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

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ABSTRACT

Environmental temperature is one of the critical factors that affect fish development. 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 *Acipenser baerii*. This was performed by assessing: i) larval survival and growth; ii) immunofluorescence localization and expression of genes involved in muscle development and growth – *myog* and *Igf1*; iii) stress status through the expression of thermal stress genes (*Hsp70, Hsp90α*, *Hsp90β*) and whole body cortisol. Overall survival rate and larval weight did not show any significant difference across temperatures. Larvae subjected to 22°C showed a faster absorption of the yolk-sac than larvae subjected to 19°C or 16°C. Both at schooling and at the end of the trial, larvae reared at 16°C showed significant lower level of cortisol than those reared at 19°C or 22°C. IGF-1 immunopositivity was particularly evident in red muscle at schooling stage in all temperatures. The expression of all *Hsps* as well as of *myog* and *Igf1* genes was statistically higher in larvae reared at 16°C, but limited to the schooling stage. Cortisol levels were higher in larvae at 22°C temperatures probably because of the high metabolism demand rather than a stress response. The observed apparent incongruity between the *Hsps* gene expression and cortisol level could be due to the lack of a mature system. 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.

Significance Statement

The best rearing temperature for Siberian sturgeon (*Acipenser baerii*) larvae remains unknown, in particular during the endogenous feeding phase. This study investigated the range of temperatures from 16° to 22°C, from hatch until the complete absorption of the yolk-sac. Higher temperatures led to a faster absorption of the yolk-sac and, within this range even if there was a poor stress response, this seemed not to compromise the growth performance of the larvae.

Introduction

The Acipenseriformes (Chondrostei) are a group of primitive fish that include both sturgeons (Acipenseridae) and paddlefishes (Polyodontidae) (Bemis *et al.*, 1997), which are commonly referred to as 'living fossils'. Along with their evolutionary significance, sturgeons are important as well, as a food resource mainly because of caviar. Italy has been the largest producer of sturgeons in the EU since 2008, followed by Poland, France and Germany, mainly for caviar production (EUMOFA, 2018). Siberian sturgeon (*Acipenser baerii*), is one of the most commercially important species of sturgeons due to the quality of its products (caviar and meat), and is one of the main farmed species of sturgeon in European countries (Gisbert & Williot, 2002). The Siberian sturgeon presents a large range of habitats when compared to other sturgeon species: the basins of all large Siberian rivers and Lake Baikal (Ruban, 1997). Since the beginning of 1990, natural stocks have suffered a sharp decline, due to overfishing, dam construction and pollution. The Siberian sturgeon is, currently, a threatened species and it is included in the IUCN Red Data List (https://www.iucnredlist.org/). In this context, the production of Siberian sturgeon in aquaculture became of great importance, as it allows to supply a growing market demand for caviar and meat and, at the same time it allows the production of juveniles that may be used for repopulation purposes.

Commentato [LA1]: Secondo il reviewer, l'introduzione sullo storione è goffa…

Commentato [CB2R1]: E partire da Siberian sturgeon saltando le prime 5 righe generali? Le avevano chieste loro? Non ricordo….
SI AVEVO CHIESTO DI INSEIRE LORO UNA PARTE SULLE ENDANGERED SPECIES

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 & Williot, 1997; Bardi *et al*., 1998). The early life stages constitute an important phase of development of fish, where important changes take place: embryonic adaptations and functions like the yolk sac nutrition and cutaneous respiration are replaced by definitive features, like exogenous feeding and branchial respiration. The relationship of the young fish with the surrounding environment is conditioned by these adaptations (Dettlaff et al., 1993), which may have a direct impact on growth and survival of fish.

In vertebrates, growth is determined by a regulatory network in which the growth hormone (GH)-insulin-like growth factor (IGF) axis has an important role in regulation of the process together with insulin, thyroid hormones and sex steroids (Jones & Clemmons 1995). 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 & Vijayan, 2015). In mammals, skeletal muscle is the main target tissue of the IGFs where these directly stimulate muscle cell proliferation and differentiation, hypertrophy and inhibition of muscle atrophy (Glass, 2003, 2005). As reviewed by Fuentes *et al.* (2013) it has been shown, in several fish species such as the barramundi (*Lates calcarifer*), rainbow trout (*Oncorhynchus mykiss*), gilthead sea bream (*Sparus aurata)* and gulf killifish, that IGFs stimulate glucose and alanine uptake, protein synthesis, and myoblast proliferation.

In aquaculture, fish are continuously exposed to environmental stress factors that are related to routine husbandry. Handling, transportation, sorting, water parameters conditions (e.g. 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).

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 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 which are necessary in various aspects of protein metabolism to maintain cellular homeostasis (Fink & Goto, 1998). The HSPs 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-90s have an active role in supporting various components of cell signalling, including the cytoskeleton, enzymes, and steroid hormone receptors. Vertebrates show two isoforms of

HSP90: alpha and beta. HSP90 beta is constitutively expressed in many tissues, such as muscle, while HSP90 alpha is overexpressed in stress condition. 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 a stress condition.

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 ectotherms (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), Danube Bleak *Alburnus chalcoides* (Stoiber *et al*., 2002), European sea bass *Dicentrarchus labrax* and gilthead sea bream (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 (*Igfs*), the myogenic regulatory factors (MRFs: *myod*, *myf5*, *mrf4* and *myog*), the myostatin (*mstn*) and the paired-box protein (*pax7*) (De-Santis & 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) and 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 *Oreochromis niloticus* (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 (Gisbert & Ruban, 2003). 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 & Williot, 2002). ~~Improving the knowledge for a more efficient production. Temperature constitutes one of the most important environmental factors affecting the development and growth in teleost fish (Moyle & Cech, 1982; Donaldson *et al.*, 2008). The larval phase is indeed quite susceptible to temperature changes (Johnston *et al.*, 1995; Kamler, 2002).~~ UUnderstanding the influence of the temperature changes in the early life-history stages of Siberian sturgeon is an important target not only for a successful and competitive expansion of the aquaculture industry of this species, but also for what concerns repopulations purposes. There may be long-term consequences on the populations' dynamics, following the physiological responses of individuals towards ecologically related factors, such as a negative impact on the recruitment of this species. Therefore, kknowing the most suitable rearing temperature in this delicate phase of development of Siberian sturgeon may be of importance not only for aquaculture production, but also to better manage re-population programs.

Commentato [CB3]: Questa cosa la cominciamo a dire alle righe 121-127. E provare a unirle? Prima di parla di come la temperatura influenzi crescita e sviluppo muscolare, poi tutto un pezzo sui geni implicati nello sviluppo muscolare, e poi di nuovo come la temperatura sia il fattore ambientale più importante nella crescita del pesce.

POSSIAMO ANCHE TOGLIERLO E STOP

In the present work the impact of different rearing temperatures on the development of yolk-sac Siberian sturgeon larvae was investigated. In order to analyse the short-term effect of temperature on the developing lateral muscle and stress status in Siberian sturgeon larvae, three rearing temperatures (16, 19 or 22°C) were tested during the endogenous feeding larval phase by using a multi-disciplinary approach. The aims of this study were thus to assess: i) larval survival and growth; ii) ontogenic development; iii) whole body cortisol, and iv) the expression of genes involved in muscle development and growth (*myog* and *Igf1*) and in the stress status (*Hsp70*, *Hsp90α*, *Hsp90β*).

Data on this temperature range may be useful not only for aquaculture production but it may also have an ecological relevance when managing the wild populations in Italy, which is actually the main European sturgeon producer (EUMOFA, 2018).

Materials and Methods

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 Siberian sturgeon 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 reared at three different water temperatures and the individuals were maintained in the experimental nurseries (three per temperature): 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)

194 or 22°C (21.9 ± 0.2 °C). This range of temperature (16-22°C) has been chosen as it reflects the
195 range usually observed in northern Italy, in the period of spawning (Spring).
196 In all nurseries, water temperature was daily monitored and kept under the target values
197 established for each treatment. Water O_2 was close to the saturation throughout the trial
198 (>8mg/l) in all nurseries and pH values were maintained inside the range described for this
199 species at this development stage 6.5-7.5, according to the FAO Technical Paper. During the
200 trial the photoperiod regime was of 12L:12D and larvae were not fed. Sampling points consisted
201 of important steps of Siberian sturgeon larval development: hatching (T0), beginning of the
202 schooling phase (T1) and complete yolk sac absorption phase (T2). In particular, schooling was
203 assessed by larvae behaviour observation: T1 took place when larvae became benthonic and
204 started to aggregate in shoals, swimming in groups. After schooling, larvae were sampled in
205 order to evaluate the pigment plug evacuation movement in the anal direction; T2 took place
206 when larvae showed the pigment plug evacuation. Larvae were killed by over-anaesthesia with
207 MS222 (Ethyl 3-Aminobenzoate, Methanesulfonic A, Sigma-Aldrich). All procedures
208 performed in the experiment were approved by the ethical committee (OPBA) of the University
209 of Milan (OPBA_20_2016).
210
211 ***Zootechnical performance***
212 Larval development period was calculated as "days post-hatch" (dph) until the yolk sac was
213 fully absorbed. Dead larvae were removed daily, and survival rate was estimated by dead larvae
214 daily recording.
215 Sampled larvae were weighed (wet weight), in order to determine body weight (BW). The
216 growth performance was described at T1 and at T2, using the following parameters:
217 1) specific growth rate (SGR) (FBW: final body weight; IBW: initial body weight)

$$(SGR) = 100 \times \left(\frac{\ln FBW - \ln IBW}{Days}\right)$$

2) thermal unit Growth Coefficient (TGC) (FBW: final body weight; IBW: initial body weight)

$$(TGC) = 100 \times \left[\frac{\left(\sqrt[3]{FBW} - \sqrt[3]{IBW}\right)}{\sum(T \times Days)}\right]$$

Micro-anatomical analyses: immunofluorescence

Samples for the micro-anatomical analyses were fixed in 4% (v/v) paraformaldehyde (N=3 sample at T0; N=3 samples for each nursery; N=9 samples per treatment, for each sampling point; N=57 in total). 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 µg/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 µg/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.

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 (N=3 sample at T0; N=3 samples for each nursery; N=9 samples per treatment, for each sampling point; N=57 in total), 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 goat anti-rabbit c-globulin serum diluted 1:1000 in 0.15 mM sodium acetate buffer, pH 9, and incubated overnight 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 µl 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%.

Gene identification and primers design

Genes involved in cellular stress reactions (*Hsp70*, *Hsp90α* and *Hsp90β*) and genes involved in myogenesis (*myog*) and growth (*Igf1*) were selected. Additionally, *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

265 BLAST), using a previously published assembled transcript of Siberian sturgeon as the
266 reference database (Song *et al*, 2016). Specific primers were *de novo* designed for the target
267 genes and the related sequences, the annealing temperatures and the amplification size of each
268 fragment are reported in Table 1.
269
270 ***RNA extraction and cDNA synthesis***
271 The sampling was performed at the beginning of T1 and at T2 phase. Larvae were immediately
272 stored at -80°C soon after the sampling procedure. Total RNA was extracted from each frozen
273 single larval sample (N=3 sample at T0; N=3 samples for each nursery; N=9 samples per
274 treatment, for each sampling point; N=57 in total) using RNeasy Mini Kit® (Qiagen), and
275 eluted in a final volume of 40 µl of RNase-free water. A double treatment with DNase enzyme
276 was performed, in order to remove any genomic DNA contamination, according to
277 manufacturer instructions. Five hundred nanograms of RNA was retro-transcribed to cDNA
278 using Quantitect Reverse Transcription Kit® (Qiagen) following manufacturer protocol. An
279 additional reaction without retrotranscriptase enzyme was performed to verify the complete
280 DNA removal. cDNAs were stored at -80°C until subsequent use.
281
282 ***Gene expression profiles***
283 The expression of genes coding for myogenesis, growth factors and stress were analysed by
284 Quantitative PCRs (qPCR) in larvae collected at the three rearing temperatures.
285 cDNA samples were used as template in qPCR using a BioRad iQ5 Real-Time PCR instrument
286 (Bio-Rad, California, USA) and Universal SYBR® Green Supermix (Bio-Rad, California,
287 USA) as fluorescent molecule. The final concentration of forward and reverse primers was 150
288 nM for each amplified gene; the thermal profile was 98 °C for 30 s, 40 cycles of 98 °C for 15
289 s, 58-60 °C for 30 s and a melting profile was also 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 at T1 and T2 was compared to the calibrator sample T0 and 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 and the obtained sequences were deposited in Gene bank.

Statistical analysis

Statistical analysis of the data was performed by SAS (version 8.1, Cary Inc., NC) using the 2-way ANOVA with temperatures and developmental stages as main factors. 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 $P < 0.01$.

Results

Larval development, survival rate and growth

Larvae subjected to ~~the highest water temperature,~~ 22ºC, showed: i) a faster yolk-sac absorption; 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 than larvae subjected to 16°C. Survival rate was unchanged among temperatures and varied from 88 and 90% from hatch to the complete yolk-sac absorption (data not shown) and no deformities were detected in larvae throughout the trial.

Commentato [CB4]: Lo togliamo? OK

Larvae body weight significantly increased from T0 to T2 stages ($P<0.05$ for stage, Figure 1). Otherwise, considering body weight at T1 and T2, no significant differences were found among temperature treatments (Figure 1). The interaction between developmental stages and temperatures was not significant (Figure 1).

Further data on growth are reported in Table 2. The growth of larvae expressed as specific growth rate (SGR) was higher for larvae reared at 16°C at both timepoints, although this was not statistically significant. Also the TGC was not significantly affected among fish, by different temperatures, at both T1 and T2. The interaction between developmental stage and temperatures was not significant.

Micro-anatomical analyses: immunofluorescence

Myogenin immunopositivity was detected in the cytoplasm of undifferentiated cells at all stages and in all temperatures considered (green staining myogenin; blue staining nuclei; Figure 2a-c). IGF-1 immunofluorescence appeared in both red and white skeletal muscle fibres at T0 (green staining IGF-1; blue staining nuclei; asterisk IGF-1 localization in white muscle fibres and arrow-heads in red muscle fibres; Figure 2d) and precisely it was present in the cytoplasm. Furthermore, IGF-1 immunofluorescence was similarly expressed in skeletal muscle at T1 and T2 in all experimental temperatures, following the correct development of skeletal muscles (Figure 2e,f representative images). A stronger immunostaining in the red muscle at T1 was observed in all temperature treatments (arrow-heads; Figure 2e).

Cortisol level

Whole body cortisol level gradually increased between different stages with higher values at T2, regardless of the rearing temperature ($P<0.01$; Figure 3). Cortisol concentration of larvae reared at 16°C was significantly lower than those reared at both the higher temperatures ($P<0.05$

339 both, Figure 3). No significant differences were found at T2 at 19°C when compared with larvae
340 of the other two experimental groups (16 and 22°C). The interaction between developmental
341 stages and temperatures was not significant.
342
343 ***Thermal stress and growth related gene expression***
344 The specificity of primers designed for the amplification of *rpl6, gapdh, Hsp70, Hsp90α,*
345 *Hsp90β, myog* and *Igf1* gene fragments of Siberian sturgeon was assessed by Sanger
346 sequencing. The obtained sequences were deposited in GenBank under the accession numbers
347 (MH702440 - MH702446). The expression of thermal stress and growth related genes at T1
348 and T2 was related to T0 considered as reference sample; results were normalized versus *rpl6*
349 and *gapdh* considered as reference genes. The relative expressions of *Hsp70, Hsp90α, Hsp90β,*
350 *myog and Igf1* genes at T1 and T2 phases are shown in Figure 4.
351 During T1 all genes resulted significantly more expressed in larvae reared at 16 °C compared
352 to the larvae reared at the other two rearing temperatures (*Hsp70*, *Hsp90α*, *Hsp90β, myog*, *Igf1*:
353 $P<0.05$). No differences were found in the expression of the analysed genes between larvae
354 reared at 19°C and 22°C. Conversely, at T2 no significant differences were found in the
355 expression of all genes among larval stages and rearing temperatures.
356 Moreover, assuming the gene expressions at T0 equal to 1, the expression of *Hsp90β* (Figure
357 4c), *myog* and *Igf1* (Figures 4d and e, respectively) genes results upregulated at all temperatures
358 for both T1 and T2 phases. Furthermore, an increased *Hsp70* (Figure 4a) gene expression is
359 observed at 16°C for T1 and at all temperatures for T2 while *Hsp90α* (Figure 4b) gene
360 expression increases only at16°C for T1.
361
362 **Discussion**

~~In the present work the impact of different rearing temperatures on the development of yolk-sac Siberian sturgeon larvae was investigated. In order to analyse the short-term effect of temperature on the developing lateral muscle and stress status in Siberian sturgeon larvae, three rearing temperatures (16, 19 or 22°C) were tested during the endogenous feeding larval phase by using a multi-disciplinary approach. Data on this temperature range may be useful not only for aquaculture production but it may also have an ecological relevance when managing the wild populations in Italy, which is actually the main European sturgeon producer (EUMOFA, 2018).~~

~~Temperature constitutes one of the most important environmental factors affecting the development and growth in teleost fish (Moyle & 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, we have observed that the three temperatures tested did not lead to differences in terms of larval growth. Perhaps the temperature range 16°C-22°C did not allow to observe any differences in growth. In green sturgeon (*Acipenser medirostris)* indeed, juveniles Mayfield & Cech (2004) observed an increase in growth only in a certain range of temperatures (between 11° and 15°C) and no differences in growth in temperatures between 15° and 19°C. It would seem that, overall, growth increases with increasing temperatures, within a certain range. The fact that in our study we found no differences in growth between temperatures may be due to the range of tested temperatures itself, which did not allow to observe any differences. ???? ~~Perhaps at temperatures higher than 22°C it would be possible to observe differences in growth in Siberian sturgeon~~. Indeed, in other studies with other fish species, higher temperatures led to higher growth in early life stages (white sturgeon *Acipenser trasmontanus*, Hung *et al*., 1993 and Bates *et al*., 2014; Atlantic sturgeon *Acipenser oxyrinchus*, Atlantic salmon *Salmo salar*, Bjørnevik *et al*., 2003; coral reef fish, *Amphiprion melanopus*,

Commentato [LA5]: First paragraph of discussion (lines 348-355) and the first two sentences of the 2nd paragraph (356-359) are more appropriate in the introduction rather than the discussion.

Commentato [LA6]: Disingenuous - too obvious, elucidate!

Commentato [CB7R6]: E se togliamo la frase?

Commentato [LA8]: missing words, unclear

Commentato [CB9R8]: Ma non lo abbiamo già detto aggiungendo la frase sopra. Forse anche questa frase si potrebbe togliere

Commentato [LA10]: repetitive

Green & Fisher, 2004; Senegalese sole Campos *et al*., 2013a; sablefish *Anoplopoma fimbria*, Cook *et al*., 2018). Further studies would be necessary in order to investigate which are the temperatures that significantly influence Siberian sturgeon growth. ~~clarify~~ ~~this issue~~.

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 temperature. Likewise, an increase of the rearing temperatures caused an increase in the developmental rate in several marine species like cod *Gadus morhua* (Pepin *et al*., 1997), Senegalese sole (Campos *et al*., 2013a,b), gilthead sea bream (Garcia de la Serrana *et al*., 2012), in freshwater species ??? like brown trout *Salmo trutta* (Réalis-Doyelle *et al*., 2016), Atlantic salmon (Ojanguren *et al*., 1999) and in several other species of sturgeon (Hardy & Litvak, 2004).

Moreover, it is also known that temperature can be associated with larval fish survival rates (Boucher *et al*., 2014). In fact, in studies conducted upon different sturgeon species, a clear effect of temperature on survival at 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 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%, and there were no significant differences among rates in the tested rearing temperatures; this ~~which~~ is in accordance with the above mentioned studies.

The results of the micro-anatomical analyses demonstrated a constant presence of myogenin-positive cells at all stages and temperatures of the experiment. This is in agreement with Sassoon (1993), who observed that in embryonic somatic muscle, myogenin is expressed prior to other muscle specific genes. Moreover, the cytoplasmic staining is in agreement to what was observed by Ferri *et al*. (2009), who showed that myogenin is already expressed in undifferentiated cells in vitro, being particularly ~~especially~~ detected in the cytoplasm. After the beginning of the differentiation, myogenin translocates into the nucleus.

Commentato [LA11]: what issue? Explain

Commentato [LA12]: "in freshwater species" is out of place - reword

Commentato [LA13]: "which is. . . " awkward and not clear

Commentato [LA14]: Somatic muscle?

Commentato [A15]: Si dice somitic e somatic, ma il secondo più usato, quindi cambiamo così evitiamo problemi

Commentato [LA16]: "especially" wrong word

Cytoplasmic retention is a mechanism that regulates 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 were myogenin-negative and probably related to quiescent myoblastic cells, identified as "resting cells" by Yoshida *et al.* (1998). In accordance with immunostaining results, *myog* gene was highly expressed at the three rearing temperatures in both T1 and T2 phases (Figure 4d), although its expression was significantly higher at 16°C at T1 compared to the other two temperatures. Therefore, these results could suggest that the expression of *myog* gene may vary with temperature and may have an influence in muscle growth. In Fernandes *et al*., 2006), in fact the authors found that myogenin expression was higher at 21 °C than at either 18 or 15°C, so that changes in the relative timing and intensity of myogenin expression can be used for explaining thermal plasticity of muscle phenotype in larvae.

The immunostaining of IGF-1 was present in the skeletal muscle at all stages of larval development. In agreement with our findings, Berishvili *et al*. (2006) observed the presence of IGF-1 in the skeletal muscle of Nile tilapia but Radaelli *et al*. (2003) did not detect it in shi drum larvae (*Umbrina cirrosa*). In our study, it is interesting to notice that, at T1, a stronger immunopositivity in red muscle was observed, irrespective of the temperature treatment. To be able to explain this interesting observation, it is necessary to focus on the particularity of larvae behaviour at schooling (T1): as Gisbert *et al.* (1999) we also observed that in this stage of development, larvae became benthonic and swam in a slow continuous way. It is known that, at these sustained swimming speeds when only the red muscle is used there is an increase in glucose uptake (Moyes & West, 1995; Baños *et al*., 1997) and we suggest that this is the reason why we observed a higher IGF-1 immunopositivity in red muscle at T1. Further studies would be necessary in order to clarify the specific effects of IGF-1 on the red muscle.

The relative expression of *Igf1* gene was 20-fold ~~circa~~ increased in T1 at 16°C compared to T0, and significantly higher at 16°C respect to the other two temperatures (Figure 4e). This finding ~~evidence~~ could be correlated with an enhanced growth (Hall *et al*., 2003; Campos *et al.*, 2013b), as also observed in rainbow trout (*Oncorhynchus mykiss)*, where *Igf1* transcript abundance in muscle increases as water temperature decreases (Gabillard *et al*., 2003; Deane & Woo, 2005). The stress status of the Siberian sturgeon larvae reared at the different temperatures was investigated by means of cortisol level and *Hsp70* and *Hsp90s* gene expression. Due to the small size of the larvae, cortisol was assessed in whole-body homogenates whose concentration reflect the hormone levels of all tissues and have been efficiently used to determine stress levels in various fish species (Yeh *et al.*, 2013; Bertotto *et al*., 2011; Simontacchi *et al.* 2009; Barry *et al.*, 1995; Hwang *et al.*, 1992). Cortisol is known to be involved in hatching, development, growth, and stress response during the early development of teleosts and the hypothalamic-pituitary-interrenal (HPI) axis develops at different times among species e.g before or soon after hatching or close to metamorphosis (de Jesus et al. 1992; Barry *et al.*, 1995; Sampath-Kumar *et al.* 1995; Stouthart *et al.* 1998; Gessner *et al.*, 2009; Simontacchi *et al.*, 2009). The low cortisol levels found at the earliest larval stages may reflect a poor stress-coping ability which protect larvae from the elevated metabolic demands involved by stress responses and promote faster growth and survival (Piccinetti *et al.*, 2017).

In the present study, cortisol in sturgeon larvae significant increased from hatch to (T0) to the (T2) indicating a gradual maturation of the HPI axis during the first days post hatch as already observed in other sturgeon species (Gessner *et al*., 2009; Simontacchi *et al*., 2009). Cortisol concentrations significantly increased in larvae reared at high temperatures (19 and 22 *vs* 16°C) but hormone concentrations remained low and comparable with no stress status levels observed in other sturgeon species (Simontacchi *et al.*, 2009; Bates *et al.*, 2014). These results may suggest that the increase in cortisol level could be due to the increased metabolic rate caused

Commentato [LA17]: misuse of circa

Commentato [LA18]: "T0" and "T2" should be referred to as days post hatch here. NON SONO MOLTO D'ACCORDO PERCHE IL T2 E' DIVERSO PER OGNI TEMPERATURA… HA PIU SENSO DI PARLARE DI T2, CREDO

462 by the rearing temperature (Mommsen *et al.* 1999) rather than to the stress response. The stress
463 copying ability at these developmental stages has therefore to be further investigated by
464 submitting larvae to a certain stress event.
465 Considering genes related to thermal stress (*Hsp70, Hsp90α, Hsp90β*; Figures 4 a-c), we
466 observed a significantly higher expression at 16°C at T1 compared to the other two rearing
467 temperatures. This could be attributed to, for example, stress-related protein damage, enhanced
468 cytoprotection (as suggested by results obtained on sliver sea bream *Sparus sarba*; Deane &
469 Woo, 2005), or to support the correct folding of proteins (Pelham, 1986). Conversely, no
470 differences in gene expression were observed among the three temperatures at T2. These genes
471 code for highly conserved proteins expressed in response to biotic and abiotic stressors and are
472 usually identified as damage biomarkers. The HSPs-response is involved in cellular processes
473 including protein synthesis, folding and translocation as well as assembly of larger protein
474 complexes, all of which can be impaired upon stress, as well as preventing the damaging effects
475 of thermal cellular stress (Airaksinen *et al.*, 2003). Additionally, differences in the ability to
476 over-express HSPs during stressful conditions may be associated with an organism's
477 vulnerability and the extent of thermal injury (Werner *et al.*, 2007).
478 Moreover, increased levels of *Hsp*70 may indicate an attempt of the cell to counteract the
479 increase in levels of damaged proteins when activity of other chaperones such as HSP90s is
480 insufficient (Ivanina *et al.*, 2008). In our study, a higher level of was only observed in the T1
481 phase at 16°C, as no differences have been found at T2 among the three temperature treatments,
482 suggesting the restoring of the stress condition.
483 The observed apparent incongruity between the *Hsps* gene expression and cortisol level at these
484 stages could be due to the lack of a mature system as already observed in other developmental
485 studies (Marlowe *et al.* 2015). Nonetheless, although the induction of HSPs is part of the stress

Commentato [LA19]: conclusion should be stated more clearly

Commentato [CB20R19]: Cioè è più da introduzione e non da complusioni?

response, contrasting results have been sometimes reported in their relationship with cortisol levels (Basu *et al*., 2001; Iwama *et al*., 2004).

Further studies focusing on assays performed at intermediate timepoints could establish the precise gene expression modulation in terms of stage of development and temperature. Moreover, the expression of genes involved in the lateral muscle development in Siberian sturgeon yolk-sac larvae and maybe the possible disjunction between *Hsp70* gene expression and measurable HSP-70 tissue levels should be examined in depth. Finally, as during the endogenous feeding phase we had no clear results regarding thermal stress, it would be interesting to assess the effect of temperature during the exogenous feeding phase, where larvae may present a stronger stress response. Moreover, tthese data could be used to create models of the early life-history stages of Siberian sturgeon. This could allow identifying key areas that may have a negative impact on the recruitment of this species: Hardy & Litvak (2004) suggest that in long-living species, such as sturgeons, the failing in the recruitment in early stages may be the reason for the population drops. Considering that the Siberian sturgeon is an endangered species, a better understanding of the factors influencing larval growth, such as temperature, might be important for conservation purposes. Indeed, early developmental stages in aquatic organisms are particularly sensitive to environmental variables. Climate changes and anthropogenic environmental alterations are expected to profoundly impact on wild animals, particular in species that characterized by larval development.

Commentato [LA21]: perhaps sum up the major points in this paragraph before discussing further projects - 3 sentences maybe with broad conclusions?

No diventa troppo pesante

Commentato [LA22]: "being interesting is not a strong enough argument for more research - can you make a stronger one

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Contributions: L.A. and A.D.G. designed the project, performed the *in vivo* experimental procedures and the micro-anatomical analyses; D.B. and G.R. performed the cortisol analyses; A.C. and C.B. performed the gene expression analyses; L.A., A.C. and A.D.G. performed the statistical analyses and wrote the paper. All authors contributed to the different draft versions of the manuscript and approved the final manuscript.

Reference list

Airaksinen, S., Jokilehto, T, Råberghm C.M.I. & Nikinmaam 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**, 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.). *Anatomy, Histology and Embryology* **29**, 235-242. doi:10.1046/j.1439-0264.2000.00262.x

Baños, N., Moon, T.W., Castejón, C., Gutiérrez, J. & Navarro, I. (1997) Insulin and insulin-like growth factor-I (IGF-I) binding in fish red muscle: regulation by high insulin levels. *Regulatory Peptides* **68**, 181-187. https://doi.org/10.1016/S0167-0115(96)02118-0.

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**, 25-31. https://doi.org/10.1577/1548-8640(1998)060<0025:FTWHPG>2.0.CO;2

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

535 Basu, N. Nakano, T. Grau, E.G. Iwama G.K., (2001) The effects of cortisol on heat shock
536 protein 70 levels in two fish species, *General and Comparative Endocrinology* **124,** 97–105.

537

538 Bates, L.C., Boucher, M.A. & Shrimpton, J.M. (2014) Effect of temperature and substrate on
539 whole body cortisol and size of larval white sturgeon (Richardson, 1836). Journal of Applied
540 Ichthyology **30**, 1259-1263. https://doi.org/10.1111/jai.12570.

541 Bemis, W.E., Findeis, E.K. & Grande, L. (1997) An overview of Acipenseriformes.
542 *Environmental Biology of Fishes* **48**, 25-71. http://dx.doi.org/10.1023/A:1007370213924

543 Berishvili, G., Shved, N., Eppler, E., Clota, F., Baroiller, J.F. & Reinecke, M. (2006) Organ-
544 specific expression of IGF-I during early development of bony fish as revealed in the tilapia,
545 *Oreochromis niloticus*, by in situ hybridization and immunohistochemistry: indication for the
546 particular importance of local IGF-I. *Cell and Tissue Research* **325**, 287-301.
547 doi:10.1007/s00441-005-0133-9

548 Bertotto, D., Poltronieri, C., Negrato, E., Richard, J., Pascoli, F., Simontacchi, C. & Radaelli,
549 G. (2011) Whole body cortisol and expression of HSP70, IGF-I and MSTN in early
550 development of sea bass subjected to heat shock. *General and Comparative Endocrinology* **174**,
551 44-50. doi:10.1016/j.ygcen.2011.08.003

552 Bjørnevik, M., Beattie, C., Hansen, T. & Kiessling, A. (2003) Muscle growth in juvenile
553 Atlantic salmon as influenced by temperature in the egg and yolk sac stages and diet protein

554 level. *Journal of Fish Biology* **62**, 1159-1175. https://doi.org/10.1046/j.1095-
555 8649.2003.00109.x

556 Boucher, M.A., McAdam, S.O. & Shrimpton, J.M. (2014) The effect of temperature and
557 substrate on the growth, development and survival of larval white sturgeon. *Aquaculture* **430**,
558 139-148. https://doi.org/10.1016/j.aquaculture.2014.03.011

559

560 Campos, C., Fernandes, J.M.O., Conceição, L.E.C., Engrola, S., Sousa, V. & Valente, L.M.P.
561 (2013a) Thermal conditions during larval pelagic phase influence subsequent somatic growth
562 of Senegalese sole by modulating gene expression and muscle growth dynamics. *Aquaculture*
563 **414-415**, 46-55. doi: 10.1016/j.aquaculture.2013.07.039

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

567 Celi, M., Vazzana, M., Sanfratello, M.A. & Parrinello, N. (2012) Elevated cortisol modulates
568 Hsp70 and Hsp90 gene expression and protein in sea bass head kidney and isolated leukocytes.
569 *General and Comparative Endocrinology* **175**, 424-431.
570 https://doi.org/10.1016/j.ygcen.2011.11.037.

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

574 Conte, F.S. (2004) Stress and the welfare of cultured fish. *Applied Animal Behaviour Science*
575 **86**, 205-223. doi:10.1016/j.applanim.2004.02.003

576 Cook, M.A., Lee, J.S., Massee, K.M., Wade, T.H. & Goetz, F.W. (2018) Effects of rearing
577 temperature on growth and survival of larval sablefish (*Anoplopoma fimbria*). *Aquaculture*
578 *Research* **49**, 422-430. https://doi.org/10.1111/are.13473
579 De Jesus, E.G. & Hirano, T. (1992) Changes in whole body concentrations of cortisol, thyroid
580 hormones, and sex steroids during early development of the chum salmon, Oncorhynchus keta.
581 *General and Comparative Endocrinology* **85**, 55-61. doi:10.1016/0016-6480(92)90171-F.

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

586 Dettlaff, T. A., Ginzburg, A. S. & Schmalhausen, O. I. (1993) Chapter 3: Development of
587 Prelarvae. In: *Sturgeon fishes: developmental biology and aquaculture* (Dettlaff, T. A.,
588 Ginzburg, A. S. & Schmalhausen, O. I. eds), pp 155-195. Springer-Verlag Berlin and
589 Heidelberg. http://dx.doi.org/10.1007/978-3-642-77057-9

590 Dabrowski, K., Kaushik, S.J. & Fauconneau, B. (1985) Rearing of sturgeon (*Acipenser baeri*,
591 Brandt) larvae I. Feeding trial. *Aquaculture* **65**, 31-41. https://doi.org/10.1016/0044-
592 8486(87)90268-7

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

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

597 EUMOFA, European Market Observatory for Fisheries and Aquaculture Product (2018)
598 The caviar market last update: Production, trade and consumption in and outside the EU.
599 Luxembourg, Publications Office of the European Union.

600 Chebanov, M.V., & Galich, E.V. (2013). Sturgeon Hatchery Manual, FAO Fisheries and
601 Aquaculture Technical Paper 558, Ankara2013.

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

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

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

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

616 Fuentes, E.N., Valdés, J.A., Molina, A. & Björnsson, B.T. (2013) Regulation of skeletal muscle
617 growth in fish by the growth hormone – Insulin-like growth factor system. *General and*
618 *Comparative Endocrinology* **192**, 136-148. https://doi.org/10.1016/j.ygcen.2013.06.009

619 Gabillard, C., Weil, C., Rescan, P.Y., Navarro, I., Gutiérrez, J. & Le Bail, P.Y. (2003) Effects
620 of environmental temperature on IGF1, IGF2, and IGF type I receptor expression in rainbow
621 trout (*Oncorhynchus mykiss*). *General and Comparative Endocrinology* **133**, 233-242.
622 https://doi.org/10.1016/S0016-6480(03)00167-9

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

626 Gessner, J., Kamerichs, C.M., Kloas, W. & Wuertz, S. (2009) Behavioural and physiological
627 responses in early life phases of Atlantic sturgeon (*Acipenser oxyrinchus* Mitchill 1815)
628 towards different substrates. *Journal of Applied Ichthyology* **25**, 83-90.
629 https://doi.org/10.1111/j.1439-0426.2009.01246.x

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

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

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

638 Gisbert, E., Williot, P. & Castello´-Orvay, F. (1999) Behavioural modifications in the early life
639 stages of Siberian sturgeon (*Acipenser baeri*, Brandt). *Journal of Applied Ichthyology* **15**, 237–
640 242

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

644 Glass, D.J. (2003) Molecular mechanisms modulating muscle mass. *Trends in Molecular*
645 *Medicine* **9**, 344–350. https://doi.org/10.1016/S1471-4914(03)00138-2

646 Glass, D.J. (2005) Skeletal muscle hypertrophy and atrophy signaling pathways. *The*
647 *International Journal of Biochemistry and Cell Biology* **37** 1974–1984.
648 https://doi.org/10.1016/j.biocel.2005.04.018

649 Green, B.S. & Fisher, R. (2004) Temperature influences swimming speed, growth and larval
650 duration in coral reef fish larvae. *Journal of Experimental Marine Biology and Ecology* **299**,
651 115-132. https://doi.org/10.1016/j.jembe.2003.09.001.

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

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

659 Hung, S.S.O., Lutes, P.B., Shqueir, A.A. & Conte, F.S. (1993). Effect of feeding rate and water
660 temperature on growth of juvenile white sturgeon (*Acipenser transmontanus*). *Aquaculture*.
661 **115**, 297-303. 10.1016/0044-8486(93)90144-N.

662 Hwang, P.P., Wu, S.M., Lin, J.H. & Wu, L.S. (1992) Cortisol content of eggs and larvae of
663 teleosts. *General and Comparative Endocrinology* **86**, 189-196

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

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

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

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

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

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

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

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

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

688 Ma, K., Mallidis, C., Bhasin, S., Mahabadi, V., Artaza, J., Cadavid, N.G., Arias, J. & Salehian,
689 B. (2003). Glucocorticoid-induced skeletal muscle atrophy is associated with upregulation of
690 myostatin gene expression. *American Journal of Physiology, Endocrinology and Metabolism*
691 **285**, E363-E371. https://doi.org/10.1152/ajpendo.00487.2002

692 Marlowe, C., Caipang, A., Fagutao, F.F., Fatira, E., Lazado, C.C., Pavlidis, M. (2015) Cortisol
693 levels and expression of selected stress- and apoptosis-related genes in the embryos of Atlantic
694 cod, Gadus morhua following short-term exposure to air.*International Aquatic Research* **7**,
695 75-84. https://doi.org/10.1007/s40071-015-0094-x

696 Mayfield, R.B. & Cech, J.J. (2004) Temperature Effects on Green Sturgeon Bioenergetics.
697 *Transactions of the American Fisheries Society* **133**, 961-970. DOI:10.1577/T02-144.1

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

701 Moyes, C.D. & West, T.G. Exercise metabolism of fish. In: Hochachka and T.P. Mommsen
702 (Eds.), Biochemistry and Molecular. Biology of Fishes, Vol. 4, Elsevier Science, Amsterdam,
703 1995, pp 367–392.

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

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

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

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

714 Philip, A.M. & Vijayan, M.M. (2015) Stress-immune-growth interactions: cortisol modulates
715 suppressors of cytokine signaling and JAK/STAT pathway in rainbow trout liver. PLOS ONE
716 **10**, e0129299. https://doi.org/10.1371/journal.pone.0129299

717 Piccinetti, C. C., Grasso, L. , Maradonna, F. , Radaelli, G. , Ballarin, C. , Chemello, G. , Evjemo,
718 J. O., Carnevali, O. & Olivotto, I. (2017), Growth and stress factors in ballan wrasse (*Labrus*
719 *bergylta*) larval development. *Aquaculture Research* **48**, 2567-2580. doi:10.1111/are.13093

720 Radaelli, G., Domeneghini, C., Arrighi, S., Bosi, G., Patruno, M. & Funkenstein, B. (2003)
721 Localization of IGF-I, IGF-I receptor, and IGFBP-2 in developing *Umbrina cirrosa* (Pisces:
722 Osteichthyes). *General and Comparative Endocrinology* **130**, 232-244. doi:10.1016/S0016-
723 6480(02)00609-3

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

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

731 Ruban, G.I. (1997) Species structure, contemporary distribution and status of the Siberian
732 sturgeon *Acipenser baerii*. In: *Sturgeon Biodiversity and Conservation Developments. Environmental Biology of Fishes* (Birstein V.J., Waldman J.R., Bemis W.E. eds). pp 221-230.
734 Springer, Dordrecht. https://doi.org/10.1007/0-306-46854-9_12

735 Sampath-Kumar, R., Byers, R.E., Munro, A.D. & Lam TJ (1995) Profile of cortisol during the
736 ontogeny of the Asian seabass, *Lates calcarifer*. *Aquaculture* **132**, 349–359

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

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

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

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

751 Stouthart, A.J., Lucassen, E.C., Van Strien, F.J., Balm, P.H., Lock, R. & Wendelaar Bonga,
752 S.E. (1998) Stress responsiveness of the pituitary-interrenal axis during early life stages of

753 common carp (*Cyprinus carpio*). *The Journal of Endocrinology* **157**,127–137.
754 DOI:10.1677/joe.0.1570127.

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

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

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

766 Weatherley, AH (1990) Approaches to understanding fish growth. *Transactions of the*
767 *American Fisheries Society* **122**, 784-796. https://doi.org/10.1577/1548-
768 8659(1993)122<0784:TAOFBM>2.3.CO;2

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

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

775 Wright, W.E., Sassoon, D.A., Lin, V.K. (1989) Myogenin, a factor regulating myogenesis, has
776 a domain homologous to MyoD. *Cell* **56**, 607-617. https://doi.org/10.1016/0092-
777 8674(89)90583-7.

778 Yeh, C., Gloeck, M., & Ryu, S. (2013). An Optimized Whole-Body Cortisol Quantification
779 Method for Assessing Stress Levels in Larval Zebrafish. *PloS one*. *2013*.
780 DOI:10.1371/journal.pone.0079406.

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