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RESEARCH PAPER

# Morphological Differences of White Muscle Fibers and Genetic Diversity of Fast and Slow Growing Atlantic Sturgeons (*Acipenser oxyrinchus*)

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**Abstract**

The aim of this study was to establish causes of variation in body weight of Atlantic sturgeons reared for reintroduction program. Substantial disproportion of individuals released into freshwaters may influence reintroduction success. Histological analysis of white muscle was conducted due to large share of this tissue in body weight. Comparison of morphological structure of white muscle tissue showed significant statistical differences between the fast and the slow growing sturgeons. Mosaic hyperplasia (MH) was observed in white muscle tissue in both sturgeon groups, but the number of proliferating myonuclei was significantly higher in slow growing individuals. Genetic analysis applying 16 microsatellite loci revealed comparable levels of polymorphism and assigned the studied individuals into kin groups consisting of both fast and slow growing sturgeons. The study revealed that the slow growing group had higher growth potential in further stages of development, but the fast growing sturgeons based on the histological analysis of muscle fiber distribution were more advanced in muscle tissue growth. Moreover, difference in growth rate could be caused by complex polygenic inheritance of the trait.

*Keywords*: Atlantic sturgeon, growth, white muscle fibers, microsatellite DNA.

## Introduction

Muscle tissue constitute up to 60 - 80% of fish body weight, and have therefore major impact on size and growth rate of fish (Johnston, Bower & Macqeen, 2011). The continuous development of muscle tissue is caused by proliferation of myogenic precursor cells (MPCs) (Brodeur, Peck & Johnston, 2002). Two processes are characteristic for muscle growth and development – hyperplasia and hypertrophy. In myogenesis two types of hyperplasia can be distinguished: stratified hyperplasia (SH), and mosaic hyperplasia (MH) (Johnston *et al*., 2011). SH, the step of myogenesis occurring after myoblast fusion is observed during embryogenesis, and shortly after hatching (Steinbacher, Haslett, Sänger & Stoiber, 2006; Valente *et al*., 2013). MH, a step following SH, is characterized by presence of muscle fibers of various sizes (Rescan, 2005; Johnston *et al*., 2011). Proliferation of somatic cells, including MPCs, can be detected using antibodies directed against proliferating cell nuclear antigen (PCNA). Synthesis of PCNA is characteristic during S phase of the cell cycle.

Growth is a quantitative trait, under polygenic control, with the most important influence on the somatotropic axis and myogenic transforming growth factor superfamily (Valente *et al*., 2013; Yue, 2013). Identification of the major quantitative trait loci (QTLs) or genes affecting growth is performed usually by linkage map construction and QTL mapping or in association studies and candidate gene approaches (De-Santis & Jerry, 2007; Yue, 2013). Heritability of growth is usually moderate to high ($h^2$=0.2 to 0.7), resulting in correlations of growth rates between closely related individuals (Ma, Wang & Lei, 2009). It is generally accepted that the loss of genetic diversity may result in inbreeding depression leading to fitness decline often manifested by decrease of growth rate (Borrell *et al*., 2011 and references therein). Genetic polymorphism and kinship can be easily measured using microsatellite loci that are commonly used as markers in fish restitution and conservation programs including sturgeons (Panagiotopoulou *et al*., 2012; Waldman *et al*., 2013; O'Leary, Dunton, King, Frisk & Chapman, 2014; Popović *et al*., 2014).

One of the most important aspects of Atlantic

sturgeon (*Acipenser oxyrinchus*) breeding is to define causes of large variation in sizes of individuals from the same cohort. Atlantic sturgeon, in contrast to other sturgeon species commonly breed in aquaculture, is difficult to rear, due to specific nutritional requirements and high mortality of larvae and juveniles (Piotrowska, Szczepkowska, Kozłowski, Wundrelich & Szczepkowski, 2013). This species is currently under restitution in Poland as its native population became extinct in the '60s of the last century. Based on population genetic studies, Atlantic sturgeons from St. John River (Canada) were chosen for the restitution purposes in Polish waters (Kolman, Kapusta, Duda & Wiszniewski, 2011; Popović *et al*., 2014). Since 2004, fry has been produced through artificial spawning of wild sturgeons, reared under artificial conditions and released into Polish waters (Kolman *et al*., 2011).

As slow growing individuals exhibit often lower fitness, disease resistance and survival rate that cause elimination from stocks (Janhunen, Kause & Järvisalo, 2012 and references therein), abilities of Atlantic sturgeon fingerlings to adapt to natural conditions might be lower for individuals with lower body weight and length. Therefore, it is essential to recognize the most important causes of this phenomenon, to minimalize the differences in growth rate. Main factors affecting fish growth described so far are: environmental conditions (Szczepkowski, Szczepkowska & Piotrowska, 2011), exercise training (Martin & Johnston, 2006), diet, including quality and availability of food (Ostaszewska, Dąbrowski, Wegner & Krawiec, 2008; Kamaszewski, Prasek, Ostaszewska & Dąbrowski, 2014) and genetic background (Valente *et al*., 2013).

During rearing of Atlantic sturgeon fingerlings, presence of large disproportions in total body length and weight between individuals belonging to the same cohorts were recorded. Aiming to compare individuals with highly diverse body weight, comparative histological analysis of white muscle tissue were performed. Additionally, genetic polymorphism and kinship of fast and slow growing sturgeons were characterized, in order to verify, if these genetic parameters correlated meaningly with the observed phenomenon.

## Materials and Methods

### Fish Rearing and Sampling Conditions

Fingerlings of Atlantic sturgeons, obtained from multiple crossing of two females with three males, were provided by the Inland Fisheries Institute in Olsztyn. All individuals used in the experiment were kept under the same rearing conditions. Larvae were reared in recirculatory tanks with two inflow points. Initial stocking density was 2000 larvae per $m^2$ of the tank bottom. Subsequently stocking density was reduced to 1000 individuals per $m^2$to average body weight of 0.5g, 750 individuals per $m^2$ to average body weight of 2-3g and 400 individuals per $m^2$ to average body weight of 20g. Water temperature was 18±0.5°C, and oxygen content was maintained above 7 mg/$dm^3$ (70%). Beginning from $9^{th}$ day post hatching (dph) larvae were feed brine shrimp (*Artemiasalina)* nauplii at the level of 30% of fish biomass per day. From $17^{th}$dph the diet was gradually switched to commercial extruded starter for marine fish, Perla Larva Proactive 6.0 (Skretting, Norway). When average body weight of fish reached 0.5gthe feed was subsequently switched to Nutra (Skretting, Norway). Daily feeding rate and granulation was set according to Kolman (2008). The survivability of sturgeons over the first $25^{th}$dph was 58.7%, after that period mortality were observed occasionally.

At $200^{th}$ dph slow and fast growing fingerlings were sampled for subsequent analyses. Sturgeons were anaesthetized using MS-222 **(e**thyl 3-aminobenzoate methanesulphonate, Sigma-Aldrich, St. Louis, MO, USA). Animals were measured (total length with ±0.1 mm accuracy and weight with ±0.001 g accuracy). Individuals were assigned to slow or fast growing group based on body weight: fast growing 10–25 g and slow growing 0.5–5 g.

### Histological and Immunohistochemical Analysis

White muscle tissue samples of 10 fast and 10 slow growing fish were collected from epaxial and caudal region according to Valente *et al*. (1999). Samples were preserved in Bouin's Fixative and subjected to standard histological procedures. The paraffin preparations were cut transversely into series of 5 µm thick sections using the Leica RM2265 microtome (Leica Microsystems, Wetzlar, Germany). Sections were stained with H&E, and trichrome Masson staining (Sigma Aldrich, St. Louise, USA) according to manufacturer recommendations.

Proliferation of MPCs was detected based on immunohistochemical reaction for PCNA with anti-PCNA antibody, 1:300(clone PC10, DAKO, Gdynia, Poland) according to Ostaszewska *et al*. (2008).

Morphometric analysis involved: the area of white muscle single fiber area (FA), the number of white muscle fiber per $1mm^2$ (FN) and the total number of white muscle nuclei per $1mm^2$ (NN)carried out in the right, upper quadrant of white muscle tissue. To determine the studied parameters $500^{th}$ measurements were carried out. The number of PCNA positive nuclei were counted per $1mm^2$ of white fiber muscles. Measurements were estimated using Nikon Eclipse 90i with Nikon Digital Sight DS-U1 camera.

### Statistical Analysis

Total length and body weight were visualized as minimum, $1^{st}$ quartile, median, $3^{rd}$ quartile and maximum values. Total length and body weight were

compared between fast and slow growing sturgeons using non-parametric Mann – Whitney's test. Variables in morphometric results were analyzed using the software STATISTICA 10.0 (StatSoft, Tulsa, OK, USA). Distribution of white muscle fibers into classes in terms of FA was illustrated by histograms. The difference in white muscle fibers distribution within and between sturgeon groups was estimated by Pearson Chi-square test. For all statistical tests, the significance level was set to $P \leq 0.05$.

### Genetic Analysis

Fin-clips, originating from the same specimens subjected to the histological and immunohistochemical analysis, were collected and preserved in 75% ethanol. DNA extractions were performed using Wizard SV96 Genomic DNA Purification System Kit (Promega). Fish genotyping was based on 16 microsatellite loci (Supplemental Table S1) amplified in four multiplex PCR reactions as described in Panagiotopoulou, Popović, Zalewska, Węglański and Stanković (2014). Allele length reads were conducted using ABI PRISM3730 Genetic Analyzer, binning and scoring with PeakScanner Software v.1.0 (Applied Biosystem).

Genetic polymorphism indices of the fast and slow growing sturgeon groups were estimated applying GenAlEx v.6.501 (Peakall & Smouse, 2012), Arlequin v.3.5.1.2 (Excoffier & Lischer, 2010), Genepop v.4.0.10 (Raymond & Rousset, 1995) and FSTAT v.2.9.3.2 (Goudet,1995) softwares. The combined over loci non-exclusion probability of a parent pair (NE-PP) of the analyzed set of individuals was calculated in Cervus v.3.0 software (Kalinowski, Taper & Marshall, 2007). Relatedness between the studied fish was attributed manually based on the allelic profiles of parental individuals. Only individuals, whose genotypes were perfectly matching to the fingerling's genetic profiles were considered as true parents. Other adult fish, used for multiple crossing, were additionally checked and excluded as potential parents based on mismatching alleles. The pair-wise genetic distances between individuals were calculated with GenAlEx v.6.501 software and visualized with the same program using Principal Coordinates Analysis (PCoA). Genepop v.4.0.10 software was used to test for significant ($P<0.05$) genic and genotypic differentiation between the two groups of sturgeons, applying the default implemented values.

## Results

### Morphological and Morphometric Analysis

At 200$^{th}$dph fast and slow growing sturgeons revealed significant variation in body weight and total length. Both parameters were significantly higher in fast growing sturgeons (Figure 1).

Histological analysis showed that white skeletal muscle was the dominant muscle tissue in all individuals. Muscle fibers were separated by the connective tissue (endomysium and perimysium) and poorly vascularized. Mosaic pattern of muscle fibers distribution was observed in both analyzed sturgeon groups (Figure 2A, Figure 2B). Range of FA in fast growing sturgeons was 27.04µm$^2$–1494.62µm$^2$ and in slow growing sturgeons 22.00µm$^2$–886.11µm$^2$. Mean FA was higher in fast growing sturgeons however NN and FN was significantly higher ($P \leq 0,05$) in slow growing sturgeons (Table 1). Empirical distribution of FA (divided in seven distribution classes) in both sturgeon groups was statistically different (Figure 3). In fast growing sturgeons' fibers <300µm$^2$ were significantly less abundant and higher participation of fibers >800µm$^2$was observed. In both, fast and slow growing sturgeon groups PCNA-positive myonuclei were observed in white muscle tissue (Figure 2C, Figure 2D). Number of PCNA-positive myonuclei

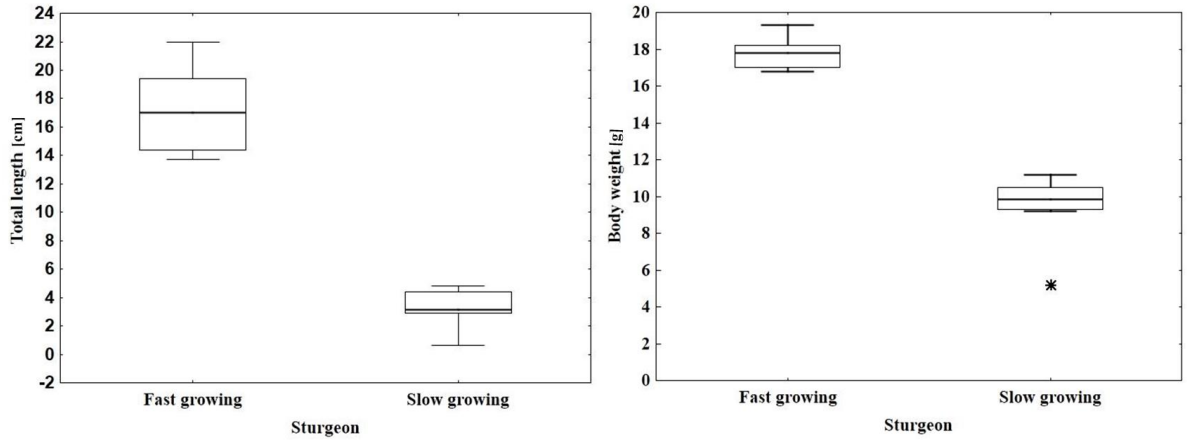


**Figure 1.** Total length (cm) and body weight (g)of fast and slow growing groups of sturgeons visualized as minimum, 1st quartile, median, 3rd quartile and minimum-maximum values. Extreme values were presented with asterisk (greater than three times the interquartile range over the third quartile).

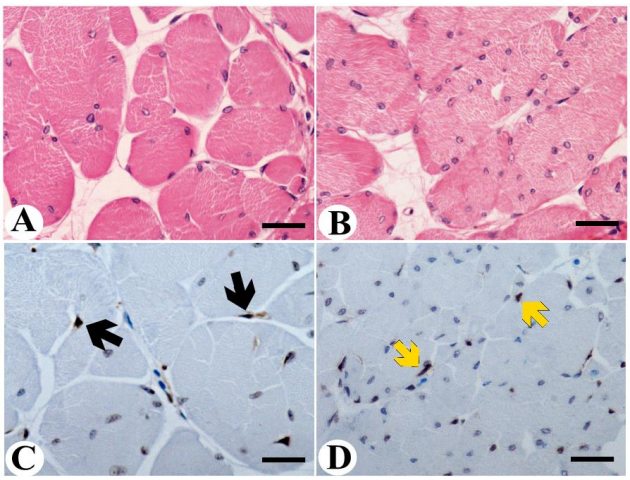


**Figure 2.** Transverse sections of myotomal white muscle in fast (A) and slow (B) growing sturgeons. PCNA-positive myonuclei in fast growing (C – black arrow) and slow (D -yellow arrow) growing sturgeons. A and B Masson Trichromes staining; C and D immunohistochemistry detection of PCNA; Scale bars = 50 µm.

**Table 1.** Histomorphometry of the white muscle fiber mean area (µm$^2$) (FA), total number of myonuclei of white muscle [mm$^2$] (TNN), total number of fibers of white muscle [mm$^2$] (TFN), number of PCNA-positive myonuclei [mm$^2$] (PCNA)of fast and slow growing group of sturgeons. The results were expressed as the mean ± one standard deviation (SD). Statistically homologous groups have been indicated with the same characters (P< 0.05).

| Group | FA | TNN | TFN | PCNA |
|---|---|---|---|---|
| Fast growing | 420.1±62,67µm$^2$ $^a$ | 157,79±17.86$^a$ | 1432.19±160.96$^a$ | 92.38±80.19$^a$ |
| Slow growing | 336.4±41.26 µm$^2$ $^b$ | 207.16±30.31$^b$ | 1778.76±154.33$^b$ | 174.78±91.96$^b$ |

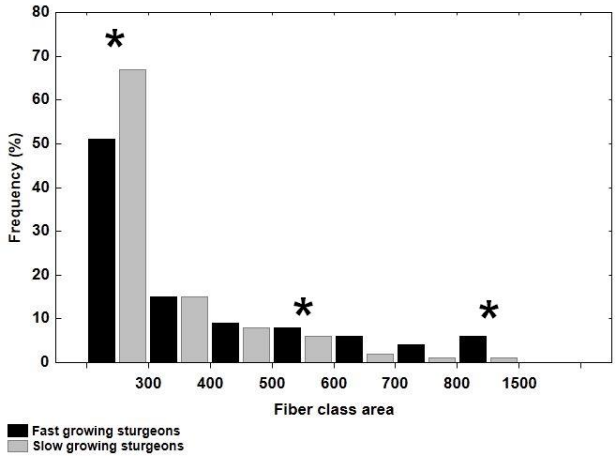


**Figure 3.** Distribution of white muscle fibers into classes in terms of area (µm$^2$) in fast and slow growing sturgeons. Bars represent class of fibers observed in a cross-sections in increasing order. Statistical significant differences were marked with asterisk.

was significantly higher in slow growing sturgeons (Table 1).

## Genetic Diversity and Relatedness

The overall microsatellite loci amplification and scoring success across all specimens was close to 98% (Supplemental Table S1). Altogether, 67 different alleles were identified in the analyzed brood stock across the 16 studied loci. This resulted in sufficient low value of non-exclusion probability of a parent pair (NE-PP=5*10$^{-6}$) and enabled confident parentage analysis, when performing assignments of the candidate parental individuals to the obtained offspring. Both groups, the fast and slow growing individuals, exhibited very comparable level of genetic polymorphism, including allelic diversity (Na and R between 3.5 and 4.0) and heterozygosity (ranging from 0.61 to 0.72) values (Figure 4). The $F_{IS}$ values were negative both in fast and slow growing groups of sturgeons (-0.135and -0.107, respectively), though not significant. The analyzed fishes were

progeny of all five spawners used for reproduction (two females: F1, F2 and three males: M1, M2, M3), resulting in five groups of full siblings (crosses F1xM1, F1xM2, F1xM3, F2xM1and F2xM3) each of them encompassing slow and fast growing fingerlings. PCoA analysis of pair-wise genetic distances grouped the individuals according to their relatedness (Figure 5). In each of the identified kin group both fast and slow growing individuals occurred. The conducted analyzes of genic and genotypic differentiation showed no statistically significant differences between these two groups.

## Discussions

The main aim of the conducted research was to characterize morphometric and morphological differences in white muscle tissue of fast and slow growing Atlantic sturgeons belonging to the same cohort. There is lack of knowledge about sturgeon fingerlings muscle growth and correlation between growth rate and muscle development. Up to date research are focused mainly on myogenesis during embryonic and larval development (Daczewska & Saczko, 2005; Steinbacher *et al*., 2006).

In the analyzed sturgeons, empirical distribution of muscle fiber area was similar to values observed in individuals of other juvenile fish species during their intensive muscle growth phase (Valente *et al*., 1999; dos Santos, Yoshirara, Marcco & Fonseca de Freitas, 2012). Transverse sections of white muscle tissue showed significant number of small muscle fibers in slow and fast growing sturgeons. This suggests that not hypertrophy but hyperplasia was the dominating muscle growth process in both sturgeon groups at 200$^{th}$dph. Formation of a new muscle fibers continues until the fish achieve approximately 40% of final size, and afterwards fibers grow in a volume, not in a numbers (Zimmerman & Lowery, 1999). An average total length of investigated sturgeons was 19.3 cm, which constitutes around 6% of adult Atlantic sturgeons' total length (Mohler, 2003). Muscle growth in sturgeons, similarly to other fish species, is a continuous process although running with different intensity in particular stages of development. Observed variations could therefore represent different phases of muscle growth, as it was shown for rainbow trout, where the fish growth rate was unaligned (Valente *et al*., 1999). Both fast and slow growing Atlantic sturgeon fingerlings ended SH phase of muscle growth while the MH phase was not completed what was revealed by histological and

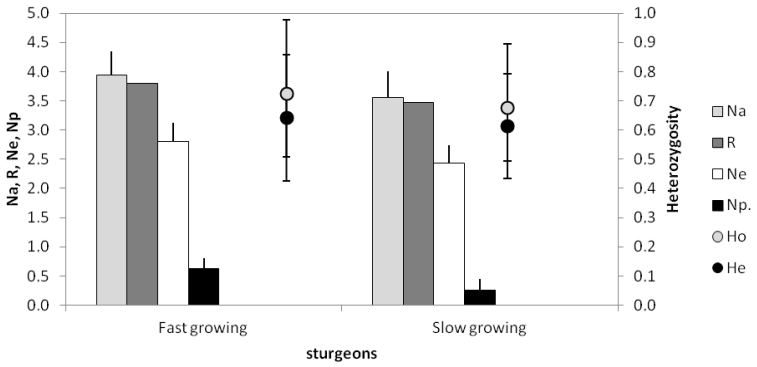


**Figure 4.** Genetic polymorphism indices values of the fast and slow growing groups of sturgeons. Na - the average number of alleles per locus R - allelic richness, Ne - effective number of alleles, Np - number of private alleles, $H_O$ and $H_E$ – observed and- expected heterozygosities.

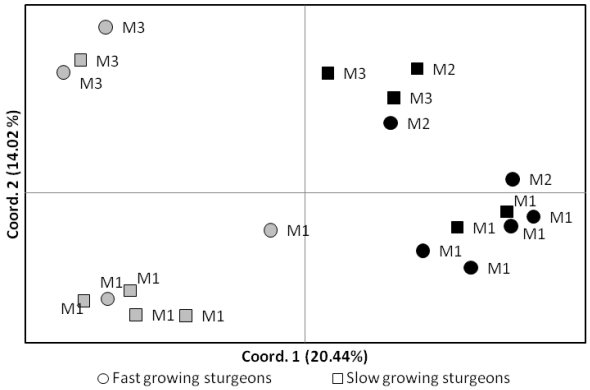


**Figure 5.** Principal coordinates analysis (PCoA) of pair-wise genetic distances between fast and slow growing fingerlings. Black indicators represent progeny of female F1, grey – F2. Symbols M1, M2, M3 indicate progeny of the males used for reproduction.

immunohistochemical analysis. High occurrence of PCNA-positive myonuclei is caused by MPCs activation and intense new muscle fibers formation (Kamaszewski *et al*., 2014). A significant higher number of PCNA-positive myonuclei observed in slow growing individuals confirms intensification of MH growth phase and may reflect on the maintenance of growth potential in the slow growing sturgeons. Similar results were obtained for fast and slow growing rainbow trout (Valente *et al*., 1999) and Nile tilapia strains (dos Santos *et al*., 2012).

The observed variation in morphometric parameters of sturgeons belonging to the same cohort result probably from interaction of genetic, environmental and social factors as it was described for many fish species (Thorpe, 1977). In the presented study rearing conditions were fully controlled and identical for all studied specimens, therefore the observed differences in growth rate resulted mostly from combined genetic factors and social interactions. We did not found though any signs of higher relatedness or elevated levels of inbreeding that could explain the differences in body size of fast and slow growing fingerlings. All analyzed sturgeons exhibited lower levels of genetic polymorphism in comparison to the wild (source) population from St. John River (King, Lubinski & Spidle, 2001; Panagiotopoulou *et al*., 2012; Popović *et al*., 2014). This reduction arose from a small number of breeders used to obtain the offspring under study, however no genetic differentiation between the slow and fast growing group of sturgeons was detected. The risk of inbreeding depression increases during captive breeding programs as a consequence of using small number of breeders for reproduction or due to their unequal reproductive success (Borrell *et al*., 2011). However, different growth rates of the studied sturgeons were likely not caused by inbreeding depression, as the values of $F_{IS}$ of the two compared groups were negative and similar. The microsatellite loci used in this study are assumed to be selectively neutral (King *et al*. 2001; Henderson-Arzapalo & King, 2002). However, as heritability of growth is relatively high, microsatellite markers were used in several studies to assess parental contributions in growth heritability in selective breeding programs. Microsatellites are also an efficient tool in family selection programs, especially when communal rearing techniques are applied (Ma *et al*., 2009; Borrell *et al*., 2011; Karahan *et al*., 2013; Ninh, Thoa, Knibb & Nuyen, 2014). Nevertheless, fast and slow growing individuals were intermixed in the identified kin groups.

The observed significant differences in the length and weight of the analyzed sturgeons may therefore result in first order from high stocking density in the breeding tanks. Several studies carried out on different fish species, including sturgeons, showed that high stocking density causes reduction in growth rate and increases variation of weight between individuals (Justice *et al*., 2009; Szczepkowski *et al*., 2011). As suggested by Biswas *et al*. (2013) high fish density adversely affects the growth rate of fish from the same cohort due to stress and increased interaction between individuals, including competition for access to food. Behavioral experiments of Sbikin and Budayev (1990) as well as Kynard and Horgan (2002) showed that Atlantic and shortnose sturgeons established hierarchy inside the group as a consequence of limited tank space. Bigger, and therefore dominating individuals restricted access to food for smaller sturgeons. Isolation of dominant individuals from the herd was sufficient enough for runt sturgeon recovery (Georgiadis, Hedrick, Johnston & Gardner, 2000). The success of sturgeon restitution program relies on high quality stocking material and it requires optimal breeding conditions development. One of the important tasks, however, is to reduce the domestication effects that favors selection of traits that are not necessary privileged in natural conditions (Williams & Hoffman, 2009). These concern physiological and behavioral changes including smaller brain development in fish reared in captivity. The domestications effects can be substantially reduced when rearing environment simulate natural conditions (Williams & Hoffman, 2009). Minimizing domestication would be important also for the sturgeon restitution project, as it remains unclear, if the fast growing sturgeons reared in unnatural high densities would outperform the slow growing ones in wild environment.

To our knowledge this is the first study comparing the muscle morphology and genetic polymorphism of fast and slow growing sturgeons reared under controlled conditions. Factors causing the observed differences in growth rate, which was manifested in differences in white muscle tissue morphology, may be caused by the genetic background, feeding conditions and social interactions between fishes or the combination of above. To determine which of them has greater impact for growth, more detailed studies using larger sample set and different rearing conditions are needed. An important task includes identification of particular genes or major QTLs influencing phenotypic variation of sturgeon growth. Recognition and controlling the main factors affecting growth will improve sturgeon rearing both for farming and restitution purposes.

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