)90190-1

Chapter 3

Effect of temperature on fatty acid composition and development of unfed Siberian sturgeon (*A. baerii*) larvae

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STURGEON PAPER

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Effect of temperature on fatty acid composition and development of unfed Siberian sturgeon (*A. baerii*) larvae

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3. Effect of temperature on fatty acid composition and development of unfed Siberian sturgeon (*A. baerii*) larvae

Abstract

The fatty acid metabolism in fish is influenced by various factors, including fish species, water temperature, water environment and diet supply. The aim of present work is to investigate the fatty acid composition of yolk-stage Siberian sturgeon larvae reared at three different temperatures. Fertilized Siberian sturgeon eggs were transferred to the Lodi Aquaculture Research Center of the University of Milan, divided in three aquaria, each containing three incubators and incubated at 16°C. After hatching the temperature was switched to 16, 19 and 22°C. Larvae sampling was performed at the end of yolk sac reabsorption. No feed was dispensed during the trial. Eggs and larvae were weighed and fatty acid profile was determined by GC-FID analysis after lipid extraction by chloroform/methanol mixture and fatty acid transesterification by methanolic hydrogen chloride. The fertilized eggs had a weight of 23.27 mg and a lipid content of 2.67 mg/egg. At hatching, the weight was 12.2 (0.17 *SD*) mg and lipid content 1.9 (0.6 *SD*) mg/larva. At the end of the trial, larvae mean weight was 33.6 (3.6 *SD*), 34.7 (1.8 *SD*) and 36.9 (1.1 *SD*) mg, while lipid content was 2.0 (0.3 *SD*), 2.1 (0.3 *SD*) and 2.0 (0.2 *SD*) mg for larvae reared at 16, 19 and 22°C respectively, without statistically significant difference. Larvae subjected to the highest water temperature showed a faster yolk-sac absorption. No differences were found across temperatures regarding survival rates and regarding ontogenic development. The fatty acid composition of larvae was affected by the temperature. Larvae reared at 16°C had the lowest amount of saturated fatty acids, mainly due to a lower palmitic acid content, that was offset by a higher level of linolenic and linoleic acid, if compared with larvae reared at 19°C and 22°C. The study suggests that at a lower temperature sturgeon spare unsaturated fatty acid consuming preferably saturated fatty acids, increasing our knowledge of the fatty acid metabolism in this species.

3.1. Introduction

Siberian sturgeon (*Acipenser baerii*) is one of the most popular sturgeon species, farmed in more than 22 countries (Bronzi, Rosenthal, & Gessner, 2011). The success of Siberian sturgeon aquaculture among other sturgeon species is due to its rapid growth and early maturity, that allows farmers to obtain earlier a good quality caviar (Gisbert & Williot, 2002). As with all species

of sturgeon, the critical stage of Siberian sturgeon farming is the larval stage, between hatching and the end of the weaning period, when larvae accept artificial diets. The bottleneck of sturgeon faming could be identified in the transition between endogenous to exogenous feeding (Boucher, McAdam & Shrimpton, 2014), when fish have finished consuming the nutrient reserves provided by the yolk and they must satisfy their requirements by assuming nutrients from the diet. Fatty acids, in particular polyunsaturated fatty acids of n-3 series, are generally known as key nutrients in fish larvae (Sargent et al., 1999). Their role has also been investigated in larvae of different sturgeon species, such as white sturgeon (*Acipenser transmontanus*) (Gawlicka, Herold, Barrows,Noüe, & Hung, 2002), Russian sturgeon (*Acipenser gueldenstaedtii*) (Sener, Yildiz, & Savaş, 2005) and Persian sturgeon (*Acipenser persicus*) (Hafezieh et al., 2010), identifying in these fatty acids as an essential element to guarantee an optimal survival rate. Temperature was identified as a possible factor that determines the different survival rates of Siberian sturgeon, as recorded in a Korean hatchery (Park, Lee, Kim, & Nam, 2013). Temperature is one of the most fundamental and critical parameter affecting sturgeon physiology, as it regulates the activity of enzymes involved in fish metabolism. The temperature recommended and therefore mostly used in the rearing of Siberian sturgeon larvae is 18°C (Gisbert & Williot, 2002); Italian sturgeon farmers, due to the increase in temperatures recorded in the last decades, use to rear Siberian sturgeon larvae at 19°C.

The aim of the present work is to investigate the influence of three different temperatures on the fatty acid metabolism and development during early stages of Siberian sturgeon larvae before the start of exogenous feedings. Results of our investigation could be useful in studying new feeding strategies for Siberian sturgeon larvae at the beginning of their external feeding, according to water temperature.

3.2. Materials and Methods

3.2.1. *Animals*

Siberian sturgeon eggs were obtained from the hatchery of Società Naviglio Agricola SS sturgeon farm. Eggs were collected from one female and fertilized with multiples males' sperm. Around 1,000 eggs were transported at approximately 24 hr post fertilization to the Centro Zootecnico Didattico Sperimentale of the University of Milan in polypropylene bags filled with water and oxygen. After acclimation, eggs were randomly divided in three experimental groups, using three

thermally independent aquaria of 60 L. Each aquarium contained three experimental incubators, volume 2 L, which constituted the triplicate for each thermal treatment. In total, nine incubators were used. Eggs and larvae were kept at the density of 50–60 larvae per liter.

Aquaria were individually aerated and a submerged pump provided an internal water flow to keep eggs in gentle motion. Eggs were disinfected by a permanent bath of 0.3 mg/L of methylene blue. Oxygen concentration and pH were monitored daily by a Hach HQ 30d Portable Meter, (Hach Lange, Dussendolf, Germany). Oxygen saturation resulted constantly superior to 96% while pH ranged from the value of 8.58–8.71. Total ammonia and nitrites, measured in each aquarium, were below 0.05 mg/L, and they were measured once a week by Hach 2,800 Portable Spectophotometer (Hach Lange, Dussendolf, Germany). The photoperiod regime was 12L:12D with a very low level of light intensity. The incubation temperature was kept at 16.2 (0.3 *SD*) °C until hatching, which occurred 7 days after fertilization. At hatching, the temperature of the aquaria was modified. Aquarium 1 maintained the incubation temperature (16°C), while aquaria 2 and 3 were slowly heated to 19 (0.3 *SD*) °C (19°C) and 22 (0.3 *SD*) °C (22°C) respectively. When present, dead eggs and larvae were removed from each incubator every day. The trial was stopped when the yolk sac of larvae appeared depleted and they started to show a benthic behavior.

3.2.2. *Sampling*

In order to follow the morphological development from egg to yolk-sac absorption larva, we decided to perform sampling on different time points: eggs at 24 hpf, moving embryo, larvae at hatching, schooling phase and larvae at the end of yolk-sac absorption. Fatty acid analysis was carried out only on eggs at 24 hpf and on larvae at the end of their yolk-sac absorption. Eggs and larvae were collected by 50 ml glass beaker and terminally euthanized by Tricaine (Tricaine Pharmaq 1,000®) at concentration of 250 mg/L. Eggs and larvae were dried with absorbent paper and then weighed. For lipid and fatty acid analysis ten eggs and larvae from each incubators were immediately stored at −32°C until analysis. Other samples were used for morphological studies.

3.2.3. *Morphological analyses*

Larvae were euthanized as mentioned above, and then fixed in 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS). Samples were subsequently dehydrated in graded ethanol

series and finally paraffin-embedded. Paraffin-embedded samples were used in order to obtain microtome sections (4 mm thick, both longitudinal and transverse), which were stained for morphological examination with Hematoxylin and Eosin (HE) and with Alcian-Blue Periodic Schiff Acid (AB-PAS), in order to highlight structural details and their progressive changes. All observations were conducted by a blind observer utilizing an Olympus BX51 microscope equipped with a digital camera and DP software (Olympus, Italy) for computer-assisted image acquirement and managing.

3.2.4. *Chemical analysis*

The extraction and determination of total lipids was performed according to the Folch Lees and Sloane (1957) method with chloroform:methanol (2:1), using a pool of 5 eggs or larvae sampled from each incubator; analysis were performed in duplicate. 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. The sample was sealed and heated at 50°C overnight; then, 2 ml of a 1 M potassium carbonate solution and 5 ml of 5% NaCl were added to each sample. FAME were extracted with 2 × 2 ml of hexane and the mixture was 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 6,890 Series GC) fitted with an automatic sampler (Model 7,683) 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 (30 m × 0.25 mm I.D., 0.25 µm film thickness; Agilent Technologies) was used to separate FAME. The oven temperature program for separation was from 100 to 180°C at 3°C/min, then from 180 to 250°C at 2.5°C/min and held for 10 min. Carrier gas was helium at 1.0 ml/min, inlet pressure 16.9 psi. Fatty acids were identified by comparison of retention times with standard 37 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.

3.2.5. *Statistical Analyses*

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 temperature. Significance was

accepted at probabilities of 0.05 or less. All the statistical analysis were performed by SPSS version 24.0 (SPSS Inc. Chicago, Illinois), data in the tables are reported as mean values ± standard deviation.

3.3. Results

The rearing temperature did not affect the survival rate and the weight at the end of yolk absorption of Siberian sturgeon larvae but it showed an effect on the duration of yolk sac absorption. Higher temperature produced a faster development, larvae reared at 22°C completed yolk absorption in 7 days, while those reared at 19°C and 16°C took an additional 1 and 2 days to reach the same development stage, respectively (Table 1). Larvae weights, measured at the same development stage, were not statistical different (*p*-value 0.317). Siberian sturgeon eggs weighed 23.2 (0.9 *SD*) mg at their arrival at the aquaculture unit facility and larvae at the end of experimental period showed a weight ranging from 31.5 to 37.9 mg. Taking into account that Sturgeon are poikilothermic vertebrates, the larval stage duration was different according to different temperatures: larvae subjected to the highest water temperature showed a faster absorption of the yolk-sac.

Table 3.1. Duration of yolk sac absorption, mortality, weight and lipid content of sturgeon larvae reared at different temperatures.

	16°C	**19°C**	**22°C**
Days to compete yolk absorption	9	8	7
Mortality (%)	10.61	11.77	9.78
Weight (mg)	33.7 ± 3.61	34.7 ± 1.77	36.9 ± 1.11
Lipid content (mg)	2.0 ± 0.03	2.1 ± 0.04	2.0 ± 0.27

Data are expressed as mean ± standard deviation. (n = 30)

3.3.1. *Morphological Analyses*

At 24 hpf, it was possible to observe the area in the egg where the embryo was developing in (Figure 3.1a, 3.2a). Samples taken at the moving embryo stage showed a clear, well formed embryo: it was possible to see the mouth still closed by a membrane (Figure 3.1c, asterisks), the eye region, the developing olfactory organs, the neural tube and the notochord (Figure 3.1c, 3.2c, NT) as well as evident myomeres (Figure 3.1b, 3.1d-f, 3.2b, arrows). At hatching, it was already possible to observe the difference between slow/white and fast/red fibres (Figure 3.1d, 3.2d). Slow fibres appeared as a monolayer and will remain as such until the yolk-sac is fully absorbed (Figure 3.1d-f, 3.2d-f, SF). On the opposite, fast fibres showed an expansion since hatching until the yolk-sac absorption stage (Figure 3.1d-f, 3.2d-f,FF). No deformities were observed in all the stages considered and notochord revealed to be always anatomically normal (Figure 3.1, 3.2, NT).

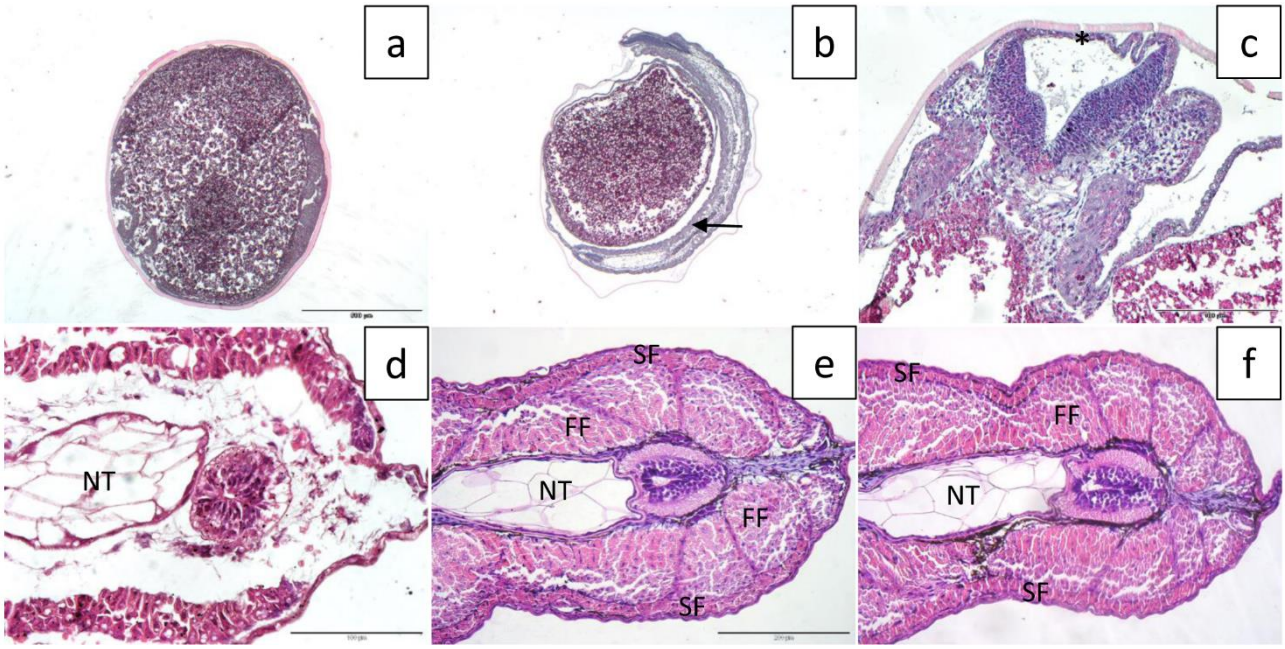

Figure 3.1. HE staining representative images of embryonic and larval development subjected to different temperatures at different timepoints.a) 24hpf, scale bar 500 µm; b) embryo moving stage, scale bar 500 µm; c) embryo moving stage, longitudinal section, scale bar 100 µm; d) hatching, cross section, scale bar 100 µm; e) schooling, cross section, scale bar 200 µm; f) yolk-sac full absorption, cross section, scale bar 200 µm; arrows, myomers; arrowheads, mouth membrane; FF, fast fibres; SF, slow fibres; NT, notochord.

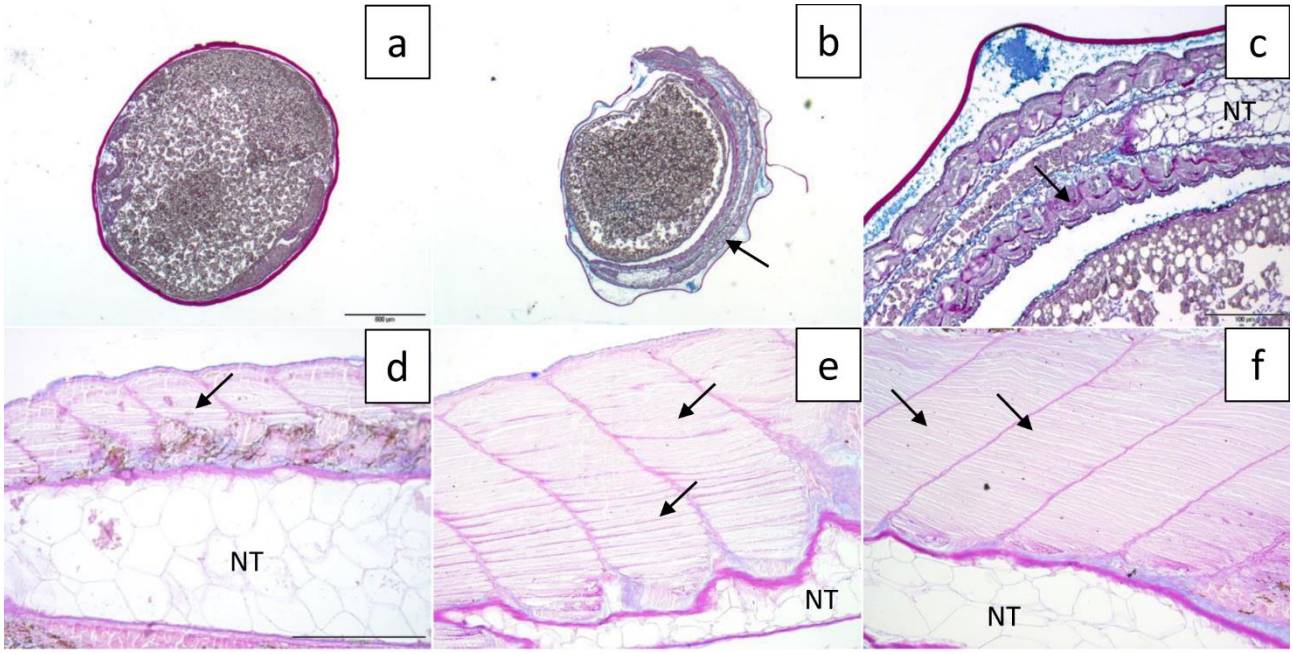

Figure 3.2. AB-PAS staining representative images of embryonic and larval development subjected to different temperatures at different timepoints, (longitudinal sections). a) 24 hpf, scale bar 500 µm; b) embryo moving stage, scale bar 500 µm; c) embryo moving stage, scale bar 100 µm; d-f) hatching, schooling, yolk-sac full absorption. Scale bar 200. Arrows, myomers; asterisks, mouth membrane; FF, fast fibres; SF, slow fibres; NT, notochord.

3.3.2. *Chemical analyses*

Lipid content decreased during the trial. Eggs had a mean lipid content of 2.61 (0.02 *SD*) mg at their arrival while lipid content of larvae at the end of the trial was found to be very similar, regardless of temperature, with value close to 2 mg (Table 3.1). Fatty acid composition of eggs and larvae is showed in Table 3.2.

Table 3.2. Fatty acid composition, expressed as g/100 g, of sturgeon larvae

	Eggs	16°C	19°C	22°C
14:0	0.47 ± 0.026	0.40 ± 0.014	0.41 ± 0.011	0.41 ± 0.024
16:0	19.85 ± 0.941	18.72 ± 0.175[a]	19.18 ± 0.205[b]	19.24 ± 0.227[b]
16:1 *n*-7	2.71 ± 0.152	2.48 ± 0.073	2.48 ± 0.043	2.44 ± 0.119
17:0	0.30 ± 0.032	0.21 ± 0.008	0.22 ± 0.004	0.22 ± 0.013
16:3 *n*-4	0.00 ± 0.064	0.15 ± 0.004	0.15 ± 0.005	0.15 ± 0.008
16:4 *n*-1	0.23 ± 0.085	0.28 ± 0.024	0.28 ± 0.015	0.28 ± 0.009
18:0	4.86 ± 0.319	4.73 ± 0.118	4.74 ± 0.079	4.87 ± 0.290
18:1 *n*-9	36.75 ± 1.954	36.64 ± 0.304	36.81 ± 0.100	36.81 ± 0.298
18:1 *n*-7	2.82 ± 0.146	2.92 ± 0.004[a]	2.95 ± 0.012[b]	2.95 ± 0.018[b]
18:2 *n*-6	10.53 ± 0.520	9.20 ± 0.062[b]	9.04 ± 0.040[a]	9.09 ± 0.047[a]
18:3 *n*-6	2.22 ± 0.131	2.20 ± 0.058	2.17 ± 0.019	2.19 ± 0.044
18:3 *n*-3	1.56 ± 0.078	1.35 ± 0.014[b]	1.31 ± 0.010[a]	1.30 ± 0.023[a]
18:4 *n*-3	0.50 ± 0.033	0.49 ± 0.012	0.49 ± 0.009	0.50 ± 0.023
20:1 *n*-11	0.88 ± 0.041	0.91 ± 0.006	0.90 ± 0.008	0.89 ± 0.019
20:2 *n*-6	0.37 ± 0.018	0.36 ± 0.003[b]	0.35 ± 0.002[a]	0.34 ± 0.008[a]
20:3 *n*-6	0.54 ± 0.032	0.61 ± 0.007	0.60 ± 0.013	0.60 ± 0.004
20:4 *n*-6	3.17 ± 0.202	3.72 ± 0.090	3.63 ± 0.038	3.60 ± 0.163
20:4 *n*-3	0.00 ± 0.000	0.19 ± 0.003	0.17 ± 0.001	0.12 ± 0.101
20:5 *n*-3	1.62 ± 0.098	1.74 ± 0.030	1.68 ± 0.018	1.66 ± 0.052
21:5 *n*-3	0.00 ± 0.087	0.18 ± 0.007	0.20 ± 0.002	0.13 ± 0.115
22:5 *n*-3	0.00 ± 0.141	0.39 ± 0.012	0.41 ± 0.016	0.39 ± 0.015
22:6 *n*-3	10.64 ± 0.549	12.13 ± 0.360	11.82 ± 0.270	11.82 ± 0.427
SFA	25.48 ± 0.211	24.05 ± 0.156[a]	24.55 ± 0.180[b]	24.74 ± 0.223[b]
MUFA	43.15 ± 0.279	42.95 ± 0.364	43.14 ± 0.124	43.09 ± 0.413
PUFA	31.37 ± 0.346	33.00 ± 0.362	32.31 ± 0.304	32.17 ± 0.371
n-3	13.82 ± 0.231	15.99 ± 0.373	15.59 ± 0.287	15.42 ± 0.319
n-6	16.82 ± 0.162	16.09 ± 0.047	15.79 ± 0.054	15.82 ± 0.100
n3/n6	0.82 ± 0.084	0.99 ± 0.025	0.99 ± 0.019	0.97 ± 0.017

Data are expressed as mean ± standard deviation. Values of larvae fatty acid composition in the same row that do not have the same superscript are significantly different at $p \leq 0.05$, ANOVA and Student-Newman-Keuls post hoc test.

Siberian sturgeon eggs used in this trial showed the prevalence of monounsaturated fatty acids (MUFA) followed by polyunsaturated fatty (PUFA) acids and saturated fatty acids (SFA) (Table 3.2). Oleic acid (OA, 18:1 n-9) was the fatty acid present in highest amount, followed by palmitic acid (PA, 16:0), docosahexaenoic acid (DHA, 22:6 n-3) and linoleic acid (LA, 18:2 n-6). The fatty acid composition of larvae changed during their development, with difference among larvae reared at different temperature. SFA decreased during larval growing; the highest decrease was found in larvae reared at 16°C while larvae reared at 19°C and 22°C had similar results. Differences among the total SFA were attributed to the different presence of PA, which

represented the 18.7% of total fatty acid in 16°C larvae and the 19.1% and 19.2% for 19°C and 22°C larvae respectively. An opposite trend was recorded concerning the presence of LA and eicosadienoic acid (20:2 n-6), which increased their relative presence in fish reared at the lower temperature, while decreased in larvae reared at 19°C and 22°C. If we consider the fatty acid profile of fertilized eggs and larvae at the end of the trial we can highlight other differences. The total amount of n-3 series of fatty acid increased with the larval development, while n-6 fatty acid slightly decreased. Larvae, opposite to what was discussed in eggs, had a small amount of eicosatetraenoic acid (20:4 n-3), heneicosapentaenoic acid (21:5 n-3) and docosapentaecnoic acid (22:5 n-3) which are intermediate compounds of n-3 series fatty acid metabolism; at the same time, a decrease of alfa linolenic acid (18:3n-3) was observed.

3.4. Discussion

The fatty acid profile of the fertilized eggs used in this trial differed from the one we determined in previously research that analyzed the fatty acid composition of caviar obtained from four different species of sturgeon farmed in Italy, including Siberian sturgeon (Borella et al., 2016). Caviar had a higher content of n-3 fatty acid compared to fertilized eggs analyzed in this study, a lower content of oleic acid and an upper of linoleic acid. These differences could be linked to different feeding strategies between sturgeon broodstock and female used for caviar production. Sturgeon farmers, with the aim to obtain a high-quality caviar, use to feed sturgeon with a finishing diet richer in fish oil than basal diet (Pierluigi Perantoni, personal communication). The rearing temperature did not influence the survival rate of larvae, but showed an effect on the rate of development. The duration of yolk sac absorption was similar to that described by Gisbert and Williot (2002), who measured an absorption period of 8–9 days with larvae reared at 18°C. As expected, the growth of larvae reared at higher temperature was faster than lower temperature. This data agrees with the results of Boucher et al. (2014), who compared the effect of temperature and substrate on the survival and growth of white sturgeon (*Acipenser transmontanus*) larvae. Authors found that temperature did not affect the survival of white sturgeon larvae at the yolk absorption phase but it showed a positive relationship with yolk absorption rate. The same findings were recorded on shortnose sturgeon *(Acipenser brevirostrum*) and Atlantic sturgeon (*Acipenser oxyrhynchus*), where the increase of temperature induced a faster yolk absorption in larvae without affecting on the survival at of complete yolk absorption (Hardy & Litvak, 2004). In all these trials, the mortality raised when sturgeon larvae

switched from the endogenous feeding to the external feeding, identifying the phase of external nutrition as the critical one in the breeding of sturgeons.

Morphological analyses have been performed with the aim of monitoring the correct development: embryonic as long as larval development both, followed the correct steps. No deformities among the three temperatures used were detected, especially regarding notochords because it is already known that non-lethal temperature stress commonly causes deformed notochords. These results are quite similar with Werner, Linares-Casenave, Eenennaa, and Doroshov (2007), who found that larval green sturgeon (*Acipenser medirostris*) developed a high percentage of curved notochords when fish were exposed to 26°C, with a recovery after transfer 16.5°C cool water. Deformities attributable to an incorrect development temperature were shown when the larvae were subjected a temperature far than their optimum range. Muscle fibres development and morphology were not affected by different temperatures, thus indicating that non-lethal temperatures do not alter sturgeon morphology in early stages.

Larvae partially consumed their lipid reserve during the first days of life. The increasing body weight was not followed by an increase in lipid content. The energy reserves constituted by lipids have been partially used to supply energy in the period from hatching to the first feeding. The source of energy used by fish larvae before their first exogenous feeding vary among species and could also involve proteins and carbohydrates (Rainuzzo, Reitan, & Olsen, 1997; Shan, Huang, Cao, & Wu, 2008), even if fish that have eggs rich in lipids, as sturgeons, consume predominantly lipids after hatching in response of increased energy demand due to the swimming activity (Kamler, 2008).

The fatty acid composition of larvae of Siberian sturgeon presented some differences if compared with the fertilized eggs from which they derived. The appearance of intermediate metabolites of fatty acid metabolism, such as eicosatetraenoic, heneicosapentaenoic and docosapentaecnoic acids, together with the increasing percentage of DHA and the decrease of the precursor (18:3n-3) of *n*-3 long chain fatty acids, suggest an activity of the metabolic pathway of fatty acid biosynthesis. Moreover, the results suggests that Siberian sturgeon larvae used differentially their body fatty acid.

Saturated fatty acid decreased, indicating a preferential use of these fatty acids rather than PUFA. The decrease was not equal in all larvae; larvae reared at 16°C consumed more SFA than the others reared at higher temperature, saving PUFAs. This reaction to different temperatures also produced a different use of PUFAs. LA decreased in all larvae but the decrease was higher in

larvae reared at 19°C and 22°C, at the same time DHA increased in all larvae. DHA is known as essential fatty acid, which is most preferentially retained and saved by fish on the detriment of other fatty acids since the essential role of this nutrient in physiological functions (Sargent, Tocher, & Bell, 2002). The oxidation of DHA in starved larvae of perch (*Perca fluviatilis*) was observed only when the energy requirement was high (Abi-ayad, Kestemont, & Mélard, 2000), while its presence increased in unfed rainbow trout (*Oncorhynchus mykiss*) larvae, even more than what it was observed in fed larvae (Zengin, Vural, & Çelik, 2013). Linoleic acid is not a fatty acid typical of marine and aquatic environments and it derives mainly from the presence of vegetable fats in the diet of farmed fish (Vasconi, Caprino, Bellagamba, & Moretti, 2017). Fish generally consume LA accumulated in their lipids when the dietary intake of this fatty acid decreases (Turchini, Torstensen, & Ng, 2009). Fish, as all poikilothermic organisms, are not able to regulate their body temperature and they must adapt their membrane lipid composition to maintain membrane fluidity in the cold (Ernst, Ejsing, & Antonny, 2016) in a mechanism known as homeoviscous adaptation, that results in an increase of PUFAs to maintain fluidity at low temperature (Charlotte & Patrick, 1994). For this reason, fish that lives in cold water oceans have a PUFA content higher than tropical fish (Tocher, 2003). The homeoviscous adaptation was also observed in white sturgeon (Buddington, Hazel, Doroshov, & Eenennaam, 1993) where larvae changed their rate of saturated and unsaturated fatty acid after hatching in response to temperature variation. The physiological mechanism that regulates this process is probably linked to a different activity, influenced by water temperature, of enzymes involved in fatty acids metabolism.The effect of temperature on the activity of staetoryl-CoA desaturase has been studied in tilapia, finding that its activity decreased with the increase of temperature so that temperature variation might affect fatty acid composition (Ma, Qiang, He, Gabriel, & Xu, 2015). A similar result was found in rainbow trout (Mellery et al., 2016) where the increase of temperature negatively affected the ability of fatty acid bioconversion by a reduction of Δ6 desaturase enzyme activity.

In conclusion, our study revealed that Siberian sturgeon larvae have an active fatty acid metabolism, working since the beginning of hatching. Moreover larvae showed the ability to react to different temperatures, modifying their fatty acid composition, by sparing essential PUFAs at the expense of SFA when exposed to low temperature. The presented results lead to better understand how larvae use energy under different temperature and how changes in fatty acid metabolism during larval stages could contribute to prevent the high mortality that normally occurs in sturgeon hatchery during the first external feedings stage.

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3.5. References

Abi-ayad, S.M.A., Kestemont, P. & Mélard, C. (2000) Dynamics of total lipids and fatty acids during embryogenesis and larval development of Eurasian perch (*Perca fluviatilis*). *Fish Physiology and Biochemistry,* **23**(3), 233-243. https://doi.org/10.1023/A:1007891922182

Borella, L., Vasconi, M., Caprino, F., Bellagamba, F., Pazzaglia, M. & Moretti, V.M. (2016) La qualità del caviale prodotto da storioni allevati in Italia. *Industrie Alimentari*. **565**, 3-8.

Boucher, M.A., McAdam, S.O. & Shrimpton, M.J. (2014). The effect of temperature and substrate on the growth, development and survival of larval white sturgeon. *Aquaculture,* 430, 139–148 http://dx.doi.org/10.1016/j.aquaculture.2014.03.011

Bronzi, P., Rosenthal, H., & Gessner, J. (2011). Global sturgeon aquaculture production: an overview. *Journal of Applied Ichthyology,* **27**(2), 169-175. https://doi.org/10.1111/j.1439-0426.2011.01757.x

Buddington, R.K, Hazel, J.R., Doroshov, S.I. & Van Eenennaam, J. (1993) Ontogeny of the capacity for homeoviscous adaptation in white sturgeon (*Acipenser transmontanus*). *Journal of Experimental Zoology,* **265**(1), 18-28. https://doi.org/10.1002/jez.1402650104

Charlotte, W. & Patrick, J.B. (1994) Thermal adaptation affects the fatty acid composition of plasma phospholipids in trout. *Lipids,* **29**(5), 373-376. https://doi.org/10.1007/BF02537193

Ernst, R., Ejsing, C.S., & Antonny, B. (2016). Homeoviscous Adaptation and the Regulation of Membrane Lipids. *Journal of Molecular Biology,* **428**(24), 4776-4791. https://doi.org/10.1016/j.jmb.2016.08.013

Folch, J., Lees, M., & Sloane, S. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *Journal of biological chemistry* 226, 497-509

Gawlicka, A., Herold, M.A., Barrows, F.T., De La Noüe, J. & Hung, S.S.O. (2002) Effects of dietary lipids on growth, fatty acid composition, intestinal absorption and hepatic storage in white sturgeon (*Acipenser transmontanus* R.) larvae. *Journal of Applied Ichthyology,* **18**(4-6), 673–681 DOI: 10.1046/j.1439-0426.2002.00371.x

Gisbert, E., & Williot, P. (2002). Advances in the larval rearing of Siberian sturgeon. *Journal of Fish Biology,* **60**(5), 1071–1092. https://doi.org/10.1111/j.1095-8649.2002.tb01705.x

Hafezieh, M., Mohd Salah Kamarudin, S., Bin Saad, C.R., Mostafa Kamal, A. S., Agh, N., Valinassab, T., Sharifian, M.& Hosseinpour, H. (2010). Effect of Enriched *Artemia urmiana* with HUFA on growth, survival and fatty acids composition of Persian sturgeon larvae (*Acipenser persicus*). *Iranian Journal Fisheries Sciences,* **9**(1), 61-72 URL: http://jifro.ir/article-1-23-en.html

Hardy, R.S.& Litvak, M.K. (2004). Effects of temperature on the early development, growth and survival of shortnose sturgeon, *Acipenser brevirostrum*, and Atlantic sturgeon, *Acipenser oxyrhynchus*, yolk-sac larvae *Environmental Biology of Fishes,* **70**(2), 145–154 https://doi.org/10.1023/B:EBFI.0000029345.97187.5b

Kamler, E., 2008. Resource allocation in yolk-feeding fish. *Reviews in Fish Biology and Fisheries,* **18**(2), 143–200. https://doi.org/10.1007/s11160-007-9070-x

Ma, X.Y., Qiang, J., He, J., Gabriel, N.N. & Xu, P. (2015) Changes in the physiological parameters, fatty acid metabolism, and SCD activity and expression in juvenile GIFT tilapia (*Oreochromis niloticus*) reared at three different temperatures. *Fish Physiology and Biochemistry,* **41**(4),937–950 https://doi.org/10.1007/s10695-015-0059-4

Mellery, J., Geay, F., Tocher, D.R., Kestemont, P., Debier, C., Rollin, X.,& Larondelle, Y. (2016) Temperature Increase Negatively Affects the Fatty Acid Bioconversion Capacity of Rainbow Trout (*Oncorhynchus mykiss*) Fed a Linseed Oil-Based Diet. *PLoS ONE,* **11**(10): e0164478. https://doi.org/10.1371/journal.pone.0164478

Park, C., Lee, S.Y., Kim, D.S. & Nam, Y.N. (2013) Effects of Incubation Temperature on Egg Development, Hatching and Pigment Plug Evacuation in Farmed Siberian Sturgeon *Acipenser baerii.. Fisheries and aquatic sciences,* **16**(1), 25-34. http://dx.doi.org/10.5657/FAS.2013.0025

Rainuzzo, J.R., Reitan, K.I. & Olsen, Y. (1998). The significance of lipids at early stages of marine fish: A review. *Aquaculture,* **155**(1-4),103-115. https://doi.org/10.1016/S0044-8486(97)00121-X

Sargent, J., McEvoy, L., Estevez, A., Bell, G., Bell, M., Henderson, J. & Tocher, D. (1999). Recent developments in the fatty acids nutrition of fish. *Aquaculture,* **177**(1-4), 191-199. https://doi.org/10.1016/S0044-8486(99)00083-6

Sargent, J.R., Tocher, D.R., Bell, J.G., (2002). The lipids, In: Halver, J.E., Hardy, R.W. (Eds.), *Fish Nutrition*, pp. 181–257. 3rd edition. San Diego: Academic Press

Sener, E., Yildiz, M., & Savaş, E. (2005). Effects of Dietary Lipids on Growth and Fatty Acid Composition in Russian Sturgeon (*Acipenser gueldenstaedtii*) Juveniles. *Turkish Journal of Veterinary and Animal Sciences*, **29**(2), 1101-1107. https://doi.org/10.1111/j.1365-2095.1996.tb00016.x

Shan, X., Huang, W., Cao, L. & Wu, Y. (2008) Advances in studies of the effects of starvation on growth and development of fish larvae. *Journal of Ocean University of China,* **7**(3), 319-326 https://doi.org/10.1007/s11802-008-0319-3

Tocher, D.R. (2003). Metabolism and Functions of lipids and fatty acids in teleost fish. *Reviews in Fisheries Science*, **11**(2), 107-184. https://doi.org/10.1080/713610925

Turchini, G., Torstensen, B.E. & Ng, W.K. (2009). Fish oil replacement in finfish nutrition. *Reviews in Aquaculture*, **1**(1), 10-57. https://doi.org/10.1111/j.1753-5131.2008.01001.x

Vasconi, M., Caprino, F., Bellagamba, F. &Moretti, V.M. (2017) Fatty acid composition of gilthead sea bream (*Sparus aurata*) fillets as affected by current changes in aquafeed formulation. *Turkish Journal of Fisheries and Aquatic Sciences,* 17, 451-459. http://doi.org/10.4194/1303-2712-v17_3_01

Werner, I., Linares-Casenave, J., Van Eenennaa, J.P.& Doroshov, S.I. (2007) The Effect of Temperature Stress on Development and Heat-shock Protein Expression in Larval Green Sturgeon (*Acipenser mirostris) Environmental Biology of Fishes,* **79**(3-4), 191–200. https://doi.org/10.1007/s10641-006-9070-z

Zengin H, Vural N, Çelik VK. (2013). Comparison of Changes in Fatty Acid Composition of Starved and Fed Rainbow Trout, (*Oncorhynchus mykiss*) Larvae. *Turkish Journal of Fisheries and Aquatic Sciences*, **13**, 397-405 http://doi.org/10.4194/1303-2712-v13_3_02

Chapter 4

How different rearing temperatures affect growth and stress status of *Acipenser baerii* larvae?

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4. How different rearing temperatures affect growth and stress status of *Acipenser baerii* larvae?

Abstract

The aim of this study was to examine the impact of three different rearing temperatures (16°C, 19°C and 22°C) throughout the endogenous feeding phase of the Siberian sturgeon. This were performed by assessing larval survival and growth; immunofluorescence localization and expression of genes involved in muscle development and growth – *Myogenin* and *Igf1*; stress status by means of expression of thermal stress genes (*hsp70, hsp90α, hsp90β*) and whole body cortisol. Results showed that there were no significant differences across temperatures regarding overall survival rate. Larvae subjected to 22°C showed a faster absorption of the yolk-sac than larvae subjected to 19°C or 16°C. There were no significant differences among temperatures regarding larval weight. Both at schooling and at the end of the trial, larvae reared at 16°C showed a lower level of cortisol than those reared at 19°C or 22°C ($P < 0.05$). Igf-1 immunopositivity was particularly evident in red muscle at schooling stage in all temperatures. Regarding gene expression, all *hsp's* as well as *myogenin* and *igf1* revealed to be statistically higher in larvae reared at 16°C ($P<0.05$ all comparisons), but limited to the schooling stage. These results suggest that larvae in the schooling stage are particularly sensitive to lower temperature regimes, as it can be seen by the high expression all *hsp's* at 16°C. Moreover, cortisol levels were higher in larvae reared at higher temperatures which may seem contradictory with the previous results. For this reason, further studies are necessary especially regarding the exogenous feeding phase in order to better understand if this species is effectively sensitive to thermal stress.

Key words: *Acipenser baerii* larvae, temperature, muscle development, gene expression, stress.

4.1. Introduction

Siberian sturgeon (*Acipenser baerii*) is a commercially important aquaculture species and is one of the main sturgeon species farmed in several European countries (Gisbert and Williot, 2002). As the demand of the grow-out production facilities is increasing, there is a growing need for the development of enhanced hatchery technologies for the production of Siberian sturgeon larvae. Larval production is one of the most critical phases in the intensive sturgeon farming and embryonic and larval stages mortality is still relevant (Gisbert and Williot, 1997; Bardi et al.,

1998). After hatching, larvae go through important steps of development that comprehend the replacement of embryonic functions by the ultimate ones. Therefore, in the early larval phases, there are important changes in the way fish relates with the surrounding environment (Dettlaff et al. 1993).

In aquaculture, fish are continuously exposed to stress factors that are related to routine husbandry. Handling, transportation, sorting, water parameters conditions (temperature, pH, salinity and oxygen) and high stocking density are stress factors that are commonly present in fish farms (Conte, 2004). The general effect of stress consists of the activation of the hypothalamic– pituitary–interrenal axis (HPI) and the production of catecholamine such as epinephrine and norepinephrine, and corticosteroid hormones such as cortisol. Cortisol leads to secondary responses that mainly regard energy requirements and is frequently used as an indicator of stress in fish (Wendelaar Bonga, 1997; Mommsen et al., 1999; Bertotto et al., 2011). Stress in fish may cause immunosuppression, and reduced growth (Wendelaar Bonga, 1997). In vertebrates, growth is determined by a regulatory network in which the growth hormone (GH)-insulin-like growth factor (IGF-1) axis has an important role in regulation of the process together with insulin, thyroid hormones and sex steroids (Jones and Clemmons 1995). Stress, among other factors, strongly influences growth. Indeed, in fish as in mammals, cortisol leads to catabolic and antianabolic effects which, in turn, delay somatic growth (Ma et al., 2003). Moreover, cortisol induces attenuation of GH signalling in hepatocytes reducing IGF-1 levels and variates IGF-binding proteins levels, contributing to the inhibitory effects on somatic growth in teleosts (Kajimura et al., 2016; Philip and Vijayan, 2015). Chronic stress has a deleterious effect on animal health and homeostasis, with somatic growth, and therefore skeletal muscle, being particularly affected (Valenzuela et al. 2018). Indeed, in fish as in mammals, cortisol leads to catabolic and antianabolic effects which, in turn, delay somatic growth (Ma et al., 2003) but it is still lacking a detailed understanding of the core endocrine and molecular mechanisms of how chronic stress affects skeletal muscle growth remains lacking (Valenzuela et al. 2018). Fish also respond to stressors at the cellular level. This response includes changes in protein as, for instance, the increased synthesis of heat shock proteins - HSPs (Iwama et al., 1998). The HSP-families are named based on the molecular mass (kDa) of the protein and three major families of HSPs – HSP-90 (85–90 kDa), HSP-70 (68–73kDa) and low-molecular-mass HSPs (16–24 kDa) have been studied broadly (Iwama et al., 1998). In the unstressed cell, there is a constitutive production of these proteins, and these are necessary in various aspects of protein metabolism to maintain cellular homeostasis (Fink and Goto, 1998). The HSP response constitutes one of the

most important cellular mechanisms used to repair proteins and in preventing the damaging effects of thermal cellular stress (Feige et al. 1996; Somero 2002). HSP-70 is involved in the folding of emerging polypeptide chains, acting as a molecular chaperone and has a role in reparation and degradation of altered or denatured proteins. HSP-90 has an active role in supporting various components of cell signalling, including the cytoskeleton, enzymes, and steroid hormone receptors. Quite recently, Iwama et al. (2004) reviewed how HSPs, in various fish tissues, respond to a wide range of stressors thus suggesting the use of these proteins as indicators of stressed conditions. There are growing indications, both in animals and in humans, that early events may cause a deep imprinting on an individual physiological memory leading to long-term effects on postnatal growth and physiological functions (Rehfeldt et al., 2011). Environmental temperature is one of the most important and critical factors that affect cold-blooded animals (such as sturgeons), development and physiology. Early thermal history may have a deep impact on the subsequent growth of a fish and can be, hence, an important instrument to modulate fish's phenotype (Johnston, 2006). Differences in size and muscle cellularity caused by temperature have been reported in studies performed with Senegalese sole, *Solea senegalensis* (Campos et al., 2013a), in Danube Bleak, *Alburnus chalcoides* (Stoiber et al., 2002), in European seabass and gilthead seabream (Ayala et al., 2000), among others. This plasticity of the phenotype may be related to changes in the expression of growth-related genes during ontogenic development. Indeed, likewise embryonic myogenesis, also in the larval stages muscle growth is a result of proliferation, fusion and differentiation of muscle fibres, which all involve a great number of genes (reviewed by Johnston et al., 2011). There are several genes involved in muscle development and growth, such as the genes coding for the IGF- system proteins (*Igf*), the myogenic regulatory factors (MRFs: *myod*, *myf5*, *mrf4* and *myog*), the myostatin (*mstn*) and the paired-box protein (*pax7*) (De-Santis and Jerry, 2007). Above all, myogenin (coded by the *myog* gene) a member of the helix-loop-helix family, is a muscle regulatory gene that acts as transcription factor during myogenesis (Wright et al. 1989), probably acts as a sequence specific DNA binding factor which interacts with other muscle-specific genes during myogenesis (Wright et al. 1989). Myogenin is a useful tool to identify the earliest signs of myogenic determination in Nile tilapia (Berishvili et al. 2006). During larval stages, fish muscle plasticity in response to the environment is usually not reversible because of the rapid pace of the ontogenetic changes in this period of development. . If the proliferative capacity of the myogenic cells is affected in early stages, this could compromise growth potential of larvae, taking into account that the number of muscle fibres in young fish determine both the ultimate size and growth rate (Weatherley, 1990). Siberian sturgeon, in the wild, can adapt to a

wide range of water temperatures (FAO species fact sheet). In aquaculture conditions, farmed fish may be exposed to relevant temperature oscillations and it seems to be of great importance for the enhancement of the commercial production to have a tighter control of water temperature. . The best temperature for rearing Siberian sturgeon larvae still remains unidentified (Dabrowski et al., 1985), but in commercial and experimental procedures, Siberian sturgeon larvae are usually reared at 18°C (Gisbert and Williot, 2002). Improving the knowledge for a more efficient *A. baerii* larvae production is a fundamental target for a successful and competitive expansion of the aquaculture industry of this species.

In the present study, the *A. baerii* larval development throughout the endogenous feeding phase was evaluated at 16 °C, 19 °C and 22 °C rearing temperatures. The aims of this study were thus to assess: i) larval survival and growth; ii) ontogenic development; iii) the expression of genes involved in muscle development and growth (*myog* and *Igf-1*) and in the stress status (*Hsp70*, *Hsp90α*, *Hsp90β*) and iv) whole body cortisol.

4.2. Materials and Methods

4.2.1. *Larval rearing and sampling*

The trial was carried on at the Experimental Animal Research and Application Centre of Lodi, University of Milan, in April 2016. Fertilized *A. baerii* eggs were transported 24 hours after fertilization, at 14°C in oxygen over-saturated water from the fish farm "Società Agricola Naviglio" (Mantua, Italy) to the experimental site. Eggs were distributed among experimental nurseries after an acclimation period. The incubation temperature in all of the experimental nurseries was according to standard procedures of 16°C (16.2 ± 0.2°C) until hatching, which occurred five days after the fertilization. After hatching, larvae were maintained in the experimental nurseries (three per temperature) and temperature was manipulated: in one group temperature remained at 16°C (16.4 ± 0.2 °C) and, in the two other groups, temperatures were gradually shifted (one degree per hour) to either 19°C (19.3 ± 0.2°C) or 22°C (21.9 ± 0.2 °C). In all nurseries, water temperature was daily monitored and kept under the target values established for each treatment. Water O_2 was close to the saturation throughout the trial (>8mg/l) in all nurseries and pH values were maintained inside the range described for this species at this development stage 6.5-7.5, according to the FAO Technical Paper). During the trial the photoperiod regime was of 12L:12D and, during the experimental period, larvae were not

fed. Sampling points consisted of important steps of *A. baerii* larval development: hatching (T0), beginning of the schooling phase (T1) and complete yolk sac absorption phase (T2). Larval development period was calculated as "days post-hatch" (dph) until the yolk sac was fully absorbed. Survival rate was estimated by dead larvae daily recording. Larvae were killed by over-anaesthesia with MS222 (Ethyl 3-Aminobenzoate, Methanesulfonic A, Sigma-Aldrich). All procedures performed in the experiment were in accordance with the ethical standards of the ethics committee (OPBA) of the University of Milan (OPBA_20_2016).

4.2.2. *Cortisol extraction and radioimmunoassay (RIA)*

Whole body cortisol analyses were performed in frozen larvae by a specific microtitre radioimmunoassay (RIA) as described by Simontacchi et al. (2009). Larvae were pooled (two larvae for each nursery; N=12 samples per treatment) weighed, thawed out and pulverized in liquid nitrogen, and the resulting powders were suspended in 1 ml phosphate-buffered saline (PBS, pH 7.2). The suspension was then extracted with 8 ml of diethyl ether and the supernatant was evaporated to dryness. The dry extracts were dissolved in 0.5 ml of PBS and varying aliquots were used for RIA. Briefly, a 96-well microtitre plate (Optiplate, Perkin Elmer Life Sciences) was coated with anti-rabbit c-globulin serum raised in a goat, incubating overnight the antiserum, diluted 1:1000 in 0.15 mM sodium acetate buffer, pH 9, at 4 °C. The plate was washed twice with PBS and incubated overnight at 4 °C with the specific antiserum solution. It was then carefully washed with PBS, standards, quality controls, unknown extracts and 3H tracers were added, and the plate was incubated overnight at 4 °C. Lastly, it was washed with PBS, added with 200 ll scintillation cocktail (Microscint 20, Perkin Elmer Life Sciences) and counted on a β-counter (Top-Count, Perkin Elmer Life Sciences). The anti-cortisol serum showed the following cross-reactions: cortisol 100%, prednisolone 44.3%, 11-deoxycortisol 13.9%, cortisone 4.95, corticosterone 3.5%, prednisone 2.7%, 17-hydroxyprogesterone 1.0%, 11-deoxycorticosterone 0.3%, dexamethasone 0.1%, progesterone < 0.01%, 17-hydroxypregnenolone < 0.01%, pregnenolone < 0.01%.

4.2.3. *Micro-anatomical analyses: immunofluorescence*

Samples for the micro-anatomical analyses were fixed in 4% (v/v) paraformaldehyde (N=3 samples for each nursery; N=9 samples per treatment). The samples were then dehydrated in a graded 50% (v/v), 70% (v/v), 95% (v/v) and 100% (v/v) ethanol series, embedded in paraffin and transversally cut into 4-µm-thick serial sections. After rehydration, sections were incubated

with the first-step primary antiserum, 1:50 anti-rabbit IGF-1 (Abcam, Cambridge,UK) or 1:50 anti-rabbit Myogenin (Santa Cruz Biotechnology) for 48 hrs at 18–20°C, then washed in PBS for 10 min and incubated with a solution of 10 lg/ml goat biotinylated anti-rabbit IgG (Vector Laboratories Inc.) for 6 hrs at 18–20°C. The sections were then washed twice in PBS, and treated with Fluorescein–Avidin D (Vector Laboratories Inc.), 10 lg/ml in NaHCO3, 0.1 M, pH 8.5, 0.15 M NaCl for 1 hr at 18–20°C. Finally, slides with tissue sections were embedded in Vectashield Mounting Medium with DAPI (H-1200, Vector Laboratories Inc.) and observed using a Confocal Laser Scanning Microscope (FluoView FV300; Olympus). The immunofluororeactive structures were excited using Argon/ Helio–Neon–Green lasers with excitation and barrier filters set for fluorescein. Images containing superimposition of fluorescence were obtained by sequentially acquiring the image slice of each laser excitation or channel.

4.2.4. *Gene identification and primers design*

Genes involved in cellular stress reactions (*Hsp70*, *Hsp90α* and *Hsp90β*) and genes involved in myogenesis (*myog*) and growth (*igf-1*) were selected. *rpl6* (coding for Ribosomal protein L6) and *gapdh* (coding for Glyceraldehyde 3-phosphate dehydrogenase) genes were used as reference.

Gene sequences from *Acipenser* spp. and some teleostean species were selected in order to perform alignments with the Basic Local Alignment Search Tool (NCBI BLAST), using a previously published assembled transcript of *A. baerii* as the reference database (Song et al, 2016). Specific primers were de novo designed for the target genes and the related sequences, the annealing temperatures and the amplification size of each fragment are reported in Table 1.

4.2.5. *RNA extraction and cDNA synthesis*

The sampling was performed at the beginning of T1 and at T2. Larvae were immediately stored at -80°C soon after the sampling procedure. Total RNA was extracted from each frozen larval sample (N=2 sample at T0; N=2 samples for each nursery; N=12 samples per treatment; N=38 samples in total) using RNeasy Mini Kit® (Qiagen), and eluted in a final volume of 40 µl of RNase-free water. A double treatment with DNase enzyme was performed, in order to remove any genomic DNA contamination, according to manufacturer instructions. Five hundred nanograms of RNA was retro-transcribed to cDNA using Quantitect Reverse Transcription Kit® (Qiagen) following manufacturer protocol. An additional reaction without retrotranscriptase enzyme was

performed to verify the complete DNA removal. cDNAs were stored at -80°C until subsequent use.

4.2.6. *Gene expression profiles*

The expression of genes coding for stress, myogenesis and growth factors were analysed by Quantitative RT-PCRs (RT-qPCR) in larvae collected at the three rearing temperatures.

cDNA samples were used as template in RT-qPCR using a BioRad iQ5 Real-Time PCR instrument (Bio-Rad, California, USA) and Universal SYBR® Green Supermix, (Bio-Rad, California, USA) as fluorescent molecule. The amplification conditions were: 150 nM (final concentration) of forward and reverse primers; 98 °C for 30 s, 40 cycles of 98 °C for 15 s, 58-60 °C for 30 s; and a melting profile was included after the last amplification cycle. Annealing temperatures were defined according to primers melting temperatures indicated in Tab. 1.

Cycle threshold (Ct) values were determined for each gene and normalized according to the reference genes. The expression of each gene at T1 and T2 was compared to the calibrator sample T0 and the relative expression values were calculated after a delta Ct-measure using *rpl6* and *gapdh* genes as references.

The amplified gene fragments were loaded on agarose gel, purified and sequenced and the obtained sequences were deposited in Gene bank.

4.2.7. *Statistical analysis*

Statistical analysis of the data was performed using the 2-way ANOVA with temperatures and developmental stages as main factors of the SAS (version 8.1, Cary Inc., NC). Each nursery was considered as the individual value. The data were presented as least squared means ± SEM. Differences were considered significant at $P < 0.05$ and $P < 0.01$.

4.3. Results

4.3.1. *Larval development, survival rate and growth*

Larvae subjected to the highest water temperature showed: i) a faster yolk-sac absorption (Figure 4.1a); ii) the schooling behaviour at 3dph; iii) the complete yolk-sac absorption at 7dph. Larvae subjected to either 16°C or 19°C presented the schooling behaviour at 5dph but larvae

subjected to 19°C fully absorbed the yolk-sac sooner that larvae subjected to 16°C (Figure 4.1a). Regarding survival rate, no differences were found among temperatures from T0 to T1 and T2 (Figure 4.1b) and no deformities were detected in larvae throughout the trial.

Larvae body weight significantly increased from T0 to the following developmental stages ($P<0.05$ for stage, Figure. 4.1c). Otherwise, considering live weight in the single stage of schooling, no significant differences were found among temperatures (Figure 4.1c). At T2 larvae subjected to 16°C and 22°C presented a significant higher body weight than larvae subjected to 19°C ($P<0.05$, Figure 4.1c). The interaction between developmental stages and temperature was not significant (Figure 4.1c).

4.3.2. *Cortisol level*

Regarding cortisol levels, a significant difference was found between T0 vs T2, regardless of the rearing temperature ($P<0.01$; Figure 4.1d). Moreover, at T1 the mean cortisol concentration for larvae subjected to 22°C and 19°C was significantly higher when compared to that of larvae subjected to 16°C ($P<0.05$ both, Figure 4.1d). A higher significant difference in cortisol level was found at T2, where larvae subjected to 16°C showed lower concentrations than larvae subjected to 22°C ($P<0.01$, Figure 4.1d). Conversely, no significant differences between the other two experimental groups was revealed at 19°C. The interaction between developmental stages and temperature was not significant.

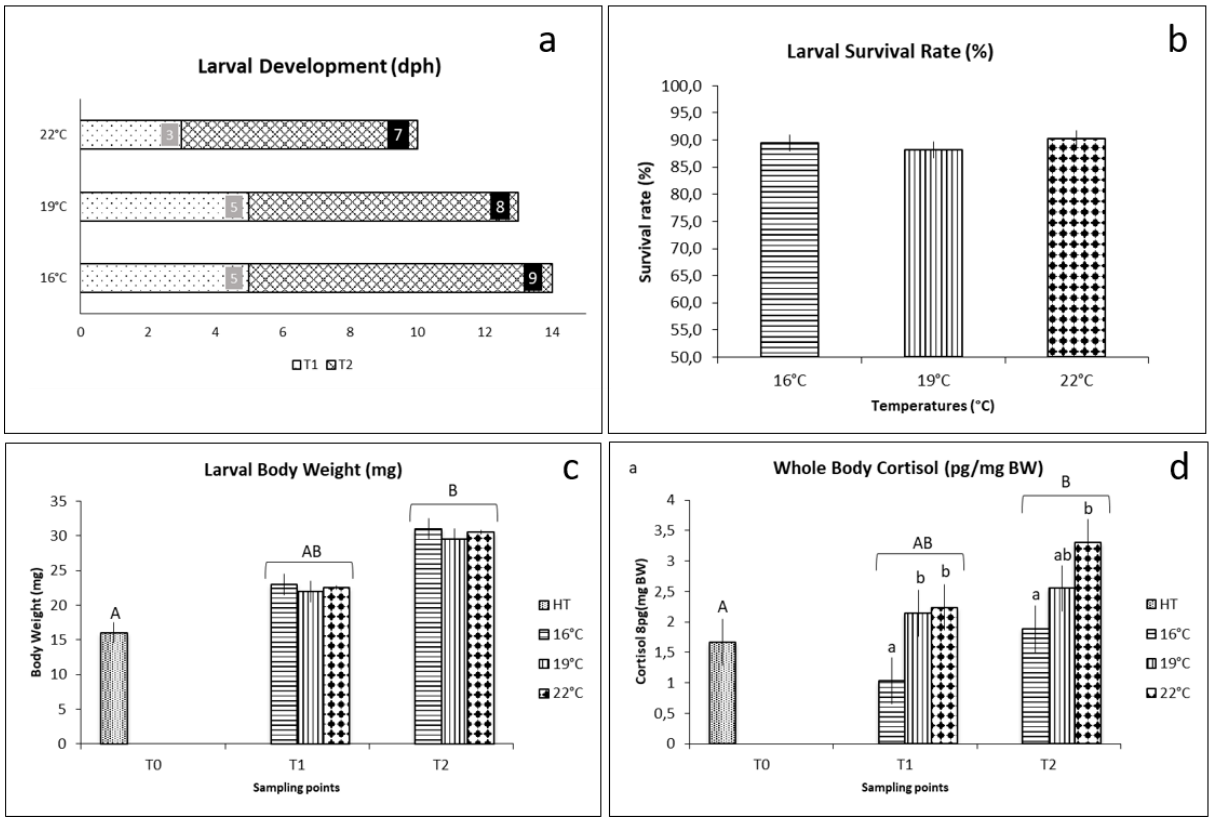

Figure 4.1. a) larval development, measured in days post-hatch (dph); b) larval survival rate (%); c) Larval growth expressed in mg of body weight; d) whole body cortisol concentrations expressed as pg per mg of body weight.. Error bars indicate the standard error of the mean for each treatment/stage of development; lowercase letters indicate significant differences between temperatures, of at least $P < 0.05$; capital letters indicate significant differences between stages of development, of at least $P < 0.05$

4.3.3. *Micro-anatomical analyses: immunofluorescence*

Myogenin immunopositive cells were detected in the cytoplasm of undifferentiated cells at all stages and in all temperatures considered (Figure 4.2a-c). IGF-1 immunofluorescence appeared in both red and white skeletal muscle fibers at T0 (Figure 4.2d) and precisely it was present in the cytoplasm. Furthermore, IGF-1 immunofluorescence was similarly expressed in white muscle at T1 and T2 in all experimental temperatures, following the correct development of skeletal muscles (Figure 4.2e,f representative images). A stronger immunostaining in the red muscle at T1 was observed in all experimental groups (Figure 4.2e).

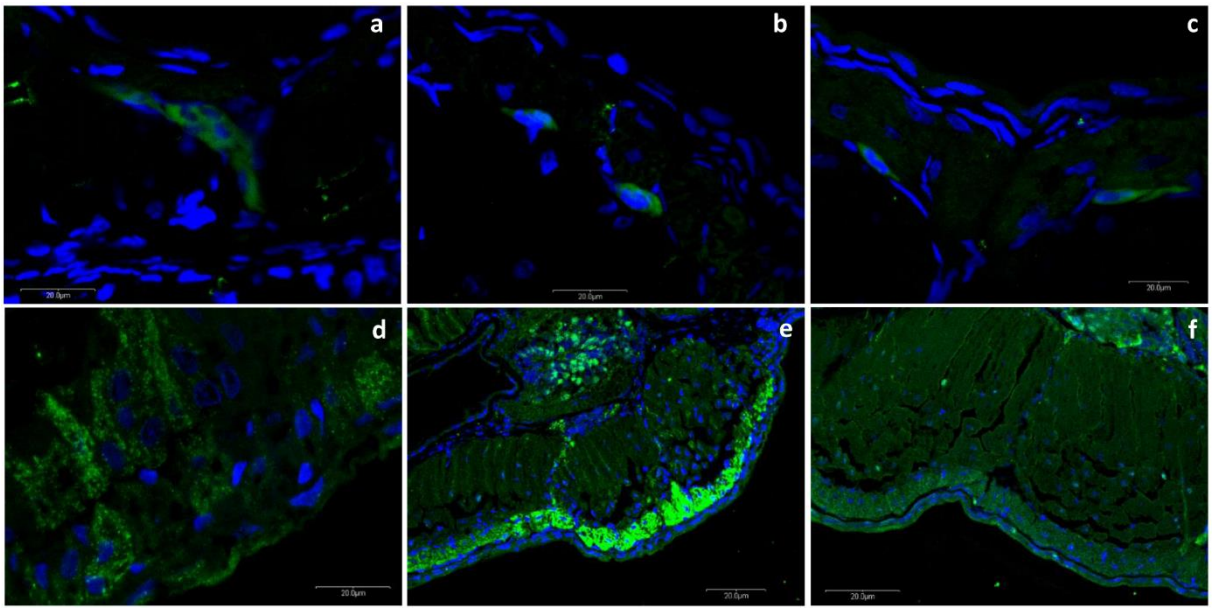

Figure 4.2. Representative images of the temperatures at different timepoints – myod immunofluorescence localization: a) at hatching; b) at 16°C, schooling; c) at 19°C, yolk-sac abscorption; Igf-1 immunofluorescence localization d) at hatching; e) at 22°C, scholing; f) at 19°C, yolk-sac full absorption; scale bar 20µm.

4.3.4. *Thermal stress and growth related gene expressions*

The specificity of primers designed for the amplification of *rpl6, gapdh, Hsp70, Hsp90α*, *Hsp90β*, *myog* and *Igf-1* gene fragments of *A. baerii*, was assessed by Sanger sequencing. The obtained sequences were deposited in GenBank under the accession numbers (MH702440 - MH702446).The expression of the selected genes at T1 and T2 was related to T0 considered as reference sample and results were normalized versus *rpl6* and *gapdh* considered as reference genes. The relative expressions of *Hsp70*, *Hsp90α*, *Hsp90β*, *myog and Igf-1* genes at T1 and T2 phases are shown in Figure 4.3.

Hsp90β, *myog* and *Igf-1* genes showed an upregulation compared to T0 at all temperatures for both T1 and T2 phase. Concerning *Hsp70*, an upregulation was observed at 16°C for T1 and at all temperatures for T2. Furthermore, an upregulation of *Hsp90α* gene was observed only at 16°C for T1.

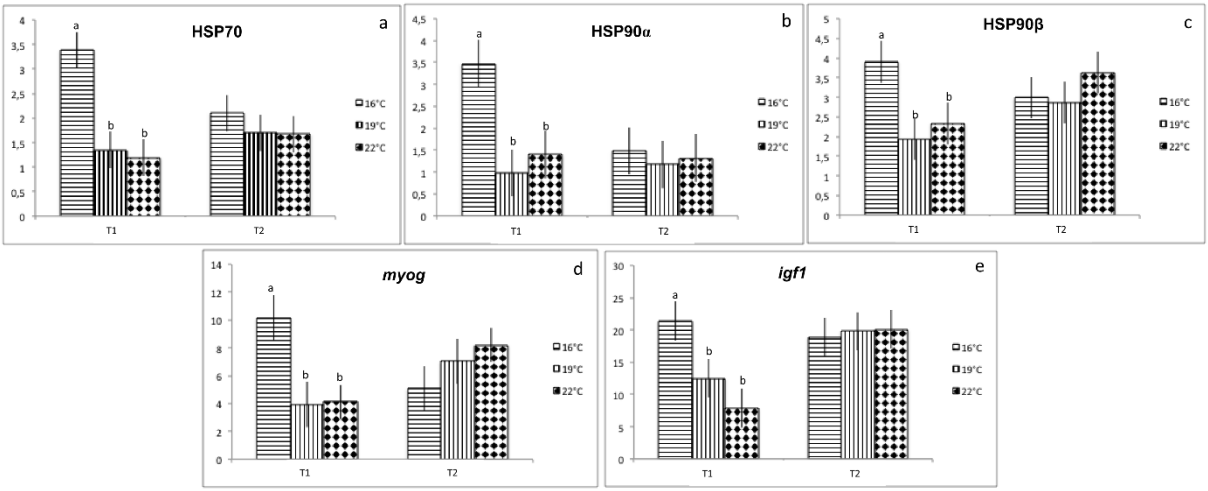

Figure 4.3 relative gene expression of: a) *hsp*70; b) *hsp* 90α, c) *hsp* 90β; d) *Myog* and e) *Igf*-1; lowercase letters indicate significant differences of at least P < 0.05

During T1 all genes resulted significantly more expressed in larvae reared at 16 °C compared to the larvae reared in the other two rearing temperatures (*Hsp70*, *hsp90α*, *hsp90β, myog*, *Igf-1*: P<0,05). No differences were found in the expression of the analysed genes between larvae reared at 19°C and 22°C. Conversely, at T2 no significant differences were found in the expression of all genes among larval stages and rearing temperatures.

4.4. Discussion

In the present work the impact of different rearing temperatures on the development of precocious (yolk sac) *A. baerii* larvae was investigated. In order to analyse the short-term effect of temperature on the developing lateral muscle, three rearing temperatures (16, 19 or 22°C) were tested during the endogenous feeding larval phase. Morphological, biochemical and gene expression approaches were combined.

Temperature constitutes one of the most important environmental factors affecting the development and growth in teleost fishes (Moyle and Cech, 1982; Donaldson et al.,2008). The larval phase is indeed quite susceptible to temperature changes (Johnston et al., 1995; Kamler, 2002). In the present study, larvae subjected to the highest rearing temperature (22°C) reached the complete yolk-sac absorption stage in 20% less time than larvae subjected to the lowest temperatures. Likewise, an increase of rearing temperatures caused an increase in the developmental rate in several marine species like Atlantic cod (Pepin et al., 1997), Senegalese sole (Campos et al., 2013),gilthead seabream (Garcia de la Serrana et al., 2012), in freshwater

species like brown trout (Réalis-Doyelle et al., 2016), Atlantic salmon (Ojanguren et al., 1999) and in several species of Sturgeon (Hardy and Litvak, 2004).

Moreover, it is also known that temperature can be associated with fish survival rates (Boucher et al., 2014). In fact, in studies conducted upon different sturgeon species a clear effect of temperature on survival to hatch was observed (Wang et al., 1987; Van Eenennaam et al., 2005), whereas during the endogenous feeding stage, temperature did not influence survival rates until the complete yolk-sac absorption (Gisbert et al., 2000; Boucher et al., 2014). In our study, survival rates from hatch to the complete yolk-sac absorption varied between 88 and 90%, but there were no significant differences among rates in the tested rearing temperatures, which is in accordance with the above mentioned studies.

Moreover, cortisol level was studied in order to assess stress status in sturgeons. Our results showed that whole body cortisol generally increased from T0 to T2 and this is probably due to the fact that organogenesis is uncomplete and these animals should not be able to exhibit a strong response to a stressor as observed by Bates et al (2014) in white sturgeon, *Acipenser trasmontanus*. The same authors showed similar results about cortisol level in *A. trasmontanus* but limited at 8 days post hatching. As cortisol induces attenuation of GH signalling in hepatocytes reducing IGF-1 levels and variates IGF-binding proteins (IGF-BPs) levels, contributing to the inhibitory effects on somatic growth in teleosts (Kajimura et al., 2016; Philip and Vijayan, 2015), we suggest that larval sturgeon exhibit a heightened sensitivity to warmer temperature, thus revealing their vulnerability to high temperature in the early larval stages. Similar susceptibilities to disturbance have been observed in salmonid larvae during development (Barry et al., 1995).

Regarding micro-anatomical analyses, our results demonstrated a constant presence of myogenin-positive cells at any stage and/or temperature of the experimental trial. This is in agreement with Sassoon et al (1993), who observed that in embryonic somitic muscle, myogenin is expressed prior to other muscle specific genes. Moreover, the cytoplasmic staining is in agreement to what observed by Ferri et al. (2009), who showed that myogenin is already expressed in undifferentiated cells, where it is especially detected in cytoplasm. After the beginning of differentiation, myogenin translocates into the nucleus. Cytoplasmic retention is a mechanism to regulate the biological activity of a protein, as revealed by Chen et al. (1996). For this reason, we suggest that the cytoplasmic staining is especially due to the high presence of undifferentiated cells. In addition we observed that some myotubes myogenin-negative were

present and they are possibly related to quiescent myoblastic cells, identified as “resting cells” by Yoshida et al (1998).

Regarding IGF-1, our study revealed that expression differs considerably among the developmental stages. IGF-1 also appears early in skeletal muscle and precisely at hatching. Similar results have been observed in Nile tilapia, *Oreochromis niloticus,* by Berishvili et al. (2006), but in contrast to the skeletal muscle of young shi drum larvae, *Umbrina cirrosa* (Radaelli et al., 2003) in which no IGF-1 immunoreactivity has been detected. In our study the onset of IGF-1 presence in *A. baerii* muscle development occurs from T0 to T2 at all experimental temperatures. These results are in agreement with Berishvili et al (2006), who suggests a key role for IGF-1 in muscle development. Considering the specific immunostaing at T1, red muscle revealed to be strong immunopositive at all temperatures. We suggest that this higher staining would be due to the particular stage of life of the sturgeons which correspond to the schooling behaviour, during which there’s an increasing of swimming. These data are in agreement with Ginsberg et al (1999) who observed in *A. baerii* a transition from pelagic to benthic swimming (4-5 days post hatch) with aggregation of prelarvae into schools until the compete absorption of yolk-sac. Similar behavioural patterns was also reported in white sturgeon (Conte et al. 1988), lake sturgeon, *Acipenser fulvescens* (Kempinger, 1988) and Russian sturgeon, *Acipenser gueldestaedtii* (Dettlaff et al. 1993).

igf-1 gene expression was 3.5-fold circa increased in T1 at 16°C compared to T0, and this could be correlated with conditions of enhanced growth and minimal stress. As observed in rainbow trout, *Igf-1* transcript abundance in muscle increases as water temperature decreases (Gabillard et al., 2003; Deane and Woo, 2005).

Considering genes related to thermal stress (*Hsp70, Hsp90α, Hsp90β*), we observed a significant higher expression at 16°C at T1. This could be attributed to, for example, stress-related protein damage, enhanced cytoprotection (as suggested by results obtained on silver sea bream; Deane and Woo, 2005), or could support the correct folding of proteins (Pelham, 1986).

Conversely, no differences in genes expression were observed among the three temperatures at T2, even though *Hsp70* and *Hsp90β* showed an upregulation compared to T0. These genes code for highly conserved proteins expressed in response to biotic and abiotic stressors and usually identified as damage biomarkers. The HSP-response is involved in cellular processes including protein synthesis, folding and translocation as well as assembly of larger protein complexes, all of which can be impaired upon stress as well as preventing the damaging effects of thermal

cellular stress (Airaksinen et al., 2003). Additionally, differences in the ability to over-express HSPs during stressful conditions may be associated with an organism's vulnerability and the extent of thermal injury (Werner et al., 2007).

Moreover, increased levels of *hsp*-70 may indicate an attempt of the cell to counteract the increase in levels of damaged proteins when activity of other chaperones such as HSP-90 is insufficient (Ivanina et al., 2008). In our study this is a condition limited to the T1, because no differences has been found at T2 suggesting a restoring of the stress condition.

In addition, we examined thermal plasticity of growth related genes and as previously described for thermal stress genes, also myogenin and *igf-1* were highly expressed at 16°C at T1 comparing to T0. There is evidence that these genes expression may vary with temperature and may have an influence in muscle growth (Campos et al., 2013b; Fernandes et al., 2006; Hall et al., 2003; Wilkes et al., 2001). Both growth rate and growth potential are definitely heritable characteristics, but growth of all species depends on environmental factors, such as temperature and food availability, regarding growth itself but also regarding the expression of morphological characteristics (Leatherland, 1994).

4.5. Conclusion

The natural s *A. baerii* populations have suffered a sharp decrease throughout the last two centuries, due to overfishing and due to the loss of its spawning grounds, because of dams construction and pollution (Ruban 1997, 1999). Siberian sturgeon is, therefore, threatened (Birstein et al., 1997) and is currently on the IUCN Red List of endangered species, which contributes not only to avoid illegal trade of sturgeons and their products, but also to implement conservation plans. Due to its characteristics such as relatively fast early growth, tolerance for farming at high densities, and the high quality of its products (caviar and meat), it seems to be of great importance to understand the mechanisms that control early muscle growth and development because this can allow to detect the temporal opportunity to eventually introduce growth variations. In the present study, we have observed that all temperatures are congruent with a correct development of cultured *A. baerii* and that a rearing temperature of 22°C leads to a higher developmental rate of *A. baerii* larvae.

These results suggest that larvae in the schooling stage are particularly sensitive to lower temperature regimes, as it can be seen by the high expression all *hsp's* at 16°C. Moreover, cortisol levels were higher in larvae reared at higher temperatures which may seem contradictory with

the previous results. Further studies are however necessary in order to investigate the expression of genes involved in the lateral muscle development in Siberian sturgeon yolk-sac larvae, with the additional aim to investigate the possible disjunction between *Hsp70* gene expression and measurable HSP-70 tissue levels. Results of this study will assist in the interpretation of future studies on thermal stress.

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4.6. Reference list

Airaksinen, S., Jokilehto, T., Råbergh, C.M.I. & Nikinmaa, M. (2003). Heat- and cold-inducible regulation of HSP70 expression in zebrafish ZF4 cells*, Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, **136**(2), 275-282. https://doi.org/10.1016/S1096-4959(03)00205-7

Ayala, M.D., López-Albors, O., Gil, F., Latorre, R., Vázquez, J.M., García-Alcázar, A., Abellán, E., Ramírez, G. & Moreno, F. (2000). Temperature Effect on Muscle Growth of the Axial Musculature of the Sea Bass (*Dicentrarchus labrax* L.). *Anatomia, Histologia Embryologia*, **29**(4), 235-242. https://doi.org/10.1046/j.1439-0264.2000.00262.x

Bardi, R.W., Chapman, F.A. & Barrows, F.T. (1998). Feeding trials with hatchery-produced Gulf of Mexico sturgeon larvae. *The Progressive Fish-Culturist*, **60**(1), 25-31. https://doi.org/10.1577/1548-8640(1998)060<0025:FTWHPG>2.0.CO;2

Barry, T.P., Malison, J.A., Held, J.A. & Parish, J.J. (1995). Ontogeny of the cortisol stress response in larval rainbow trout. *General and Comparative Endocrinology*, **97**(1), 57-65. https://doi.org/10.1006/gcen.1995.1006

Bates LC, Boucher MA, Shrimpton JM (2014), Effect of temperature and substrate on whole body cortisol and size of larval white sturgeon (*Acipenser transmontanus* Richardson, 1836). *Journal of Applied Ichthyology*, **30**(6), 1259-1263. https://doi.org/10.1111/jai.12570

Berishvili, G., Shved, N., Eppler, E., Clota, F., Baroiller, J.F. & Reinecke, M. (2006). Organ-specific expression of IGF-I during early development of bony fish as revealed in the tilapia, *Oreochromis*

niloticus, by in situ hybridization and immunohistochemistry: indication for the particular importance of local IGF-I. *Cell and Tissue Research*, **325**(2), 287-301. doi:10.1007/s00441-005-0133-9

Bertotto, D., Poltronieri, C., Negrato, E., Richard, J., Pascoli, F., Simontacchi, C. & Radaelli, G. (2011) Whole body cortisol and expression of HSP70, IGF-I and MSTN in early development of sea bass subjected to heat shock. *General and Comparative Endocrinology*, **174**(1), 44-50. https://doi.org/10.1016/j.ygcen.2011.08.003

Birstein, V.J., Hanner, R. & DeSalle, R. (1997). Phylogeny of the Acipenseriformes: cytogenetic and molecular approaches. *Environmental Biology of Fishes*, **48**(1-4), 127-155. http://dx.doi.org/10.1023/A:1007366100353

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

Campos, C., Fernandes, J.M.O., Conceição, L.E.C., Engrola, S., Sousa, V. & Valente LMP. (2013). Thermal conditions during larval pelagic phase influence subsequent somatic growth of Senegalese sole by modulating gene expression and muscle growth dynamics. *Aquaculture,* 414–415, 46–55. https://doi.org/10.1016/j.aquaculture.2013.07.039

Campos, C., Valente, L.M., Conceição, L.E.,Engrola, S.,Sousa, V., Rocha, E. & Fernandes, J.M. (2013a) Incubation temperature induces changes in muscle cellularity and gene expression in Senegalese sole (*Solea senegalensis*).*Gene,* **516**(2):209-217. doi: 10.1016/j.gene.2012.12.074

Chen, C-M.A., Kraut, N., Groudine, M. & Weintraub, H. (1996). I-mf, a novel myogenic repressor, interacts with members of the MyoD family. *Cell*, **86**(5), 731–741. https://doi.org/10.1016/S0092-8674(00)80148-8

Conte, F. S. (2004). Stress and the welfare of cultured fish*. Applied Animal Behaviour Science*, **86**(3-4), 205-223. https://doi.org/10.1016/j.applanim.2004.02.003

Conte, F., Doroshov, S. & Lutes, P. (1988). Hatchery manual for the white sturgeon *Acipenser transmontanus* with application to other North American Acipenseridae. Publ. #3322. Cooperative Extension, Division of Agriculture and Natural Resources, University of California, Davis

Deane, E.D. & Woo, N.Y.S. (2005). Cloning and characterization of the hsp70 multigene family from silver sea bream: Modulated gene expression between warm and cold temperature acclimation. *Biochemical and Biophysical Research Communications*, **330**(3), 776-783. https://doi.org/10.1016/j.bbrc.2005.03.039

Dettlaff, T. A., Ginzburg, A. S. and Schmalhausen, O. I. (1993). Chapter 3: Development of Prelarvae. In: *Sturgeon fishes: developmental biology and aquaculture*.1st ed., Springer-Verlag Berlin and Heidelberg. http://dx.doi.org/10.1007/978-3-642-77057-9

Dabrowski, K., Kaushik, S.J. & Fauconneau, B. (1985). Rearing of sturgeon (*Acipenser baeri*, Brandt) larvae I. Feeding trial. *Aquaculture*, **65**(1), 31-41. https://doi.org/10.1016/0044-8486(87)90268-7

De-Santis, C. & Jerry, D.R. (2007). Candidate growth genes in finfish — where should we be looking? *Aquaculture*, **272**(1-4), 22-38. https://doi.org/10.1016/j.aquaculture.2007.08.036

Donaldson, M.R., Cooke, S.J., Patterson, D.A. & MacDonald, J.S. (2008). Cold shock and fish. *Journal of Fish Biology*, **73**(7), 1491-1530. https://doi.org/10.1111/j.1095-8649.2008.02061.x

FAO Technical PaperSturgeon Hatechery Manual, Ankara 2013.

Feige, U., Morimoto, R.I., Yahara, I. & Polla, B.S. (eds) (1996). *Stress-inducible cellular responses*. Birkhauser Verlag, Basel, Switzerland. Doi: 10.1007/978-3-0348-9088-5

Ferri, P., Barbieri, E., Burattini, S., Guescini, M., D'Emilio, A., Biagiotti, L., Del Grande, P., De Luca, A., Stocchi, V. & Falcieri, E. (2009). Expression and subcellular localization of myogenic regulatory factors during the differentiation of skeletal muscle C2C12 myoblasts. *Journal of Cellular Biochemistry*, **108**(6), 1302-1317. https://doi.org/10.1002/jcb.22360

Fernandes, J.M., Mackenzie, M.G., Wright, P.A., Steele, S.L., Suzuki, Y., Kinghorn, J.R. & Johnston, I.A. (2006). Myogenin in model pufferfish species: comparative genomic analysis and thermal plasticity of expression during early development. *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics*, **1**(1), 35-45. https://doi.org/10.1016/j.cbd.2005.09.003

Fink, A.L. & Goto, Y. (1998). In: Fink, A.L. & Goto, Y. (eds), *Molecular Chaperones in the Life Cycle of Proteins: Structure, Function, and Mode of Action*. New York: Marcel Dekker.

Gabillard, C., Weil, C., Rescan, P.Y., Navarro, I., Gutiérrez, J. & Le Bail, P.Y. (2003) Effects of environmental temperature on IGF1, IGF2, and IGF type I receptor expression in rainbow trout

(*Oncorhynchus mykiss*). *General and Comparative Endocrinology*, **133**(2), 233-242. https://doi.org/10.1016/S0016-6480(03)00167-9

Garcia de la Serrana, D., Vieira, V.L.A., Andree, K.B., Darias, M., Estévez, A., Gisbert, E. et al. (2012). Development Temperature Has Persistent Effects on Muscle Growth Responses in Gilthead Sea Bream. *PLoS ONE*, **7**(12), e51884. http://doi.org/10.1371/journal.pone.0051884

Gisbert, E. & Williot, P. (1997). Larval behaviour and effect of the timing of initial feeding on growth and survival of Siberian sturgeon larvae under small scale hatchery production. *Aquaculture*, **156**(1-2), 63-76. https://doi.org/10.1016/S0044-8486(97)00086-0

Gisbert, E. & Williot, P. (2002). Advances in the larval rearing of Siberian sturgeon. *Journal of Fish Biology,* **60**(5), 1071–1092. https://doi.org/10.1111/j.1095-8649.2002.tb01705.x

Gisbert, E., Williot, P. & Castellò-Orvay, F. (2000). Influence of egg size on growth and survival of early stages of Siberian sturgeon (*Acipenser baeri*) under small scale hatchery conditions. *Aquaculture,* **183**(1-2), 83–94. https://doi.org/10.1016/S0044-8486(99)00287-2

Hardy, R.S.& Litvak, M.K. (2004) Effects of temperature on the early development, growth and survival of shortnose sturgeon, *Acipenser brevirostrum*, and Atlantic sturgeon, *Acipenser oxyrhynchus*, yolk-sac larvae *Environmental Biology of Fishes,* **70**(2), 145–154 https://doi.org/10.1023/B:EBFI.0000029345.97187.5b

Hall, T.E., Cole, N.J. & Johnston, I.A. (2003). Temperature and the expression of seven muscle specific protein genes during embryogenesis in the Atlantic cod *Gadus morhua* L. *Journal of Experimental Biology*, **206**(18), 3187-3200. https://doi.org/10.1242/jeb.00535

Ivanina, A.V., Cherkasov, A.S. & Sokolova, I.M. (2008). Effects of cadmium on cellular protein and glutathione synthesis and expression of stress proteins in eastern oysters,*Crassostrea virginica* Gmelin. *Journal of Experimental Biology,* **211**, 577-587. doi: 10.1242/jeb.011262

Iwama, G.K., Thomas, P.T., Forsyth, R.B. & Vijayan, M.M. (1998). Heat shock protein expression in fish. *Reviews in Fish Biology and Fisheries*, **8**(1), 35-56. https://doi.org/10.1023/A:1008812500

Iwama, G.K., Afonso, L.O., Todgham, A., Ackerman, P. & Nakano, K. (2004). Are hsps suitable for indicating stressed states in fish? *Journal of Experimental Biology*. **207**, 15-19. doi:10.1242/jeb.00707

Johnston, I.A. (2006). Environment and plasticity of myogenesis in teleost fish. *Journal of Experimental Biology*, **209**(12), 2249-2264. http://jeb.biologists.org/cgi/doi/10.1242/jeb.02153

Johnston, I., Vieira, V. & Abercromby, M. (1995). Temperature and myogenesis in embryos of the Atlantic herring *Clupea harengus*. *Journal of Experimental Biology*, **198**(Pt6), 1389-1403. http://www.ncbi.nlm.nih.gov/pubmed/9319285

Johnston, I.A., Bower, N.I. & Macqueen, D.J. (2011). Growth and the regulation of myotomal muscle mass in teleost fish. *Journal of Experimental Biology,* **214**(10), 1617-1628. http://jeb.biologists.org/cgi/doi/10.1242/jeb.038620

Jones, J. & Clemmons, D.R. (1995). Insulin-Like Growth Factors and Their Binding Proteins: Biological Actions. *Endocrine Reviews,* **26**(1), 3-34. https://doi.org/10.1210/edrv-16-1-3

Kajimura, S., Hirano, T., Visitacion, N., Moriyama, S., Aida, K. & Grau, G. (2016). Dual mode of cortisol action on GH/IGF-I/IGF binding proteins in the tilapia, *Oreochromis mossambicus. Journal of Endocrinology,* **178**(1), 91-99. doi:10.1677/joe.0.1780091

Kamler, E. (2002). Ontogeny of yolk-feeding fish: an ecological perspective. *Reviews in Fish Biology and Fisheries*, **12**(1), 79-103. https://doi.org/10.1023/A:1022603204337

Kempinger, J.J. (1988). Spawning and early life history of lake sturgeon in the Lake Winnebago system, Wisconsin. pp 110-122. In: R. D. Hoyt, ed. *11° Annual Larval Fish Conference*. American Fisheries Society, Symposium 5, Bethesda, Maryland

Leatherland, J.F. (1994). Reflections on the thyroidology of fishes: from molecules to mankind. *Guelph Ichthyology Reviews* **2**, 1–67

Ma, K., Mallidis, C., Bhasin, S., Mahabadi, V., Artaza, J., Cadavid, N.G., Arias, J. & Salehian, B. (2003). Glucocorticoid-induced skeletal muscle atrophy is associated with upregulation of myostatin gene expression. *American Journal of Physiology, Endocrinology and Metabolism*, **285**(2), E363-E371. https://doi.org/10.1152/ajpendo.00487.2002

Mommsen, T.P., Vijayan, M.M. & Moon, T.W. (1999). Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Reviews in Fish Biology and Fisheries*, **9**(3), 211-268. https://doi.org/10.1023/A:1008924418720

Moyle, P.B. & Cech, JR. (1982). In: *Fishes: An Introduction to Ichthyology*, 5th edition. London: Prentice Hall, Inc.

Ojanguren, A.F., Reyes-Gavilán, F.G. & Muñoz, R.R. (1999). Effects of Temperature on Growth and Efficiency of Yolk Utilisation in Eggs and Pre-feeding Larval Stages of Atlantic Salmon. *Aquaculture International*, **7**(2), 81-87. https://doi.org/10.1023/A:1009214804949

Pepin, P., Orr, D.C. & Anderson, J.T. (1997). Time to hatch and larval size in relation to temperature and egg size in Atlantic cod (*Gadus morhua). Canadian Journal of Fisheries and Aquatic Sciences*, **54**(s1), 2-10. https://doi.org/10.1139/f96-154

Pelham, H.R. (1986). Speculations on the functions of the major heat shock and glucose-regulated proteins. *Cell*, **46**(7), 959-61.

Philip, A.M. & Vijayan, M.M. (2015). Stress-immune-growth interactions: cortisol modulates suppressors of cytokine signaling and JAK/STAT pathway in rainbow trout liver. PLOS ONE 10(6): e0129299. https://doi.org/10.1371/journal.pone.0129299

Radaelli, G., Domeneghini, C., Arrighi, S., Bosi, G., Patruno, M. & Funkenstein, B. (2003). Localization of IGF-I, IGF-I receptor, and IGFBP-2 in developing *Umbrina cirrosa* (Pisces: Osteichthyes). *General and Comparative Endocrinology*, **130**(3), 232-244. doi:10.1016/S0016-6480(02)00609-3

Réalis-Doyelle, E., Pasquet, A., De Charleroy, D., Fontaine, P. & Teletchea, F. (2016). Strong effects of temperature on the early life stages of a cold stenothermal fish species, brown trout (*Salmo trutta* L.). *PLoS One*, **11**(5) e0155487. https://doi.org/10.1371/journal.pone.0155487

Rehfeldt, C., Te Pas, M.F.W., Wimmers, K., Brameld, J.M., Nissen, P.M. & Berri, C. (2011). Advances in research on the prenatal development of skeletal muscle in animals in relation to the quality of muscle-based food. I. Regulation of myogenesis and environmental impact. *Animal*, **5**(5), 718–730. https://doi.org/10.1017/S1751731110002089

Ruban, G.I. (1997). Species structure, contemporary distribution and status of the Siberian sturgeon *Acipenser baerii*. *Environmental Biology of Fishes*, **48**(1-4), 221-230. https://doi.org/10.1023/A:1007372932

Ruban, G.I. (2005). In: *The Siberian Sturgeon Acipenser baerii Brandt. Species structure and ecology*. World Sturgeon Conservation Society, New Wulmstorf (Germany)

Sassoon, D.A. (1993). Myogenic regulatory factors: dissecting their role and regulation during vertebrate embryogenesis. *Developmental Biology*, **156**(1), 11-23. https://doi.org/10.1006/dbio.1993.1055

Simontacchi, C., Negrato, E., Pazzaglia, M., Bertotto, D., Poltronieri, C. & Radaelli, G. (2009). Whole-body concentrations of cortisol and sex steroids in white sturgeon (*Acipenser transmontanus*, Richardson 1836) during early development and stress response. *Aquaculture International,* **17**(1), 7-14. https://doi.org/10.1007/s10499-008-9174-x

Somero, G.N. (2002). Thermal physiology and vertical zonation of intertidal animals: optima, limits and costs of living. *Integrative and Comparative Biology*, **42**(4), 780-789. https://doi.org/10.1093/icb/42.4.780

Stoiber, W., Haslett, J.R., Wenk, R., Steinbacher, P., Gollmann, H.P. & Sänger, A. M. (2002). Cellularity changes in developing red and white fish muscle at different temperatures: simulating natural environmental conditions for a temperature freshwater cyprinid. *Journal of Experimental Biology,* **205**(Pt16), 2349–2364. http://dev.biologists.org/content/134/7/1253.abstract

Valenzuela, C.A., Zuloaga, R., Mercado, L., Einarsdottir, I.E., Björnsson, B.T., Valdés, J.A. & Molina, A. (2018). Chronic stress inhibits growth and induces proteolytic mechanisms through two different nonoverlapping pathways in the skeletal muscle of a teleost fish. *American Journal of Physiology, Regulatory, Integrative and Comparative Physiology*, **314**(1), R102-R113. https://doi.org/10.1152/ajpregu.00009.2017

Van Eenennaam, J.P., Linares-Casenave, J., Deng, X. & Doroshov, S.I. (2005). Effect of incubation temperature on green sturgeon embryos, *Acipenser medirostris*. *Environmental Biology of Fishes*, **72**(2), 145-154. https://doi.org/10.1007/s10641-004-8758-1

Wang, Y.L., Buddington, R.K. & Doroshov, S.I (1987). Influence of temperature on yolk utilization by the white sturgeon, *Acipenser transmontanus. Journal of Fish Biology,* **30**(3), 263–271. https://doi.org/10.1111/j.1095-8649.1987.tb05751

Weatherley AH (1990). Approaches to understanding fish growth. *Transactions of the American Fisheries Society*, **122**(5), 784-796. https://doi.org/10.1577/1548-8659(1993)122<0784:TAOFBM>2.3.CO;2

Wendelaar Bonga, S. (1997). The stress response in fish. *Physiological Reviews*, **77**(3), 591-625. https://doi.org/10.1152/physrev.1997.77.3.591

Werner, I., Linares-Casenave, J., Van Eenennaa, J.P.& Doroshov, S.I. (2007). The Effect of Temperature Stress on Development and Heat-shock Protein Expression in Larval Green

Sturgeon (*Acipenser mirostris) Environmental Biology of Fishes,* **79**(3-4), 191–200. https://doi.org/10.1007/s10641-006-9070-z

Wilkes, D., Xie, S.Q., Stickland, N.C., Alami-Durante, H., Kentouri, M., Sterioti, A., Koumoundouros, G., Fauconneau, B. & Goldspink, G. (2001). Temperature and myogenic factor transcript levels during early development determines muscle growth potential in rainbow trout (*Oncorhynchus mykiss)* and sea bass (*Dicentrarchus labrax*). *Journal of Experimental Biology*, **204**(16), 2763–2771. http://jeb.biologists.org/content/204/16/2763

Wright, W.E., Sassoon, D.A., Lin, V.K. (1989). Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD. *Cell*, **56**(4), 607-617. https://doi.org/10.1016/0092-8674(89)90583-7

Yoshida, N., Yoshida, S., Koishi, K., Masuda, K. & Nabeshima, Y. (1998). Cell heterogeneity upon myogenic differentiation: down-regulation of MyoD and Myf-5 generates 'reserve cells'. *Journal of Cell Science*, **111**(6), 769-779. http://jcs.biologists.org/content/111/6/769.abstract

Table 4.1 Primers used in qPCR

Gene	Fwd sequence (5' → 3')	Rev sequence (3' → 5')	T F (°C)	T R (°C)	Size (bp)
miog	CACGGTGGACAGGAGGA	GGTTCATCAGGGTGCTTCT	62	60	101
igf-1	TCCAGCAGGCATTCAGTC	TCAGCTCCGCACAGAGTC	60	62	158
hsp70	CCCGTGGAGAAGTCC	CCCGTTGAAGAAATCCTG	59	57.6	124
hsp 90β	GGTCATCTTGCACCTGA	TTCTGCTTCATCATCGCTG	60	58	154
hsp 90α	CAGAGGCCGACAAGAATGA	CTGTAGATGCGGTTGGAATG	60	60	121
ef1α	GTGAAGCAGCTCATCATC	GTTGTAGCCGATCTTCTTG	58	58	120
β_actin	ATGAAGTGTGACGTTGACATC	CATTGTGCTCGGTGCCAG	58	62	130
rpl6	GATGCTGTGCTAATGAGTG	ACAGCTCCACTCACTATGA	58	58	119
gapdh	ACACCCGCTCATCTATCTTT	AGGTCCACGACTCTGTTGC	56	56	115

For each gene, its temperature both forward (TF) and reverse (TR) and amplicon size (bp) are indicated.

Chapter 5

Effects of stocking density on Siberian sturgeon (*Acipenser baerii*) larval growth, muscle development and fatty acids composition

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5. Effects of stocking density on Siberian sturgeon (*Acipenser baerii*) larval growth, muscle development and fatty acids composition

Abstract

This study evaluated the effects of rearing density on muscle growth and development in Siberian sturgeon larvae. Three different stocking densities were tested: low (LD, 30 larvae/l), mid (MD, 80 larvae/l) and high (HD, 150 larvae/l). 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 GC-FID analysis. LD 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 and presented a higher proliferation rate of the muscle fibres. Moreover, larvae reared at the higher density, showed an acceleration in muscle development.

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

5.1. 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 and 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, therefore, have an impact on the performance in aquaculture settings. The major stressors in aquaculture are stocking density, temperature and low dissolved oxygen. Stocking density in intensive aquaculture directly influences physiology, welfare and behavior of reared fish (Schreck et al., 1997; Montero et al., 1999; Ellis et al., 2002; Schram et al., 2006). In aquaculture systems, the efficiency is maximized by increasing the stocking densities. Previous studies demonstrated that high densities may lead to lower welfare of some fish species (Lupatsch et al., 2010; Yvette et al., 2011). However, in species such as wedge sole (*Dicologlossa cuneata*) and winter flounder (*Pseudopleuronectes americanus*) there were no negative effects caused by high densities (Fairchild et al., 2001 and Herrera et al., 2009 respectively). However, in sturgeons high rearing density was shown to be an environmental stressor; Wuertz et al. (2006) and Jodun et al. (2002) found that the growth of the Atlantic sturgeon was suppressed if reared in a high stocking density. The cause of the growth suppression is not fully known, in particular for what regards its impact on muscle development. In acipenseridae, muscle growth is the result of the fusion of myoblasts derived from somite, leading to the formation of multinucleated muscle lamellae and later of polygonal cells (Steinbacher et al., 2006). Fatty acids, in particular polyunsaturated fatty acids of n-3 series, are generally known as key nutrients in fish larvae (Sargent et al., 1999). Their role has been investigated in larvae of several sturgeon species, such as white sturgeon (*Acipenser transmontanus*) (Gawlicka et al., 2002), Russian sturgeon (*Acipenser gueldenstaedtii*) (Sener et al., 2005) and Persian sturgeon (*Acipenser persicus*) (Hafezieh et al., 2010), where these fatty acids were 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. Leatherland

and Cho (1987) described an effect of the rearing density on the hepatosomatic index (HSI) and on the concentration of plasma free fatty acids. 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 was to assess the rearing density that allows good farming conditions without compromising larval morpho-functional aspects. Specifically, we quantified the effects of rearing conditions on free-embryo's weight, total length, survival, muscle development and fatty acid profile in Siberian sturgeon until the complete yolk-sac absorption.

5.2. Materials and Methods

5.2.1. *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: low (LD, 30 larvae/l), mid (MD, 80 larvae/l) and high (HD, 150 larvae/l). 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 replicate (analysis were performed in duplicate; total n=30 per treatment) for fatty acid composition were picked up with a wide pipette and killed by over-anaesthesia with Ethyl 3-Aminobenzoate, Methanesulfonic A (Sigma-Aldrich). This research was approved by the Ethic Committee of the University of Milan (OPBA_22_2017).

5.2.2. *Scanning Electron Microscope (SEM)*

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

5.2.3. *Histological and immunohistochemical analyses*

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

5.2.4. *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.

5.2.5. *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 $p < 0.05$.

5.3. Results

5.3.1. *Development, survival and growth*

Water parameters are reported in figure 5.1. O_2 was constantly close to the saturation value (Figure 5.1a); temperature ranged from 17 to 19.7°C (Figure 5.1b); pH values were within the physiological range described for this species (Figure 5.1c). The duration of the endogenous feeding phase was of 8 days across treatments, and fish exhibited schooling behaviour in synchrony among density treatments.

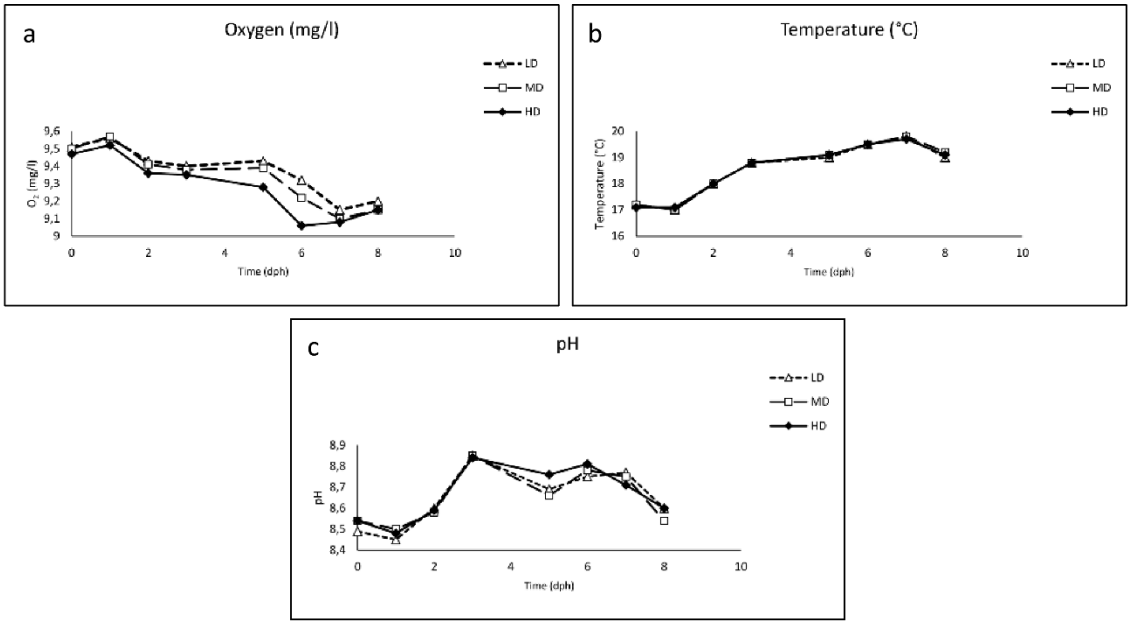

Figure 5.1. Water parameters from T0 until the end of the trial for each stocking density: a) dissolved oxygen (mg/l); b) temperature (ºC); c) ph.

Mortality from schooling to the yolk sac absorption significantly decreased (Figure 5.2a; P<0.01), and within the schooling stage the LD group showed lower mortality than MD and HD groups (Figure 5.2a; P<0.05). At the end of the trial no differences were found between treatments (Figure 5.2a).

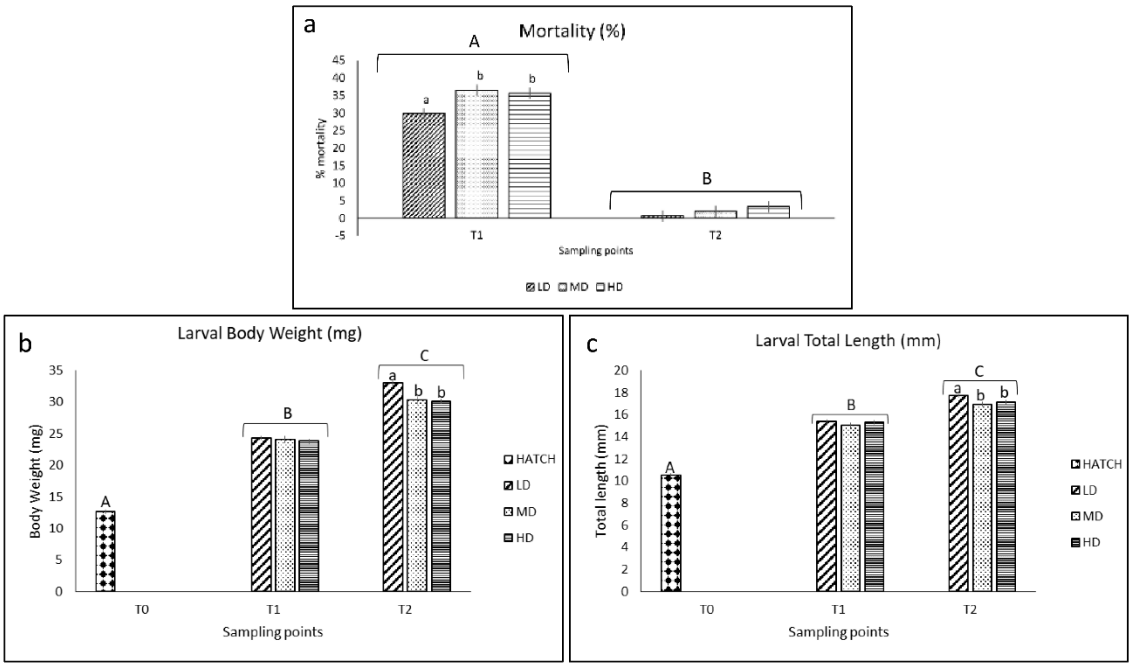

Figure 5.2. a) mortality (%); b) Larval growth expressed in mg of body weight; c) Larval length expressed in mm of body length for stocking density,; Error bars indicate the standard error of the mean for each treatment/stage of development; ^{A,B}Means with different superscripts differ significantly between stages of development (P< 0.05); ^{a,b}Means with different superscripts differ significantly between treatments (P< 0.05).

Body weight significantly increased from one stage of development to the other, regardless of the density (Figure 5.2b; $P<0.001$). 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 5.2b).

Total length (TL) significantly increased from one stage of development to the other, regardless of the density ($P<0.001$; Figure 5.2c). 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 5.2c). The interaction between developmental stages and temperature was not significant.

5.3.2. *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 (Figure 5.3a-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 5.3a, 5.3b, 5.3c respectively). No damaged fins, looped tail or change in the form of yolk-sac was observed.

5.3.3. *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 5.3d-f). Histometrical results are presented in Figure 3g-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 5.3g); there were highly significantly differences from one stage to the other regarding TMA ($P<0.01$; Figure 5.3g). 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 3h). Also regarding the FMA, there was a highly significant increase from hatch to the yolk-sac absorption stage ($P<0.01$; Figure 5.3i). However, no differences were

found across treatments. The interaction between developmental stages and density was not significant.

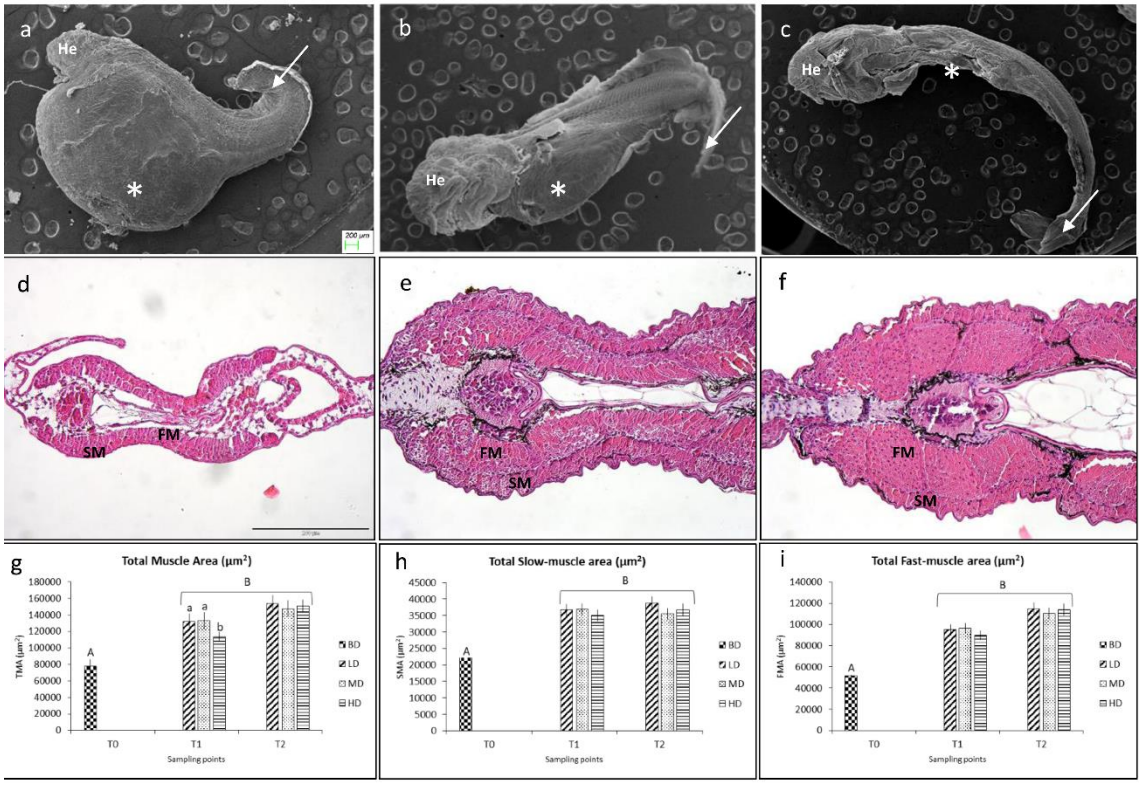

Figure 5.3. Images of the three stocking densities at different time points – SEM representative figures at HD; a) at hatching; b) at schooling; c) yolk-sac full absorption. The figures have same scale bar as located in Figure 3a: 200 µm; asterisks, yolk-sac; thin arrows, tail; He, head. d-f) HE staining 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 ($P< 0.05$).

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, 5.4a and b). Regarding the areas occupied by LF and PC, no differences were found between treatments at the schooling stage (Figure 5.4c,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 5.4c). On the opposite, the area occupied by the polygonal cells, was significantly higher for larvae subjected to HD ($P<0.05$, Figure 5.4d). The interaction between developmental stages and density was not significant.

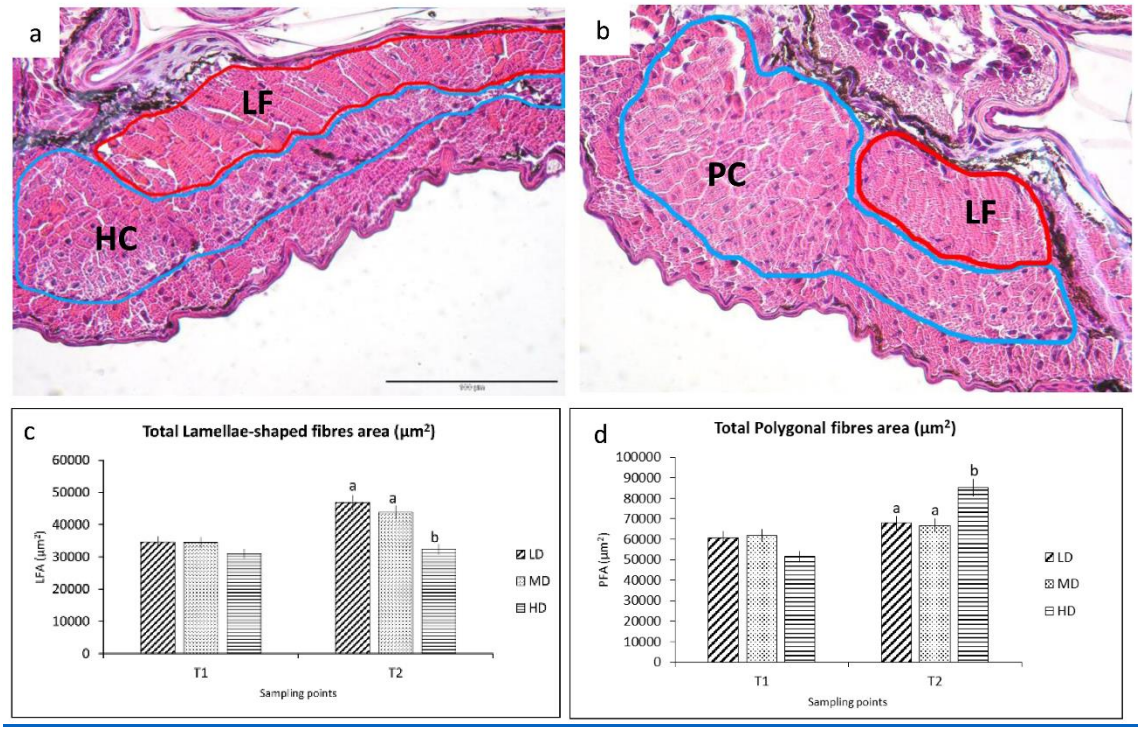

Figure 5.4. HE-staining with representative images at MD for a,b) lamellae-shaped fibres (LF) and polygonal cells (PC); c) quantitative representation of lamellae-shaped fibres area; d) quantitative representation of and polygonal cells area. Area expressed in µm^2; n=9/group; ^{a,b}Means with different superscripts differ significantly between treatments (P< 0.05).

Regarding the anti-PCNA of the lateral muscle, immunostaining was observed in FMA nuclei (arrowheads; Figure 5.5a-c); quantification of the proliferating cells is reported in Figure 5.5d. Proliferating cells of the FMA, revealed that the T0 group was significantly higher than all the other groups (Figure 5.5d; 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.

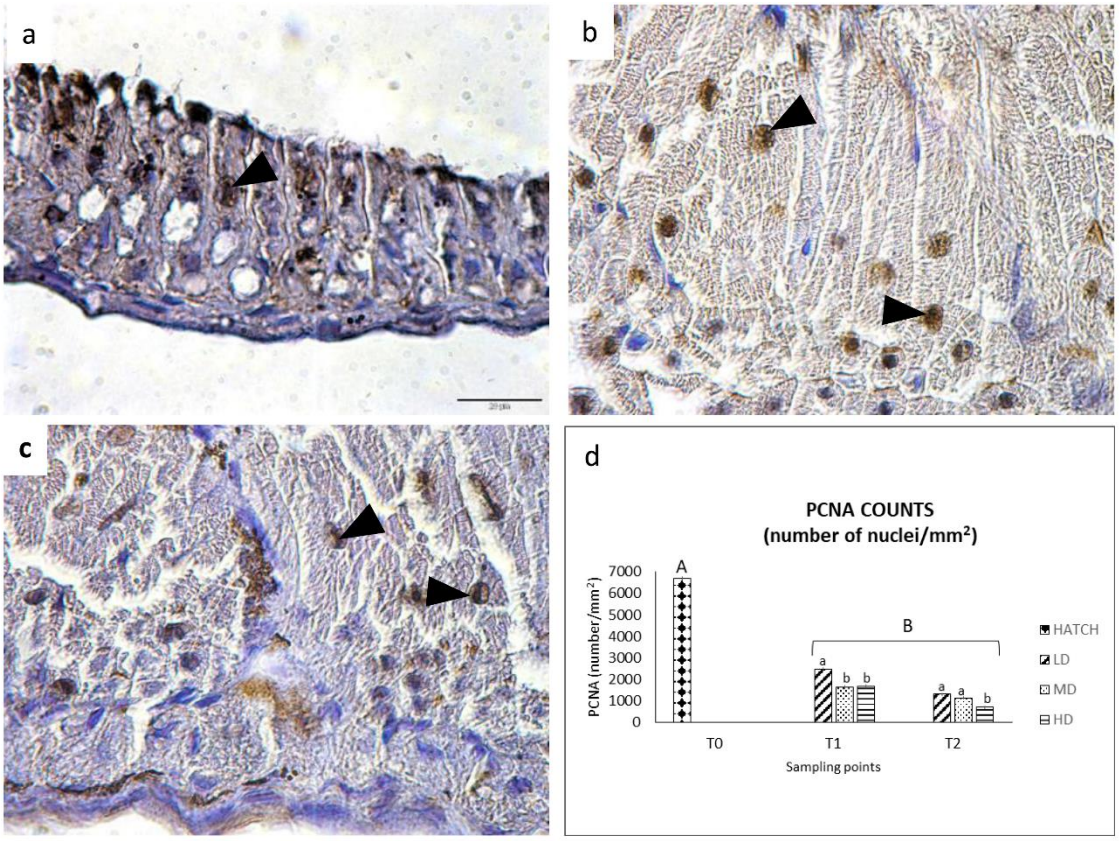

Figure 5.5. a,b,c) Representative images of PCNA-immunolocalization (arrowheads); for LD at different timepoints; d) quantitative representation of PCNA counts. Area expressed in number/mm^2; n=9/group; ^{A,B}Means with different superscripts differ significantly between stages of development (P< 0.05); ^{a,b}Means with different superscripts differ significantly between treatments (P< 0.05

5.3.4. *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 5.1. 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.

TABLE 5.1. Fatty acid composition of Siberian sturgeon reared at three different density sampled at three different stages of development

	Hatching	Schooling LD	Schooling MD	Schooling HD	YSA LD	YSA MD	YSA HD	p-value
16:0	16.07 ±0.96	16.03 ±0.47	15.93 ±0.11	15.83 ±0.08	16.74 ±0.40	16.97 ±0.33	16.54 ±0.15	0.001
16:1 n-7	1.75 ±0.09	1.61 ±0.02	1.65 ±0.02	1.65 ±0.03	1.41 ±0.03	1.36 ±0.08	1.37 ±0.04	0.000
16:4 n-1	0.12 ±0.17	0.00 ±0.00	0.00 ±0.00	0.11 ±0.20	0.50 ±0.01	0.56 ±0.05	0.54 ±0.02	0.000
18:0	4.85 ±1.14	5.39 ±0.43	5.20 ±0.12	5.21 ±0.09	6.45 ±0.36	6.74 ±0.48	6.35 ±0.14	0.000
18:1 n-9	37.09 ±0.98	36.74 ±0.50	36.84 ±0.27	36.71 ±0.26	34.85 ±0.48	34.73 ±0.61	34.82 ±0.27	0.000
18:1 n-7	2.87 ±0.05	2.93 ±0.02	2.94 ±0.01	2.95 ±0.00	3.06 ±0.01	3.08 ±0.02	3.08 ±0.01	0.000
18:2 n-6	13.10 ±0.46	12.63 ±0.24	12.76 ±0.09	12.71 ±0.06	11.33 ±0.33	11.12 ±0.28	11.30 ±0.09	0.000
18:3 n-6	3.36 ±0.12	3.21 ±0.05	3.20 ±0.01	3.16 ±0.02	2.77 ±0.07	2.73 ±0.13	2.77 ±0.02	0.000
18:3 n-3	2.68 ±0.11	2.55 ±0.07	2.58 ±0.02	2.56 ±0.03	2.09 ±0.08	2.02 ±0.11	2.04 ±0.04	0.000
18:4 n-3	1.13 ±0.03	1.06 ±0.02	1.06 ±0.02	1.05 ±0.02	0.86 ±0.03	0.84 ±0.06	0.85 ±0.02	0.000
20:1 n-11	1.02 ±0.00	1.00 ±0.03	0.99 ±0.01	0.99 ±0.02	0.97 ±0.02	0.95 ±0.03	0.95 ±0.01	0.000
20:2 n-6	0.51 ±0.10	0.32 ±0.28	0.47 ±0.06	0.48 ±0.02	0.16 ±0.28	0.00 ±0.00	0.00 ±0.00	0.000
20:3 n-6	0.47 ±0.01	0.48 ±0.02	0.49 ±0.00	0.49 ±0.00	0.57 ±0.01	0.37 ±0.32	0.58 ±0.02	0.901

20:4 n-6	2.95 ±0.05	3.29 ±0.09	3.25 ±0.07	3.28 ±0.07	3.87 ±0.12	4.03 ±0.21	3.99 ±0.04	0.000
20:5 n-3	2.26 ±0.01	2.24 ±0.06	2.25 ±0.03	2.29 ±0.00	2.29 ±0.03	2.30 ±0.06	2.33 ±0.01	0.050
22:5 n-3	0.57 ±0.04	0.57 ±0.11	0.59 ±0.03	0.59 ±0.01	0.58 ±0.01	0.36 ±0.31	0.59 ±0.02	0.529
22:6 n-3	9.20 ±0.04	9.95 ±0.30	9.82 ±0.10	9.94 ±0.25	11.50 ±0.39	11.84 ±0.67	11.89 ±0.17	0.000
SFA	20.92 ±2.10	21.42 ±0.89	21.12 ±0.22	21.04 ±0.16	23.19 ±0.76	23.71 ±0.80	22.89 ±0.21	0.000
MUFA	42.72 ±1.13	42.28 ±0.51	42.41 ±0.29	42.30 ±0.27	40.29 ±0.50	40.12 ±0.68	40.22 ±0.31	0.000
PUFA	36.35 ±0.97	36.30 ±0.39	36.47 ±0.08	36.66 ±0.15	36.53 ±0.28	36.17 ±0.27	36.88 ±0.19	0.847
n-3	15.85 ±0.16	16.37 ±0.20	16.30 ±0.05	16.43 ±0.20	17.33 ±0.32	17.36 ±0.29	17.70 ±0.15	0.000
n-6	20.39 ±0.64	19.93 ±0.41	20.17 ±0.12	20.11 ±0.10	18.70 ±0.58	18.26 ±0.51	18.64 ±0.05	0.000
n3/n6	0.78 ±0.02	0.82 ±0.02	0.81 ±0.01	0.82 ±0.01	0.93 ±0.04	0.95 ±0.04	0.95 ±0.01	0.000

p-values refer to the significant differences between stages of development. Differences between densities were never significant for any FA. Values are expressed as g/100g of fatty acid and are presented as mean ± standard deviation

5.4. Discussion

In the present study we analysed the effects of three stocking density rearing conditions on free-embryo's 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 in growth rate in high densities (between 264 and 792 juveniles/litre; Fajfer et al. al 1999, Mohler et al., 2000). However, many studies indicate that high stocking density increases stress (Barton 2002; Barton and Iwama 1991; Leatherland and Cho 1985; Pickering and Duston 1983; Wedemeyer, 1976) and to date, it has been shown that high density is an environmental stress factor for sturgeons (Wuertz et al., 2006). In our study, in fact, larvae reared at a low density were significantly heavier and longer than those reared at medium and high densities. Mortality was significantly lower in the LD group at the schooling stage, while no differences were observed at the end of the trial. In addition, Li et al. (2011) in their study on the effects of stock density on growth of the sturgeon Amur, found that growth rate decreased significantly by increasing stocking density, with a significant negative impact. The strong reduction in growth in high-density reared fish is often related to a decrease in food consumption and a reduction in the efficiency of conversion (Papoutsoglou et al., 1998; Vijayan and Leatherland 1990). Even if in our study we evaluated only the endogenous feeding conditions, we still can compare this data in terms of the efficiency in converting yolk-sac resources. Recent studies on sturgeon (Falahatkar and Barton 2007, Rafatnezhad et al 2008, Falahatkar et al 2009) suggest that this species is relatively

resistant to disturbances caused during aquaculture practices, but is still relatively sensitive. A reduction in the growth rate in medium and high density reared sturgeons also corresponds to a different muscular development. Considering muscle growth, we have to point out that in teleosts, muscle growth is the result of two processes: hypertrophy and hyperplasia. Muscle fibres grow by hypertrophy during post-embryonic life to reach a functional maximum (Rowlerson and Vegetti 2001) and have been described for different species, such as carp (Alami-Durante et al., 1997), cod (Galloway et al, 1999), salmon (Nathanailides et al., 1995) and in several other marine species, 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 Rowlerson and 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 of muscle development in its final form. According to Rowlerson and Vegetti 2001 we assessed the rate of replication of the cells. PCNA counts revealed to be higher in the fast fibres at LD at both T1 and T2, suggesting that the turnover was decreased in HD group and this is consistent with the higher final weight and length reached by larvae subjected to the lowest density. Up to date the mechanism of polygonal cells formation is still unclear, but we suggest

that in the HD group, a faster differentiation from lamellae-shaped fibres into polygonal cells was present, thus revealing a correlation with the lowest growth parameters in the same group.

The fatty acid composition has not been influenced by stocking density during our trial. Density could act as a stressor and induce some alteration of fatty acid metabolism in fish, as found in gilthead sea bream (Montero et al., 1999). The modification of fatty acid composition was observed after long time experiments, and it has been linked to the utilization of polyunsaturated fatty acids (PUFA) in liver in response to a stress situation and plasma cortisol release. Other authors found similar results in rainbow trout, *Oncorhynchus mykiss* (Bayir and Bayir, 2017), where the modification of fatty acids profile was also linked with the increase of Δ-6 desaturase in the liver and muscle of trout reared ad high density. All these studies were performed on fed fish and for longer periods than that used in our study, so it is possible that the modification of fatty acids profile in response to density could appear only after a long exposure to longer density-rearing conditions than the one experimented in our trial. Not taking into account the rearing density but only the evolution of fatty acids profile in time, during the first days of life of Siberian sturgeon larvae, we could observe how it changes according to the larval development. Oleic acid and linoleic acid decreased during the development, probably because larvae rather used 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.

5.5. Conclusions

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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5.6. References

Aidos, L., Valente, L. M. P., Sousa, V., Lanfranchi, M., Domeneghini, C. & Di giancamillo, A. (2017). Effects of different rearing temperatures on muscle development and stress response in the early larval stages of Acipenser baerii. *European Journal of Histochemistry*, **61**(4), 287-294. https://doi:10.4081/ejh.2017.2850

Alami-Durante, H., Olive, N. & Rouel, M. (2007). Early thermal history significantly affects the seasonal hyperplastic process occurring in the myotomal white muscle of Dicentrarchus labrax juveniles. *Cell and Tissue Research*, **327**(3), 553-570. https://doi 10.1007/s00441-006-0321-2

Balon, E. K. (2001). Saltatory ontogeny and the life-history model: neglected processes and patterns of evolution. *Journal of Bioeconomics*, **3**(1), 1–26. https://doi.org/10.1023/A:1016305005962

Barton; B.A. (2002) Stress in Fishes: A Diversity of Responses with Particular Reference to Changes in Circulating Corticosteroids. *Integrative and Comparative Biology*, **42**(3), 517–525. https://doi.org/10.1093/icb/42.3.517

Barton ,B. A. & Iwama, G. K. (1991). Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Annual Review of Fish Diseases*, **1**, 3 -26. https://doi.org/10.1016/0959-8030(91)90019-G

Bates, L. C., Boucher, M. A., & Shrimpton, J. M. (2014). Effect of temperature and substrate on whole body cortisol and size of larval White Sturgeon (Acipenser transmontanus Richardson, 1836). *Journal of Applied Ichthyology*, **30**(6), 1259–1263. https://doi.org/10.1111/jai.12570

Bayir, M. & Bayir, A. (2017). A comparison of growth, antioxidant levels and fatty acid metabolism in juvenile rainbow trout (Oncorhynchus mykiss) reared in two different stocking densities: low and high. *Marine and Freshwater Behaviour and Physiology*, **50**(5-6), 345-357. https://doi: 10.1080/10236244.2017.1412652

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

Christie, W. W. (2003).Lipid Analysis. 3rd ed. Bridgwater: The Oily Press

Crossman, J. A., Forsythe, P. S., Scribner, K. T. & Baker, E. A. (2011). Hatchery rearing environment and age affect survival and movements of stocked juvenile Lake Sturgeon. Fi*sheries Management and Ecology*, **18**(2), 132–144. https://doi.org/10.1111/j.1365-2400.2010.00762.x

Dettlaff, T. A., Ginzburg, A. S. and Schmalhausen, O. I. (1993). Chapter 3: Development of Prelarvae. In: *Sturgeon fishes: developmental biology and aquaculture*.1st ed., Springer-Verlag Berlin and Heidelberg. http://dx.doi.org/10.1007/978-3-642-77057-9

Di Giancamillo A., Rossi R., Vitari F., Pastorelli G., Corino C.& Domeneghini C. (2009). Dietary conjugated linoleic acids decrease leptin in porcine adipose tissue. *Journal of Nutrition*, **139**(10), 1867 – 1872. https://doi.org/10.3945/jn.109.110627

Ellis, T., North, B., Scott, A. P., Bromage, N. R., Porter, M. & Gadd, D. (2002). The relationships between stocking density and welfare in farmed rainbow trout. *Journal of Fish Biology*, **61**(3), 493-531. https://doi.org/10.1111/j.1095-8649.2002.tb00893.x

Fairchild, E. A.& Howell, W. H. (2001). Optimal stocking density for juvenile winter flounder Pseudopleuronectes americanus. *Journal of the World Aquatic Society*, **32**(3), 300-308. https://doi.org/10.1111/j.1749-7345.2001.tb00453.x

Fajfer, S., Meyers, L., Willman, G., Carpenter, T. & Hansen, M. J. (1999). Growth of Juvenile Lake Sturgeon Reared in Tanks at Three Densities. *North American Journal of Aquaculture*, **61**(4), 331-335. https://doi.org/10.1577/1548-8454(1999)061<0331:GOJLSR>2.0.CO;2

Falahatkar, B.,Poursaeid, S.,Shakoorian, M.& Barto, B. (2009). Responses to handling and confinement stressors in juvenile great sturgeon Huso huso. *Journal of Fish Biology*, **75**(4), 784-796. https://doi.org/10.1111/j.1095-8649.2009.02334.x

Falahatkar, B. & Barton, B. A. (2007). Preliminary observations of physiological responses to acute handling and confinement in juvenile Beluga Huso huso. *Aquaculture Research,* **38**(16), 1786-1789. https://doi.org/10.1111/j.1365-2109.2007.01855.x

Galloway, T. F., Kjorsvik, E. & Kryvi, H. (1999) Muscle growth and development in Atlantic cod larvae (Gadus morhua L.), related to different somatic growth rates. Jou*rnal of Experimental Biology*, **202**, 2111-2120

Gawlicka, A., Herold, M. A., Barrows, F. T., De La Noüe, J. & Hung, S. S. O. (2002) Effects of dietary lipids on growth, fatty acid composition, intestinal absorption and hepatic storage in white sturgeon (Acipenser transmontanus R.) larvae. *Journal of Applied Ichthyology*, **18**(4-6), 673–681. https://doi.org/10.1046/j.1439-0426.2002.00371.x

Hafezieh, M., Mohd Salah Kamarudin, S., Bin Saad, C.R., Mostafa Kamal, A. S., Agh, N., Valinassab, T., Sharifian, M. & Hosseinpour, H. (2010). Effect of Enriched Artemia urmiana with HUFA on growth, survival and fatty acids composition of Persian sturgeon larvae (Acipenser persicus). *Iranian Journal Fisheries Sciences,* **9**(1), 61-72. http://aquaticcommons.org/id/eprint/22355

Heming, T. A. & Buddington, R. K. (1988). Yolk absorption in embryonic and larval fishes. *Fish Physiology Part A*, **11**, 407–446. https://doi.org/10.1016/S1546-5098(08)60203-4

Herrera, M., Vargas-Chacoff, L., Hachero, I., Ruíz-Jarabo, I., Rodiles, A.& Navas, J. I. (2009). Physiological responses of juvenile wedge sole Dicologoglossa cuneata (Moreau) to high stocking density. *Aquaculture Research*, **40**(7), 790-797. https://doi.org/10.1111/j.1365-2109.2008.02162.x

Jodun, W. A., Millard, M. J.& Mohler, J. (2002) The effect of rearing density on growth, survival, and feed conversion of juvenile Atlantic sturgeon. *North American Journal of Aquaculture*, **64**(1), 10–15. https://doi.org/10.1577/1548-8454(2002)064<0010:TEORDO>2.0.CO;2

Kamler E. (2002). Ontogeny of yolk-feeding fish: an ecological perspective. *Reviews in Fish Biology and Fisheries*, **12**(1), 79-103. https://doi.org/10.1023/A:1022603204337

Kamler, E. (2008). Resource allocation in yolk-feeding fish. *Reviews in Fish Biology and Fisheries*, **18**(2), 143–200. https://doi.org/10.1007/s11160-007-9070-x

Leatherland, J. F. & Cho, C. Y. (1985). Effect of rearing density on thyroid and interrenal gland activity and plasma and hepatic metabolite levels in rainbow trout, Salmo gairdneri Richardson. *Journal of Fish Biology*, **27**(5), 583–592. https://doi.org/10.1111/j.1095-8649.1985.tb03203.x

Li, D., Liu, Z. & Xie, C. (2012). Effects of rearing density on juvenile Acipenser schrenckii digestibility, feeding rate and growth. *Fish Physiology and Biochemistry*, **38**(2), 511–520. doi 10.1007/s10695-011-9531-y

Liu, C., Wang, J., Ma, Z., Li, T., Xing, W., Jiang, & Luo, L. (2018). Effects of totally replacing dietary fish oil by linseed oil or soybean oil on juvenile hybrid sturgeon, Acipenser baerii Brandt♀×A. schrenckii Brandt♂. *Aquaculture Nutrition*, **24**(1),184–194. https://doi.org/10.1111/anu.12546

Lupatsch, I., Santos, G. A. & Schrama, J. W. (2010). Effect of stocking density and feeding level on energy expenditure and stress responsiveness in European sea bass Dicentrarchus labrax. *Aquaculture*, **298**(3-4), 245-250. https://doi.org/10.1016/j.aquaculture.2009.11.007

Mambrini, M., Sanchez, M. P., Chevassus, B., Labbe, L., Quillet, E.& Boujard, T. (2004). Selection for growth increases feed intake and affects feeding behavior of brown trout. *Livestock Production Science*, **88**(1-2), 85-98. https://doi.org/10.1016/j.livprodsci.2003.10.005.

Mohler, J. W., King, M. K.& Farrell, P. R. (2000). Growth and survival of first-feeding and fingerling Atlantic Sturgeon under culture conditions. *North American Journal of Aquaculture*, **62**(3), 174-183. https://doi.org/10.1577/1548-8454(2000)062<0174:GASOFF>2.3.CO;2

Mohseni, M., Pourkazemi, M., Kazemi, R., Norooz Foshkhomi, M. R., Mojazi Amiri, B., Kaladkova, L. N. (2000). Study on the effects of stocking density of eggs and larvae on the survival and frequency of morphological deformities in Persian sturgeon, great sturgeon and stellate sturgeon. *Iranian Journal of Fisheries Sciences*, **2**(1), 75-90 http://jifro.ir/article-1-3175-en.html

Montero, D., Izquierdo, M. S., Tort, L., Robaina, L., & Vergara, J. M. (1999). High stocking density produces crowding stress altering some physiological and biochemical parameters in gilthead sea bream, Sparus aurata, juveniles. *Fish Physiology and Biochemistry*, **20**(1), 53–60. https://doi.org/10.1023/A:1007719928905

Nathanailides, C., Stickland, N. C. & Albors, O. L. (2011). Influence of prehatch temperature on the development of muscle cellularity in posthatch Atlantic salmon (Salmo salar*). Canadian Journal of Fisheries and Aquatic Sciences*, **52**(4), 675-680. https://doi.org/10.1139/f95-068

Papoutsoglou, S. E., Tziha, G., Vrettos, X. & Athanasiou, A. (1998). Effect of stocking density on behavior and growth rate of European sea bass (Dicentrarchus labrax) juveniles reared in a closed circulated system. *Aquacultural Engineering*, **18**(2), 135-144. https://doi.org/10.1016/S0144-8609(98)00027-2

Pickering, A. D. & Duston, J. (1983). Administration of cortisol to brown trout Salmo trutta L., and its effects on the susceptibility to Saprolegnia infection and furunculosis. *Journal of Fish Biology*, **23**(2), 163-175. https://doi.org/10.1111/j.1095-8649.1983.tb02891.x

Sargent, J., McEvoy, L., Estevez, A., Bell, G., Bell, M., Henderson, J. & Tocher, D. (1999). Recent developments in the fatty acids nutrition of fish. *Aquaculture*, **177**(1-4). 191-199. https://doi.org/10.1016/S0044-8486(99)00083-6.

Rafatnezhad, S., Falahatkar, B. & Tolouei Gilani, M. H. (2008). Effects of stocking density on haematological parameters, growth and fin erosion of great sturgeon (Huso huso) juveniles. *Aquaculture Research*, **39**(14), 1506-1513. https://doi.org/10.1111/j.1365-2109.2008.02020.x

Rowlerson, A. & Vegetti, A. (2001). Cellular mechanism of post-embryonic muscle growth in aquaculture species. In: Johnston IA, ed. *Muscle development and growth*. 1st ed. San Diego: Academic Press, pp 103-140

Sener, E., Yildiz, M., & Savaş, E. (2005). Effects of Dietary Lipids on Growth and Fatty Acid Composition in Russian Sturgeon (Acipenser gueldenstaedtii) Juveniles. *Turkish Journal of Veterinary and Animal Sciences*, **29**(5), 1101-1107

Schram, E., Van der Heul, J. W., Kamstra, A.& Verdegem, M. C. J. (2006). Stocking density dependent growth of Dover sole (Solea solea). *Aquaculture*, **252**(2-4), 339-347. https://doi.org/10.1016/j.aquaculture.2005.07.011.

Schreck, C. B., Olla B. L. & Davis M. W. (1997). Behavioral response to stress. In: Iwama, G. K., Pickering, A. D., Sumpter, J. P.& Schreck, C. B., eds, *Fish stress and health in aquaculture*. 1st ed. Cambridge: Cambridge University Press, pp 62, 145-170

Steinbacher, P., Haslett, J. R., Sänger, A. M.& Stoiber, W. (2006). Evolution of myogenesis in fish: a sturgeon view of the mechanisms of muscle development. *Anatomy and Embryology*, **211**(4), 311-322. https://doi.org/10.1023/A:1007719928905

Yvette, S., Wunderink, S. E. & Silke, H. (2011). Chronic and acute stress responses in Senegalese sole (Solea senegalensis): the involvement of cortisol, CRH and CRHe BP. *General and Comparative Endocrinology*, **171**(2), 203-210. https://doi.org/10.1016/j.ygcen.2011.01.010.

Valente, L. M. P., Moutou, K. A., Conceicao, L. E. C., Engrola, S., Fernandes, J. M. O. & Johnston, I. A. (2013). What determines growth potential and juvenile quality of farmed fish species? *Reviews in Aquaculture*, **5**(S1), S168–S193. https://doi.org/10.1111/raq.12020

Vasconi, M., Aidos, L., Di Giancamillo, A., Bellagamba, F., Domeneghini, C. & Moretti, V. (2018). Effect of temperature on fatty acid composition and development of unfed Siberian sturgeon (A. baerii) larvae. *Journal of Applied Ichthyology*, 00:1-7. https://doi.org/10.1111/jai.13725

Vijayan, M. M. & Leatherland, J. F. (1990). High stocking density affects cortisol secretion and tissue distribution in brook charr, Salvelinus fontinalis. *Journal of Endocrinology*, **124**(2), 311-318. https://doi.org/10.1677/joe.0.1240311

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

Wuertz, S., Lutz, I., Gessner, J., Loeschau, P., Hogans, B., Kirschbaum, F.& Kloas, W. (2006). The influence of rearing density as environmental stressor on cortisol response of Shortnose sturgeon (Acipenser brevirostrum). *Journal of Applied Ichthyology*, **22**(1), 269-267. https://doi.org/10.1111/j.1439-0426.2007.00966.x

Xu, R., Hung, S. S.& German, J. B. (1993). White sturgeon tissue fatty acid compositions are affected by dietary lipids. *Journal of Nutrition*, **123**(10), 1685-1692. https://doi.org/10.1093/jn/123.10.1685

Chapter 6

How different stocking densities affect growth and stress status of *Acipenser baerii* larvae?

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6. How different stocking densities affect growth and stress status of *Acipenser baerii* larvae?

Abstract

In the present study a multidisciplinary approach was used in order to evaluate muscle development and stress response in Siberian sturgeon larvae at schooling (T1) and complete yolk sac absorption (T2), reared at three different stocking densities (low, medium and high). Larvae growth was accessed, as well as whole body cortisol levels. Moreover, levels of relative expression of different genes involved in the growth process (*igf1* and *igf2*), in the myogenesis process (*myog*) and in the regulation of cellular stress (*glut1*, *glut2* and *hsp70*) were analysed using real time PCR approach. Larvae reared at lower densities showed a higher body weight. Cortisol levels did not differ significantly, both in time and across densities. All the genes examined have shown an up-regulation in both development stages at all the rearing densities considered, with the exception of the *myog* gene which was, instead, always down-regulated. This down-regulation is significantly greater in larvae reared in high-density in the phase of the complete absorption of the yolk sac. The significant up-regulation observed for the gene regulator of the cellular stress *glut2* in the conditions of greater density of the phase of complete absorption of the yolk sac, confirms what reported in the literature. The data presented in this study was analysed by evaluating the ΔCt of each target with respect to the reference genes, comparing the relative expressions between different densities without normalizing the data towards the HT phase. In this way, it was possible to study the significance of expression between development phases for each gene. Considering all of the results, it would seem that lower densities should be used in these stages of development, as these showed a higher growth potential and lower stress levels.

6.1. Introduction

The Siberian sturgeon *Acipenser baerii* has been included, since 1996, in the IUCN red list for endangered species and in 2010 it passed from the "vulnerable" state to the "endangered" state, meaning it is considered at risk of extinction in the near future. The species began to undergo a sharp decline in the 1930s (years in which the demand was significantly higher) and continues to decline today. The reasons for the deterioration of the status of natural populations are mainly due to the destruction of its natural habitat due to the construction of dams, pollution and

excessive fishing (FAO, 2013). *A. baerii* is included in the "Cultured Aquatic Species Information Program" of the FAO and therefore considered among the most important species in aquaculture. The success and diffusion of *A. baerii* is to be found in the great rusticity, in the adaptability to high density housing conditions, in the reduced oxygen demand already in the juvenile phase and in the quality of the derived products (caviar, meat and skin) (Gisbert and Williot, 2002).

In aquaculture, stress is defined as any external stimulus that changes the fish homeostasis and results in neuroendocrine, physiological and behavioural alterations (Eissa and Wang, 2016). Density is a key factor in determining the profitability and economic sustainability of a company (Rafatnezhad et al., 2008) and farmers often tend to increase it to favour production (Iguchi et al., 2003) causing, eventually, chronic stress to the animal (Ramsay et al., 2006). As in all farms, even in aquaculture, fish are exposed to continuous stress factors related to the rearing routine. Handling, transport, changes in water parameters (temperature, pH, salinity and oxygen) and high storage density are the most common stressors (Conte, 2004). The stress causes, as a general effect, an activation of the hypothalamic-pituitary-interrenal axis (HPI) with the production of corticosteroid hormones. Chronic stress, such as overcrowding, can induce a prolonged increase in plasma cortisol levels (Pickering and Pottinger, 1989) leading to harmful consequences (Barton and Iwama, 1991) such as the continuous release of glycocorticoids; these may have a marked immunodepressive action, negatively affecting the immune defences and the growth of the individuals and may also have repercussions on reproduction. Moreover, in fish as in mammals, cortisol leads to catabolic and antanabolic effects which, in turn, delay somatic growth (Ma et al., 2003). The response to various stressors is one of the main fish survival mechanisms and involves protein changes that include the increase in heat-shock protein synthesis, HSP (Iwama et al., 1998). HSPs are a family of highly conserved proteins used to prevent cell damage in the case of external (abiotic) or biotic factors. HSPs have been shown to have a relatively short half-life, but they present a long permanence in fish tissue cells (Morimoto and Santoro, 1998) demonstrating that they may play an important role in the long-term adaptation of fish to their habitats (Russotti et al., 1996). For example, Gornati et al. (2005) have shown an alteration in the gene expression of several genes and in particular an up-regulation of the *Hsp70* (heat-shock protein) gene in Seabass (*Dicentrarchus Labrax*). As already mentioned above, high density in fish farming induces stress that in turn elevates plasma cortisol and glucose levels. The resulting glucose is distributed in various tissues thanks to the Glucose transporter (GLUT) proteins in order to restore normal levels. The isoforms of GLUT proteins

have been categorized according to their sequence and their form in: class I (GLUT1-4); class II (GLUT5, 7, 9 and 11); class III (GLUT6, 8, 10, 12 and the myo-inositol transporter HMIT1) (Joost and Thorens, 2001; Joost et al., 2002; Wood et al., 2003). The proteins belonging to class I have been completely characterized in mammals and in recent years have also been identified in fish. In particular, the GLUT1 protein has been identified in tilapia, rainbow trout, *Oncorhynchus mykiss*, common carp. GLUT1, a transmembrane protein found in erythrocytes, is responsible for the import and export of glucose into red blood cells. However, knowledge of how chronic stress affects glucose and *glut*1 in fish under high-density conditions is not yet clear (Aketch et al., 2014). The effect of high density on the expression of the *glut1* gene has been studied in *Oreochromis niloticus* (L.) demonstrating an increase in plasma cortisol, glucose, erythrocyte count, and *glut1* levels that can be used as a cellular stress biomarker in aquaculture (Aketch et al., 2014). Regarding the *glut2* gene, coding for the sodium-independent glucose transporter, a study was conducted by Terova and collaborators (2009) in which the changes induced by acute and chronic hypoxia in the expression of *Glut2* mRNA in the liver are analysed using RT-PCR, demonstrating a significant increase in the number of *Glut2* mRNA copies in response to stress conditions.

Myogenesis, an event that characterizes the differentiation of a myogenic precursor into a skeletal muscle fibre, is a complex event that starts from the first weeks of embryonic development (Williams and Tsang, 1994). The whole locomotor apparatus originates from the somites, roundish masses of paraxial mesoderm, whose cells are multipotent. The miotome, belonging to the dorso-medial part of the somite, is a functional structure formed by mononuclear differentiated muscle cells called miocytes that differ first in myoblasts, and later, by fusion in myotubes, they evolve into mature muscle fibres (Salvatori et al., 1995). From this moment, the new muscle fibres are different in different body positions due to a process called stratified hyperplasia (Devoto et al, 1996). During the late segmentation and in the initial larval stages a secondary myotome originates: the mesenchymal cells of the dorsal fin, the fins muscles and the dermis (Stellabotte and Devoto, 2007). Muscle development in fish is a multi-stage process that combines two mechanisms: hyperplasia, which the formation of new fibres and hypertrophy, the increase in size of pre-existing muscle fibres. The balance between these two mechanisms defines the total mass and growth in fish (Rowlerson and Vegetti, 2001). Embryonic development in teleosts is profoundly influenced by the environmental conditions that determine the rate of myogenesis, the distribution of the number and size of muscle fibres and gene expression (Johnston, 2006). During the larval phases, the plasticity of fish muscles in

response to the environment is usually not reversible due to the rapid rate of ontogenetic changes in this developmental period. If the proliferative capacity of myogenic cells is compromised in these phases, this could compromise the growth potential of the larvae since both the maximum size and the growth rate are related to the number of muscle fibres in young fish (Weatherley et al., 1988).

There are several genes involved in muscle development and growth, such as the insulin growth factor gene complex (*igf*), the muscle regulatory factors (MRF*: myod, myf5, mrf4* and *myog*), the paired box protein (*pax7*) and myostatin (*mstn*) (De-Santis and Jerry, 2007). *Myog* and *mrf4* are activated during myoblast differentiation and cell fusion (Cornelison et al., 2000), while the *igf* system regulates myoblast proliferation and the balance between protein synthesis and muscle degradation (Seiliez et al. , 2011). In a study conducted by Salas-Leiton and collaborators (2010) the effect of the density and ratio of food of *S.senegalensis* was investigated through the quantification of the expression of the *igf1* and *igf2* genes, demonstrating a down-regulation.

Cultivating fish for food requires knowledge of white muscle ontogeny, functional anatomy and the role in swimming, within the limits of the ecological niche of each species. For this reason the objective of this study was to evaluate, through the monitoring of physiological parameters and the use of a molecular approach, muscle growth and stress in larvae of *A. baerii* reared in three different density conditions. This evaluation was performed by quantifying the expression of the Insuline-like growth factor 1 (*igf1*) and 2 (*igf2*) genes (involved in the growth process); *myog* (involved in myogenesis processes), Glucose transporter *glut1* and *glut2* (involved in the regulation of cellular stress); Heat-Shock Protein 70 (*hsp70*) (involved in the regulation of cellular stress) in the larval stages, until the complete absorption of the yolk sac. Genes relative expression was shown as ΔΔCt using the newly hatched larvae as a reference sample and the *Gapdh* and *Rpl6* genes as normalizing genes.

6.2. Materials and Methods

6.2.1. *Experimental set-up*

The fertilized eggs of *A. baerii*, provided by the sturgeon farm Società Agricola Naviglio (Mantova), were transported at 14°C in water saturated with oxygen, 24 hours after fertilization.

After a period of acclimatization, the fertilized eggs were incubated at 16°C and, at hatching, temperature was gradually increased to 19°C. After hatching, which took place 5 days after fertilization, larvae were randomly distributed in experimental rearing units (3 replicates per treatment), at three different densities: low density (LD; 30 larvae/litre), medium density (MD; 60 larvae/litre) and high density (HD; 120 larvae/litre), until the yolk-sac was fully absorbed. Chosen densities are representative of the protocols currently used in Siberian sturgeon production farms.

Water quality parameters as oxygen, temperature and pH were measured every day by Hach HQ 30d Portable Meter, (Hach Lange, Dussendolf, Germany); Water in all the groups was saturated with O_2 (> 8mg / l) and the pH value was within the range described for this species (Kamler, 2002). Eggs and larvae were exposed to an artificial photoperiod of 12L: 12D. Fish were reared in a recirculating aquaculture unit, composed by a sand filter, a biological submerged filter and a UV lamp sterilization unit. 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. For the whole trial it was not necessary to supply any kind of exogenous feeding, because larvae only used the nourishment of the yolk sac for their maintenance. Sampling time points consisted of important steps of Siberian sturgeon larvae development: hatching (T0), beginning of the schooling phase (T1) and complete yolk-sac absorption phase (T2). For each sampling time-point, larvae were picked up with a becker and killed by over-anaesthesia with Ethyl 3-Aminobenzoate, Methanesulfonic A (Sigma-Aldrich).

All procedures performed in studies involving animals were in accordance with the ethical standards of the OPBA committee of the University of Milan (OPBA_22_2017).

6.2.2. *Zootechnical performance*

Dead larvae were removed daily, and survival was estimated by dead larvae daily recording.

Sampled larvae were weighed and measured, in order to determine body weight (BW), and total Length (TL) respectively. The growth performance was described for at T1 and at T2, using the following parameters:

- Specific growth rate (SGR) = 100 · ((LnFBW-LnIBW)/Days;
- Condition factor (K) = FBW/Length^3 · 100

6.2.3. *Micro-anatomical analyses: immunofluorescence (actin)*

In order to follow muscle development, immediately after sampling, larvae underwent micro-anatomical analyses and were fixed in 4% (v/v) paraformaldehyde (N=3 samples for each nursery; N=9 samples per treatment). The samples were then dehydrated in a graded 50% (v/v), 70% (v/v), 95% (v/v) and 100% (v/v) ethanol series, embedded in paraffin and transversally cut into 4-µm-thick serial sections. After rehydration, sections were incubated with the first-step primary antiserum, 1:1000 anti-rabbit Skeletal Muscle Actin Antibody (ACTA1, LifeSpan BioSciences, Seattle, USA) for 48 hrs at 18–20°C, then washed in PBS, and subsequently washed in PBS for 10 min and incubated with a solution of 10 lg/ml goat biotinylated anti-rabbit IgG (Vector Laboratories Inc.) for 6 hrs at 18–20°C. The sections were then washed twice in PBS, and treated with Fluorescein–Avidin D (Vector Laboratories Inc.), 10 lg/ml in NaHCO3, 0.1 M, pH 8.5, 0.15 M NaCl for 1 hr at 18–20°C. Finally, slides with tissue sections were embedded in Vectashield Mounting Medium with DAPI (H-1200, Vector Laboratories Inc.) and observed using a Confocal Laser Scanning Microscope (FluoView FV300; Olympus). The immunofluororeactive structures were excited using Argon/ Helio–Neon–Green lasers with excitation and barrier filters set for fluorescein. Images containing superimposition of fluorescence were obtained by sequentially acquiring the image slice of each laser excitation or channel.

6.2.4. *Cortisol extraction and radioimmunoassay (RIA)*

Whole body cortisol analyses were performed in frozen larvae by a specific microtitre radioimmunoassay (RIA) as described by Simontacchi et al. (2009). Larvae were pooled (two larvae for each sample; N.12 samples per treatment) weighed, thawed out and pulverized in liquid nitrogen, and the resulting powders were suspended in 1 ml phosphate-buffered saline (PBS, pH 7.2). The suspension was then extracted with 8 ml of diethyl ether and the supernatant was evaporated to dryness. The dry extracts were dissolved in 0.5 ml of PBS and varying aliquots were used for radioimmunoassay (RIA). Briefly, a 96-well microtitre plate (Optiplate, Perkin Elmer Life Sciences) was coated with anti-rabbit c-globulin serum raised in a goat, incubating overnight the antiserum, diluted 1:1000 in 0.15 mM sodium acetate buffer, pH 9, at 4 °C. The plate was washed twice with PBS and incubated overnight at 4 °C with the specific antiserum solution. It was then carefully washed with PBS, standards, quality controls, unknown extracts and 3H tracers were added, and the plate was incubated overnight at 4 °C. Lastly, it was washed with PBS, added with 200 ll scintillation cocktail (Microscint 20, Perkin Elmer Life Sciences) and counted on a beta-counter (Top-Count, Perkin Elmer Life Sciences). The anti-cortisol serum

showed the following cross-reactions: cortisol 100%, prednisolone 44.3%, 11-deoxycortisol 13.9%, cortisone 4.95, corticosterone 3.5%, prednisone 2.7%, 17-hydroxyprogesterone 1.0%, 11-deoxycorticosterone 0.3%, dexamethasone 0.1%, progesterone < 0.01%, 17-hydroxypregnenolone < 0.01%, pregnenolone < 0.01%.

6.2.5. *Gene identification and primers design*

Genes involved in cellular stress reactions (*hsp70*, *Glut1* and *Glut2*) and genes involved in myogenesis (*myog*) and growth (*igf1* and *igf2*) were selected. The *rpl6* (coding for Ribosomal protein L6) and *gapdh* (coding for Glyceraldehyde 3-phosphate dehydrogenase) genes were used as reference.

Gene sequences from *Acipenser* spp. and some teleostean species were selected in order to perform alignments with the Basic Local Alignment Search Tool (NCBI BLAST), using a previously published assembled transcript of *A. baerii* as the reference database (Song et al, 2016). Specific primers were de novo designed for the target genes *glut1*, *glut2* and *igf2* and the related sequences, the annealing temperatures and the amplification size of each fragment are reported in Table 6.3. For information on *hsp70*, *myog*, *igf1*, *rpl6* and *gapdh* genes amplification see chapter 4.

Table 6.3 - *glut1*, *glut2* and *igf2* sequences, annealing temperatures and amplification size of each fragment

Genes	Primer Forward	Primer Reverse	Tm F	Tm R	Size (bp)
Igf2	GCTGAAACGCTATGTGGTG	GTGACCTTCGGATGTTTG	59	60	109
glut1	AGCCCATTCCTCCAACCTC	GAGTTTCGCCTCCCAAAGC	62	62	124
glut2	CTATCGTGGTGCCTTGGGA	GCCCCTGACAAGCCCAGAA	62	64	132

6.2.6. *RNA extraction and cDNA synthesis*

The sampling was performed at the beginning of T1 and at T2. Larvae were immediately stored at -80°C soon after the sampling procedure. Total RNA was extracted from each frozen larval sample (N=2 sample at T0; N=2 samples for each replicates; N=12 samples per treatment; N=38 samples in total) using RNeasy Mini Kit® (Qiagen), and eluted in a final volume of 40 µl of RNase-free water. A double treatment with DNase enzyme was performed, in order to remove any genomic DNA contamination, according to manufacturer instructions. Five hundred nanograms

of RNA was retro-transcribed to cDNA using Quantitect Reverse Transcription Kit® (Qiagen) following manufacturer protocol. An additional reaction without retrotranscriptase enzyme was performed to verify the complete DNA removal. cDNAs were stored at -80°C until subsequent use.

6.2.7. *Gene expression profiles*

The expression of genes coding for stress, myogenesis and growth factors were analysed by Quantitative RT-PCRs (RT-qPCR) in larvae collected at the three rearing densities.

cDNA samples were used as template in RT-qPCR using a CFX connect Real-Time PCR instrument (Bio-Rad, California, USA) and Universal SYBR® Green Supermix, (Bio-Rad, California, USA) as fluorescent molecule. The amplification conditions were: 150 nM (final concentration) of forward and reverse primers; 98°C for 30 s, 40 cycles of 98°C for 15 s, 58-62°C for 30 s; and a melting profile was included after the last amplification cycle. Annealing temperatures were defined according to primers melting temperatures indicated in Table 1.

Cycle threshold (Ct) values were determined for each gene and normalized according to the reference genes. The expression of each gene in T1 and T2 was compared to the calibrator sample T0. The relative expression values were calculated after a ΔΔCt-measure using *rpl6* and *gapdh* genes as references.

The amplified gene fragments were loaded on agarose gel, purified and sequenced in order to confirm the specificity of the amplification fragment

6.2.8. *Statistical analysis*

Statistical analysis of the data was performed using the 2-way ANOVA with densities and developmental stages as main factors of the SAS (version 8.1, Cary Inc., NC). Each rearing unit was considered as the individual value. The data were presented as least squared means ± SEM. Differences were considered significant at $P < 0.05$ and $P < 0.01$.

6.3. Results

6.3.1. *Water parameters*

In all treatments, the level of oxygen dissolved in the water was above the physiological value described for this species in this phase of development (the recommended value, according to the "Sturgeon hatchery manual" published by the United Nations Organization for food and agriculture (FAO, 2013), is of 6 mg/l). Values of pH were between 8.5 and 8.8 throughout the trial, which fall within the physiological range described for this species in this developmental phase. Temperature ranged from 17 to 19.7°C. Total ammonia and nitrites were below 0.05 mg/L throughout the trial. Some authors suggest that the negative influence of high stocking densities on growth is due to a deterioration of the water conditions (Kebus et al., 1992), in this study water quality parameters were deliberately maintained stable, thus eliminating the effect of water quality on the growth performance of the fish.

6.3.2. *Larval mortality, development and growth*

From T1 toT2, mortality significantly decreased (Table 6.1; P<0.01); within T1 the LD group showed lower mortality than both MD and HD groups (Table 6.1; P<0.05). At the end of the trial no differences were found between treatments (Table 6.1).

TABLE 6.1 – Mortality rate (%)

	LOW-DENSITY	MID-DENSITY	HIGH-DENSITY
T1	29.83% ± 1.66 [a]	36.50% ± 1.86 [b]	35.66% ± 2.35 [b]
T2	0.66% ± 0.35	2.0% ± 0.88	3.3% ± 2.3

Within a row, means without a common superscript letter differ significantly (P<0.05). Absence of superscript indicates no significant difference between treatments. Values are means ± Standard error

A. baerii larvae development from T0 to T2 was not affected by rearing density: schooling occurred at 5dph and the yolk-sac was fully absorbed at 8 dph, irrespective of the rearing density.

Data on growth are reported in Table III. Both body weight and total length of larvae reared at the lowest density was higher at the end of the trial (Table 6.2). The growth of larvae expressed as specific growth rate (SGR) was significantly improved at the yolk-sac absorption stage for

larvae reared at LD (P<0.05). The condition factor apparently was not significantly affected among fish by different densities at both T1 and T2. The interaction between developmental stage and density was not significant.

Table 6.2 - Growth performance of *A. baerii* larvae reared at different densities in the three timepoint (T0, T1 and T2)

		LOW-DENSITY	MID-DENSITY	HIGH-DENSITY
T0	**Body Weight mg**	12.65±1.56	12.65±1.56	12.65±1.56
	Total Length mm	10.5±0.17	10.5±0.17	10.5±0.17
	Condition Factor K	1.13±0.05	1.13±0.05	1.13±0.05
T1	**Body Weight mg**	24.29±0.92	24.08±0.98	23.80±0.95
	Total Length mm	15.35±0.10	15.07±0.18	15.33±0.20
	Condition Factor K	0.67±0.03	0.71±0.03	0.67±0.03
	Specific Growth Rate SGR	5.58±0.28	5.51±0.30	5.43 ±0.30
T2	**Body Weight mg**	33.01±0.87^{a}	30.31±0.74^{b}	30.15±0.87^{b}
	Total Length mm	17.73±0.13^{a}	16.96±0.13^{b}	17.11±0.16^{b}
	Condition Factor K	0.59±0.03	0.62±0.02	0.61±0.03
	Specific Growth Rate SGR	3.72±0.10 ^{a}	3.27±0.11 ^{b}	3.36±0.17 ^{b}

Within a row. means without a common superscript letter differ significantly (P<0.05). Absence of superscript indicates no significant difference between treatments. Values are means ± Standard error

6.3.3. *Micro-anatomical analyses: immunofluorescence (actin)*

ACTA1 immunofluorescence was detected in the cytoplasm of muscle cells (green staining) in all developmental stages (Figure 6.1). Moreover, no morphological differences among densities were detected regarding muscle structure at all stages: at hatching red and white muscle showed a monolayer, while at schooling and yolk-sac absorption the white muscle revealed the presence of both lamellar shaped fibres as well as polygonal cells (Figures 6.1 a, b and c respectively).

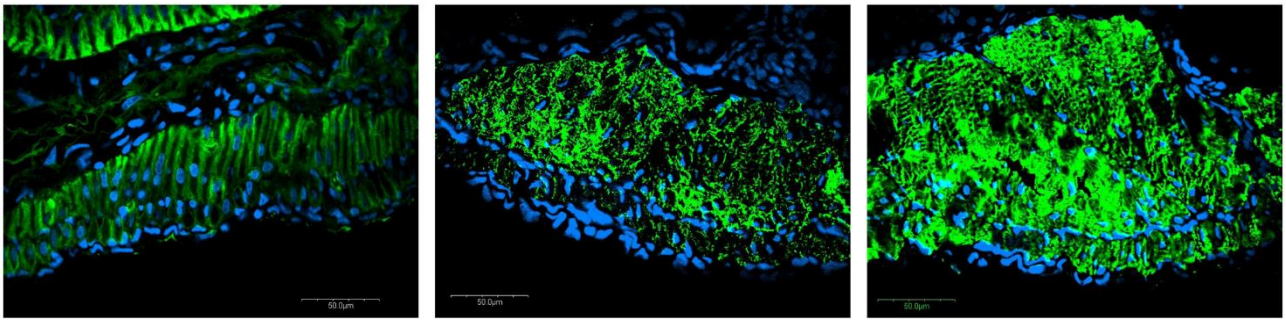

Figure 6.1. Representative images of the rearing densities at different timepoints – actin immunofluorescence localization: **a)** at hatching; **b)** at LD. schooling; **c)** at MD. yolk-sac absorption

6.3.4. *Whole body cortisol*

Regarding cortisol levels, no significant differences were found between stages of development (Figure 6.2). Both at T1 and at T2, no differences were found across densities and the interaction between developmental stage and density was not significant.

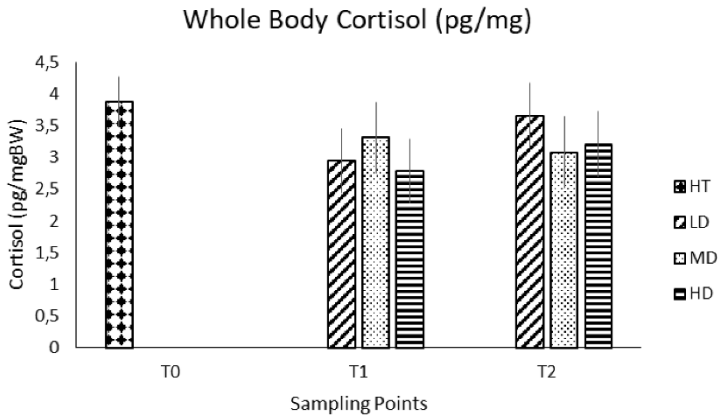

Figure 6.2. whole body cortisol concentrations expressed as pg per mg of body weight. Error bars indicate the standard error of the mean for each treatment/stage of development

6.3.5. *Stress and growth related gene expressions*

The expression of *Hsp70, myog, igf1, igf2, glut1 and glut 2* genes at T1 and T2 was related to T0 considered as reference sample and results were normalized versus *rpl6* and *gapdh* considered as reference genes as described in Material and methods and are shown in Figure 6.3.

The results show an up-regulation of all the genes analysed both in T1 and/or T2, except that for the *myog* gene which is instead down-regulated. Within the T1 the *myog* gene shows a significant difference in expression across the three different rearing conditions, with a greater down-regulation in correspondence with the LD and MD conditions respect to HD ($P < 0.05$). In T2, the

evolution of gene expression levels is reversed, since *myog* is more down-regulated in the HD condition. Regarding the genes involved in the growth process (*igf1* and *igf2*), an up-regulation of gene expression levels in both sampling timepoints was observed for both genes. However, no statistically significant differences in the expression levels was observed across treatments.

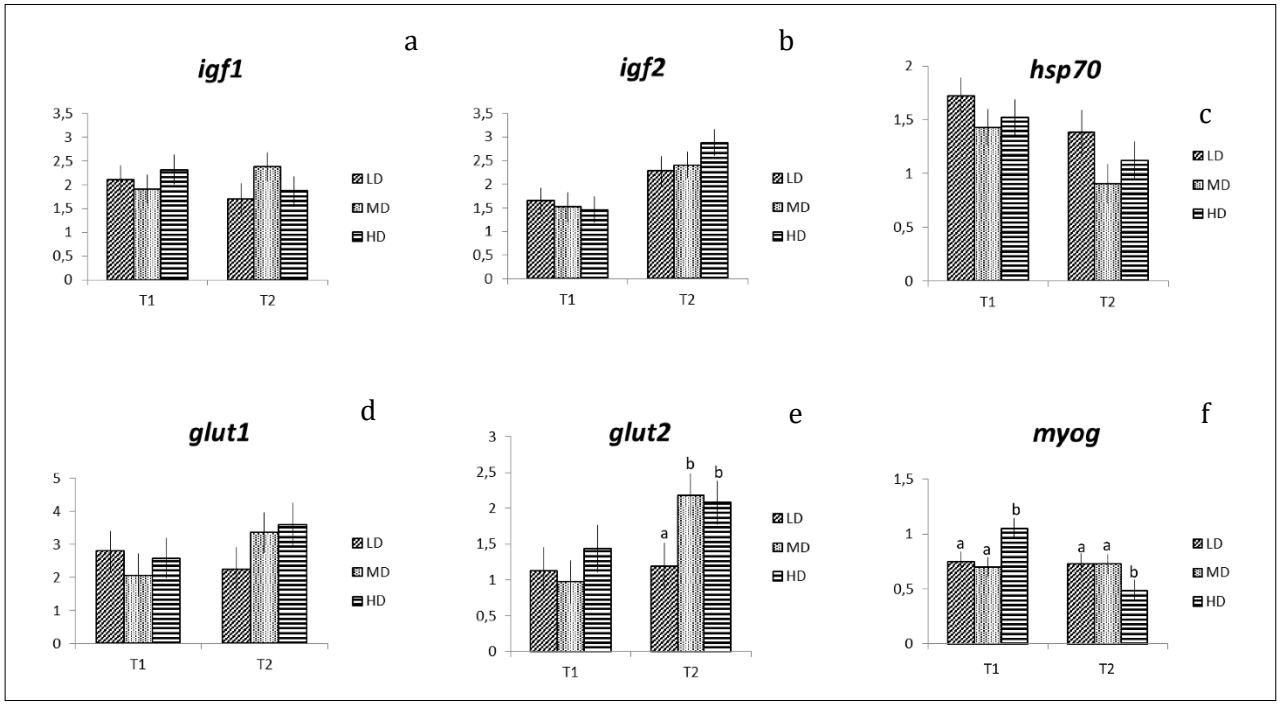

Figure 6.3. relative gene expression of: a) *igf*1; b) *igf2;* c) *Hsp*70; d) *glut1;* e) *glut2* and f) *Myog;* lowercase letters indicate significant differences of at least P < 0.05

Genes involved in regulating cell stress (*glut1*, *glut2* and *Hsp70*) also showed up-regulation in all treatments except for *glut2* gene in T1 were the expression is similar to T0 phase. The *glut1* gene reported no significant differences between the different rearing densities at both sampling points. The *glut2* gene did not show a statistically significant difference in expression between treatment during T1; however, at the end of the experiment, it showed a significant up-regulation in larvae subjected to MD and HD conditions. when compared to larvae reared in LD (P <0.05).

The *Hsp70* gene also showed no statistically significant differences between gene expression levels across the three rearing densities, showing an up-regulation in all densities at T1.

6.4. Discussion

In teleost bony fish, the environment is crucial for several factors including the rate of myogenesis, the composition of the subcellular organelles, the patterns of gene expression and the number and size of muscle fibres (Johnston, 2006). Many studies indicate that high rearing density increases stress and, at the same time, induces the inhibition of density-dependent growth in fish (Barton 2002; Barton and Iwama 1991; Wedemeyer et al., 1990). To date, it has been shown that high rearing density is an environmental stress factor for sturgeons (Wuertz et al., 2006).

Understanding the mechanisms that underlie the development and growth of muscle and the state of stress are, therefore, essential in order to identify optimal strategies for rearing and conserve *A. baerii*.

For this purpose, in this study the effect of different rearing densities in early larval stages of *A. baerii* was analysed, in terms of early muscular development and stress condition through the evaluation of physiological parameters and the use of molecular methods.

From the results obtained, it can be stated that the growth performances have not been influenced by the deterioration of the water conditions, as the main water parameters (dissolved O_2. pH and temperature) remained stable throughout the trial. Some authors report that sturgeons are quite sensitive to high rearing densities. Jodun et al (2002) observed that an increased rearing density in the Atlantic sturgeon resulted in a weight reduction; similar results have been obtained by Rafatnezhad et al. (2008) in juveniles of beluga, Huso huso (L.). On the contrary. Fajfer et al. (1999) reported that high rearing densities had no impact on the final weight of *Acipenser fulvescens*. In this study, indeed, larvae reared at a low stocking density presented a significantly higher body weight and length than those reared at medium and high densities. Mortality was significantly lower in larvae reared at a low density at the schooling stage, whereas no differences were observed at the end of the trial. Moreover, in our study also the stress response has not been influenced by high or low stocking densities. Actually, we know that stocking density can cause stress and decrease the ability of the fish to react to it. Although this is usually noted when stocking density is too high (Caipang et al., 2008), sometimes this occurs when stocking densities are too low (Szczepkowski et al., 2011)

Stocking density can also impact the muscle development. For this reason, we evaluated the skeletal muscle morphology at all stages in all experimental group. Actin is a cytoskeletal protein which exerts a broad range of functions in almost all eukaryotic cells and examples for actin

functions include maintenance of the cytoskeleton, cell motility and muscle contraction. Quite recently, Bartolo et al. (2008) revealed that ACTA1 was expressed in skeletal muscle of fish. In our study ACTA1 immunofluorescence was used to follow morphological development of the skeletal muscle. Considering different stages, it can be noticed that already at the time of hatching it was possible to observe the difference between slow / red fibres and fast / white fibres in all the treatments. The red fibres appeared as a cell monolayer from the time of hatching until the whole sac was absorbed. In contrast, the white fibres showed expansion from hatching up to the absorption of the yolk sac. In agreement with Steinbacher et al. (2006) muscle development appeared therefore regular for all studied rearing densities.

Analysis of the expression patterns of some genes in *A. baerii* showed some statistically significant differences in the three different density conditions analysed. In particular, the *myog* gene involved in the differentiation of muscle cell precursors, is down-regulated with respect to T0 in all the density conditions at both timepoints. In detail, it has been observed that this down-regulation is significantly greater in the HT when the yolk-sac is fully absorbed. This result is in agreement with what was observed regarding larvae' body weight: in fact, at the end of the trial, larvae subjected to HT were lighter than those subjected to LD. The *glut2* gene, involved in the regulation of cellular stress, showed an up-regulation at MD and HD for T2. This up-regulation showed statistically significant differences between the different densities, exclusively in the phase of complete yolk-sac absorption, with significantly higher up-regulation values in the MD and HD. This result confirms what was observed in a study conducted by Terova and collaborators (2009) on Sea Bass (*Dicentrarchus labrax*), in which it was shown an increase in the expression levels of the *glut2* gene under stress conditions. For a better interpretation of the influence exerted by the rearing density on the stress and muscle growth of *A. baerii*, it would seem necessary to evaluate the expression levels of other genes involved in the same regulations.

To conclude, taking into account the SGR and the gene expression results, we suggest that lower densities are used in these stages of development, as these showed a higher growth potential and lower stress levels.

6.5. References

Aketch, B., Ang'ienda, P., Radull, J. & Waindi, E. (2014). Effect of stocking density on the expression of glucose transporter protein 1 and other physiological factors in the Lake Victoria

Nile tilapia. *Oreochromis niloticus* (L.). *International Aquatic Research*, **6**, 69. https://doi.org/10.1007/s40071-014-0069-3

Barton; B.A. (2002) Stress in Fishes: A Diversity of Responses with Particular Reference to Changes in Circulating Corticosteroids. *Integrative and Comparative Biology*, **42**(3), 517–525. https://doi.org/10.1093/icb/42.3.517

Barton, B.A. & Iwama, G.K. (1991). Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Annual Review of Fish Diseases*, **1**, 3–26. https://doi.org/10.1016/0959-8030(91)90019-G

Caipang, C.M.A., Brinchmann, M.F., Berg, I., Iversen, M., Eliassen, R. & Kiron, V. (2008). Changes in selected stress and immune–related genes in Atlantic cod, *Gadus morhua*, following overcrowding. *Aquaculture Research,* **39**(14), 1533-1540. https://doi.org/10.1111/j.1365-2109.2008.02026.x

CITES (Convention on International Trade in Endangered Species of Flora and Fauna). (2014). *Acipenser baerii* Brandt. 1869. Doc. AC16.7.2 Annex. http://www.cites.org/eng/com/ac/16/16-7-2a1.pdf

Conte, F. S. (2004). Stress and the welfare of cultured fish*. Applied Animal Behaviour Science*, **86**(3-4), 205-223. https://doi.org/10.1016/j.applanim.2004.02.003

Cornelison, D.D.W., Olwin, B.B., Rudnicki, M.A. & Wold, B.J. (2000). MyoD (-/-) satellite cells in single fiber culture are differentiation defective and MRF4 deficient. *Developmental Biolology,* **224**(2), 122-137. https://doi.org/10.1006/dbio.2000.9682

De-Santis, C. & Jerry, D.R. (2007). Candidate growth genes in finfish — where should we be looking? *Aquaculture*, **272**(1-4), 22-38. https://doi.org/10.1016/j.aquaculture.2007.08.036

Devoto. S.H.. Melançon. E.. Eisen. J.S. and Westerfield. M. (1996). Identification of separate slow and fast muscle precursor cells in vivo. prior to somite formation. *Development,* **122**(11), 3371-3380. http://dev.biologists.org/content/122/11/3371.abstract

Eissa, N. & Wang, H.W. (2016). Transcriptional stress responses to environmental and husbandry stressors in aquaculture species. *Reviews in Aquaculture*, **8**(1), 61–88. https://doi.org/10.1111/raq.12081

Fajfer, S., Meyers, L., Willman, G., Carpenter, T. & Hansen, M. J. (1999). Growth of Juvenile Lake Sturgeon Reared in Tanks at Three Densities. *North American Journal of Aquaculture*, **61**(4), 331-335. https://doi.org/10.1577/1548-8454(1999)061<0331:GOJLSR>2.0.CO;2

FAO (Food and Agriculture Organization of the United Nations). (2013). Fisheries and Aquaculture Department - Species Fact Sheets. Website: www.fao.org.

Gisbert, E. & Williot, P. (2002). Duration of synchronous egg cleavage cycles at different temperatures in Siberian sturgeon (*Acipenser baerii*). *Journal of Applied Ichthyology*, 18:271–274.

Gornati, R., Papis, E., Rimoldi, S., Chini, V., Terova, G., Prati, M., Saroglia, M. & Bernardini, G. (2005). Molecular markers for animal biotechnology: sea bass (*Dicentrarchus labrax. L.)* HMG-CoA reductase mRNA*. Gene*, **344**, 299-305. https://doi.org/10.1016/j.gene.2004.11.007

Iguchi, K., Ogawa, K., Nagae, M. & Ito, F. (2003). The influence of rearing density on stress response and disease susceptibility of ayu (*Plecoglossus altivelis). Aquaculture,* **220**(1-4), 515–523. https://doi.org/10.1016/S0044-8486(02)00626-9

IUCN (International Union for Conservation of Nature and Natural Resources) (2013). The IUCN Red List of threatened species. *Website: www.iucnredlist.org*.

Iwama. G.K. (1998). Stress in fish. *Annals of the New York Academy of Sciences*, **851**(1), 304-310. https://doi.org/10.1111/j.1749-6632.1998.tb09005.x

Jodun, W.A., Millard, M.J. & Mohler, J.W. (2002). The effect of rearing density on growth. survival. and feed conversion of juvenile Atlantic sturgeon. *North American Journal of Aquaculture,* 64(1), 10-15. https://doi.org/10.1577/1548-8454(2002)064<0010:TEORDO>2.0.CO;2

Johnston, I.A. (2006). Environment and plasticity of myogenesis in teleost fish. *Journal of Experimental Biology*, **209**(12), 2249-2264. http://jeb.biologists.org/cgi/doi/10.1242/jeb.02153

Joost, H.G. & Thorens, B. (2001). The extended GLUT-family of sugar/polyol transport facilitators: nomenclature. sequence characteristics. and potential function of its novel members (review). *Molecular Membrane Biology*, **18**(4), 247–256. https://doi.org/10.1080/09687680110090456

Joost, H.G., Bell, G.I., Best, J.D., Birnbaum, M.J., Charron, M.J., Chen, Y.T., Doege, H., James, D.E., Lodish, H.F., Moley, K.H., Moley, J.F., Mueckler, M., Rogers, S., Schurman, A., Seino, S. & Thorens,

B. (2002). Nomenclature of the GLUT/SLC2A family of sugar/polyol transport facilitators. *American Journal of Physiology – Endocrinology and Metabolism*, **282**(4), 974-976. https://doi.org/10.1152/ajpendo.00407.2001

Kamler, E. (2002). Ontogeny of yolk-feeding fish: an ecological perspective. *Reviews in Fish Biology and Fisheries*, **12**(1), 79-103. https://doi.org/10.1023/A:1022603204337

Kebus, M., Collins, M., Brownfield, M., Amundson, C., Kayes, T. & Malison, J. (1992). Effects of rearing density on the stress response and growth of rainbow trout. *Journal Aquatic Animal Health*, 4(1), 1-6. https://doi.org/10.1577/1548-8667(1992)004<0001:EORDOT>2.3.CO;2

Kottelat, M. & Freyhof, J. (2007). In: Kottelat (ed), *Handbook of European freshwater fishes*. Berlin: 646 pp.

Ma, K., Mallidis, C., Bhasin, S., Mahabadi, V., Artaza, J., Cadavid, N.G., Arias, J. & Salehian, B. (2003). Glucocorticoid-induced skeletal muscle atrophy is associated with upregulation of myostatin gene expression. American Journal of Physiology, Endocrinology and Metabolism, **285**(2), E363-E371. https://doi.org/10.1152/ajpendo.00487.2002

Morimoto, R.I. & Santoro, M.G. (1998). Stress–inducible responses and heat shock proteins: new pharmacologic targets for cytoprotection. *Nature biotechnology*, **16**, 833-838. https://doi.org/10.1038/nbt0998-833

Pickering, A. & Pottinger, T. (1989). Stress responses and disease resistance in salmonid fish: effects of chronic elevation of plasma cortisol. Fish Physiology and Biochemistry 7(1-6), 253-258. https://doi.org/10.1007/BF00004714

Rafatnezhad, S., Falahatkar, B. & Tolouei Gilani, M.H. (2008). Effects of stocking density on haematological parameters. growth and fin erosion of great sturgeon (*Huso huso*) juveniles. *Aquaculture Research*, **39**(14), 1506-1513. https://doi.org/10.1111/j.1365-2109.2008.02020.x

Ramsay, J.M., Feist, G.W., Varga, Z.M., Westerfield, M., Kent, M.L. & Schreck, C.B. (2006). Whole-body cortisol is an indicator of crowding stress in adult zebrafish, *Danio rerio. Aquaculture*, **258**(1-4), 565–574. https://doi.org/10.1016/j.aquaculture.2006.04.020

Rowlerson, A. & Vegetti, A. (2001). Cellular mechanism of post-embryonic muscle growth in aquaculture species. In: Johnston, I.A., ed. *Muscle development and growth,* pp103-140. Academic Press. London.

Russotti, G., Brieva, T.A., Toner, M. & Yarmush, M.L. (1996). Induction of tolerance to hypothermia by previous heat shock using human fibroblasts in culture. *Cryobiology,*. **33**(5), 567-580. https://doi.org/10.1006/cryo.1996.0060

Salas-Leiton, E., Anguis, V., Martın-Antonio, B., Crespo, D., Planas, J.V., Infante, C., Cañavate, J.P. & Manchado, M. (2010). Effects of stocking density and feed ration on growth and gene expression in the Senegalese sole (*Solea senegalensis*): potential effects on the immune response. *Fish and Shellfish Immunology*, **28**(2), 296–302. https://doi.org/10.1016/j.fsi.2009.11.006

Salvatori, G., Lattanzi, L., Coletta, M., Aguanno, S., Vivarelli, E., Kelly, R., Ferrari, G., Harris, A.J., Mavilio, F. & Molinaro, M. (1995). Myogenic conversion of mammalian fibroblasts induced by differentiating muscle cells. *Journal of Cell Science*, **108**(8), 2733-2739. http://jcs.biologists.org/content/108/8/2733.abstract

Seiliez, I.. Panserat. S.. Lansard. M.. Polakof. S.. Plagnes-Juan. E.. Surget. A.. Dias K.. Larquier M.. Kaushik S. and Skiba-Cassy. S. (2011). Dietary carbohydrate-to-protein ratio affects TOR signalling and metabolism-related gene expression in the liver and muscle of rainbow trout after a single meal. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology*, **300**(3), R733-R743. https://doi.org/10.1152/ajpregu.00579.2010

Simontacchi, C., Negrato, E., Pazzaglia, M., Bertotto, D., Poltronieri, C. & Radaelli, G. (2009). Whole-body concentrations of cortisol and sex steroids in white sturgeon (*Acipenser transmontanus*. Richardson 1836) during early development and stress response. *Aquaculture International*, **17**(1), 7-14. http://dx.doi.org/10.1007/s10499-008-9174-x

Steinbacher, P., Haslett, J.R., Sänger, A.M. and Stoiber, W. (2006). Evolution of myogenesis in fish: a sturgeon view of the mechanisms of muscle development. *Anatomy and Embryology*, **211**(4), 311–322. https://doi.org/10.1007/s00429-006-0082-4

Stellabotte, F. & Devoto, S.H. (2007) The teleost dermomyotome. *Developmental Dynamics,* **236**(9), 2432–2443. https://doi.org/10.1002/dvdy.21253

Song, W., Jiang, K., Zhang, F., Lin, Y. & Ma, L. (2016). RNA-sequencing of the sturgeon *Acipenser baeri* provides insights into expression dynamics of morphogenic differentiation and developmental regulatory genes in early versus late developmental stages. *BMC genomics*, **17**, 564. https://doi.org/10.1186/s12864-016-2839-3

Szczepkowski, M., Szczepkowska, B. & Piotrowska, I. (2011). Impact of higher stocking density of juvenile Atlantic sturgeon. *Acipenser oxyrinchus* Mitchill. on fish growth. oxygen consumption. and ammonia excretion. *Archives of Polish Fisheries,* **19**, 59-67. doi 10.2478/v10086-011-0007-6

Terova, G., Gornati, R., Rimoldi, S., Bernardini, G. & Saroglia, M. (2009). Quantification of a glucocorticoid receptor in sea bass (*Dicentrarchus labrax. L.*) reared at high stocking density. *Gene,* **357**(2), 144-151. https://doi.org/10.1016/j.gene.2005.06.016

Weatherley, A.H.; Gill, H.S.; Lobo, A.F. (1988). Recruitment and maximal diameter of axial muscle fibres in teleosts and their relationship to somatic growth and ultimate size. *Journal of Fish Biology,* **33**(6), 851-859. https://doi.org/10.1111/j.1095-8649.1988.tb05532.x

Wedemeyer, G.A., Barton, B.A. & McLeay, D.J. (1990). Stress and acclimation. In: Schreck, C.B. & Moyle, P.B. (eds), *Methods for fish biology,* pp451–489. American Fisheries Society. http://pubs.er.usgs.gov/publication/70180779

Wuertz, S., Lutz, I., Gessner, J., Loeschau, P., Hogans, B., Kirschbaum, F.& Kloas, W. (2006). The influence of rearing density as environmental stressor on cortisol response of Shortnose sturgeon (Acipenser brevirostrum). *Journal of Applied Ichthyology*, **22**(1), 269-267. https://doi.org/10.1111/j.1439-0426.2007.00966.x

Williams, B.A. & Tsang, A. (1994). Analysis of multiple classes of abscisic acid responsive genes during embryogenesis in *Zea mays. Developmental Genetics*, **15**(5), 415–424. https://doi.org/10.1002/dvg.1020150504

Wood, I.S. & Trayhurn, P. (2003). Glucose transporters (GLUT and SGLUT): expanded families of sugar transport proteins*. The British Journal of Nutrition,* **89**(1), 3-9. https://doi.org/10.1079/BJN2002763

Chapter 7

Environmental enrichment for the early larval stages of *Acipenser baerii* in captive environments: impact on behaviour, growth and muscle development

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7. Environmental enrichment for the early larval stages of *Acipenser baerii* in captive environments: impact on behaviour, growth and muscle development

Abstract

The morpho-functional and behavioural responses of early life phases in Siberian sturgeon (*Acipenser baerii*) towards two types of substrate (Bioballs type 1: ⌀35mm; Bioballs type 2: ⌀38mm BB1 and BB2 respectively) vs. no substrate (CTR), were examined from hatching and during the endogenous feeding period, in two timepoints: schooling (T1) and full yolk-sac absorption (T2). Until 3dph the number of larvae swimming in the water column was significantly higher in group CTR. From 4-5dph the number of larvae swimming in the water column was significantly higher in the BB2 group. The number of swimming larvae from group BB1 was in between the other two groups throughout the trial. At T1 and at T2, larvae reared with in BB1 showed a significantly higher weight and higher total length than larvae reared in either CTR or BB2 ($P<0.05$). Histometrical analyses were also performed in order to evaluate muscle development: at the end of the trial, total muscle area, slow muscle area and fast muscle area were significantly higher for larvae reared in BB1 ($P<0.05$). It would seem more favourable to provide a substrate rather than a bare bottom, for Siberian sturgeon in these early phases of development, in particular a substrate with characteristics similar to those of BB1.

Key words: *Acipenser baerii*, muscle development, swimming behaviour, histometry

7.1. Introduction

The Siberian sturgeon, *Acipenser baerii* Brandt 1869 is the most widely farmed sturgeon species. This species is farmed mainly in China, Russia federation, France, Spain and Italy (Bronzi and Rosenthal, 2014). Nowadays, Siberian sturgeon is a threatened species (Birstein 1993) and it is included in the IUCN Red Data List. Taking into account the continuous decrease of its natural populations, due to the reduction of spawning spots, overfishing and water pollution (Ruban 1997, 1999), it is necessary to gather as much information as possible in order to properly manage the natural populations of this species and to enhance farming practises.

During Siberian sturgeon ontogenesis there are several behavioural changes regarding rheotaxis, phototaxis, and swimming ability, which are the result of both extrinsic (e.g. water

temperature, current intensity, substrate typology, predation, food resources) and intrinsic (morpho-physiological development) parameters. These ontogenic changes in behaviour may have an influence on how the different life stages forms (free embryo, larva, and juvenile) use different river habitats, which may affect this species survival, distribution and recruitment. The main factors affecting Siberian sturgeon's free embryo and larvae seem to be the intensity of the rivers current, the type of substrate and the food resources, while for juveniles and adults, food availability plays a major role in its distribution.

Until 3 days post hatch (dph), free embryos show positive phototaxis, the preference for white substrates, vertical swimming and drift behaviour: they move upward by active movements and then, in a passive way, settle to the bottom (Gisbert et al. 1999). This swimming passive behaviour allows free embryos to save yolk-sac energy that may be allocated, instead, for the development of the sensorial, locomotor and feeding systems before the yolk-sac in completely absorbed. At 4 dph, pectoral fins have developed as well as several sensorial structures in the head and trunk and the eyes appear differentiated; consequently, at this point, free embryos' behaviour shift from pelagic to benthic (Gisbert et al., 1999). At 5-6 dph free embryos are effective benthic swimmers and show a positive rheotactic response. At 7-8 dph free embryos show schooling behaviour and look for covered habitats preferably with dark bottoms (Gisbert et al. 1999). Just before the beginning of the exogenous feeding, there is a proliferation of the taste buds on barbels and the development of extraoral taste, which allows free embryos to sense feed chemical stimuli (Devitsina and Kazhlayev 1993).

Most behavioural observations in fish have been performed in the absence of an adequate substrate and, therefore, may not reflect the behaviour of larvae in their natural environment (McAdam, 2011). To date, the habitat used by early larval stages of this species has not been described yet and, taking into account the sensitivity of larval fish to environmental conditions (Barton and Iwama, 1991), it seems important to evaluate the potential contribution of environmental enrichment on growth and muscle development.

Gravel substrates are known to be essential to the life histories of many riverine fish species, particularly in the early life stages. The main influence of rough substrate is on behaviour, as it allows young fish to satisfy their 'innate righting response' (Barns, 1969; Barns and Simpson, 1976), and remaining immobile. Indeed, in several studies performed with several fish species, there was an evident effect of the substrate presence. Peterson and Martin-Robichau (1995) tested the effect of substrate at different temperatures and found that gross yolk utilization

efficiency was higher for salmon alevins reared on substrate regardless of the temperature. A positive effect of the presence of a substrate in early life stages was also found in American Atlantic sturgeon (*Acipenser oxyrinchus*) (Gessner et al., 2009), white sturgeon, *Acipenser transmontanus* (Baker et al., 2014; Bates et al., 2014) and in lake sturgeon, *Acipenser fulvescens* (Zubair et al 2012).

Little information is available on habitat preferences of the free embryos of *Acipenser baerii* upon hatch. Therefore, the aim of this study was to assess the environment enrichment that allows good rearing conditions without compromising larval morpho-functional aspects. Specifically, we quantified the effects of two substrate conditions on free-embryo's weight, total length, survival, muscle development through the use of histometrical analyses, of Siberian sturgeon until the complete yolk-sac absorption.

7.2. Material and Methods

7.2.1. *Experimental set-up*

The experiment was held during April/May 2018 at the Experimental Animal Research and Application Centre of Lodi, of the University of Milan. Siberian sturgeon fertilized eggs were transported 24 hours after fertilization from the "Società Agricola Naviglio" fish farm to the experimental unit. 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, larvae were randomly transferred to the experimental units (three replicates per treatment) until the yolk-sac was completely absorbed. The bottom of the experimental units was covered by two different rearing substrates: Bioballs type 1 (Figure 7.1a.; BB1; ⌀35mm; specific surface area: 395.64 m^2/m^3) and Bioballs type 2 (Figure 7.1b.; BB2; ⌀38mm; specific surface area: 410,36 m^2/m^3). A group reared without additional substrate served as a control (CTR). Rearing density was of 130 larvae /1.6-L tank corresponding to 612 individuals/m^2 : also in this case fish density was chosen on the basis of a previous experimental trial (Unpublished data, already presented in this thesis). Fish were reared in a recirculating aquaculture unit, composed by a sand filter, a biological submerged filter and a UV lamp sterilization unit. Every substrate condition 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 permanently close to the

saturation value and pH values were within the range described for this species in this stage of development (Kamler, 2002). Mortality was estimated by dead larvae daily counting and 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. This study was approved by the Ethic Committee of the University of Milan (OPBA_15_2018).

Figure 7.1. a) Bioballs type 1 (BB1); b) Bioballs type 2 (BB2)

7.2.2. *Larval sampling*

Sampling time points were chosen according to important steps of Siberian sturgeon larvae development: hatching (T0), beginning of the schooling phase (T1) and complete yolk sac absorption phase (T2).

Behaviour of the free embryos was assessed by daily counting of the larvae swimming in the water column and therefore, not hiding in the substrate, using image analyses according to Gessner et al. (2009). Relative ratios of substrate use were calculated based upon these counts in relation to the total number of the free embryos in the rearing units.

To determine growth, 18 free embryos were sampled per treatment and were weighed (BW) and measured for total length (TL) at each sampling point.

For histological analyses, for each sampling time point 6 larvae per replicate were collected (total n=18 larvae per treatment) with a becker and killed by over-anaesthesia with Ethyl 3-Aminobenzoate, Methanesulfonic A (Sigma-Aldrich, Milan, Italy).

7.2.3. *Muscle Histometry*

After sampling, whole larvae were immediately fixed in fresh 4% para-formaldehyde in 0.01M phosphate-buffered saline (PBS) pH 7.4 for 24h at 4°C, then dehydrated in a graded series of ethanol, cleared with xylene and embedded in paraffin. Serial transverse microtome sections (5 µm-thick) were obtained from each sample.

The sequential Haematoxylin/eosin (HE) stain was performed for the evaluation of the structural aspects of the developing lateral muscle tissues and for histometry.

Briefly, the histometric variables were measured on HE-stained sections of transversal body sections of individual fish larvae, at a peri-anal localization with the transverse sections conducted in a cranio-caudal direction with the first section rostral to the anus. Standard histometrical techniques were applied using an Olympus BX51 light microscope equipped with a DP-software program (Cell^B, Basic Imaging Software, Olympus, Italy) for describing: i) total muscle cross-sectional area (TMA), ii) red muscle area (slow muscle cross-sectional area, SMA), and iii) white muscle area (fast muscle cross-sectional area, FMA), iv) lamellae fibres area (LFA) and v) polygonal fibres area (PFA) at the three analysed developmental stages: hatching (T0), schooling (T1) and yolk-sac full absorption (T2).

7.2.4. *Statistical analysis*

Statistical analysis was performed with SAS statistical software (version 9.3, Cary Inc., NC). Data from the histometrical analyses (TMA, SMA, FMA) were analysed using 2-way ANOVA with substrates (CTR, BB1 and BB2) and developmental stages (T0, T1 and T2) as main factors; moreover LFA and PFA were also co-variated for the area corresponding to the FMA. The data are presented as least-square means (SEM). Differences between means were considered significant at $P < 0.05$.

7.3. Results

7.3.1. *Water parameters*

Water parameters are reported in Figure 7.2. O_2 was constantly close to the saturation value (Figure 7.2a); temperature ranged from 16.9 to 18.6°C (Figure 7.2b); pH values were within the physiological range described for this species (Figure 7.2c).

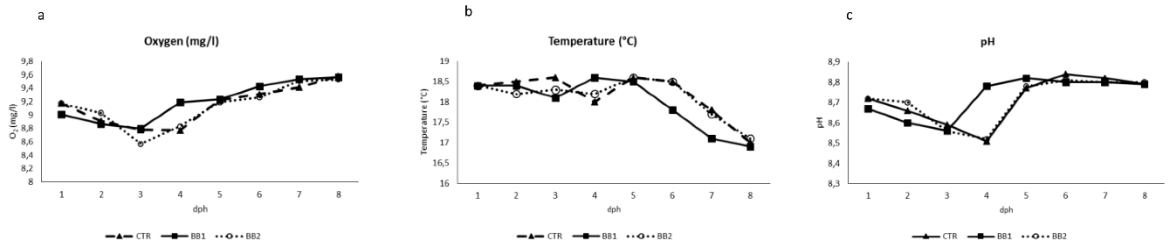

Figure 7.2. Water parameters from T0 until the end of the trial for each environmental enrichment condition: a) dissolved oxygen (mg/l); b) temperature (°C); c) pH.

7.3.2. *Development, behaviour, survival and growth*

The duration of the endogenous feeding phase was of 8 days post-hatch (dph) and schooling behaviour took place at 5 dph across treatments, irrespective of the treatment.

Once released in the units without any substrate (CTR group), newly-hatched larvae exhibited swim-up and drift behaviour, swimming upwards in the water column and falling down to the bottom, in a continuous movement. Larvae released in the BB1 or BB2 units showed the same behaviour as the CTR group but only for a limited amount of time, until the substrate was encountered. At this point, most of the larvae from these groups remained in the bottom, mainly the ones from group BB1. Few larvae from groups BB1 and BB2 showed schooling behaviour while larvae from the CTR group all swam in schools at 4-5 dph. After schooling, larvae from the CTR group remained mostly in the bottom until the yolk-sac was fully absorbed. On the opposite, larvae from groups with a substrate (BB1 or BB2) kept on swimming in the water column from day 4-5 post-hatch until the yolk-sac was fully absorbed. Figure 7.3 shows the records of the larvae that swam in the water column from hatch until the end of the trial. Until 3dph the number of larvae swimming in the water column was significantly higher in group CTR. From day 4-5 post-hatch the situation started to invert: the number of larvae swimming in the water column

was significantly higher in the BB2 group. The number of swimming larvae from group BB1 was in between the other two groups throughout the trial.

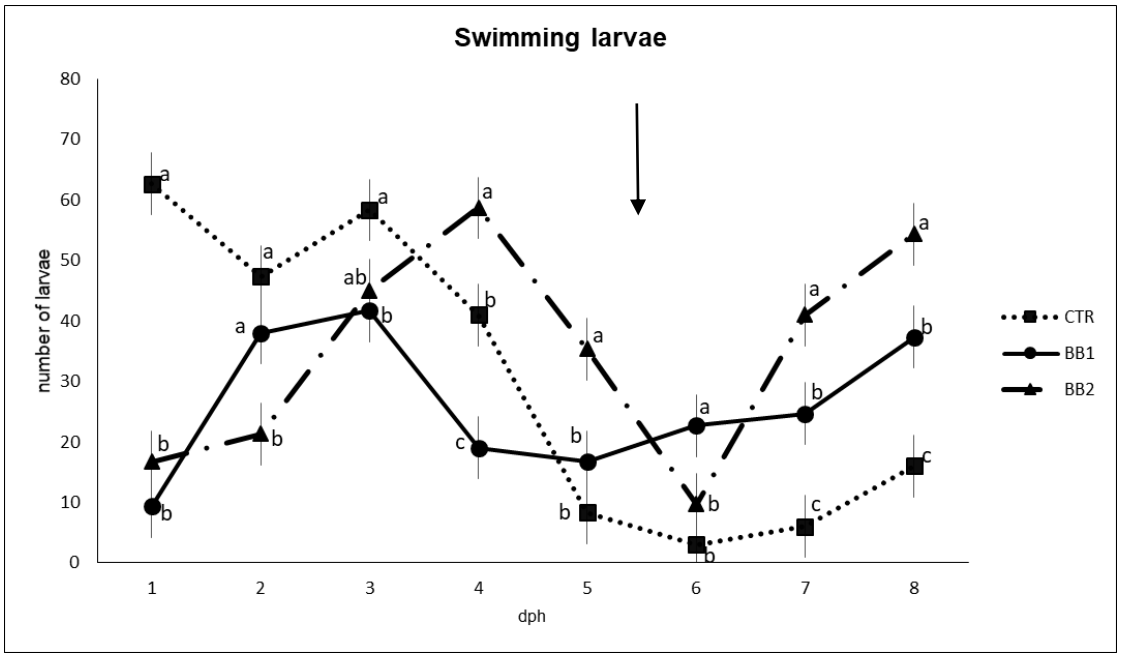

Figure 7.3. Number of larvae of *A. baerii* swimming in the water column for each treatment, from hatching until the full yolk-sac absorption. Error bars indicate the standard error of the mean for each treatment/stage of development; [a,b]Means with different superscripts differ significantly between treatments (P< 0.05); arrow indicating schooling phase.

There were no significant differences regarding survival rate between treatments (data non shown).

Body weight significantly increased from one stage of development to the other, irrespective of the treatment (Figure 7.4a; P<0.001). At T1 larvae reared with substrate BB1 showed a significantly higher weight than larvae subjected to the other two treatments and this difference became bigger in T2 (Figure 7.4a; P<0.05).

Total length (TL) significantly increased from one stage of development to the other, irrespective of the treatment (Figure 7.4b; P<0.001). Both at T1 and T2 larvae reared in the substrate BB1 were longer than larvae from groups CTR and BB2 (Figure 7.4b; P<0.05). The interaction between developmental stages and substrate was not significant nor for body weight nor for total length.

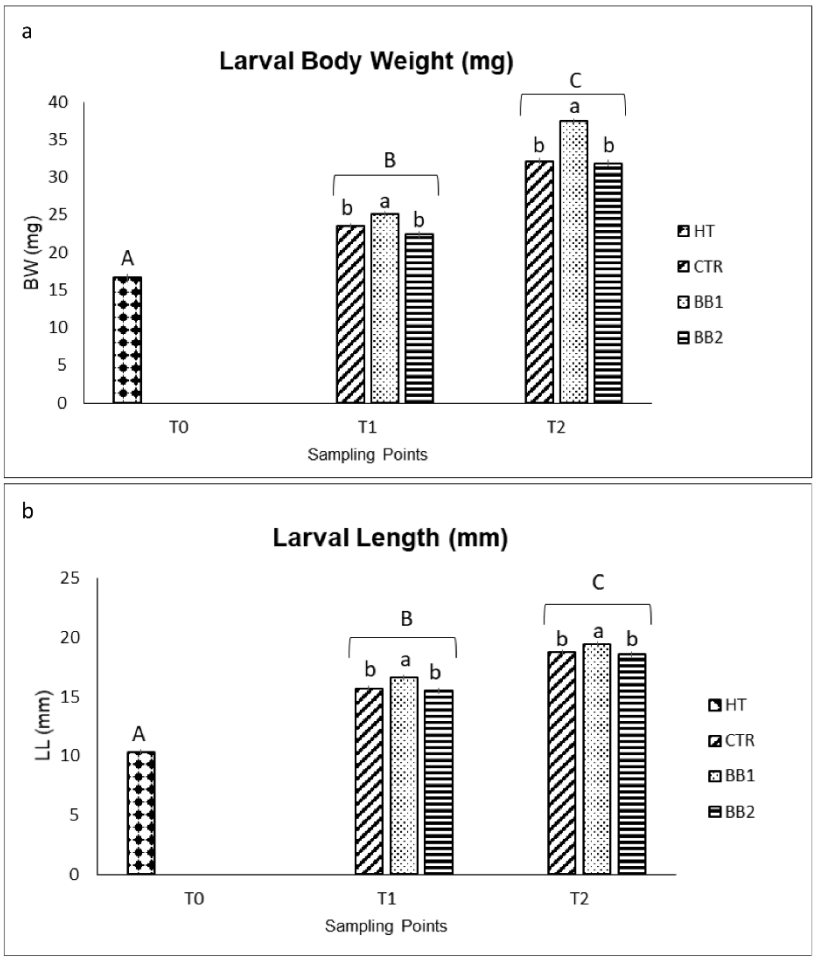

Figure 7.4. a) Larval growth expressed in mg of body weight; b) Larval length expressed in mm of body length; Error bars indicate the standard error of the mean for each treatment/stage of development; ^{A,B}Means with different superscripts differ significantly between stages of development ($P < 0.05$); ^{a,b}Means with different superscripts differ significantly between treatments ($P < 0.05$).

7.3.3. *Histological and histometrical analyses*

Histological analyses revealed an anatomically regular muscle development: at hatching larvae presented an outer monolayer of slow muscle cells (SM) and an inner monolayer of fast muscle cells (FM). From hatching to the yolk-sac absorption stage there was an expansion of both layers (SM and FM), from monolayer to multilayers (arrowheads and arrows, respectively; Figure 7.5a-c and d-f).

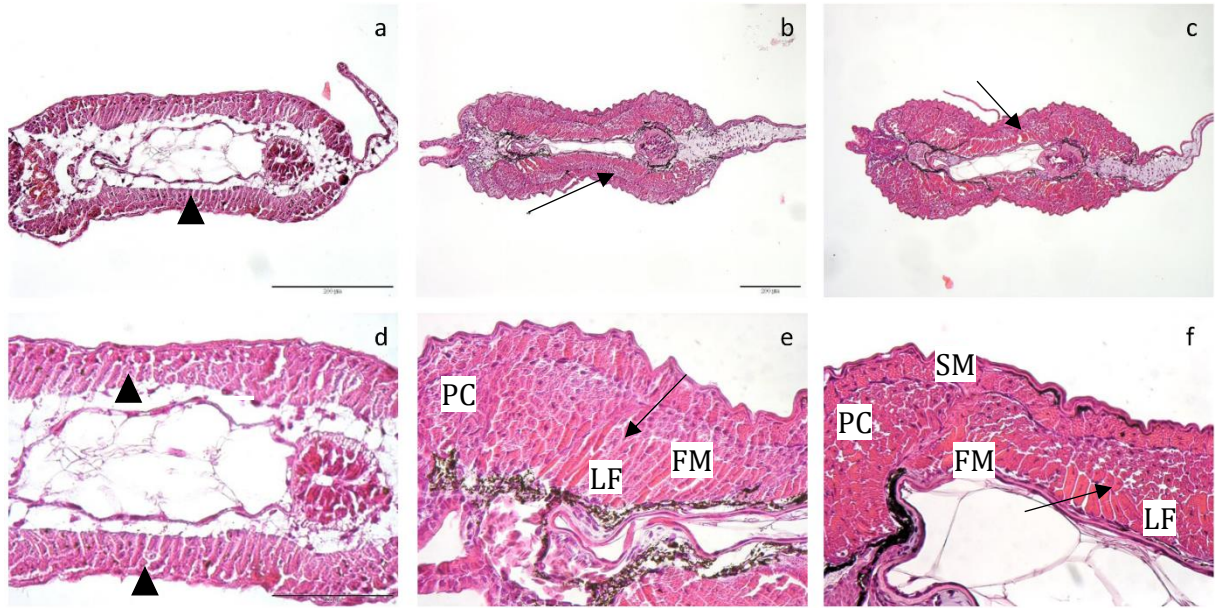

Figure 7.5. HE staining representative figures for: a, d) hatching; b,e) schooling and c,f) yolk-sac full absorption, at BB1. FM, fast muscle; SM, slow muscle; monolayer, arrowheads; multilayers, arrows; LF, lamella-shaped fibre; PC, polygonal cells. Figure 5a as the scale bas as indicated in the figure itself: 200 µm; Figure 5c has the same scale bar as located in Figure 5b: 200 µm. Figures 4e,f have the same scale bas as indicated in Figure 5d: 100 µm

Histometrical results are presented in Figure 7.6a-c. TMA, SMA and FMA significantly increased from one stage of development to the other (Figures 7.5a-c; $P<0.0001$). At the schooling stage, there were no significant differences regarding TMA; at the end of the trial, though, TMA was significantly higher for larvae reared in substrate BB1 (Figure 7.6a; $P<0.05$). Also for the SMA, there were no significant differences at T1 but, at T2, SMA was significantly higher for larvae reared in substrate BB1 when compared to larvae reared without any substrate (CTR) (Figure 7.6b; $P<0.05$). At the schooling stage there were no differences between treatments concerning the FMA; At T2 FMA was significantly higher for larvae reared with substrate BB1 (Figure 7.6c; $P<0.05$). The interaction between developmental stages and substrate was not significant for TMA, SMA or FMA.

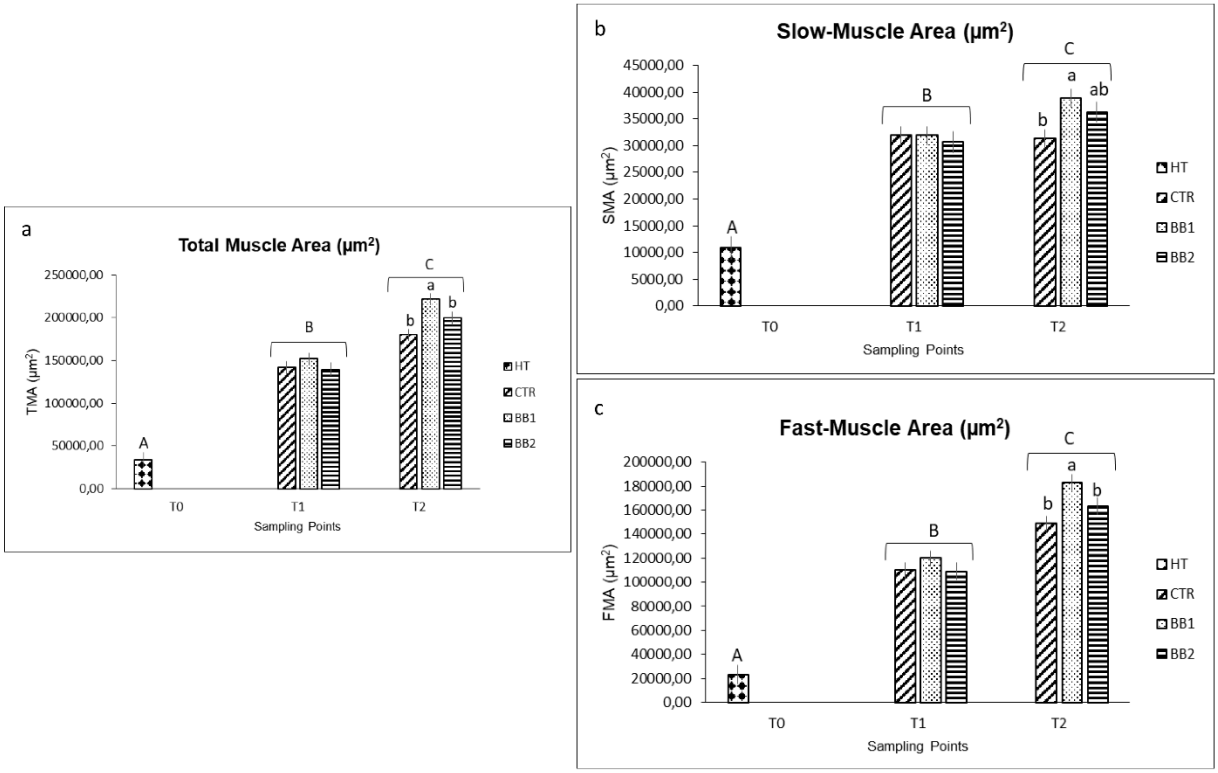

Figure 7.6. a) quantitative representation of TMA: area expressed in µm²; n=9/group; b) quantitative representation of SMA: area expressed in µm²; n=9/group; c) quantitative representation of FMA: area expressed in µm²; n=9/group; ^{A,B}Means with different superscripts differ significantly between stages of development (P< 0.05); ^{a,b}Means with different superscripts differ significantly between treatments (P< 0.05).

In the FMA at T1 and T2 two different types of cells were identified: lamella-shaped fibres (LF), that occupy an inner position and polygonal cells (PC), which are located externally (Figure 7.5). As for the areas occupied by LF and PC, no differences were found between treatments at the schooling stage (Figure 7.7a, b respectively). At the yolk-sac absorption stage, however, the area occupied by the lamellae-shaped fibres was significantly higher in larvae reared in substrates BB1 and BB2 rather than larvae reared without any substrate (CTR) (Figure 7.7a; P<0.05). As for the area occupied by the polygonal cells, this was significantly higher for larvae reared without any substrate (CTR) than in larvae from group BB1 or BB2 (Figure 7.7b; P<0.05,). The interaction between developmental stages and substrate was not significant for LF or PC, too.

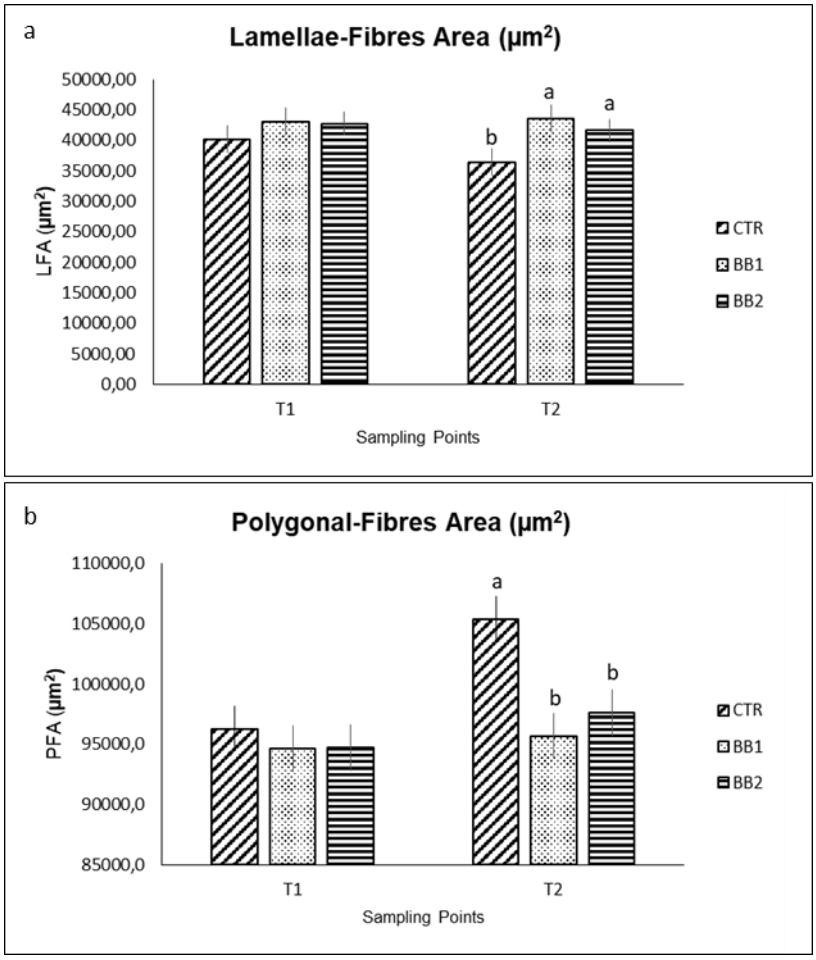

Figure 7.7. a) quantitative representation of lamella-shaped fibres area; b) quantitative representation of and polygonal cells area. Area expressed in µm^2; n=9/group; [a,b]Means with different superscripts differ significantly between treatments (P< 0.05).

7.4. Discussion

The present work investigated the impact of the presence and type of a rearing substrate on behaviour, growth and muscle development of Siberian sturgeon yolk-sac larvae. To do so, two rearing substrates (BB1 or BB2) plus a control group without any substrate (CTR) were tested during Siberian sturgeon endogenous feeding larval phase. Both the behavior observations and the morpho-functional data confirmed performance differences among the substrate treatments.

In our study we found that, in the presence of a substrate, Siberian sturgeon free-embryos, during the first 5 days-post hatch, preferred to hide in the interstitial spaces of the BioBalls, rather than swimming in the water column. On the opposite, in the same period, free-embryos reared with no substrate were always swimming in the water column. This situation, from the schooling stage on, was inverted, when larvae left the Bioballs and started to swim in the water

column. These results are in agreement with a study of Gessner et al. (2009) on the impact of different substrates on Atlantic sturgeon's larvae behaviour: until day 6 post-hatch, the big majority of the free-embryos preferred to hide in the middle of the gravel substrate than standing on the sand, where there were not hiding spots whereas, from this day on, the majority of the larvae started to actively swim in the water column. Also McAdam (2011) found that White Sturgeon yolk-sac larvae hid when exposed to porous substrates while nearly all larvae drifted in response to nonporous substrates. It would seem that in early life stages of sturgeon larvae seek for shelter. On the opposite Gisbert et al., (1996) found that *A:baerii* during pre-larval development were positively phototactic and preferred white bottoms and did not show any preference for bottom concealment, and this fact can probably be explained by the different substrate employed.

In our study we found differences both in weight and in length among substrates. Type 1 BioBalls produced heavier and longer larvae. There are several studies on the impact of environmental enrichment on larval and juvenile performance and physiology of various fish species. Peterson and Martin-Robichaud (1993) found an increased yolk-sac efficiency in *Salmo salar* larvae when reared with a substrate. Kihslinger and Nevitt (2006) showed the importance of environmental enrichment on brain development. European sea bream reared in blue substrate showed higher growth performance, in terms of body mass, total length and specific growth rate (Batzina et al 2014). Gessner et al (2009) studied the effect of two different substrates (sand and gravel) in American Atlantic sturgeon (*Acipenser oxyrinchus*) free embryos and found significant differences in terms of total length and wet mass. White sturgeon (*Acipenser transmontanus*) larvae reared on unenriched substrates tended to grow more slowly, showed a lower condition factor, and also exhibited delayed gut development and reduced rate of yolk sac absorption (at 15 dph) than those reared with enriched substrates (Baker et al., 2014). Bates et al. (2014) observed increased growth and reduced stress hormone levels in White sturgeon yolk-sac larvae reared in substrate. Still in white sturgeon, Boucher et al. (2014) studied the joint effect of temperature and substrate (gravel) and found that these factors affected size; yolk absorption efficiency was independent of temperature but was significantly higher in gravel-reared larvae; survival was higher in YSL reared in gravel. Boucher et al. (2017), in another study with white sturgeon, found that larvae reared in gravel and artificial substrate were larger than those reared without substrate; additionally, gravel-reared larvae had higher whole-body glycogen concentrations relative to bare-tank-reared larvae. Zubair et al. (2012) in a study on Lake sturgeon larvae found that there was an impact of substrate on the HPI stress axis.

The energy contained in the yolk sac is used for growth, development, and metabolism. As the energy of the yolk is limited, the more energy goes to one process, the less will be available for another one. Since larvae move less when settled in a substrate, more yolk's energy is available for growth. In the wild, substrate would also likely give these newly hatched fish a safe place to hide, with the added bonus of lots of invertebrates close-by for food.

Considering growth, it is actually known that in teleosts, muscle growth is the result of hypertrophy and hyperplasia. Hypertrophy takes place during post-embryonic life, when it reaches a functional maximum (Rowlerson and Vegetti 2001) and has been described for several species, such as carp (Alami-Durante et al., 1997), cod (Galloway et al, 1999), salmon (Nathanailides et al., 1995) and in several other marine species, as reviewed by Valente et al. (2013). A considerable difference between sturgeons and teleosts is that these present the white muscle composed entirely of cylindrical cells from hatching to the adult life. Instead, in sturgeons, white muscle is initially composed of multinucleated muscle lamellae which, later on, give place to polygonal cells (Steinbacher et al., 2006), in a process that is not yet fully clear. In this study it was possible to observe, in all treatments, a profound change in the three timepoints considered: from hatching through schooling, until the full yolk-sac absorption stage, fast fibres undergo a phenomenon of hypertrophy / hyperplasia, increasing its number and size. There were no qualitative nor morphological differences among the three tested substrate treatments regarding the stages of development. According to Rowlerson and Vegetti (2001), a broadly used method for measuring muscle growth consists in calculating the cross-sectional muscle areas, that provide an index of hypertrophic or hyperplastic growth. Larvae reared in the BB1 substrate showed higher cross-sectional areas of total muscle (TMA), slow-muscle (SMA) and fast-muscle (FMA), which is in agreement with the fact that these larvae were also significantly heavier and longer than larvae belonging to group CTR or BB2. Differences regarding muscle development have been noticed in the definitive polygonal cells and in the primary lamellae-shaped fibres: larvae reared in bare units had more definitive polygonal cells than larvae from groups BB1 or BB2. In these latter, instead, there were more lamellae-shaped fibres. It would seem that an increase in the conversion / differentiation of the primitive lamellae in definitive polygonal cells, is related to the reduction in both length and weight of the CTR group. These preliminary results give an indication that larvae reared with a substrate are similar in terms of muscle development, while in the group of larvae raised with no substrate at all, it would seem that there is an acceleration of muscle development in its final form.

Taking into account that the rearing environment during these early stages of development can influence the following muscle growth potential, animal well-being and the quality of the final flesh (Johnston, 2006), it is important to optimize the larval rearing conditions. Considering this fact, according to our results, it would seem more favourable to provide a substrate rather than a bare bottom, for Siberian sturgeon in these phases of development, in particular a substrate with characteristics similar to those of BB1, even if other analyse are still ongoing about this trial.

Understanding the effect of the rearing environment on larval sturgeon development is vital for effective hatchery practices, particularly in conservation aquaculture, but also for habitat restoration to enhance natural propagation.

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7.5. References

Alami-Durante, H., Olive, N. & Rouel, M. (2007). Early thermal history significantly affects the seasonal hyperplastic process occurring in the myotomal white muscle of Dicentrarchus labrax juveniles. *Cell and Tissue Research*, **327**(3), 553-570. https://doi 10.1007/s00441-006-0321-2

Baker, D. W., McAdam, D.S.O., Boucher, M., Huynh, K.T. and Brauner, C. J. (2014). Swimming performance and larval quality are altered by rearing substrate at early life phases in white sturgeon, *Acipenser transmontanus* (Richardson, 1836) *Journal of Applied Ichthyology*, **30**(6), 1461–1472. https://doi.org/10.1111/jai.12642

Bams, R. A. and Simpson, K. S. (1976). Substrate incubators workshop - 1976. Report on current state-of-the-art. *Fkh. Mar. Serv. Res. Dev. Tech. Rep., 689, 67* PP.

Barton, B.A. & Iwama, G.K. (1991). Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Annual Review of Fish Diseases*, **1**, 3–26. https://doi.org/10.1016/0959-8030(91)90019-G

Barns, R. A. (1969). Adaptations of sockeye salmon associated with incubation in stream gravels. In: *Symposium on Salmon and Trout in Streams,* ed. T. G. Northcote. H. R. MacMillan Lectures in Fisheries, p. 71-88. Institute of Fisheries, University of British Columbia, Vancouver.

Bates, L. C., Boucher, M. A., & Shrimpton, J. M. (2014). Effect of temperature and substrate on whole body cortisol and size of larval White Sturgeon (Acipenser transmontanus Richardson, 1836). *Journal of Applied Ichthyology*, **30**(6), 1259–1263. https://doi.org/10.1111/jai.12570

Batzina, A., Kalogiannis, D., Dalla, C., Papadopoulou-Daifoti, Z., Chadio, S., Karakatsouli, N. (2014). Blue substrate modifies the time course of stress response in gilthead seabream *Sparus aurata*. *Aquaculture*, **420–421**, 247–253.

Birstein,V.J. 1993. Sturgeons and paddlefishes: Threatened fishes in need of conservation. Cons. Biol. 7: 773–787. https://doi.org/10.1016/j.aquaculture.2013.11.016

Bronzi, P. & Rosenthal, H. (2014). Present and future sturgeon and caviar production and marketing: A global market Overview. *Journal of Applied Ichthyology,* **30**, 1536–1546

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

Boucher, M.A., Baker, D., Brauner, C. J. and Shrimpton, J. M. The effect of substrate rearing on growth, aerobic scope and physiology of larval white sturgeon *Acipenser transmontanus*. December 2017. *Journal of Fish Biology,* **92**(6), 1731-1746. https://doi.org/10.1111/jfb.13611

Devitsina, G.V. & Kazhlayev, A.A. (1993). Development of chemosensory organs in Siberian sturgeon, *Acipenser baeri*, and stellate sturgeon, *A. stellatus*. *Journal of Ichthyology* **33,** 9–19.

Di Giancamillo A., Rossi R., Vitari F., Pastorelli G., Corino C.& Domeneghini C. (2009). Dietary conjugated linoleic acids decrease leptin in porcine adipose tissue. *Journal of Nutrition*, **139**(10), 1867 – 1872. https://doi.org/10.3945/jn.109.110627

Fuss, H.J., Johnson, C., (1982). Quality of chum salmon fry improved by incubation over artificial substrates. *The Progressive Fish Culturist,* 44(4), 170–172. https://doi.org/10.1577/1548-8659(1982)44[170:QOCSFI]2.0.CO;2

Galloway, T. F., Kjorsvik, E. & Kryvi, H. (1999) Muscle growth and development in Atlantic cod larvae (Gadus morhua L.), related to different somatic growth rates. *Journal of Experimental Biology*, **202**, 2111-2120

Gisbert, E. (1999). Early development and allometric growth patterns in Siberian sturgeon and their ecological significance. *Journal of Fish Biology,* **54**(4), 852–862. https://doi.org/10.1111/j.1095-8649.1999.tb02037.x

Gessner, J., Kamerichs, C. M., Kloas, W. and Wuertz, S. (2009). Behavioural and physiological responses in early life phases of Atlantic sturgeon (*Acipenser oxyrinchus* Mitchill 1815) towards different substrates. *Journal of Applied Ichthyology,* **25**(2), 83–90. https://doi.org/10.1111/j.1439-0426.2009.01246.x

Gisbert, E., Williot, P. & Castello´-Orvay, F. (1999). Behavioural modifications in the early life stages of Siberian sturgeon (*Acipenser baeri*, Brandt). *Journal of Applied Ichthyology,* **15**, 237–242.

Iguchi, K. and N. Mizuno. (1999). Early starvation limits survival in amphidromous fishes*. Journal of Fish Biology*, **54**(4), 705–712. https://doi.org/10.1111/j.1095-8649.1999.tb02027.x

Johnston, I.A. (2006). Environment and plasticity of myogenesis in teleost fish. *Journal of Experimental Biology*, **209**(12), 2249-2264. http://jeb.biologists.org/cgi/doi/10.1242/jeb.02153

Kamler, E. (1992). In: *Early life history of fish: an energetics approach*. Chapman and Hall, London, 288 pp. DOI: 10.2307/1446775

Kihslinger, R. L. and Nevitt, G. A. (2006). Early rearing environment impacts cerebellar growth in juvenile salmon. *Journal of Experimental Biology,* **209**(3), 504-509. http://jeb.biologists.org/cgi/doi/10.1242/jeb.02019

McAdam, S.O. (2011). Effects of substrate condition on habitat use and survival by white sturgeon (*Acipenser transmontanus*) larvae, and potential implications for recruitment. *Canadian Journal of Fisheries and Aquatic Sciences*, **68**(5), 812–821. https://doi.org/10.1139/f2011-021

Nathanailides, C., Stickland, N. C. & Albors, O. L. (2011). Influence of prehatch temperature on the development of muscle cellularity in posthatch Atlantic salmon (Salmo salar*). Canadian Journal of Fisheries and Aquatic Sciences*, **52**(4), 675-680. https://doi.org/10.1139/f95-068

Peterson, R.H., Martin-Robichaud, D.J., (1995). Yolk utilization by Atlantic salmon (*Salmo salar* L.) alevins in response to temperature and substrate. *Aquacultural Engineering*, **14**(1), 85–99. https://doi.org/10.1016/0144-8609(94)P4428-E

Rowlerson, A. & Vegetti, A. (2001). Cellular mechanism of post-embryonic muscle growth in aquaculture species. In: Johnston, I.A., ed. *Muscle development and growth,* pp103-140. Academic Press. London

Ruban, G.I. (1997). Species structure, contemporary distribution and status of the Siberian sturgeon *Acipenser baerii*. *Environmental Biology of Fishes*, **48**(1-4), 221-230. https://doi.org/10.1023/A:1007372932

Ruban, G.I. (2005). In: *The Siberian Sturgeon Acipenser baerii Brandt. Species structure and ecology*. World Sturgeon Conservation Society, New Wulmstorf (Germany)

Steinbacher, P., Haslett, J.R., Sänger, A.M. and Stoiber, W. (2006). Evolution of myogenesis in fish: a sturgeon view of the mechanisms of muscle development. *Anatomy and Embryology*, **211**(4), 311–322. https://doi.org/10.1007/s00429-006-0082-4

Valente, L. M. P., Moutou, K. A., Conceicao, L. E. C., Engrola, S., Fernandes, J. M. O. & Johnston, I. A. (2013). What determines growth potential and juvenile quality of farmed fish species? *Reviews in Aquaculture*, **5**(S1), S168–S193. https://doi.org/10.1111/raq.12020

Zubair, S.N., Peake, S.J., Hare, J.F. & Anderson, W.G. (2012). The effect of temperature and substrate on the development of the cortisol stress response in the lake sturgeon, *Acipenser fulvescens*, Rafinesque (1817). *Environmental Biology of Fishes*, **93**(4), 577–587. https://doi.org/10.1007/s10641-011-9951-7

Chapter 8

Discussion and Conclusions

8. Discussion and Conclusions

Large amounts of larvae hatch from fertilized eggs but, nevertheless, mortality in the early stages still remains quite high or present a high variability. For this reason, production of high quality fish larvae is of relevant importance in aquaculture. Moreover, during the embryonic and larval stages, muscle plasticity towards the environment is normally irreversible because in larval stages, the ontogenic changes show a rapid pace. Larval stages are particularly sensitive to environmental conditions, since survival and potential to grow can be severely affected. Muscle development and growth, during the larval period, can affect locomotory performance and behaviour, which may potentially affect larval survival. From a commercial hatchery point of view, it is of great importance to recognise the environmental factors that might have a relevant effect on the hyperplastic processes that occur in fish myotomal muscle.

For the aquaculture industry it is of great importance to improve hatchery technology in order to maintain high survival rates and produce good quality larvae.

Research on the larval development regarding myogenesis in Sturgeon may have positive consequences on the final product quality but also on the success in farming for re-population purposes. In addition, The edible part of the fish is composed by the skeletal muscle. For this reason, it is important to deepen the study on muscle growth since early stages, in order to reach for an optimal development and assessment of fish farms for both protective and productive aims.

This Thesis focused on the effect of several environmental factors on muscle development and growth of Siberian sturgeon larvae, during the endogenous feeding phase, that constitutes a critical period in this species development. Temperature and stocking density effects were examined, as well as the effect of the environmental enrichment.

Results concerning temperature were not completely conclusively, as it was not possible to clearly identify a temperature that presented the best results in terms of muscle development, growth and stress status. From one side, a temperature of 19°C seems to lead to a greater growth potential but no differences in body weight were found. Stress indicators were particular high in the schooling stage especially in larvae reared at the lowest temperature but at the end of the trial, a restoring of the stress condition was observed. These results, altogether, suggest that this species is not particularly sensitive to temperature variations within the examined range.

However, it would be interesting to assess the effect of temperature at the onset of the exogenous feeding, another critical period in this species development.

Regarding stocking density, our results were much clearer: lower stocking densities seem to be the most favourable ones in this stage of development. Indeed, larvae reared at the lowest density presented higher body weight, Specific Growth Rate and total length, muscle areas and lower stress levels. However, in commercial hatcheries, rearing larvae in such low densities may not be economically feasible. Therefore, choosing an intermediate stocking density could be a good compromise between larval performance and economical feasibility. On the opposite, when the production purpose is natural populations restoring, rearing pre-larvae in very low densities may be important in increasing the successful introduction of larvae in their natural habitat.

According to our results, environmental enrichment definitely plays an important role in this early stage of development in this species. The presence of a substrate produced heavier and longer larvae, and led to larger muscle areas, indicating a higher growth potential. On the opposite, larvae reared with no substrate showed an acceleration of muscle differentiation, which can have negative consequences on its growth potential. The use of an artificial substrate is suggested in hatchery guidelines for sturgeon but only to be used as a spawning ground. Indeed, in commercial hatcheries rearing tanks are bare but, according to our results it could be advantageous to take into account to use a substrate with similar characteristics to the ones used in this study.

The in-vivo trial concerning substrate, was performed in April/May of the current year and, therefore, analyses are still ongoing, which may eventually add more information to the data already presented in this Thesis.

As a conclusion, in order to ensure maximum fitness upon larval growth and vitality, the rearing conditions have to provide environmental clues that enable the fish to be farmed as its best. Environmental conditions are not the key target of this thesis, but rather environments that reveal comparable fluctuations in accordance with the rearing systems utilized. Therefore, the first question is which parameters have to fluctuate? Second, what are the amplitude and the interval of fluctuations?

Answering the two questions, in my PhD Thesis I have focused the attention on three selected parameters and I have tried to understand the correct modulation and influence of these parameters on muscle growth and development and, consequently, on potential growth. For this

reason, it is possible to suggest that it could be more favourable for commercial hatcheries to subject newly-hatched larvae to an intermediate density of 80 larvae/litre in tanks provided with a bottom covered by bioballs. Regarding temperature, it seems that commercial hatcheries may subject larvae to any temperature, within the physiological range of the Siberian sturgeon, even if a range from 16 to 19 °C seems less stressful and indicates higher growth potential.

I hope that my Thesis may possibly increase the knowledge about the best practices in sturgeon hatchery management by providing scientific evidences, as well, as practical tools for modern sturgeon hatchery practices and management.

Chapter 9

PhD student scientific activities (2015-2018)

9. PhD student scientific activities (2015-2018)

Name: Maria Lucia Matela da Silva Aidos (R11181)

Tutor: Prof. Alessia Di Giancamillo

Cicle: XXXI

9.1. Publications

Aidos, L., Valente L.M.P., Sousa V., Lanfranchi, M., Domeneghini, C. and Di Giancamillo, A. (2017). Effects of different rearing temperatures on muscle development and stress response in the early larval stages of *Acipenser baerii*. European Journal of Histochemistry 61:2850

Di Giancamillo, A., Rossi, R., Martino P., **Aidos, L.**, Maghin, F., Domeneghini, C. and Corino, C. (2018). Copper sulphate forms in piglets diet: microbiota, intestinal morphology, and enteric nervous system glial cells. Animal Science Journal. 89, 616–624

Vasconi, M., **Aidos, L.**, Di Giancamillo, A., Bellagamba, F., Domeneghini, C. and Moretti, V.M. (2018) Effect of temperature on fatty acid composition and development of unfed Siberian sturgeon (*A. baerii*) larvae. Journal of Applied Ichthyology 2018;1–7. DOI: 10.1111/jai.13725

Aidos, L., Vasconi, M., Abbate, F., Valente, L.M.P., Lanfranchi, M. and Di Giancamillo, A. Effects of stocking density on Siberian sturgeon (*Acipenser baerii*) larval growth, muscle development and fatty acids composition. Manuscript submitted to Aquaculture Research.

Aidos, L., Serra, V., Cafiso, A., Berttoto, D., Bazzocchi, C., Radaelli, G. and Di Giancamillo, A. How different stocking densities affect growth and stress status of *Acipenser baerii* larvae? Manuscript in preparation.

Aidos, L., Cafiso, A., Berttoto, D., Bazzocchi, C., Radaelli, G. and Di Giancamillo, A. How different rearing temperatures affect growth and stress status of *Acipenser baerii* larvae? Manuscript in preparation.

Aidos, L., Vasconi, M., Valente, L.M.P., Lanfranchi, M. and Di Giancamillo, A. Impact of different rearing substrates on behaviour, growth and muscle development in the early larval stages of *Acipenser baerii.* Manuscript in preparation.

9.2. Conferences

Aidos, L., Di Giancamillo, A., Lanfranchi, M. and Domeneghini, C. Oxidative stress and lateral muscle development in Siberian Sturgeon (Acipenser baerii): preliminary observations. **.** Vas-Days 2016, 6th-8th June 2016, Milan (ORAL COMMUNICATION)

Polito, U., Deponti, D., Tessaro, I., **Aidos, L.**, Andreis, M.E., Peretti, G.M., Domeneghini, C. and Di Giancamillo, A., Meniscus maturation in the swine model: role of endostatin in cellular differentiation. XI Congresso Nazionale Associazione Italiana Morfologi Veterianari. May 25th – 26th 2017 - Rome

Aidos, L., Di Giancamillo, A., Lanfranchi, M., Bertotto, D., Radaelli, G. and Domeneghini, C. 2017. Rearing temperature effect on the skeletal muscle fibres of *Acipenser baerii* yolk-sac larvae**.** Vas-Days 2017, 6th-8th June 2017, Milan (ORAL COMMUNICATION).

Aidos, L., Di Giancamillo, A., Sousa, V., Valente, L.M.P., Vasconi, M., Lanfranchi, M., Bertotto, D., Radaelli, G. and Domeneghini, D. 2017. Environmental temperature variation on reared *Acipenser baerii* yolk-sac larvae: effect on cortisol and igf-1 expression in muscle fibres. Larvi'17, 7th symposium, 4th-7th September 2017, Ghent –Belgium (POSTER).

Vasconi, M., **Aidos, L.**, Di Giancamillo, A., Bellagamba, F., Domeneghini, C. and Moretti, V.M. 2017. Effect of temperature on fatty acid composition of unfed Siberian sturgeon larvae. 8th International Symposium on Sturgeons, September 10th - 16th, 2017 - Vienna/Austria (POSTER).

Aidos L., Lanfranchi, M., Domeneghini, C. and Di Giancamillo, A.2017. Temperature effect on muscle growth in *Acipenser baerii* yolk-sac larvae. 37° Congresso Nazionale della Società Italiana di Istochimica, 22nd-23rd September 2017, Taormina – Italy (ORAL COMMUNICATION).

Aidos L., Di Giancamillo, A., Cafiso, A., Lanfranchi, M., Domeneghini, C., Bazzocchi, C. Gene expression in Siberian sturgeon larvae in response to different rearing temperatures. Aquaculture Europe, International Conference & Exposition, 17th-20th October 2017, Dubrovnik – Croatia (POSTER).

Aidos, L., Vasconi, M., Lanfranchi, M., Di Giancamillo, A. Effect of different stocking densities on growth, muscle development and fatty acid profile of *Acipenser baerii* larvae. Vas-Days 2018, 6th-8th June 2018, Milan (ORAL COMMUNICATION).

Aidos, L., Lanfranchi, M., Abbate, F and di Giancamillo, A. Effect of rearing density on Siberian sturgeon larval development. EAVA Congress 2018, 25th-28th July 2018, Hannover – Germany (POSTER)

9.3. Attended courses

General Linear Model and Experimental Design using "R". Workshop Fondazione Cariplo, 14-18 March 2016, Department of Clinical Sciences and Community Health, University of Milan;

Digital Imaging and Image Integrity in Scientific Publication. 9-13 May 2016, University of Milan.

Pathology of Laboratory Animals. May 2017, University of Milan.

CAL-AQUA, Laboratory Aquatic Organisms Sciences. The program of this course was in accordance with the criteria established by the DGAV and Federation of European Laboratory Animal Science (**FELASA**) for courses in Laboratory Animal Science, **category B**. The frequency of this course allows to obtain the certification as "Researcher" or "Research Technician" to conduct animal experiments. 7th-11th May 2018, CIIMAR - Interdisciplinary Centre of Marine and Environmental Research, Porto – Portugal.

9.4. Externship

Training period at CIIMAR - Interdisciplinary Centre of Marine and Environmental Research, University of Porto – Portugal. (27th of June to the 29th of July 2016; 12th of June to the 21st of July: 2017; 16th to the 24th July 2018, for a total of 3 months.

9.5. Awards

Involved in tutoring, orientation and didactics supplementary activities regarding the courses of Domestic Animals Anatomy, enrolled after public selection from University of Milan;

Winner of an award conference attendance grant for the participation at the "37° Congresso Nazionale della Società Italiana di Istochimica (22nd-23rd September 2017, Taormina), after public selection from the board of directors of the "Società Italiana di Istochimica".

9.6. Other activities

Activity as **co-advisor** of the final thesis of the students:

- Angela Vecchi (graduation course of "Scienze e Tecnologie delle Produzioni Animali"), with the title "Ruolo della temperatura di allevamento in larve ad alimentazione endogena di *Acipenser baerii*: effetti su marcatori dello stress (HSP70 - HSP90) e cortisolo";
- Giuditta Spinelli (graduation course of "Scienze e Tecnologie delle Produzioni Animali"), with the title "Larvicoltura in *Acipenser baeri*: effetti di diverse densità di stock su aspetti istometrici del muscolo laterale in via di sviluppo";
- Francesca Mor (graduation course of "Scienze Biotechnologiche Veterinarie"), with the title "Molecular analyses for the study of temperature effects on muscle development and oxidative stress status of *Acipenser baerii*";
- Federica Maffeo (graduation course of"Scienze Biotechnologiche Veterinarie"), with the title "Effetto della densità sullo sviluppo degli stadi larvali di *Acipenser baerii:* un approccio molecolare";

Activity as a **reviewer** for:

- European Journal of Histochemistry
- Journal of Applied Ichthyology