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Doctoral thesis

Polyploidization capacity of sturgeons and its influence on fitness

Martin Hubálek

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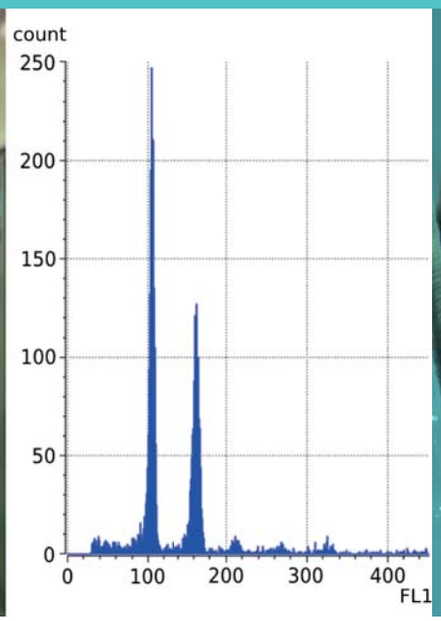

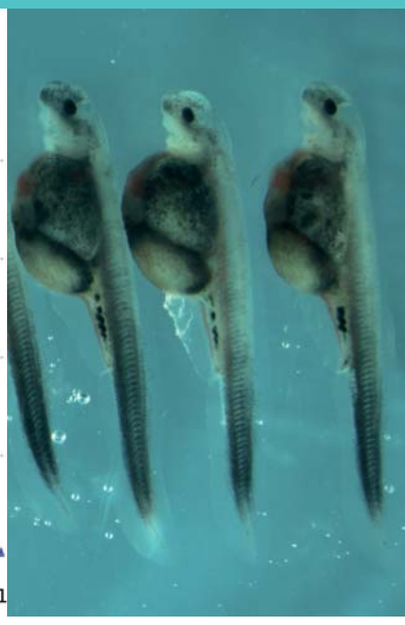

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Supervisor:
Prof. Martin Flajšhans
University of South Bohemia in České Budějovice (USB)
Faculty of Fisheries and Protection of Waters (FFPW)
Research Institute of Fish Culture and Hydrobiology (RIFCH)
Zátiší 728/II, 389 25 Vodňany, Czech Republic

Consultants:
Vojtěch Kašpar, Ph.D.
M.Sc. Tomáš Tichopád
University of South Bohemia in České Budějovice (USB)
Faculty of Fisheries and Protection of Waters (FFPW)
Research Institute of Fish Culture and Hydrobiology (RIFCH)
Zátiší 728/II, 389 25 Vodňany, Czech Republic

Head of Laboratory of Molecular, Cellular and Quantitative Genetics:
Prof. Martin Flajšhans

Dean of Faculty of Fisheries and Protection of Waters:
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CONTENT

CHAPTER 1

GENERAL INTRODUCTION

1.1. Introduction to polyploidy

The lability of genome structure could be found as one of its most surprising and remarkable characteristics. Changes in the genome could vary from small-scale to large-scale. These large-scale changes include polyploidization, which means the increase in genome size resulting from the inheritance of an additional chromosome set or sets (Otto and Whitton, 2000; Otto, 2007). The presence of more than two identical chromosome sets in somatic cells, thus genome multiplication, is called polyploidy (in the case of three sets – triploidy, four sets – tetraploidy, six sets – hexaploidy etc.). Polyploidy appears to be very frequent, especially in the plant kingdom, where it is considered one of the main driving forces of evolution (Wakachaure and Ganguly, 2016; Bourke et al., 2018). However, recent investigations have demonstrated that there is also a large number of polyploids in animals, particularly in fishes and amphibians, in which polyploidy could play an important role for example with regard to patterns of speciation (Le Comber and Smith, 2004; Suda, 2009). Nowadays it is more than clear that polyploids do not necessarily have to become 'evolutionary dead ends' (Soltis et al., 2004; Otto, 2007), as initially hypothesized by Stebbins (1947) and Wagner (1970).

Recent genetic and genomic studies have muddied the terminological waters of polyploidy. There is evidence suggesting that most plants have undergone one or more polyploidization events (Soltis et al., 2004). Moreover, for nonplant lineages, the ancient episodes of polyploidization have been hypothesized as well, for example, two genome-wide duplications events for vertebrates (Ohno, 1970; Sidow, 1996; Spring, 1997). Based on the evidence provided, it is assumed that lineages can undergo repeated cycles of polyploidization followed by extensive genome reduction causing their return to a diploid mode of chromosome pairing (Wolfe, 2001; Albertin and Marullo, 2012). In view of these findings, it seems useful to classify and discuss polyploids in general as neopolyploids and paleopolyploids (Soltis et al., 2004). The term neopolyploid refers to a newly generated polyploid individual which can be also induced through artificial means and is very similar and closely related to extant diploids (or lower polyploids; Ehrendorfer, 1980; Albertin and Marullo, 2012), whereas the genome of paleopolyploid organism was duplicated millions of years ago and the diploid and lower polyploid ancestors are now extinct or have diverged beyond recognition (Ehrendorfer, 1980; Auer and Doerge, 2010). The suitable classification was introduced by Flajšhans et al. (2013), who used the terms 'biological' and 'evolutionary' polyploidy. These authors mentioned that all the organisms with diploid/haploid cycles perceptible in every generation are biological diploids, irrespective of the chromosome number and DNA content in the nuclei of their cells. In this sense, polyploidy means the multiplication of the whole chromosome set over the common number of diploid or haploid sets. If this change resulting from the polyploidization event is fixed in evolution, it can lead to the creation of new taxa having evolutionary polyploid origin. The term evolutionary polyploidy is only meaningful in a phylogenetic context; for example, the genome of common carp may be classified as evolutionary tetraploid (2n = 100), since there is evidence for another species of cyprinids having half the number of chromosomes and, consequently, evolutionary diploid genomes (2n = 48–50) (Flajšhans et al., 2013).

1.1.1. Incidence of polyploidization

Systematists and evolutionary biologists usually distinguish two basic general categories of polyploidy called autopolyploidy and allopolyploidy (Chenuil et al., 1999; Soltis et al., 2007). The creation of autopolyploids is associated with the multiplication of structurally similar, homologous genomes (*AAAA*) within a single species, while allopolyploids arise via hybridization between two different species and subsequent multiplication of nonhomologous

genomes (*AABB*). As a result, autopolyploids show multivalent formation during meiosis, while allopolyploids mainly create bivalents, as nonhomologous chromosomes do not pair (Jackson, 1982; Ramsey and Schemske, 2002). However, worth mentioning is the presence of a considerable range of intermediate types between true autopolyploids and allopolyploids, which previously led to the introduction of more sophisticated classifications (Stebbins, 1947).

While allopolyploidy occurs strictly as a result of interspecific (or intergeneric) hybridization, there are several possible causes of autopolyploidy – e.g. errors during gametogenesis (for example premeiotic endoreduplication of chromosome set followed by two meiotic divisions, suppression/failure of the first or second phase of meiotic division or meiotic chromosome separation failure), anomalous fertilization process (polyspermic fertilization) and changes of cytoskeletal organization in oocytes leading to the suppression of the second phase of meiotic division and retention of the second polar body (Flajšhans et al., 2013). Autopolyploid genomes could arise also from environmental effects causing errors in the process of gamete fertilization (Ihssen et al., 1990; Pandian and Koteeswaran, 1998).

1.1.2. The consequences of polyploidy

Polyploidization leads to dramatic changes in the structure of genome and cell organisation. Newly formed polyploids are forced to face the alterations in cell physiology (e.g. metabolism, growth, stoichiometry), cell cycle processes (e.g. mitoses, meiosis), regulation of gene expression and genome stability (Wertheim et al., 2013; Schoenfelder and Fox, 2015).

The volume of cells and nuclei rises with increasing genome size (Cavalier-Smith, 1978; Gregory, 2001). Therefore, polyploids usually possess larger cells and nuclei than their non-polyploid counterparts (Ihssen et al., 1990; Benfey, 1999), although the exact relationship between ploidy and cell volume can vary among environments or taxa (Mable, 2001). Larger cells tend to have a lower surface-area-to-volume ratios than smaller cells, which is valid also for their nuclei (Cavalier-Smith, 1978; Levin, 1983; Bytyutskyy et al., 2012). The analysis of differences in the size of cells or nuclei is the basic principle of several methods for ploidy determination, for example, an assessment of mean erythrocyte volume using traditional haematological methods (Maxime and Labbé, 2010), analysis of cell volume based on impedance using Coulter counter (Benfey and Sutterlin, 1984), computer-assisted image analysis of the cell main axis length, nuclear area or perimeter (Cormier et al., 1993; Flajšhans et al., 2011). Nevertheless, the cell and nuclei sizes do not always increase linearly with increasing ploidy, as demonstrated by Bytyutskyy et al. (2012), who reported that the DNA in erythrocyte nuclei of sturgeons was more densely packed with the increase in ploidy in series of ploidy levels from 2n to 14n.

Although polyploids typically possess larger cells, their growth performance and overall size may or may not differ from individuals od normal ploidy – it is more frequent that polyploidy increases size in plants or invertebrates than in vertebrates (reviewed in Otto and Whitton, 2000; Gregory and Mable, 2005). Gregory et al. (2000) suggested that elevation of ploidy should directly increase the adult size if there is a fixed number of cells in the adult. However, as widely documented for example in fishes, the increase in cell size led to decreased cell numbers in different organs and tissues, larger cells thus did not provide any growth advantage due to the concomitant decrease in their number (reviewed by Benfey, 1999).

Polyploidy has a bearing on all the genome levels, from the chromosome to the DNA sequence (Leitch and Bennett, 1997). Newly generated polyploid genomes are often highly unstable, which leads to radical reshuffling of genes (Soltis and Soltis, 1995; Song et al., 1995). Therefore, the genome restructuring of polyploids appears to be more rapid and extensive in comparison to non-polyploids (Lagercrantz and Lydiate, 1996; Hu et al., 1998). Some studies

on plants suggested that reorganizations are more extensive if parental genotypes are very different (Song et al., 1995; Liu et al., 1998). Last but not least, the increase of ploidy can affect gene expression patterns and genomic network interactions. It can alter both the dose and the relative expression patterns of all or some genes and cause a 'transcriptomic shock' via changes in the relative gene interactions. Duplicated genes may become silenced and decay or diversify and gain or subdivide functions (Wertheim et al., 2013).

1.1.3. Advantages of polyploidy

As suggested by Comai (2005), polyploidy can provide long-term evolutionary flexibility instead of freezing organisms in a static state. Although polyploidy is a widespread phenomenon, its direct effect on evolutionary success remains unexplained. Nowadays, many attractive hypotheses are trying to clarify the functionality of increased genome content. There is an assumption that polyploid genomes confer unique advantages allowing polyploids to thrive in environments that pose challenges to their diploid progenitors (reviewed by Madlung, 2013).

The first potential advantage of polyploids may be linked to the level of heterozygosity. There is an idea that increased heterozygosity of polyploids can provide flexibility to cope with a broader array of conditions (Otto and Whitton, 2000). As pointed out by Comai (2005), heterozygosity and heterosis decrease in the progeny of a diploid F1 hybrid, whereas the enforced pairing of homologous chromosomes in allopolyploids hinders intergenomic recombination and results in the fixation of the same heterozygosity level through the generations. Autopolyploids are expected to maintain higher levels of heterozygosity than their diploid progenitors, simply due to polysomic inheritance (Moody et al., 1993; Osborn et al., 2003). For example, when assuming simple tetrasomic inheritance, heterozygous autotetraploid with *aabb* genotype is expected to produce progeny in the ratio of 1 *aaaa*:34 heterozygotes (of various genotypes):1 *bbbb*. This ratio differs from diploids with disomic inheritance (i.e., 1*aa*:2*ab*:1*bb*) (Soltis and Soltis, 2000).

The next advantage of polyploidy is conferred by gene redundancy. It is based on the assumption that the number of alleles of a given gene is increased in polyploids, which allows the masking of recessive alleles by dominant wild-type alleles. The masking of recessive mutations may protect polyploids against the loss of fitness (Gu et al., 2003; Rosche et al., 2017). Comai (2005) suggests that this genetic buffering might become particularly important when isolated and severely bottlenecked populations are forced to inbreed, at a time when the purging of deleterious alleles is made difficult by the reduced number of breeding individuals. Is noteworthy that the 'masking advantage' could be transient based on two assumptions: 1) individuals of higher ploidy have also a higher chance of owning a mutation and 2) deleterious mutations persist in populations of higher ploidy level for longer time. On the other hand, the deleterious mutations, which tend to be more effectively masked and more frequent in population of higher ploidy, may become beneficial in response to environmental change (Otto and Whitton, 2000).

The next possible benefit of polyploids is also related to gene redundancy: gene duplicates resulting from polyploidization can evolve to assume new or slightly modified functions (neofunctionalization or subfunctionalization) (Lynch and Force, 2000; Soltis and Soltis, 2000). In polyploids, all genes have a duplicated copy or copies accessible to 'evolutionary experimentation', whereas in diploids, the ability to diversify gene functions depends on the occurrence of rare segmental duplication event (Adams and Wendel, 2005; Wang et al., 2005). The presence of altered gene copies with diversified functions could allow ecological niche expansion or increase flexibility in responsiveness to environmental changes (Adams and Wendel, 2005; Moore and Purugganan, 2005).

1.1.4. Disadvantages of polyploidy

For the sake of completeness, it should be pointed out that polyploids do not necessarily have to get an advantage over their diploid counterparts. As suggested by Arrigo and Barker (2012), polyploidy is a condition that occurs frequently but does not usually lead to a further speciation event, evolutionary success of neopolyploids is possible but not the norm, and recently formed polyploid species have higher extinction rates than their diploid relatives. There are several possible causes.

Changes in the cell architecture in polyploids can be disadvantageous. The alteration of the surface-area-to-volume ratio (as mentioned above) can affect the rate of metabolic processes, especially those in which membranes are involved (Cavalier-Smith, 1978; Levin, 1983). In fishes, the enlarged heads of spermatozoa from polyploid males have more difficulty in passing through oocyte micropyle, which can result in reduced fertility (Chourrout et al., 1986; Blanc et al., 1993).

Another disadvantage of polyploidy may be related to the problems with the mechanics of pairing and separation of chromosomal homologues during mitosis and meiosis, possibly leading to aneuploidy (McCombie et al., 2005). The frequency of occurrence of this phenomenon may differ among species and polyploidy type (Comai, 2005). On the other hand, the sterility resulting from the production of aneuploid gametes causes more energy to be available for somatic growth, which can be economically exploitable as is the case of some artificially induced triploid fishes (Benfey, 1999; Piferrer et al., 2009).

Last but not least, polyploidy alters optimized patterns of gene expression and epigenetic modifications that are inherited from the parents (Hegarty and Hiscock, 2008). Polyploidy causes changes in the regulatory networks that were established and optimized before polyploidization occurred. Therefore, both increasing and decreasing in gene expression is necessary to re-establish transcriptional balance across the genome (Wertheim et al., 2013). Comai (2005) suggested that changes in regulatory networks and output pathways are thought to be deleterious and classified them as disadvantages of polyploidy, however, he also accepted that changes in gene expression can contribute to heterosis and provide variation that might allow adaptation to different conditions.

1.1.5. The fate of polyploids

Newly generated polyploids have to pass through the bottleneck of instability (Mayer and Aguilera, 1990; Comai et al., 2000) before becoming adapted and joining the evolutionary fray as efficient competitors of their diploid counterparts (Comai, 2005). The genomic reorganisations, such as diploidization, may take place to help the polyploid genome to become stable (Wertheim et al., 2013). Duplicated genes could be lost or highly modified during this process, duplicated chromosomes may rearrange and reduce their numbers. Interestingly, diploidization does not have to be simultaneous for all chromosomes or all loci on a particular chromosome. Single species can, for example, harbour a mixture of tetraploid and diploidized loci (Wolfe, 2001).

The probability that new polyploids establish successfully is related to the viability and fertility of new cytotypes, as well as their phenotypic characteristics and tness in various environments (Ramsey and Schemske, 2002). As suggested by Otto (2007), surviving polyploid lineages are only a biased subset of those that have been generated. Polyploidization can result in different evolutionary scenarios. Extinction happens when the genomic mutation level is too high, the effect of mutation on fitness is too severe, and survival or reproduction

is considerably lowered (Otto, 2007; Wertheim et al., 2013). However, if polyploid organisms successfully overcome above mentioned challenges, the formation of new species could be the consequence of polyploidy. The persistence and diversification of polyploid lineages are subsequently influenced by the fitness and long-term evolutionary effects of polyploidization (Wertheim et al., 2013). In some lineages, polyploidy can be a transient state because of chromosomal reorganization and gene silencing (diploidization) (Soltis and Soltis, 1999). Nonetheless, if duplicated genes are not lost, they can evolve independently and acquire specialized functions, possibly increasing the complexity of the species and enhancing the potential for fitness improvement (Pikaard, 2001).

1.2. Occurrence of polyploidy in fishes

It is generally believed that the ancestral vertebrate genome underwent two rounds (addressed 1R and 2R) of whole-genome duplications (WGDs) and that additional WGD took place in teleost fishes (addressed 3R or teleost-specific WGD – TSGD) (Lynch, 2002; McLysaght et al., 2002). As the result of these polyploidization events, the majority of diploid genomes in ray-finned fishes consist of 48 or 50 chromosomes (Ohno et al., 1968; Mank and Avise, 2006). However, additional WGDs occurred in several teleostean (as well as in non-teleostean) lineages and resulted in changes in chromosome counts and polyploidy. Karyotype analyses help reveal the ancient polyploidizations and polyploid origin of inspected lineages (Braasch and Postlethwait, 2012).

Polyploidization events in fishes seem to be phylogenetically restricted, that is, unevenly distributed across the actinopterygian tree. With few exceptions, polyploidy appears to be a widespread phenomenon among early diverging teleosts, occurring independently and often repeatedly in many groups of these fishes. On the contrary, more derived lineages (for example percomorphs) look like having only a few or no genome duplications after TSGD (Leggatt and Iwama, 2003; Mable et al., 2011).

It is not too surprising that the highest number of evolutionary polyploid fish species – over 400 – could be found in the species-rich subfamily Cyprininae (Arai, 2011; Yang et al., 2015). However, polyploidy occurs naturally also in other fish lineages such as Acipenseriformes (Birstein et al., 1997), Salmoniformes (Allendorf and Thorgaard, 1984) and Catostomidae (Uyeno and Smith, 1972) – altogether, these lineages contain less than 250 polyploid species (Yang et al., 2015). Almost simultaneously with the discovery of natural polyploidy in fishes, the first attempts to induce polyploidy artificially were being made. Nowadays, it may be concluded that polyploidy can be experimentally induced in almost all fishes, and the polyploids of some fish species can be used in aquaculture for their desirable features (Piferrer et al., 2009).

The presence of polyploidy in some fishes along with the possibility of its quite easy artificial induction indicates that fishes can be a useful model for polyploid research (Le Comber and Smith, 2004). Especially one group of fish represents suitable candidates for studying this phenomenon because of its ancient, paleopolyploid origin, enormous ploidy diversity and well-preserved predisposition to polyploidization – sturgeons (Havelka et al., 2016).

1.3. Polyploidy in sturgeons

1.3.1. Polyploidization events in sturgeons

As suggested by Mable et al. (2011), the susceptibility to polyploidization is higher in the case of the combination of the following factors: production of large numbers of gametes, external fertilization, community breeding, propensity to hybridization, and environmental variability during the breeding season. Trifonov et al. (2016) highlighted that all these conditions are presented in sturgeons which helps to explain their tendency to create polyploid genomes. The researchers dealing with this issue agree that sturgeons, although not participating in TSGD, underwent numerous genome duplication events during their evolution (e.g. Ludwig et al., 2001; Birstein et al., 2005; Fontana et al., 2007). Nevertheless, the number of these events remains unclear and full of conflicting options (Havelka et al., 2011).

Fontana et al. (2007) hypothesized that three polyploidization events took place during the evolution of sturgeons. They suggested that the first genome duplication occurred 200 million years ago and increased the chromosome number from 2n = 60 (Birstein et al., 1997; Fontana et al., 2007) to 4n = 120. Newly generated tetraploids underwent diploidization and the diploids with 120 chromosomes evolved into different species. These species hybridized and some of the hybrids participated in second genome duplication and became 240-chromosome species, this process might have independently occurred several times. The hybridization between species with 120 and 240 chromosomes followed by third genome duplication may be responsible for the creation of species with 360 chromosomes – shortnose sturgeon (*Acipenser brevirostrum*) (Fontana et al., 2007).

Similarly to Fontana et al. (2007), Vasil'ev et al. (2010) proposed three polyploidization events – one in the common ancestor of the Pacific species group and two in the Atlantic species group. Both studies accepted that even more events might occur in the evolution of sturgeon species. On the other hand, Ludwig et al. (2001) suggested the presence of four polyploidizations. The characterization of the karyotype of shortnose sturgeon, which has been reported by Kim et al. (2005), has increased the number of events in this scenario to five (Vasil'ev et al., 2010). Birstein et al. (2005) recognized seven polyploidization events within Acipenseridae, however, as suggested by Vasil'ev et al. (2010), reclassification of kaluga (*Huso dauricus*) karyotype entailed that eight polyploidization events should be assumed in conformity with this hypothesis. Five polyploidization events were hypothesized by Peng et al. (2007), but the more recent investigations of chromosome numbers in kaluga and Sakhalin sturgeon (*A. mikadoi*) (Vasil'ev et al., 2010) further indicated that this theory needs to be revised as well.

One of the most actual works about this issue, the study of Rajkov et al. (2014), accepted all the reclassifications of karyotypes reported to date. The findings of the previously mentioned authors support the polyploidization scenario proposed by Vasil'ev et al. (2010), according to which three or more events occurred – two in the Atlantic group species and one in the Pacific group species. The conclusions of Rajkov et al. (2014) are summarized in Figure 1.

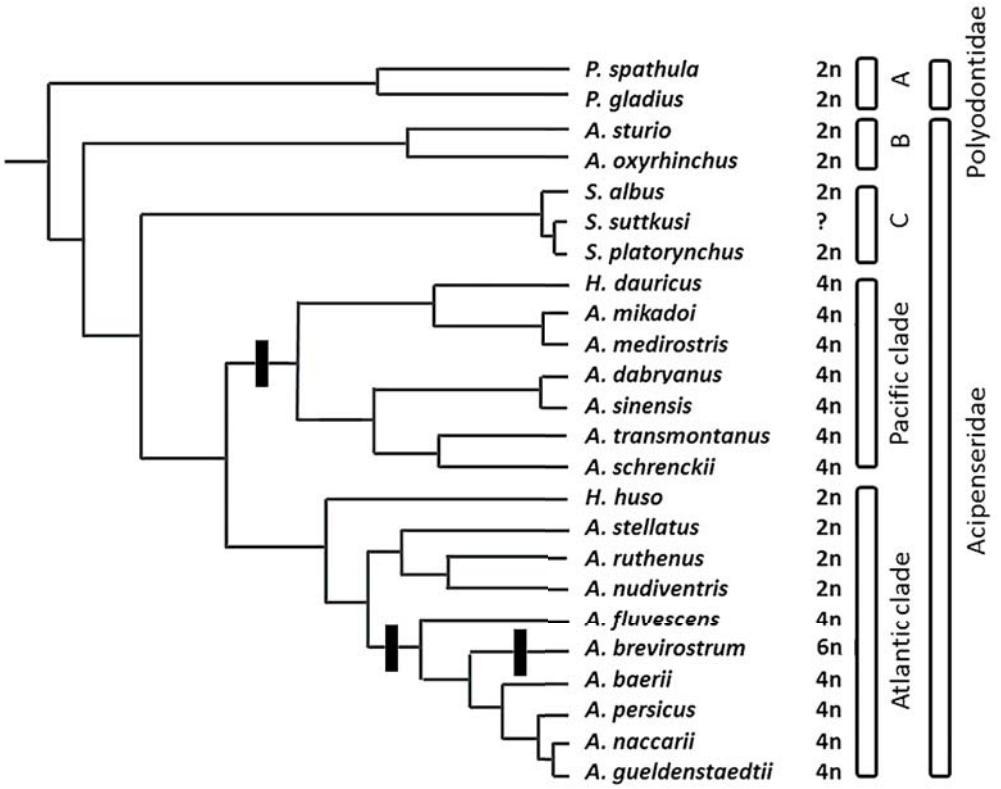

Figure 1. *Phylogenetic tree of Acipenseridae with dedicated duplication events (black filled vertical bars). A = genus Pseudoscaphirhynchus, B = sea sturgeon clade, C = genus Scaphirhynchus. Ploidy levels belonging to individual species listed in functional ploidy scale. The figure modified from Rajkov et al. (2014), who edited it from Peng et al. (2007).*

The origin of polyploidy in sturgeons is not entirely clear as well. It is generally believed that interspecific hybridization has played a key role, and so the allopolyploidy looks like a very probable scenario in sturgeons (Vasil'ev, 2009). However, the number of evidence of spontaneous autopolyploidy in sturgeons is still getting higher which is a little bit disturbing for this theory (see Chapter 1.3.3.). Concerning these observations, some authors emphasise that autopolyploidy cannot be excluded from consideration (Trifonov et al., 2016; Fontana et al., 2008).

1.3.2. Karyology and ploidy levels in sturgeons

As mentioned above, the order Acipenseriformes represents a group where independent genome duplications repeatedly occurred (Ludwig et al., 2001; Havelka et al., 2011). As a result of these events, current representatives of sturgeons are separable into three well-divided groups differing in chromosome numbers presented in their somatic cells: species with ~120 chromosomes (including all taxa with 110–130 chromosomes; Group A); species with ~250 chromosomes (including all taxa with 220–276 chromosomes; Group B); and a single species having ~360 chromosomes (Group C) (Ludwig et al., 2001; Kim et al., 2005). As mentioned by Mank and Avise (2006), the number of chromosomes is directly

correlated with genome size, which means that the groups differ also in the amount of DNA in their cell nuclei, as confirmed by flow cytometric data: Group A exhibits DNA content 2.5–4.9 pgDNA nucleus^{-1}, Group B 7.8–8.9 pgDNA nucleus^{-1} (Birstein et al., 1993; Blacklidge and Bidwell, 1993) and Group C 13.08 pgDNA nucleus^{-1} (Blacklidge and Bidwell, 1993). Havelka et al. (2011) have summarized the clustering of some sturgeon species based on karyological data available: Group A – shovelnose sturgeon (*Scaphirhynchus platorynchus*), ship sturgeon (*A. nudiventris*), beluga (*Huso huso*), European Atlantic sturgeon (*A. sturio*), sterlet, stellate sturgeon (*A. stellatus*), American paddlefish (*Polyodon spathula*), North American Atlantic sturgeon (*A. oxyrinchus*); Group B – Siberian sturgeon, Adriatic sturgeon (*A. naccarii*), white sturgeon, Sakhalin sturgeon, Russian sturgeon (*A. gueldenstaedtii*), green sturgeon (*A. medirostris*), Persian sturgeon (*A. persicus*), lake sturgeon (*A. fulvescens*), Chinese sturgeon (*A. sinensis*), kaluga; Group C – shortnose sturgeon.

It is obvious that the karyotypes of the Acipenseridae members are relatively large when compared to karyotypes of other fish species (Vasil'ev, 2009), and exhibit a considerable level of diversity. Furthermore, sturgeons are very prone to interspecific hybridization which results in the creation of hybrids with intermediate karyotypes to those of parental species (Gorshkova et al., 1996). Another peculiarity of sturgeon karyotypes is the presence of a high number of microchromosomes (Birstein et al., 1997), whose difficult identification was the main source of errors at the beginning of karyotype studies on these fishes – as was the case, for example, in sterlet or beluga (Serebryakova, 1972). Taken together, all these features contribute to the complicated interpretation of results of karyological studies and inconsistent opinions about ploidy levels of sturgeon species, as mentioned by Havelka (2013).

In the field of sturgeon genetics, the ploidy levels of current representatives appear to be a controversial topic. Based on the studies published so far, two ploidy classifications can be taken into consideration: the evolutionary and functional scale of ploidy levels (Havelka, 2013). The evolutionary scale supposes tetraploid-octoploid-dodecaploid relationships (4n-8n-12n) for species from Group A, B, and C, respectively, while the functional scale assumes diploid-tetraploid-hexaploid relationships (2n-4n-6n) (Vasil'ev, 2009). In the past, there were divergent opinions concerning ploidy levels even in studies that used the same general methods (Ludwig et al., 2001). For example, Birstein and Vasiliev (1987) suggested tetraploidy for species from Group A and octaploidy for Group B based on the analysis of stained active nuclear organizer regions (NORs). However, Fontana (1994) and Fontana et al. (1998), who investigated NORs as well, concluded that species from Group A should be classified as diploids and these from Group B as tetraploids. The studies of expressed gene products have not reached the same conclusion either: Birstein et al. (1997) observed duplicated protein loci in species from Group A and, consequently, suggested tetraploidy for them, whereas Kuzmin (1996) assigned diploidy to the same group based on the densitometric study of serum albumins. As pointed out by Havelka et al. (2011), new insight on the issue was enabled by methods of molecular biology. Ludwig et al. (2001) suggested that in vitro amplification of polymorphic nuclear markers, such as microsatellite loci, permits a direct view of the genome, and used this method for their research. They concluded that the distribution of alleles within microsatellites showed an ongoing process of functional genome copy reduction in sturgeons and pointed out that this process was nearly finished in Group A species and more active in groups of species with higher chromosome numbers. Based on the allelic patterns obtained, Ludwig et al. (2001) accepted the functional scale of ploidy levels in sturgeons. In one of the most recent works dealing with this issue, Rajkov et al. (2014) analysed the maximum number of alleles at 20 microsatellite loci per individual while working with 10 sturgeon species. They confirmed that functional diploidization is an ongoing process in sturgeons and observed also various levels of this phenomenon in different sturgeon species. Based on this

finding, they respected both the scales of ploidy classification – evolutionary and functional. The similar conclusion was provided also by Romanenko et al. (2015) whose localization of sterlet chromosome specific probes revealed different ploidy states of extended genomic regions, assuming that both the hypotheses about ploidy levels could be partly correct and the genomes of sturgeons might be more complex than proposed earlier. This theory was also corroborated by Symonová et al. (2017), who studied the karyotype of American paddlefish using a combination of karyological and molecular cytogenetic markers and distinguished chromosome quadruplets or pairs as labelled with different markers. This transient state on the rediploidization way from whole-genome duplication towards diploidy is called segmental polyploidy (Romanenko et al., 2015).

1.3.3. Spontaneous polyploidy in sturgeons

The presence of evolutionary polyploidy and great karyotypic diversity in extant sturgeons are not the only peculiarities related to genome plasticity of these fishes. The high propensity to the creation of spontaneously autopolyploid genome states *do novo* at each generation could be considered as another interesting feature of sturgeons. Until now, the occurrence of this phenomenon has been documented in number of species: European Atlantic sturgeon (Flajšhans, Gessner, Kohlmann, Lebeda, unpublished recent data), Gulf sturgeon (*A. oxyrinchus desotoi*) (Blacklidge and Bidwell, 1993), kaluga (Zhou et al., 2011), lake sturgeon (Blacklidge and Bidwell, 1993), Sakhalin sturgeon (Zhou et al., 2011; Zhou et al., 2013; Havelka et al., 2017), sterlet (*A. ruthenus*) (Zhou et al., 2011; Havelka et al., 2013), Siberian sturgeon, Russian sturgeon (Bytyutskyy et al., 2012), and white sturgeon (*A. transmontanus*) (Schreier et al., 2011). These studies reported spontaneous polyploidy in cultured sturgeons, but the first evidence of its occurrence in the wild has been recently provided (Schreier et al., 2021). Almost all spontaneous polyploids detected to date were biologically triploid (genome size 1.5 times larger than in individuals of normal ploidy), nevertheless, there were observed also spontaneously arisen biological tetraploids (genome size 2 times larger than in individuals of normal ploidy) in Sakhalin sturgeon (Zhou et al., 2011) and sterlet (Hubálek and Flajšhans, unpublished recent data). The hybridization of polyploids with their non-polyploid counterparts could lead to obtaining of progeny with next abnormal ploidy levels (as detailed in 1.3.4.6. Fertility) and it seems that polyploidization can occur again in this progeny as documented in Siberian sturgeon by Havelka et al. (2016). In the light of these findings, Schreier et al. (2021) recommended a series of best hatchery practices to help sturgeon aquaculturists eliminate the incidence of spontaneous autopolyploidy. For clarity of interpretation, biological ploidy will be used in the following section.

The origin of spontaneous triploidization in sturgeons was discussed in some articles (e.g. Omoto et al., 2005; Zhou et al., 2011; Schreier et al., 2013; Havelka et al., 2017). In general, there is a number of natural mechanisms that could be responsible for creation of spontaneous autopolyploidy in fishes: production of unreduced sperm or oocytes through apomixis or premeiotic endomitosis, the penetration of an oocyte by more than one sperm (polyspermy), retention of the second polar body in meiosis II or unequal cleavage or suppression of the first cleavage after fertilization (Dawley, 1989; Piferrer et al., 2009; Nomura et al., 2013). Based on their observations, Schreier et al. (2013) initially hypothesized that spontaneous triploidy in sturgeons may be associated with the male parent. Sturgeons' eggs possess number of micropyles, which theoretically increases the probability of polyspermic fertilization (Cherr and Clark, 1985). However, Pšenička et al. (2010) concluded that creation of cytoplasmatic projection in the sturgeon egg after fusion with the spermatozoon works as effective polyspermy prevention. In spite of this finding, Iegorova et al. (2018a,b) obtained viable

sturgeon progeny after polyspermic fertilization, but this progeny was mosaic (composed of cells of differing ploidy) and not uniformly triploid.

In accordance with recent investigations, the failure in extrusion of the second polar body in meiosis II seems to be the most likely cause of spontaneous autotriploidy (Gille et al., 2015; Havelka et al., 2016; Havelka et al., 2017). The occurrence of this phenomenon causing spontaneous diploidization of the maternal chromosome set could be influenced by ageing of eggs (Omoto et al., 2005; Zhou et al., 2011; Gille et al., 2015), as has been shown not exclusively in sturgeons but also in another fish species (e.g. Yamazaki et al., 1989; Aegerter and Jalabert, 2004; Flajšhans et al., 2007). Prolongation of period between ovulation, stripping and fertilization (which can occur easily during artificial spawning as a logistic delay) could therefore lead to increasing of incidence of spontaneous triploidy in newly generated progeny (Omoto et al., 2005; Gille et al., 2015). However, this may not always be the case, as demonstrated by Schreier et al. (2013) who found no correlations among spawning conditions such as time to ovulation and spawning temperature and the tendency to produce spontaneous autopolyploids in white sturgeon. Van Eenenaam et al. (2020) observed that post-ovulatory ageing increased the incidence of spontaneous polyploidy in progeny obtained from several white sturgeon females, but they overall concluded that the lack of a consistent association between ageing and spontaneous autopolyploidy indicates that post-ovulatory ageing is not the primary cause of second polar body retention in cultured sturgeon. Van Eenenaam et al. (2020) also reported that for nine out of twelve white sturgeon females, the 'vigorous' mixing of eggs during the 60 min de-adhesion treatment caused a significant increase in the proportion of autopolyploid progeny when compared to 'gentle' mixing. Based on this finding, the authors suggested that another mechanism contributing to spontaneous autopolyploidy in culture setting could be mechanical shock, which probably disrupted the spindle apparatus and resulted in second polar body retention. Last but not least, Van Eenennaam et al. (2020) spawned the same female white sturgeon twice with two years gap and detected a high incidence of spontaneously autopolyploid progeny after 'vigorous' stirring for each spawn (80 and 64%), assuming that a genetic predisposition for the production of this progeny could exist in sturgeons (Van Eenenaam et al., 2020). The presence of this predisposition was previously hypothesised by Thorgaard and Gall (1979) or Flajšhans et al. (1993) in other fishes. Further examination is needed to confirm this theory (Schreier et al., 2021).

1.3.4. The role and consequences of polyploidy in sturgeons

Although the polyploidy has played an important role in the evolution of sturgeons and many other fishes, the reason behind this remains unclear (Leggatt and Iwama, 2003). The persisting tendency of sturgeons to create polyploid genomes raises questions about the effect of autopolyploidy on sturgeons´ performance and fitness, whose answering would suggest the possible consequences of spontaneous polyploidization for both cultured and natural populations of sturgeon (Leal et al., 2019, 2020). The viability and growth of early developmental stages of polyploid sturgeons determine the probability of their recruitment into a new population and may be considerably hindered in the systems with altered temperature profiles (Van Eenennaam et al., 2005). However, the effect of unfavourable temperature conditions on these stages of sturgeons' autopolyploids has not been investigated so far. The impact of polyploidy on performance in older individuals has previously received more attention, but the studies were conducted on limited number of sturgeon species. Several studies dealt with the effects of autopolyploidy on sturgeons´ physiology, but they focused solely on North American species, with the vast majority of research conducted on white sturgeon (Leal et al., 2018, 2019, 2020, 2021) and one study conducted on shortnose sturgeon (Beyea et al., 2005). These

studies only examined biologically triploid sturgeons (Beyea et al., 2005; Leal et al., 2019, 2020, 2021), and individuals obtained from hybridization between biological diploids and triploids (evolutionary ploidy 10n, listed as intermediate ploidy in the following text; Leal et al., 2018). Current knowledge regarding haematology of autopolyploid sturgeons is limited to previously mentioned works on white sturgeon and shortnose sturgeon, and one study on Siberian sturgeon (Rożyński et al., 2015). Apart from the effects of polyploidy on physiology and haematology, several studies addressed the impact of biological triploidy on reproductive performance in sturgeons – these were conducted on bester, white sturgeon and Siberian sturgeon (Omoto, 2002, 2005; Schreier et al., 2011; Havelka et al., 2014; Gille et al., 2015). It is worth noting that some of the fitness-related traits of autopolyploid sturgeons have not been examined yet, for example, swimming performance. Broadening the scope of research on polyploid sturgeons is of particular interest since it may help to uncover the possible consequences of spontaneous polyploidization for both sturgeon aquaculture and conservation, and significantly contribute to scientific knowledge about the impact of polyploidy on the fish organism (Lebeda et al., 2020).

To study the effect of polyploidy on sturgeons, it is necessary to obtain a sufficient amount of polyploid material. Artificial induction of polyploidy may help to cover the demands for this kind of research (Beyea et al., 2005; Lebeda et al., 2020). Since sturgeon polyploids are visually indistinguishable from individuals of normal ploidy, the efficiency of polyploidization treatment must be verified using cytological methods, and flow cytometry and Coulter counter has proven to be the most accurate and time-efficient techniques for this purpose (Fiske et al., 2019; Schreier et al., 2021). The utilization of allopolyploids for polyploid research is complicated due to the general difficulty in separating the effects of polyploidy from those of hybridization (Chelaifa et al., 2010). This is not the case with autopolyploids which can be artificially induced by treatments causing fusion of the second polar body or suppression of the first mitotic division (Vassetzky, 1967; Lebeda and Flajšhans, 2015; Lebeda et al., 2020). As mentioned by Le Comber and Smith (2004), work on induced polyploidy suggests the same type of genome rearrangements and ecological consequences as in the case of naturally occurring polyploidy. In the following section, there will be pointed out the characteristics of artificially produced and spontaneous polyploids of sturgeon. For the sake of simplicity, biological ploidy will be used in the following text, unless otherwise indicated.

1.3.4.1. Haematology

Triploid sturgeons and individuals of intermediate ploidy had substantially lowered total erythrocyte counts in comparison to diploids, but their erythrocytes were larger and kept a higher amount of haemoglobin, as was obvious from higher mean erythrocyte volume (MEV) and mean erythrocyte haemoglobin (MEH), respectively (Beyea et al., 2005; Rożyński et al., 2015; Leal et al., 2018, 2019). These observations are in accordance with Benfey´s (1999) general conclusions about the impact of polyploidy on the number and size of somatic cells. Unlike erythrocyte count and dimensions, mean erythrocytic haemoglobin concentration (MEHC) did not seem to be affected by the increase in ploidy, although Rożyński et al. (2015) reported higher MEHC in triploid Siberian sturgeon compared to diploids of the same species. The effect of polyploidy on blood haemoglobin concentrations (Hb) and haematocrit (PCV) appeared to be less predictable than in the case of previously mentioned haematological parameters. While resting triploids of Siberian sturgeon exhibited higher Hb and similar PCV as diploids (Rożyński et al., 2015), resting triploids of shortnose sturgeon had lower Hb and similar PCV as their diploid counterparts (Beyea et al., 2005). Triploids of white sturgeon showed consistently lower levels of both parameters (Leal et al., 2019) compared to diploids

irrespective of water temperature (18, 22 or 24 °C) and exposition to an acute stressor (stressed or non-stressed) (Leal et al., 2019, 2020). Resting white sturgeon of intermediate ploidy had reduced PCV and same Hb when compared to diploids both before and following 6-weeks acclimation to ambient (18 °C) or elevated (22 °C) temperature (Leal et al., 2018). The reported decreases in Hb and/or PCV levels of polyploid sturgeons suggest lowered total oxygen-carrying capacity and may pose a limitation to whole-organism performance. Reduced oxygen-carrying capacity can, together with a decrease in surface-area-to-volume ratio of erythrocytes and nuclei which has been previously reported in triploid sturgeons (Leal et al., 2020), contribute to a reduction in aerobic scope (Leal et al., 2020, 2021).

1.3.4.2. Aerobic scope

Leal et al. (2020, 2021) reported differences in the aerobic scope of diploid and triploid white sturgeon. Three-month-old triploids at both ambient and acutely increased (24 °C) temperature exhibited reduced scope compared to diploids (Leal et al., 2020), and the same trend was observed in 5.5-month-old sturgeons acclimated to ambient temperature (Leal et al., 2021). In the secondly mentioned study, the younger individuals of both ploidies did not differ, so the authors hypothesized that parental inheritance might impact the timing of the manifestation of ploidy-related differences in aerobic scope. Leal et al. (2020, 2021) concluded that the reduced aerobic scope of triploid white sturgeon suggests a reduced capacity to fuel biological processes such as growth, reproduction and activity. The evidence for reduced growth in triploids was simultaneously provided by Leal et al. (2021), whose growth trial with diploid and triploid white sturgeon started 2 months after hatching and lasted 15 weeks. Beginning at about 4 months post-hatch and continuing through about 5.5 months post-hatch, both weights and lengths were lower in triploid white sturgeons, which also exhibited reduced specific growth rate (SGR) and feed efficiency (FE). Moreover, at the end of the 6-weeks trial with 12-month-old white sturgeon, triploids acclimated to ambient or elevated temperature also showed lower weight, length and SGR than diploids (Leal et al., 2019). On the other hand, 14.5-month-old white sturgeons of intermediate ploidy exhibited higher weights and lengths than diploids, and similar trend was, together with higher SGR in intermediate ploidy, observed after 6 weeks-acclimation to ambient or elevated temperature (Leal et al., 2018).

1.3.4.3. Stress response

Plasma cortisol is a useful indicator of primary stress response in fishes (Martínez-Porchas et al., 2009; Ellis et al., 2012) and the effect of increased ploidy on this parameter has been previously investigated in sturgeons. The levels of plasma cortisol in diploid and triploid shortnose sturgeon exposed to an acute stressor of 15-min chasing did not differ 0, 2 and 6 h post-stress (Beyea et al., 2005). Similar concentrations of cortisol in plasma were also observed in triploids and diploids of white sturgeon, which were acclimated to either ambient or elevated temperature and sampled before and 30 min after a 10-min water reduction stress. When kept at ambient temperature, both ploidies exhibited similar kinetics of primary response, as obvious from cortisol levels examined prior to and 15, 30, and 60 min after exposure to a 5- min netting stress (Leal et al., 2019). In white sturgeons of intermediate and normal ploidy, which were kept for six weeks in ambient or elevated temperature, there were observed some nuances in plasma cortisol levels, however, the authors overall concluded that both ploidies appeared comparable in their stress parameters (Leal et al., 2018).

Secondary stress response in fishes arises from increased concentrations of stress hormones (cortisol and catecholamines) in blood and includes changes in haematological parameters and blood chemistry (Wendelaar-Bonga, 1997; Siebel et al., 2021). Remarkable differences in haematological response to acute stress were observed in diploids and triploids of white sturgeon, as triploids exhibited reduced magnitude of change in Hb and PCV levels when subjected to manual chasing at both ambient and acutely increased temperature (Leal et al., 2020). However, similar trend was not observed in other study with diploid and triploid white sturgeon, which were stressed by water reduction (Leal et al., 2019). Additionally, Beyea et al. (2005) reported that diploids of shortnose sturgeon did not differ from triploids in Hb and PCV 0, 2 and 6 h post manual chasing, except for lower Hb observed in triploids 6 h after stress.

Blood chemistry indicators of secondary stress response include plasma osmolality, glucose and lactate (Wendelaar-Bonga, 1997; Siebel et al., 2021). The levels of these parameters did not differ between triploid and diploid white sturgeon after their acclimation to ambient and elevated temperatures and before or after water reduction stress (Leal et al., 2019). Triploidy also did not impact glucose and lactate response to chasing stress in white sturgeon at ambient and acutely increased temperature (Leal et al., 2020). Intermediate ploidy white sturgeon had higher plasma lactate concentration than diploid of the same species prior to acclimation to ambient or elevated temperature, but the differences no longer existed after 6 weeks-acclimation (Leal et al., 2018). In the lastly mentioned study, plasma glucose did not differ between ploidies at any sampling time, as was the case also for diploid and triploid shortnose sturgeon exposed to chasing stress (Beyea et al., 2005). However, triploid shortnose sturgeon exhibited consistently higher plasma osmolalities and chloride ion concentrations than diploids of the same species, which (together with the presence of multiple atypical cells in blood smears of triploids and elevated plasma lactate, although non-significantly) led Beyea et al. (2005) to the conclusion that triploids were chronically stressed.

1.3.4.4. Metabolic enzyme activity

In the articles which dealt with the effects of triploidy and intermediate ploidy on the physiology of white sturgeon, there were investigated differences in lactate dehydrogenase (LDH) and citrate synthase (CS) activities between polyploids and diploids (Leal et al., 2018, 2019). The LDH is a metabolic enzyme, which is responsible for converting pyruvate and lactate, and is frequently used as an anaerobic metabolism indicator; CS participates in the citric acid cycle and is measured as a bioindicator of cellular aerobic metabolism (Chandel, 2015). Leal et al. (2019) observed some dissimilarities in enzyme activities (in response to warm acclimation and acute stress) between triploid and diploid sturgeons, which led the authors to the conclusion that triploids may have a reduced cellular metabolic capacity under chronic and acute stress, possibly impacting their performance in suboptimal conditions. As reported by Leal et al. (2018), the LDH and CS activities did not appear to be similar also in the case of diploids and intermediate ploidy individuals, and the authors suggested that the aerobic capacity of intermediate ploidy may be impaired at higher water temperature.

1.3.4.5. Immune parameters

It was reported that polyploid sturgeons had fewer leukocytes than diploid sturgeons (Rożyński et al., 2015; Leal et al., 2019), but the decrease in cell number was probably accompanied by increase in cell size as discussed above in case of erythrocytes. Rożyński et

al. (2015) compared the leukograms of diploid and triploid Siberian sturgeon and found a lower percentage share of lymphocytes and an increased percentage share of monocytes and granulocytes (neutrophils, eosinophils) in triploids. Based on this observation, the authors hypothesized disturbances in the immune response, particularly in the adaptive immune system, and greater susceptibility to stress in triploid sturgeon (Rożyński et al., 2015). Another study related to immunity of polyploid sturgeons reported that white sturgeon of intermediate ploidy did not differ from diploids in respiratory burst and plasma lysozyme activity before and/or after 6-week acclimation to 18 and 22 °C, and thus did not provide further evidence for increased ploidy altering immune parameters (Leal et al., 2018).

1.3.4.6. Fertility

One interesting feature of induced and spontaneous autopolyploids of sturgeons is related to the fertility of triploid individuals (Havelka et al., 2014; Leal et al., 2019). In general, triploid teleost fishes are supposed to be sterile (there are exceptions in some species or sexes of fishes) because they do not have the normal process of gonadal development and if triploids produce gametes, these are usually aneuploid (Flajšhans et al., 2013). The results of the studies conducted on bester (Omoto et al., 2002, 2005) and preliminary data on Atlantic sturgeon suggested that triploid females in Group A may also be sterile, or exhibit delayed reproductive maturity (Schreier et al., 2021). In Group B, however, the experiments of hybridization between triploids and individuals of normal ploidy led to obtaining viable progeny with predominant ploidy levels intermediate to those of the parents (Schreier et al., 2013; Havelka et al., 2016). Fertility has been proven in both sexes of sturgeon autotriploids, for example, in a male Siberian sturgeon (Havelka et al., 2014) and female white sturgeon (Schreier et al., 2011; Gille et al., 2015). Nonetheless, little is known about the fertility in next generations, which is questionable especially in the case of backcrossing. For explanation, it is suitable to use the evolutionary scale of ploidy classification: in Group B species, normal individuals possess evolutionary ploidy 8n and triploids 12n. The offspring has intermediate ploidy level 10n, backcrosses to either 8n or 12n individuals would produce aneuploids with ploidy 9n or 11n. These odd ploidy levels would lead to uneven distribution of homologous chromosomes among daughter cells during meiosis and sterility as a result of such a meiotic mismatch (Schreier et al., 2011; Gille et al., 2015).

1.4. Aims of the thesis

The specific aims of this thesis were as follows:

- To evaluate the suitability of hydrostatic pressure shock for artificial SPBR-induction in sterlet as a model species of sturgeon.
- To develop field-feasible protocols for extended storage of various sturgeon tissues for subsequent determination of ploidy level by flow cytometry.
- To investigate how biological triploidy, tetraploidy and hexaploidy affects the survivability of early developmental stages of sterlet under both optimal and suboptimal temperature conditions.
- To examine the effects of SPBR-induced polyploidy on haematology, primary and secondary stress response and swimming performance in sterlet, Siberian sturgeon and their reciprocal hybrids.

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CHAPTER 2

APPLICATION OF HYDROSTATIC PRESSURE SHOCK FOR RETENTION OF THE SECOND POLAR BODY IN STERLET (*Acipenser ruthenus*)

Flajšhans, M., Havelka, M., Lebeda, I., Rodina, M., Gela, D., Hubálek, M., 2020. Application of hydrostatic pressure shock for retention of the second polar body in sterlet (*Acipenser ruthenus*). Aquaculture 520, 734947.

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Application of hydrostatic pressure shock for retention of the second polar body in sterlet (*Acipenser ruthenus*)

M. Flajšhans[a,*], M. Havelka[b], I. Lebeda[a], M. Rodina[a], D. Gela[a], M. Hubálek[a]

[a] University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Zátiší 728/II, 389 25 Vodňany, Czech Republic
[b] South Ehime Fisheries Research Center, Ehime University, 1289-1 Funakoshi, Ainan, Ehime 798-4292, Japan

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ABSTRACT

Induced retention of the second polar body (SPBR) is used for production of triploid fish and/or for restoration of diploidy during induced meiotic gynogenesis (MeiG). Using sterlet, *Acipenser ruthenus* as model species of sturgeon, a protocol was developed for SPBR with hydrostatic pressure shock, commonly found more considerate for treated eggs of teleosts, instead of heat shock conventionally used hitherto. In the first trial, hydrostatic pressure shock of 55 MPa for 3 min duration applied to eggs 18 min post gamete activation gave the highest fertilization rate (62.14 ± 8.90%) and hatching rate (24.59 ± 8.35%) both on Petri dishes and after pilot testing in Zuger jars (78.55%; 45.75%, respectively). Flow cytometric analysis of relative DNA content confirmed 100% triploidy in all sampled prelarvae from shocked eggs. In the second trial comparing the effectiveness of 55 MPa hydrostatic pressure shock for SPBR with conventional 35 °C heat shock for 3 min duration in order to induce triploidy and/or MeiG, pressure – induced triploids exhibited fertilization rate 62.14 ± 8.90% significantly lower than the control group (79.17 ± 7.64%) but indifferent from that in heat -shocked triploids (60.08 ± 4.65%). In MeiG groups, rediploidization with pressure shock or with heat shock did not reveal significant differences in fertilization rate, triploidization with pressure shock provided significantly higher value (46.54 ± 7.47%) than that obtained with heat shock (13.93 ± 3.46%), while similar to the control group (52.71 ± 1.9%). Hatching rates in both MeiG groups did not differ significantly each other but from values in the control group and in the pressure-induced triploids. Flow cytometric analysis of relative DNA content confirmed 100% and 80% triploidy in prelarvae sampled from the pressure- and heat shocked triploids, respectively. Among the MeiG fish, 26 and 25 analyzed prelarvae out of 30 from each the pressure- and heat shock-rediploidized groups were diploid and the remaining prelarvae were haploid. This experiment confirmed the suitability of 55 MPa hydrostatic pressure shock for SPBR in sturgeons.

1. Introduction

Induced retention of the second polar body is a widely used chromosomal manipulation technique in contemporary aquaculture, mainly for production of triploid fish and/or for restoration of diploidy during induced meiotic gynogenesis. Because fish females ovulate eggs at the metaphase stage of meiosis II and future development is induced by entry of the spermatozoon (Piferrer et al., 2009; Mable et al., 2011), prevention of extrusion of the second polar body can be easily induced by physical treatments such as temperature- or hydrostatic pressure shocks. Moreover, if the shock for retention of the second polar body is applied for restoration of diploidy during induced meiotic gynogenesis where the genome of spermatozoa used for fertilization is firstly inactivated, the resulting progeny will contain only maternal genes. For black caviar production from farmed sturgeons, such approach might be promising to increase the proportion of females in the stock as meiotic gynogenesis provides around 65–86% of females in treated populations, probably due to female heterogametic sex determination (Keyvanshokooh and Gharaei, 2010; Fopp-Bayat et al., 2018). Most authors working with induced retention of the second polar body in sturgeons or paddlefish either for triploidisation, or for diploidy restoration in meiotic gynogenesis chose temperature shocks (heat shock e.g. by Van Eenennaam et al., 1996, Mims and Shelton, 1998; Omoto et al., 2005; Fopp-Bayat, 2007, 2010; Fopp-Bayat et al., 2007a, 2007b, 2018, 2013; Wlasow and Fopp-Bayat, 2011; Zou et al., 2011; cold shock by Saber et al., 2008, 2014; Saber and Hallajian, 2014). Havelka and Arai (2019) reviewed that for sturgeon meiotic gynogenesis, heat shocks usually brought 94–100% retention of the second polar body

* Corresponding author.
E-mail address: flajshans@frov.jcu.cz (M. Flajšhans).

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while cold shock still needs more detailed studies. Recently, Leal et al. (2019) also used mechanical shock and/or let the eggs for ageing in coelomic fluid prior to fertilization.

Generally, the effectiveness of temperature- and hydrostatic pressure shocks for retention of the second polar body was critically reviewed e.g. by Piferrer et al. (2009), showing that hydrostatic pressure shocks often appeared more considerate to the treated eggs. According to Benfey (2009), heat shocks did not routinely yield in 100% triploids, as the temperature might not be uniformly distributed to all treated eggs. Currently reviewing the polyploidization techniques in salmonids, Nagler (2019) reminded this fact and pointed out that in a closed hydrostatic pressure chamber, the pressure is applied to all eggs equally, thus being more reliable at producing 100% triploids. As currently reviewed by Havelka and Arai (2019), only Flynn et al. (2006) successfully used hydrostatic pressure shock of 8500 psi (i.e. 58.6 MPa) for diploidy restoration during gynogenesis in shortnose sturgeon, *Acipenser brevirostrum*.

The main goal of this study was to develop a protocol for retention of the second polar body using pressure shock treatment in sterlet, *A. ruthenus* using a mobile hydrostatic pressure shock unit, as well as to compare the effectiveness of pressure- and high temperature shock.

2. Material and methods

2.1. Broodstock and artificial propagation

Parental fish of eight-year old sterlet, *Acipenser ruthenus* originated from pond aquaculture of the Genetic Fisheries Centre, Faculty of Fisheries and Protection of Waters in Vodňany, Czech Republic. Altogether 6 females (2.15 ± 0.22 kg mean body weight, b.w.) and 8 males (1.77 ± 0.26 kg mean b.w.) were used, 3 females and 4 males for each of the both trials. The study was carried out in strict accordance with the Czech Law No. 246/1992 about "Animal welfare" in wording of next amendments. Authors possessed a testimony according to §15d of this law. Protocols have undergone the ethical review process by the University of South Bohemia animal care committee. All surgery was performed under the clove oil anesthesia, and all efforts were made to minimize suffering. Spermiation of males was induced, sperm was sampled and its quality including the percentage of motility was assessed according to a previously published method (Linhart et al., 2000). Ovulation was induced in females by means of hormonal stimulation with a carp pituitary suspension, ovulated eggs were collected using microsurgical incisions in the oviducts, as described previously by Gela et al. (2008). Equal proportions of eggs of three females were taken, pooled and divided into four aliquots of 80 g of eggs each. Each aliquot was fertilized by 2 ml of sperm pooled from four males which exhibited at least 80% spermatozoa motility (0.5 ml each), and activated with 320 ml of 15 °C hatchery water. In 3 min after gamete activation, initial removal of egg stickiness was performed in repeated 0.04% tannic acid bath firstly for 40 s., then for 30 s. and 20 s. with rinsing in hatchery water meanwhile, followed by bath in clay suspension. Immediately before the shock, eggs were again rinsed with hatchery water. All procedures prior to hydrostatic pressure shock were carried out at 15 °C. One aliquot was let unshocked and served as a control group.

2.2. Intensity of hydrostatic pressure shock

The first trial was focused on finding the optimal intensity of hydrostatic pressure for retention of the second polar body (SPBR hereafter). Hydrostatic pressure shock was performed using a mobile unit (Fig. 1) described by Flajšhans et al. (2012). Briefly, an air bottle as a source of compressed air (≤ 40 MPa) was switched through reduction valve to high-pressure pneumatically operated multiplier for hydrostatic pressure adjustable up to 70 MPa. Tap water intake hose was also connected to the multiplier. Output of the multiplier consisted of a high-pressure hose attached to the pressure chamber. For safe supply of water under high hydrostatic pressure to the pressure chamber, the pressure line involved a safety valve, a high-pressure ball valve, manometer, pressure switch and the high-pressure hose. Pressure chamber was made of a 7 l pressure bottle fixed in a safety housing of stainless steel. Prior to shocking, the pressure chamber was filled with hatchery water at 15 °C.

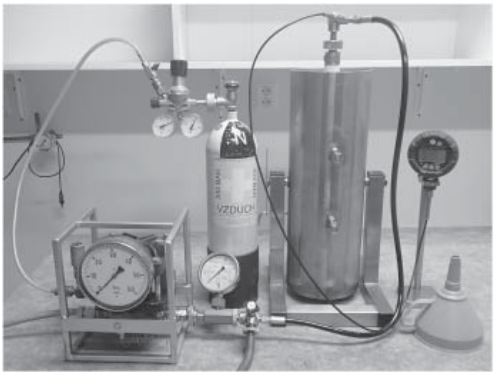

Fig. 1. Prototype of a mobile unit developed for mass application of hydrostatic pressure shock up to 65 MPa for induction of polyploidy in fish (Flajšhans et al., 2012; producer SZDT servis, Lišov, Czech Republic). Pressure chamber of 7 l volume (right, in safety metal housing) can be tilted to spill out the shocked eggs after the treatment. (Photo by M. Flajšhans).

Three different hydrostatic pressure intensities 45 MPa, 55 MPa and/or 65 MPa (i.e. 6526.69, 7977.07 and/or 9427.45 psi, respectively) were tested. The pressure shocks (each hydrostatic pressure intensity for one trial) were applied to already desticked eggs 18 min. Apost fertilization with duration of 3 min, following the optimal span for shock initiation in sturgeons (see review by Havelka and Arai, 2019). Subsequently, 15 g of shocked eggs were weighed into three Petri dishes placed into 200 cm^3 incubator cages and incubated as described by Havelka et al. (2014). Remaining 65 g of eggs were used for pilot testing, placed in clay suspension for final desticking for 45 min and then incubated in Zuger jars until hatching at 15 °C. During incubation, eggs and hatched prelarvae (period from hatching till the end of endogenous feeding) were counted, and dead eggs were counted and removed. Live embryos were counted after the second cleavage division at 4 h post-fertilization to calculate fertilization rate, and hatched prelarvae at the end of hatching to calculate hatching rate. Fertilization and hatching rates were expressed as the proportion of live embryos or prelarvae at corresponding post-fertilization times of the initial number of eggs incubated, following recommendations for sturgeon fishery practices according to Dettlaff et al. (1993).

2.3. Efficiency of pressure and heat shocks

The second trial was carried out to reveal the efficiency of pressure shock in comparison with heat shock. The experiment was performed equally but only 55 MPa pressure shock was applied identically and compared with heat shock. Its variables (timing, duration, intensity) were chosen according to our previous experience with sterlet (Lebeda et al., 2014b, 2018).

Both shock treatments were used for SPBR in order to i) induce triploidy and ii) restore diploidy during induction of meiotic gynogenesis after fertilization of eggs with UV-C irradiated sperm of sterlet following the protocols used by Lebeda et al. (2014a, 2014b) and Lebeda and Flajšhans (2016). Accordingly, the irradiation was

conducted using a UV crosslinker CL-1000 (254 nm, Ultra-Violet Products Limited; www.uvp.com) with light intensity of 45 W.m^{-2}. Sperm was diluted 1: 4 in a medium of pH 8.1, with ionic concentrations of Ca^{2+} 0.16 mM, Na^{+} 20.1 mM, K^{+} 1.5 mM and osmolality adjusted by Tris base to 80 mOsmol. kg^{-1}. Pluronic F-127 at a concentration of 0·2% was added to this medium to facilitate creating a thin sperm layer. Aliquots of diluted sperm (500 µl) were irradiated with UV-C light in 90 mm Petri dishes at 200 J. m^{-2}.

The eggs to be heat shocked were transferred into hatching trays and kept in water at 35 °C for 3 min. Heat shock was applied 18 min after fertilization. Next incubation was carried out under the same conditions as described for the first experiment.

2.4. Assessment and statistical analysis

Fertilization- and hatching rates were assessed by Pearson's chi-squared test in Statistica 12 (StatSoft). Efficacy of triploidization and gynogenesis induction treatment were evaluated by comparison of relative ploidy level of progeny from each group according to adapted method proposed by Lebeda et al. (2018). In order to evaluate ploidy level thirty samples of swimming-up prelarvae from each trial were processed for flow cytometry according to Lecommandeur et al. (1994) and analyzed following Lebeda and Flajšhans (2015). A Partec CCA I flow cytometer (Partec GmbH, Germany) was used to estimate the relative DNA content per cell and diploid (control) sterlet gave relative DNA content of 2n standard.

3. Results

3.1. Intensity of hydrostatic pressure shock

In the first experiment (Fig. 2), fertilization rate of eggs placed on Petri dishes did not significantly differ between the control group not subjected to any hydrostatic pressure change and the group treated with 55 MPa while the groups treated with 45 and/or 65 MPa exhibited values 26.46% and 32.63% less, respectively (all at $P < 0.05$). Fertilization rate of eggs placed in Zuger jars for pilot testing revealed ca. 62.1%, 93.4% and 57.4% of the value gained for the control in groups treated with 45, 55 and 65 MPa, respectively.

Concerning the hatching rate, the group treated by 55 MPa showed significantly higher mean values after incubation on Petri dishes compared to other experimental groups (6.6fold and 1.49fold more than in 45 MP and 65 MPa groups, respectively) but 2.67fold less than the control group (all at $P < 0.05$). The hatching rate after mass incubation in Zuger jars for pilot testing showed 12.8%, 55.8% and 35.2% of the value gained for the control in groups treated with 45, 55 and 65 MPa, respectively.

The mean relative DNA content in cells of prelarvae of the control group was 96.72 ± 1.84 with mean coefficient of variation (CV) 5.12 ± 0.76%. In the 45, 55 and 65 MPa groups, mean relative DNA content gained 140.5 ± 2.32; 148.3 ± 2.76 and 146.08 ± 5.60 with CVs 3.86 ± 0.93%; 3.34 ± 0.87% and 4.91 ± 1.60%, respectively. Ratio of DNA content of the pressure-shocked sterlet prelarvae to the diploid ones were 1.45, 1.53 and 1.51 for the respective 45, 55 and 65 MPa groups, confirming their triploid status. All the three hydrostatic pressure intensities tested induced 100% triploidization in the analyzed samples.

Based on these results and with respect to significantly the best hatching rate, 55 MPa was suggested to be most suitable intensity for hydrostatic pressure shock in the second experiment comparing the efficiency of pressure and heat shock to trigger SPBR for triploidization and/or induced meiotic gynogenesis (MeiG).

3.2. Efficiency of pressure and heat shocks

In the second experiment (Fig. 3), the triploids induced with 55 MPa hydrostatic pressure shock exhibited fertilization rate significantly lower than the control group (17.03% less) but insignificantly different

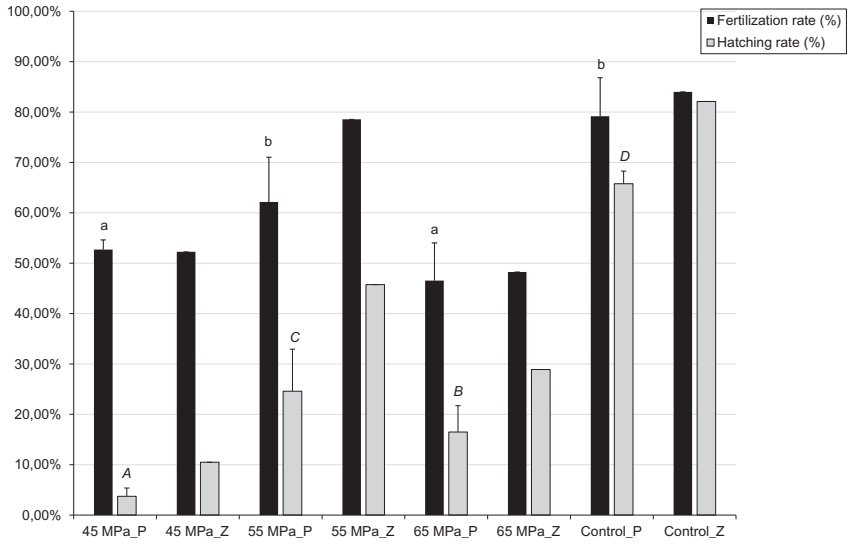

Fig. 2. Comparison of fertilization- and hatching rates (%) in the control group and in groups treated with different intensities of hydrostatic pressure shock (45, 55 and/or 65 MPa), both from experimental variants on Petri dishes (_P) and from pilot testing in Zuger jars (_Z), for retention of the second polar body (SPBR) during triploidization in sterlet (*Acipenser ruthenus*). Different alphabetic supercripts (lower-case letters for fertilization rate; block letters in Italics for hatching rate) denote statistically significant differences at $P < .05$.

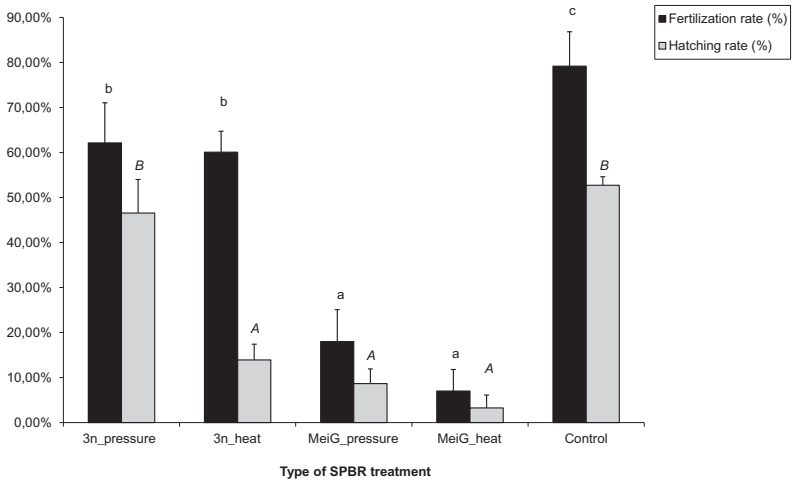

Fig. 3. Comparison of fertilization- and hatching rates (%) in the control group and in groups after hydrostatic pressure shock (55 MPa) and heat shock (35 °C) treatment for retention of the second polar body (SPBR) during triploidization and/or induction of meiotic gynogenesis (MeiG) in sterlet (*Acipenser ruthenus*). Different alphabetic supercripts (lower-case letters for fertilization rate; block letters in Italics for hatching rate) denote statistically significant differences at $P < .05$.

from that in triploids from heat shocked group (2.06% more). In the MeiG groups, rediploidization with hydrostatic pressure shock provided 11% higher fertilization rate than that reached with heat shock but this difference was insignificant, due to high standard deviation in the group rediploidized with hydrostatic pressure. With respect to the hatching rate, triploidization with 55 MPa hydrostatic pressure shock provided significantly higher mean value, i.e. 3.34fold of that obtained with heat shock. Simultaneously, mean hatching rate of the pressure-induced triploids was similar to the mean value in the control group. Hatching rates in both MeiG groups did not differ significantly each other due to high standard deviations, attaining 37.6% and 87.16% of the mean in the groups rediploidized with hydrostatic pressure- and heat shock, respectively. Also, hatching rate in both MeiG groups significantly differed from values in the control group and in the hydrostatic pressure-induced triploids (Fig. 3).

In this experiment, the mean relative DNA content in cells of prelarvae of the control group was 97.33 ± 2.85 with mean CV 4.11 ± 0.81%. Among the induced triploids, all 30 analyzed prelarvae from the pressure-treated group were found to be triploid (Fig. 4) with mean relative DNA content in cells 148.12 ± 2.45 (CV 2.46 ± 0.85%) while among 30 analyzed prelarvae in the heat-treated group, only 24 (80%) triploid specimens were found with mean relative DNA content in cells 147.08 ± 3.60 (CV 2.19 ± 1.21%). The remaining six specimens in the latter group were found to be diploid (Fig. 4).

Among the MeiG fish, 26 and 25 analyzed prelarvae out of 30 from each the pressure- and heat shock-rediploidized groups gave the mean relative DNA content 99.5 ± 3.54 with mean CV 3.68 ± 0.69% indicating diploidy while 4 and 5 analyzed prelarvae from the above samples exhibited the mean relative DNA content 49.26 ± 7.06 with mean CV 4.11 ± 1.43%, indicating haploid level (Fig. 4).

4. Discussion

The idea behind this method was based on the application of hydrostatic pressure shock, evaluated by Piferrer et al. (2009) as an approach more considerate to the treated eggs for retention of the second polar body (SPBR) than usually applied thermal shock, and to test both approaches to SPBR in sturgeons either to induce triploidy, or meiotic gynogenesis. To the best of our knowledge up-to-date, recently confirmed by Havelka and Arai (2019) in their comprehensive review, there has been only a single paper dealing with application of hydrostatic pressure shock in sturgeons (Flynn et al., 2006). It could be only partly used for comparison with data obtained in this study, as Flynn et al. worked with another species, the shortnose sturgeon, used slightly lower incubation temperature (13 °C vs. 15 °C under study) and thus used later shock initiation at 20 min with 5 min duration vs. 18 min. and 3 min under study, respectively.

4.1. Intensity of hydrostatic pressure shock

In this experiment checking solely the shock intensity, the best fertilization and hatching rates of eggs on Petri dishes were obtained after treating the eggs with 55 MPa, while both these rates obtained after treating the eggs with 45 and 65 MPa were lower. These findings were confirmed by results of pilot testing the remaining treated eggs in Zuger jars, where the variant treated with 55 MPa shock also revealed the best fertilization and hatching rates from all three variants treated. This result, supported by finding all the sampled prelarvae from all shocked groups to be triploid indicating 100% triploidization success, led us to the conclusion that the 55 MPa shock might provide the highest triploid yield. Both the 45 and 64 MPa intensities, although giving 100% triploidy also, might be considered less considerate to the eggs and thus suboptimal.

Our optimal intensity 55 MPa was found rather close to the only published hydrostatic pressure shock intensity 58.6 MPa by Flynn et al. (2006) for shortnose sturgeon but hatching rates gained in both experiments could not be compared directly as the latter authors have only mentioned very low number of survivors in both the diploid and triploid groups at hatching. Fopp-Bayat et al. (2007a) who worked with eggs of the same species as in this study and used sperm of bester hybrid (beluga, *Huso huso* x sterlet), also reported on similar hatching rate of triploids induced by heat shock.

Triploidy was not confirmed among prelarvae of the control group, showing that there was no spontaneous SPBR that might lead to

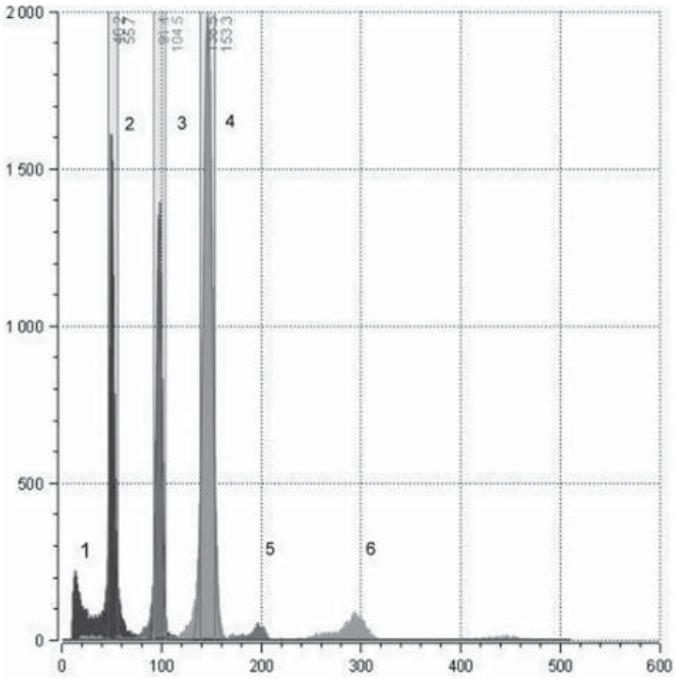

Fig. 4. Flow cytometric histogram of relative DNA content in haploid (peak 2), control diploid (peak 3) and induced triploid (peak 4) sterlet (*Acipenser ruthenus*). Peak 1 represents background noise in the haploid nuclei suspension, peaks 5 and 6 represent doublets of diploid and triploid nuclei, respectively. Combined histogram was produced by overlaying raw data in *.fcs format using CYTO-SW 0.3 software (Wolf & Danniel s.r.o., Czech Republic).

spontaneous autopolyploidization due to egg overmaturation.

4.2. Efficiency of pressure and heat shocks

The fertilization rates of both the hydrostatic pressure- and heat shocked groups for induction of triploidy were similar to each other. The same could be said about fertilization rates of both the hydrostatic pressure- and heat shocked groups for rediploidization in MeiG. Thus, it could be hypothesized that the type of shock under study had no effect on fertilization rate. Considering the hatching rate, triploidization with pressure shock provided > 3.3fold better value than that obtained with heat shock and still similar to the mean value in the control group. Thus the pressure shock, as it was used under study, might confirm the statement of Piferrer et al. (2009) to be more considerate to the treated eggs than the heat shock also for sturgeons in terms of hatching rate. With respect to the hatching rate in MeiG, Havelka and Arai (2019) summarized the average value for MeiG prelarvae to be around 20%. Results obtained in this study for both MeiG groups rediploidized either with hydrostatic pressure- or heat shock were much lower than this reported value (see Fig. 2) indicating that this approach still needs extensive improvement. These results were found contradictory to conclusions of Fopp-Bayat et al. (2007b) and Lebeda et al. (2018) who stated that diploidy restoration treatment did not substantially affect gynogenote survival rates. However, these authors worked on different maternal species, the Siberian sturgeon. On the other hand, hatching rate of pressure-rediploidized MeiG prelarvae gained in this study gave similar figure to that in the only paper known so far to deal with hydrostatic pressure - induced SPBR during gynogenesis in shortonse sturgeon by Flynn et al. (2006).

Again, all the sampled prelarvae from the pressure-treated group were found to be triploid, indicating 100% triploidization success. The heat-treated group exhibited only 80% triploidization success and the remaining prelarvae were diploid. In the MeiG groups, rediploidization by pressure- and/or heat induced SPBR yielded in ca. 87% and 83% of diploids, respectively, while the remaining prelarvae were haploid. Presence of the haploids, along with absence of any triploid prelarvae in the MeiG samples indirectly confirmed the success of inactivation of paternal genome by UV irradiation of sperm during the MeiG procedure. It also provided the first evidence that the diploid MeiG specimens did not originate as a result of fertilizing the eggs with spermatozoa with non-inactivated DNA, followed by failure of the rediploidization shock. Macroscopically, these haploids were malformed, exhibiting the "haploid syndrome" that has been described in several sturgeon species/hybrids e.g. by Van Eenennaam et al. (1996), Omoto et al. (2005), Fopp-Bayat et al. (2007) and Lebeda et al. (2018).

Similarly to the first experiment, analysis of prelarvae of the control group did not exhibit any triploidy showing that neither here was any spontaneous SPBR that might lead to spontaneous autopolyploidization. Excluding the spontaneous SPBR, we could also exclude a failure of the rediploidization shock during the MeiG procedure because the resulting diploid MeiG specimens could only arise due to duplication of the maternal genome. This provided the second evidence that the diploid MeiG specimens did not originate as a result of fertilizing the eggs with spermatozoa with non-inactivated DNA, followed by failure of the rediploidization shock.

5. Conclusions

This study successfully demonstrated that pressure shock might be used as an efficient tool for retention of the second polar body in sturgeon, similarly as it was reviewed for farmed teleost fish species (Piferrer et al., 2009; Nagler, 2019) and recently also for sturgeons

(Havelka and Arai, 2019). Moreover, pressure shock of 55 MPa intensity seemed to be more effective for this purpose in comparison to heat shock also with regards to the hatching rate. Such approach to somatic ploidy level restoration in sturgeons might be also helpful during the procedure of induced meiotic gynogenesis.

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Author contributions

MF and MHa conceptualized and designed the experiments. DG and MR were responsible for broostock management and artificial propagation. MF, MHa, IL and MHu performed the experiments, flow cytometric analyses and analyzed the data. MF and MHa drafted the initial manuscript with detailed edits from all authors. All authors reviewed and commented on the manuscript. The authors declare no competing interests.

Declaration of Competing Interests

The authors do not declare any conflict of interests.

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CHAPTER 3

SIMPLE FIELD STORAGE OF FISH SAMPLES FOR MEASUREMENT OF DNA CONTENT BY FLOW CYTOMETRY

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My share on this work was about 80%.

TECHNICAL NOTE

ISAC CYTOMETRY Journal of Quantitative Cell Science PART A

MIFlowCyt

Simple Field Storage of Fish Samples for Measurement of DNA Content by Flow Cytometry

Martin Hubálek,* Martin Flajšhans

University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Zátiší 728/II, 389 25, Vodňany, Czech Republic

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*Correspondence to: Martin Hubálek, University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Zátiší 728/II, 389 25 Vodňany, Czech Republic.
Email: mhubalek@frov.jcu.cz

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Abstract

Flow cytometry is an effective and widely used tool for determination of ploidy in fish, but it is not always possible to access the fresh samples for analysis. We investigated the potential for extended storage of fish tissue with sterlet and tench as representative species of Chondrostei and Teleostei, using blood and fin of subadult/adult specimens and tail of larvae. Thirteen procedures for extending storage, selected for rapidity and simplicity in both field and laboratory conditions, were tested for each tissue sample. Flow cytometry was applied to fresh tissue immediately after sampling and to tissue subjected to experimental protocols, always along with species-specific standard, after 1, 5, and 10 days storage at 0–4°C or freezing at −80°C. The fluorochrome 4′,6-diamidine-2′-phenylindole dihydrochloride was used with excitation/emission maximum 358/461 nm. Based on the measurability of stored samples, evaluation of directly measured coefficients of variation of their DNA peaks and the changes in fluorescence intensity compared to fresh tissue, optimal procedures for extended storage of the selected tissue types of the model species are suggested. © 2020 The Authors. *Cytometry Part A* published by Wiley Periodicals LLC. on behalf of International Society for Advancement of Cytometry.

Key terms

fixation; preservation; sterlet; tench; blood; fin tissue; larva tail tissue; coefficient of variation; fluorescence intensity

Flow cytometry is widely used in basic and applied research in biology (1–3). In fish, flow cytometry is standard method for quantification of relative DNA content to determine ploidy (4–6), which aids in identifying spontaneous polyploids; for evaluation of effectiveness of chromosome manipulation; and for investigation of diploid–polyploid complexes (7). It is also used to determine nuclear genome size (8), a fundamental feature of all species (6) and an informative parameter of taxonomic (9) and evolutionary studies (10).

Flow cytometry is suitable for analysis of body fluids in which cells are suspended as well as for solid tissue after processing by mechanical and/or enzymatic disaggregation (11). The choice of tissue to be sampled depends on objectives of the investigation and features of the observed organism. Common tissue types in fishery practice are blood, fin, and larvae (12–14). For highest quality cytometric output (low coefficient of variation, accurate level of emitted fluorescence, and peak position), analyzing samples in their native state is ideal (15) to avoid artifact or other complications of sample processing and measurement such as cell clumping, lysis, or loss (16).

It is not always possible to access fresh material for analysis. Samples may be obtained at a distance from the laboratory and need to be transported, precluding rapid analysis (17). Transportation time becomes a more complicating factor when more than one institution is involved (18). Lack of a process allowing immediate and rapid analysis can make processing large numbers of specimens or specimens at

the same developmental stage unfeasible (19). When it is necessary to work with a sample used as a standard over the course of several days, it may be a challenge to obtain a consistent supply (18).

The extension of tissue sample storage can be achieved with chemical or physical fixation (20) or freezing in a cryoprotectant (18, 20, 21). Several protocols for fixation/preservation of fish cells and tissues have been introduced and successfully tested (16, 18, 19, 22–25), many of which are time consuming, particularly those requiring a large number of centrifugation steps (26). In some cases, centrifugation is stipulated shortly after sample collection and prior to storage, meaning the protocol is not easily applicable to field conditions (27).

Vindelov et al. (18) introduced a method without centrifugation, based on freezing the sample in a solution containing dimethyl sulphoxide DMSO, sucrose, and trisodium citrate at −80°C. The protocol was successfully used for fish blood (26, 28–30), but, to the best of our knowledge, not for other fish tissue.

We aimed to identify optimal procedures, appropriate for field conditions, of extending fish tissue storage for quantification of DNA by flow cytometry. Thirteen selected protocols were evaluated for use with blood, fin, and larva tail tissue of sterlet *Acipenser ruthenus* and tench *Tinca tinca*.

Material and Methods

Ethics Statement

Sampling and sample fixation and preservation were carried out at the Genetic Fisheries Centre, Faculty of Fisheries and Protection of Waters (FFPW), University of South Bohemia in České Budějovice, Czech Republic, with storage and analysis of samples conducted at laboratories in Vodňany, Czech Republic. All experiments were carried out in compliance with criteria of the Animal Research Committee of the FFPW. Fish were maintained according to the principles of the EU harmonized animal welfare act of the Czech Republic and principles of laboratory animal care in compliance with national law (Act 246/1992 on the protection of animals against cruelty).

Experimental Design

Two model fish species were used, sterlet *Acipenser ruthenus* (Chondrostei) and tench *Tinca tinca* (Teleostei), with three types of tissue collected from each: blood, fin clips, and larva tail tissue. Blood and fin clip samples were obtained from previously determined diploid 1–2 year-old sterlet and 5–8 year-old tench. Larvae were obtained from artificial reproduction conducted according to standard methodology (31, 32) and sampled before onset of exogenous feeding. Different procedures of fixation and preservation were applied for all the samples. Flow cytometry analysis was conducted immediately (day 0) on fresh tissue and at days 1, 5, and 10 postfixation/preservation. Immediately prior to assessment, a standard was added to each sample. Our goal was to obtain two DNA peaks with one DNA peak having approximately twofold or higher fluorescence intensity compared to the second. Fresh cells from tench fin tissue (1C-value = 1.02 pg DNA) were used as a standard for all sterlet samples (1C-value = 1.86 pg DNA) (33). Commercially available fluorescently labeled fixed trout erythrocytes (DNA Control UV, Sysmex Partec GmbH, Germany; 1C-value = 2.4 pg DNA) (34) were used as standard for tench samples. This precluded peak overlaps in cases of fluorescence affected by the fixation/preservation procedure.

Sampling

One milliliter of whole blood was drawn from each fish by caudal venipuncture into heparin treated insulin syringes following the protocol of Svobodová et al. (35). Blood was placed in 1.5 ml Safe-Lock Eppendorf tube (1 drop per tube) containing 1 ml fixation/preservation solution and mixed immediately. An $\sim$0.5 cm^2 fragment of caudal fin was snipped and divided into $\sim$1 mm^2 pieces, which were placed into separate Eppendorf tubes containing the fixation/preservation solution. Larvae were killed with an overdose of CO_2 and placed in individual Eppendorf tubes with test solution.

Fixation/Preservation and Storage Protocols

Blood, fin tissue, and larva tail tissue of both sterlet and tench were subjected to fixation/preservation (Table 1).

Fixation in paraformaldehyde and in ethanol in saline. One milliliter fresh 1% paraformaldehyde (PFA) or 1, 5, 10, 15, or 20% ethanol in physiological saline was added to each of five Eppendorf tubes for each tissue type of each species and placed on ice. Tissue samples were collected, added to the tubes, transported to the laboratory, and refrigerated. The following day, the quantity needed for flow cytometry was removed and the remainder refrigerated immediately. All handling was carried out on ice. The flow cytometry process was repeated 5 and 10 days after the initial sampling.

Table 1. Fixation/preservation protocols used for blood, fin, and larva tail of both sterlet and tench

FIXATION/PRESERVATION SOLUTION	FIXATION/PRESERVATION AND STORAGE TEMPERATURE (°C)
1% EtOH in saline	0–4
5% EtOH in saline	0–4
10% EtOH in saline	0–4
15% EtOH in saline	0–4
20% EtOH in saline	0–4
Saline	−80
1% EtOH in saline	−80
5% EtOH in saline	−80
10% EtOH in saline	−80
15% EtOH in saline	−80
20% EtOH in saline	−80
DMSO-citrate buffer	−80
1% paraformalehyde (PFA) in saline	0–4

Physiological saline = a solution of 0.9% w/v of NaCl.

Freezing in saline only and in ethanol in saline. Fifteen Eppendorf tubes containing 1 ml of physiological saline and 15 containing 1, 5, 10, 15, or 20% ethanol in physiological saline were prepared for each tissue type of each species and placed on ice. Tissue samples were added, and tubes were immediately placed in an ultra-low freezer at −80°C. The following day, five samples of each tissue type were removed from the freezer and thawed in a water bath at 37–38°C for cytometric analysis and repeated with new samples after 5 and 10 days.

Freezing with cryoprotectant. Fifteen Eppendorf tubes for each tissue type were prepared with 1 ml of DMSO-citrate buffer (18). The procedure was identical to freezing in saline with the exception that sampling was followed by freezing on dry ice, after which samples were placed in an ultra-low freezer and stored at −80°C.

Fresh samples. The collection of blood or fin tissue samples from each experimental fish was completed by preparing five samples each of blood and fin tissue in physiological saline for immediate analysis in a fresh state. Five larvae were placed in physiological saline and analyzed as soon as possible.

Tissue Analysis

Blood. A 2 µl aliquot of each blood sample was placed in 1.5 ml 4′,6-diamidino-2-phenylindole (DAPI; Cystain DNA 1 step Staining Solution, Partec GmbH, Germany). The mixture was homogenized (Minishaker MS2, IKA, Germany), incubated for 10 min, and passed through a 30 µm filter (CellTrics, Partec GmbH, Germany) in a cuvette. The standard was added and cytometric analysis was conducted.

Fin and larva tail tissue. The tissue samples were placed in Eppendorf tubes with 200 µl of nuclei extraction buffer (CyStain UV Precise T: Nuclei Extraction Buffer+Buffer Reagent, Partec GmbH, Germany). Frozen fin clips were used whole, one-third of other fin samples was snipped, and the remaining tissue was returned to the fixation solution and refrigerated. Larval tissue was obtained by separating pieces smaller than 0.5 mm^2 from the tail using a scalpel. Samples were processed with a hand-held homogenizer for 5 s (cordless pellet pestle, Kimble, USA), incubated for 10 min, and 100 µl was removed to decrease the number of cells in the prepared suspension. One ml DAPI (CyStain UV Precise T: Staining Buffer, Partec GmbH, Germany) was added to the remaining cell suspension. The solution was homogenized, passed through a 30 µm nylon filter, and the standard was added.

Standard. A commercially available suspension of fixed trout erythrocytes was used immediately with no additional preparation. For the suspension of tench fresh cells, tail fin tissue was cut into small pieces, placed in 200 µl nuclei extraction buffer, minced 5 s with a homogenizer, and incubated 10 min. One milliliter DAPI stain was added, and the solution was passed through a 30 µm filter, using the same reagents as for fin and larva tail samples.

For each sample, the quantity of standard that provided histograms with two distinguishable cell populations was used. Initially, 200 µl of the standard was added to each sample. If the cell population was not differentiated, the quantity of standard was increased. When only the peak of the standard was observed, the sample was prepared again with a lower volume of standard.

Flow Cytometry

Measurements were conducted using a flow cytometer Partec CCA I (Partec GmbH, Germany) with the gain set at 440.5. The velocity of cell suspension flow was based on the concentration of cells in the suspension. The first measurement was conducted with 100 cells s^{-1} passing through the cytometric chamber. This measurement was discarded, and the flow rate was reduced to 20 cells s^{-1} in the erythrocyte suspension and to 30 cells s^{-1} for fin and larva tissue. A final histogram was recorded based on analysis of more than 1,000 cells. Mean channel number and coefficient of variation (CV) of the standard and the sample were used to assess the relative DNA content of samples. After analysis, the flow cytometer chamber and tubing were rinsed three times with sheath fluid to prevent contamination of the subsequent sample.

Evaluation of Results

Measurability, CV, and fluorescence levels were recorded for evaluation and comparison of fixation/preservation protocols within each sample type and species. Data were analyzed using Statistica 12 software.

Coefficient of variation. Mean CV of each experimental procedure was calculated for the fresh samples and the samples on days 1, 5, and 10 postfixation/preservation. When the analysis did not result in a sample histogram peak distinguishable from the peak of the standard, or when the CV was greater than 10%, the sample was designated unmeasurable and was omitted from analysis of mean CV. The number found measurable was expressed as a percentage of the five analyzed samples.

Fluorescence. Fluorescence was determined for the protocols that enabled analysis of 100% of samples of a given tissue type on days 1, 5, and 10 postfixation/preservation. Since the peak can shift during analysis despite a consistent instrument setting (36), the ratio of fluorescence of the standard to fluorescence of the sample (F_{st}/F_{sa}) was used, as opposed to absolute fluorescence values. The values of fluorescence of standard to fluorescence of fresh samples (F_{st}/F_{fresh}) and fluorescence of standard to fluorescence of fixed/preserved samples (F_{st}/F_{fixed}) were averaged for each protocol and day of analysis.

The F_{st}/F_{fresh} and F_{st}/F_{fixed} were compared for each protocol and day of analysis. Statistical analysis was conducted using Statistica 12 software. When results of Bartlett's, Cochran's, and/or Hartley's tests showed that the criterion for

homogeneity of variance was not met, the non-parametric Kruskal–Wallis test was used for F_{st}/F_{sa} difference comparison.

Criteria for Selecting Optimal Protocols

When a fixation/preservation protocol met the following criteria for given species and tissue type, the protocol was considered optimal.

1. Measurability of all fixed/preserved samples
2. Mean CV <3% on days 1, 5, and 10 postfixation/preservation
3. No significant difference between F_{st}/F_{fresh} and F_{st}/F_{fixed} on days 1, 5, and 10 post fixation/preservation.

When no protocol met criterion 2, that meeting criteria 1 and 3 and giving the lowest CV was designated optimal for a given species and tissue type. When the protocols met only criteria 1 and 2, that with the lowest number of samples showing difference between F_{st}/F_{fresh} and F_{st}/F_{fixed} was considered optimal.

Changes in fluorescence. Significant differences between F_{st}/F_{fresh} and F_{st}/F_{fixed} in protocols that met criteria 1 and 2 were considered to be the result of fixation/preservation and/or storage procedure and were quantified using the formula

$$\frac{\sum_{n=1}^{5}\left(\frac{F_{standard}}{F_{fresh}}\right)_n}{\sum_{n=1}^{5}\left(\frac{F_{standard}}{F_{fixed}}\right)_n} = X.$$

$$(X-1)*100 = \% \text{ difference in fluorescence}$$

in which F_{fixed} = fluorescence of fixed/preserved sample, F_{fresh} = fluorescence of fresh samples, $F_{standard}$ = fluorescence of standard, and X = ratio of mean fluorescence of fixed/preserved to fresh sample.

Results

A total of 1,170 measurements were made. Representative histograms are shown in Figure 1. Measurability and mean CV of fresh and fixed/preserved material of each sample type in both species are presented in Supplementary Table 1. Mean F_{st}/F_{sa} values of the analysis of fresh and fixed/preserved samples are presented in Figure 2 for blood, Figure 3 for fin tissue, and Figure 4 for larva tail tissue samples. All procedures allowing to measure 100% samples with average CVs under 3% are verbally commented in the following section divided by tissue type and species examined.

Blood

Sterlet. Fixation with 1, 5, and 15% ethanol and freezing of samples in cryoprotectant allowed measurement of all the samples and did not exceed the mean CV of 3% on any sampling day. Use of 15% ethanol produced F_{st}/F_{fixed} values that did not significantly differ from F_{st}/F_{fresh}.

With fixation in 1% ethanol, a significant difference in fluorescence ratio was observed on day one, with 4.33% greater mean fluorescence of the fixed samples compared to fresh. A difference in fluorescence ratio was also observed in the group preserved in 5% ethanol after 10 days. The average fluorescence of fixed samples was 9.78% higher than that of fresh samples. Freezing in cryoprotectant led to significant differences in F_{st}/F_{fixed} on days 1 and 10, when the average fluorescence of fixed samples was 7.72 and 10.76% greater than fresh, respectively.

Tench. Measurability of all samples and CV <3% was met by fixation in 1% ethanol, freezing in DMSO, and fixation with 1% PFA.

Freezing of samples in DMSO was considered optimal, because F_{st}/F_{fixed} and F_{st}/F_{fresh} differed only on day 1 post-sampling, when the mean level of fluorescence of the preserved sample was 11.96% higher than observed in fresh samples.

Use of 1% ethanol led to significant differences in fluorescence level at days one and five postfixation, with mean fluorescence 11.46 and 11.89% lower than in the fresh samples, respectively.

With 1% PFA, F_{st}/F_{fixed} significantly differed from the fresh samples on days 1, 5, and 10 postfixation. The average fluorescence of fixed samples was 23.84, 25.44, and 22.55%, respectively, lower than that of fresh samples.

Fin Tissue

Sterlet. No protocol allowed measuring all samples and concurrently obtained average CV <3% at all sampling times. The lowest CVs were obtained with samples frozen in DMSO (3.50 ± 1.49, 2.83 ± 0.02, and 3.47 ± 1.55%). This procedure enabled measurement of all preserved samples, and its application did not lead to significant differences between F_{st}/F_{fresh} and F_{st}/F_{fixed}.

Tench. The criteria of measurability and CV <3% were met only for samples frozen in DMSO. This procedure did not show an impact the level of fluorescence emitted, so was considered optimal.

Fish Larva Tail Tissue

Sterlet. Freezing in 1% ethanol, saline, and DMSO produced sterlet larva tail tissue samples with mean CV <3% on days 1, 5, and 10 postfixation/preservation. The F_{st}/F_{fixed} of samples frozen in 1% ethanol and saline did not significantly differ from F_{st}/F_{fresh} at any sampling time.

Freezing in DMSO led to a significant difference between F_{st}/F_{fixed} and F_{st}/F_{fresh} after 10 days storage. However, mean fluorescence was only 2.6% lower than that of fresh samples.

Tench. With fixation in 5% ethanol and freezing in 1 and 5% ethanol, all samples were successfully measured, and CV <3% was obtained. With these protocols, fluorescence did not differ from fresh samples at 1 and 5 days postfixation, but F_{st}/F_{fixed} and F_{st}/F_{fresh} differed significantly after 10 days. The smallest difference was observed with 5% ethanol fixation with fluorescence 5.29% lower than that of fresh samples,

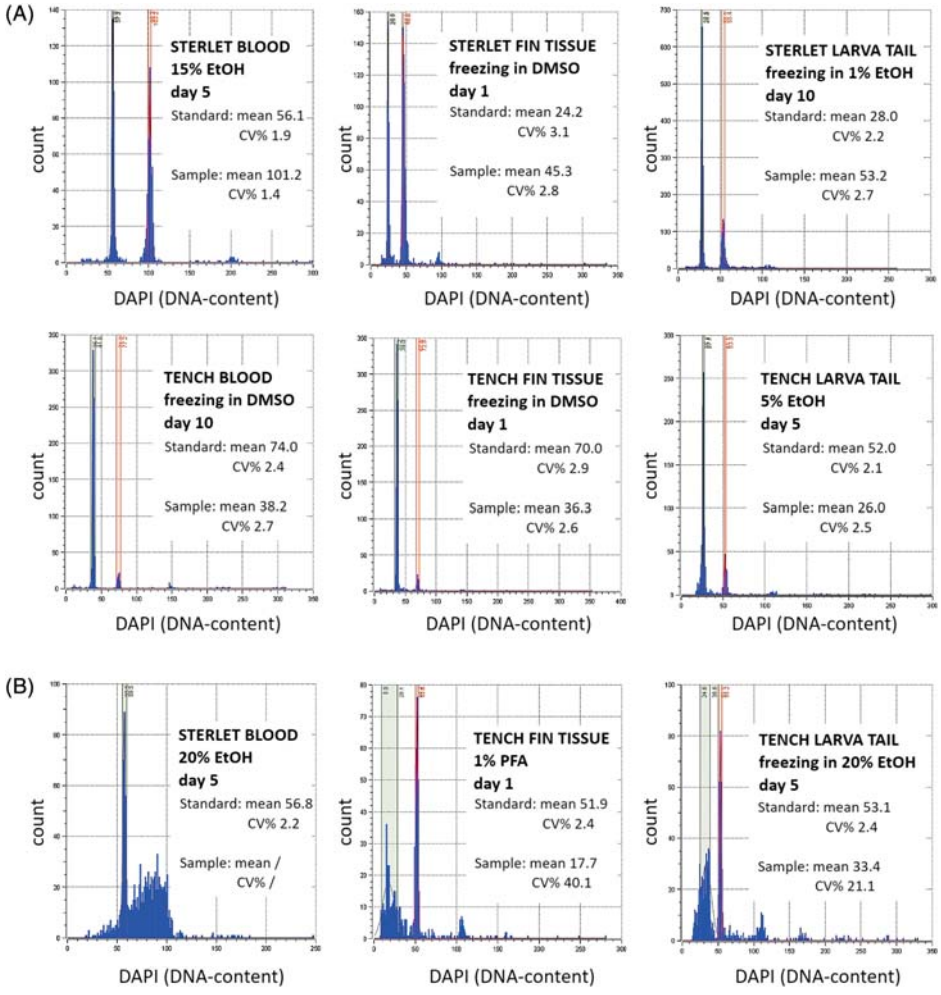

Figure 1. Representative examples of nuclear DNA content histograms obtained after flow cytometric analysis of samples, in which storage extension was successful (**A**) and unsuccessful (**B**). [Color figure can be viewed at wileyonlinelibrary.com]

while freezing in 1 and 5% ethanol yielded reductions of 6.77 and 5.96%, respectively. Samples frozen in saline and in the cryoprotectant met the criteria of measurability and similarity of F_{st}/F_{sa} to that of fresh tissue, and average CVs did not exceed 0.1% above the 3% limit.

Discussion

Storage Extension of Sterlet and Tench Tissue

We assumed that the optimal storage extension procedures for sterlet and tench could differ slightly because of potential dissimilarity of chondrostean and teleostean fish of differing evolutionary age, morphology, histology, and physiology.

While working exclusively with fresh sterlet fin samples stored in physiological saline and during the testing of the fixation/preservation procedures, we often observe formation of a thick mucus layer covering the surface of the tissue within 24 h of sampling, which could potentially be a medium for bacterial growth (37) or affect the epithelial permeability (38) and thus the fixative penetration. The effectiveness of protocols for long-term storage of sterlet versus tench fin samples

TECHNICAL NOTE

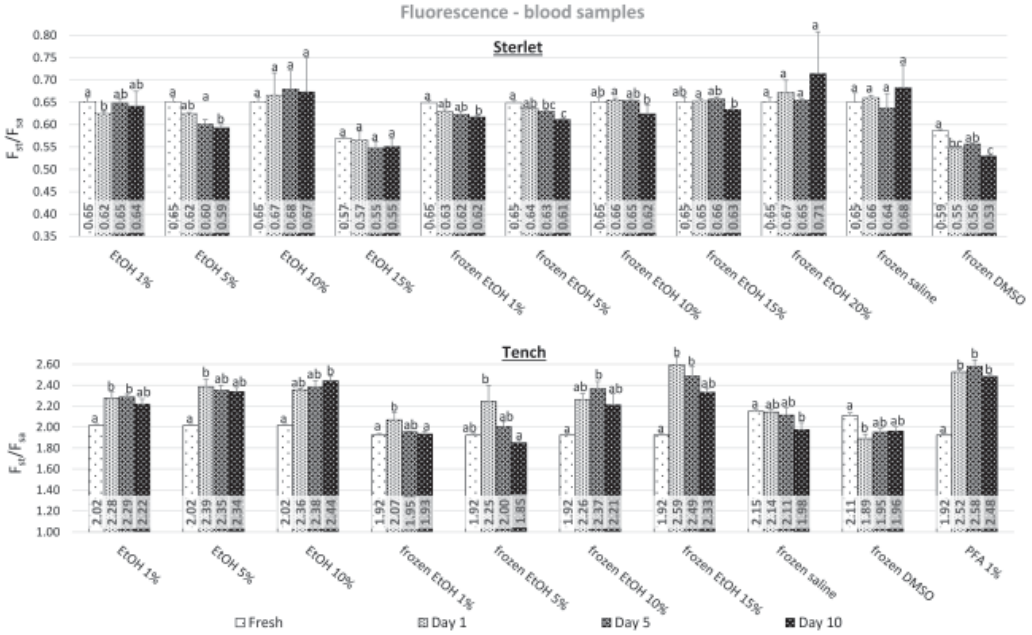

Figure 2. Mean values of fluorescence of standard/fluorescence of sample (F_{st}/F_{sa}) of sterlet and tench blood, fresh, and stored 1, 5, and 10 days. Statistical differences ($P < 0.05$) were evaluated for each fixation/preservation protocol separately.

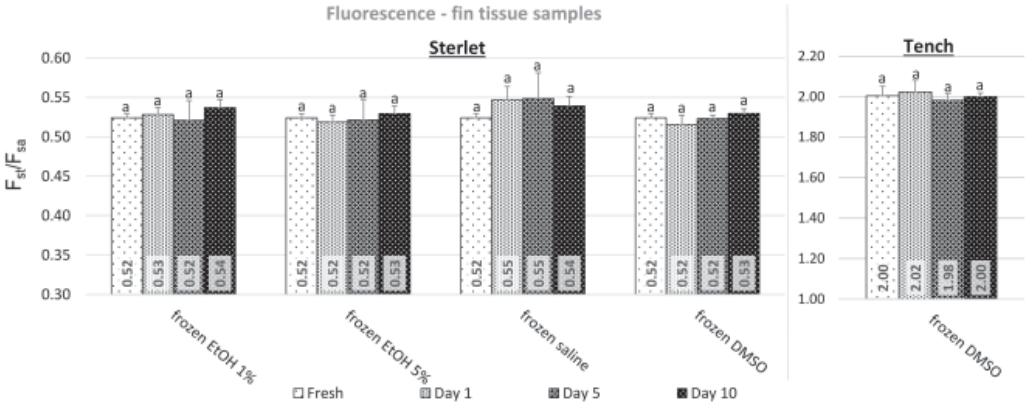

Figure 3. Mean values of fluorescence of standard/fluorescence of sample (F_{st}/F_{sa}) of sterlet and tench fin tissue, fresh, and stored 1, 5, and 10 days. Statistical differences ($P < 0.05$) were evaluated for each fixation/preservation protocol separately.

could be affected by the differences in their fin structure. The fins of sturgeon possess an elaborate endoskeleton overlapped along its distal margin by dermal lepidotrichia, while teleost fins generally have small endoskeletal radials articulating with the dermal fin skeleton terminally, with little or no proximodistal overlap (39).

The storage life of larvae could be associated with characteristics of their integument. Shute et al. (40) identified two

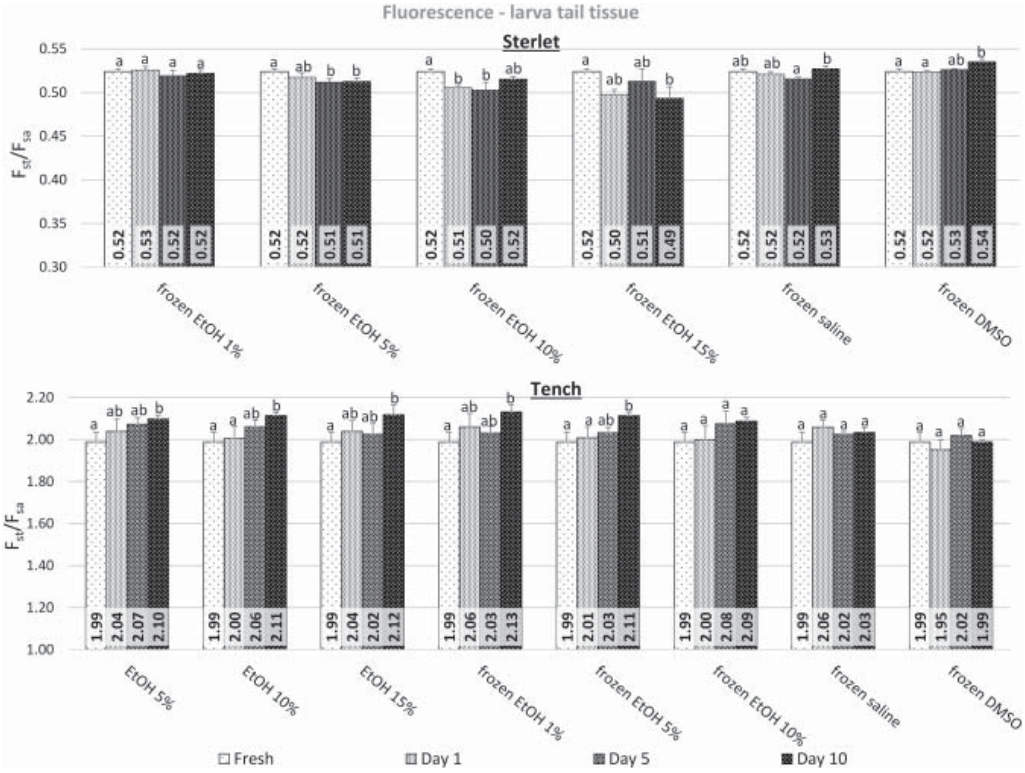

Figure 4. Mean values of fluorescence of standard/fluorescence of sample (F_{st}/F_{sa}) of sterlet and tench larva tail tissue, fresh, and stored 1, 5, and 10 days. Statistical differences ($P < 0.05$) were evaluated for each fixation/preservation protocol separately.

novel cell types in the integument of larval lake sturgeon *Acipenser fulvescens*, ampullary and superficial cells, with characteristic morphology and high abundance of secretory vesicles, which could be involved in the release of semiochemicals that may act as a pheromone, alarm substance, or chemical defense mechanism.

The optimal storage extension procedures for tissue of given species could vary with properties of their cell membranes. The differences in the integrity of the plasma membrane and its selective permeability play a fundamental role in long-term cryopreservation of sperm (41).

Blood

Most previous flow cytometry fish studies have been conducted on erythrocyte nuclei, which enable precise analysis (19). Successful blood sampling requires an animal of sufficient size. Gold et al. (19) recommended a minimum length of 40 mm when heart puncture is used. For noninvasive sampling, fish must be heavier than 20 g, and blood is taken from the caudal vein (42).

Current information suggests that the method of Vindelov et al. (18) based on freezing in DMSO is the most commonly used protocol for long-term storage of fish blood for flow cytometry determination of both relative and absolute DNA content (20, 21, 28–30, 43–45). We found the method to produce the best results for tench blood, and can be recommended. On the other hand, fixation in 15% ethanol appeared to be a more effective approach to 10-day storage of sterlet blood, because, in contrast to freezing in DMSO, it did not induce differences in the level of fluorescence emitted from that of fresh samples.

Fixation of sterlet blood at 1 and 5% ethanol also met the criteria of 100% measurability and CVs <3%, but significantly affected fluorescence. The highest concentration of ethanol used, 20%, did not allow measuring all samples. Birstein et al. (46) used 45% ethanol for sturgeon erythrocyte fixation and did not mention effects on sample measurability or fluorescence; however, they analyzed the samples fewer than 3 days postfixation and observed increased CVs compared to fresh samples.

Freezing of sterlet and tench blood samples in saline and in ethanol allowed measurement of all samples with the exception of one frozen in 20% ethanol, but the mean CVs usually exceeded 3% and were particularly high in sterlet.

Aldehydes have been used for fish blood fixation in the past. Allen et al. (27) successfully fixed blood samples of Atlantic salmon *Salmo salar* and grass carp *Ctenopharyngodon idella* x silver carp *Hypophthalmichthys molitrix* hybrids using treatment with 10% formalin, but his protocol contained five centrifugation steps and is not applicable to field conditions. A field modification for grass carp blood and tissue samples was introduced by Brown et al. (24), but still required four centrifugation steps, and prolonged storage of samples led to changes in fluorescence. Changes in fluorescence have also been demonstrated with formalin (final concentration of 1–2%) fixation of rainbow trout *Oncorhynchus mykiss* erythrocytes (26). In the abovementioned studies, propidium iodide (PI) was used as the fluorescent stain. Aldehydes decrease fluorescence intensity of the complex PI-DNA (16). With DAPI, the stoichiometry of DNA staining should be affected by crosslinks to a lesser extent (47). However, we found 1% PFA fixation to be associated with significantly lower fluorescence in tench blood compared to fresh tissue, and, in sterlet blood, the histograms contained indistinguishable peaks. Thus, we do not recommend 1% PFA fixation for sterlet or tench blood.

Fin Tissue

Fin tissue sampling is simple, rapid, and minimally invasive (16, 43, 44) and is therefore suitable for small specimens without sacrificing. Lamatsch et al. (12) obtained fin clips from more than 300 fish of mean size 3–5 cm with no mortality after repeat treatment and no effect on swimming behavior.

We found freezing in DMSO to be the most appropriate technique for extended storage of fin clips of both model species. Although the procedure is reported as universal (18), this is likely to be the first report of its use in fin tissue preservation.

Freezing without cryoprotectant was successfully applied by Xavier et al. (25) who froze fin tissue and isolated nuclei of yellowtail tetra *Astyanax altiparanae* fin cells in physiological saline at −20°C. Since we found no other report of using this procedure for fish tissue, freezing without cryoprotectant was also included in this study, although at a lower temperature (−80°C), because it can be produced in field conditions using dry ice. However, the procedure did not give satisfactory results for fin tissue, in general strongly impacting measurability and, in samples in which measurability was not affected, mean CVs exceeded the 3% level.

Lamatsch et al. (12) determined the DNA content of 14 fish species using cell suspension prepared from fin samples and fixed in 70% ethanol. With slight modifications, their protocol was suitable for field conditions. However, it included centrifugation, and our goal was to identify the simplest possible procedure applicable to field conditions. We omitted centrifugation and used lower ethanol concentrations, but, based on the results, this procedure cannot be recommended for a 10 day storage period. Xavier et al. (25) was also unsuccessful with ethanol fixation for fin tissue, despite the fact that they used the same ethanol concentration as Lamatsch et al. and a step for fixation removal. Fixation of samples with an aldehyde did not lead to satisfactory results in our study or in that of Xavier et al. (25).

Fish Larva Tissue

It is sometimes necessary to carry out analysis of fish tissue shortly after hatching (48, 49), for instance, in evaluation of the efficacy of chromosome manipulation, as prolonged rearing of fry before sampling and analysis can lead to changes in the ploidy proportions of observed populations or families (50, 51). Moreover, early developmental stages of fish are sensitive, and large losses are risked when analysis is not immediate. An essential drawback to using these animals in flow cytometry is that they must be sacrificed (52).

No method specific to fixation/preservation of larvae for flow cytometry has been published. Lecommandeur et al. (23) reported success in fixation of early developmental stages of rainbow trout and brown trout *Salmo trutta* embryos for flow cytometry analysis using 1% PFA and subsequent DAPI staining, while fixation with 70% ethanol failed. The authors did not provide all details of fixation, length of sample storage, or statistical analysis of the data. We found PFA to be ineffective and cannot recommend it for larva fixation. Nonetheless, some variations with ethanol seemed promising, particularly 5% ethanol for tench and freezing in 1% ethanol for sterlet. The established criteria were also met by freezing sterlet larvae in saline. None of these mentioned procedures affected measurability or fluorescence, and all produced average CVs <3%.

Implications

Ploidy determination based on estimate of relative DNA content is a common application of flow cytometry in fish research. Recent examples include study of the sex determination system and the reproductive ability of a synthetic octoploid male arising from two *Carassius* species (53) and triploidy induction by hydrostatic pressure shock in Mandarin fish *Siniperca chuatsi* (54) and sterlet (55).

Although the presented protocols of storage extension were intended primarily for quantification of relative DNA content, the possibility of their use for the evaluation of DNA content in absolute units is not excluded. In particular, the protocols yielding CVs <3% that did not affect measurability or F_{st}/F_{sa} after 1, 5, and 10 days storage show the potential for this application. However, all analyses in this study were conducted using DAPI as a fluorescent stain. DAPI shows adenine-thymine base preference, which significantly affects the DNA content estimate (56), and the results should be interpreted with caution. For the estimate of DNA content, the authors (56) recommended fluorochromes showing no base preference, such as PI, recently used in Russian sturgeon *Acipenser gueldenstaedtii* x American paddlefish *Polyodon spathula* hybrids (57). Since the levels of accessibility of a

given fluorochrome to DNA can vary with fixation protocol (16), verification of the suitability of our protocols for subsequent PI staining is necessary for their future application in DNA content evaluation.

The procedures of storage extension presented in this study were investigated exclusively in sterlet and tench tissue samples. We assume that protocols optimal for sterlet tissue can be effective in other chondrosteans, and those approved for tench tissue fixation/preservation are feasible for other teleostean species. We have successfully used the presented protocols in tissue samples obtained from Siberian sturgeon *Acipenser baerii*, Russian sturgeon, European sturgeon *Acipenser sturio*, brook trout *Salvelinus fontinalis*, Arctic charr *Salvelinus alpinus*, and pikeperch *Sander lucioperca*. However, we recommend the verification of the protocols in other fish species, since fish diversity is high, and species-specific differences cannot be discounted.

Conclusions

The most effective procedures for extended sterlet tissue storage are fixation in 15% ethanol for blood, freezing in DMSO for fin, and freezing in 1% ethanol or in physiological saline for larva tail tissue. In tench, freezing in DMSO is the optimal procedure for blood and fin and fixation in 5% ethanol for larva tail tissue. The use of ice and dry ice for cooling and freezing makes the presented protocols feasible under field conditions. The range of possible applications may expand if it is shown that the proposed methods can be effectively applied to other species.

Author Contributions

Martin Hubálek: Conceptualization; data curation; formal analysis; investigation; methodology; project administration; writing-original draft; writing-review and editing. **Martin Flajšhans:** Conceptualization; funding acquisition; investigation; methodology; project administration; resources; supervision; writing-review and editing.

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CHAPTER 4

HOW DO SUBOPTIMAL TEMPERATURES AFFECT POLYPLOID STERLET *Acipenser ruthenus* DURING EARLY DEVELOPMENT?

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How do suboptimal temperatures affect polyploid sterlet *Acipenser ruthenus* during early development?

Martin Hubálek | **Vojtěch Kašpar** | **Tomáš Tichopád** | **Marek Rodina** | **Martin Flajšhans**

Faculty of Fisheries and Protection of Waters, South Bohemian Research Centre of Aquaculture and Biodiversity of Hydrocenoses, University of South Bohemia in České Budějovice, Vodňany, Czech Republic

Correspondence
Martin Hubálek, Faculty of Fisheries and Protection of Waters, South Bohemian Research Centre of Aquaculture and Biodiversity of Hydrocenoses, University of South Bohemia in České Budějovice, Zátiší 728/II, 389 25 Vodňany, Czech Republic.
Email: mhubalek@frov.jcu.cz

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Abstract

Sturgeons are ancient fish exhibiting unique genome plasticity and a high tendency to produce spontaneously autopolyploid genome states. The temperature profiles of the rivers in which sturgeon live and reproduce have been severely altered by human intervention, and the effect of global warming is expected to cause further temperature shifts, which may be detrimental for early developmental stages with narrow windows of thermal tolerance. The comparison of the performance of diploid and autopolyploid sturgeon kept at unfavourable temperatures contributes to scientific knowledge of the effects of polyploid genome states on organisms and can shed light on the ability of polyploids to cope with human-induced alterations to natural conditions. Using the sterlet *Acipenser ruthenus* as a model species, we carried out conventional artificial fertilization, as well as the induction of the second polar body retention (SPBR), of the first mitotic division suppression (FMDS) and of the second polar body retention followed by the first mitotic division suppression (SPBR+FMDS). Two experiments were conducted to evaluate the effect of polyploidy on two basic performance parameters, survival and growth. In Experiment 1, fish belonging to untreated, SPBR-, FMDS- and SPBR+FMDS-induced groups were kept at 10, 16 and 20°C from the neurula stage until the end of endogenous feeding. In Experiment 2, larvae from the untreated and SPBR-induced groups were reared at 10, 16 and 20°C after their endogenous feeding transition for 3 weeks. Based on our findings, we report that the embryos, prelarvae and larvae of triploid *A. ruthenus* do not differ from diploids in their ability to survive, grow and develop under suboptimal temperature conditions, while the survival of tetraploids was significantly reduced even at the optimal temperature and even more so at temperatures far from the optimum. This was also the case in the 2*n*/4*n* mosaics observed in FMDS-induced group. Thus, we assume that in tetraploid and 2*n*/4*n* individuals, the limits of thermal tolerance are closer to the optimum than in diploids. We also conclude that the hexaploid genome state is probably lethal in *A. ruthenus* since none of the hexaploids or 3*n*/6*n* mosaics arising from the SPBR+FMDS induction survived the prelarval period.

KEYWORDS
hexaploidy, mosaicism, polyploidy, sturgeon, tetraploidy, triploidy

1 | INTRODUCTION

Sturgeons (order Acipenseriformes) are members of an ancient evolutionary lineage whose history is intrinsically connected to polyploidization events (Fontana *et al*., 2007; Ludwig *et al*., 2001; Rajkov *et al*., 2014). Probably as a result of at least three whole-genome duplications (Rajkov *et al*., 2014), the current representatives of this group of fish are separable into three well-divided classes based on chromosome numbers: (A) species with ∼120, (B) species with ∼250 (Ludwig *et al*., 2001) and (C) one species with ∼360 (Kim *et al*., 2005) chromosomes. There are two scales proposed for the classification of ploidy levels in these classes: 'evolutionary', which refers to ancient ploidy and supposed tetraploid-octoploid-dodecaploid relationships, and 'functional', which deals with functional genome rediploidization and assumes diploid-tetraploid-hexaploid relationships (Vasil'ev, 2009). Another no less interesting fact related to the unique genomic plasticity of sturgeons is that they surprisingly often produce spontaneous autopolyploids *de novo* at each generation [see the reviews of Havelka & Arai (2019) and Schreier *et al*. 2021)]. At this point, it is important to note that all metazoan organisms that reproduce *via* the fusion of haploid gametes into a new diploid organism are *biologically diploid*, irrespective of the evolutionary ploidy level of the genome. In this sense, biological polyploidy refers to phenomena that differ from evolutionary and functional polyploidy, and evolutionary and functional polyploidy are only meaningful in a phylogenetic context (Flajšhans *et al*., 2013). Taking this into account, 1.5×, 2× and 3× increases in genome size compared to the fusion of normal gametes are known as triploidy, tetraploidy and hexaploidy, respectively, and so the individuals possessing these ploidy levels are referred to as triploids, tetraploids and hexaploids in the text to avoid any misinterpretation. The vast majority of spontaneous autopolyploids observed in sturgeons are triploids (Havelka & Arai, 2019; Schreier *et al*., 2021), although spontaneous tetraploids have been observed in sterlet *Acipenser ruthenus* L. 1758 (Hubálek and Flajšhans, unpublished recent data), a species belonging to chromosome class A, and in Sakhalin sturgeon *A. mikadoi* Hilgendorf 1892 (Zhou *et al*., 2011), a species belonging to chromosome class B. The overwhelming majority of detected sturgeon spontaneous autopolyploids arise as a result of aquaculture and may be linked to hatchery practices (Havelka *et al*., 2016; Van Eenennaam *et al*., 2020), although evidence of its occurrence in the wild has been reported (Schreier *et al*., 2021). These observations confirm the unique plasticity of sturgeon genomes and raise questions regarding the fitness and performance of sturgeon autopolyploids.

In general, autopolyploids are expected to possess higher levels of heterozygosity than their diploid progenitors simply as a result of polysomic inheritance (Moody *et al*., 1993). This may provide them with increased metabolic flexibility, thereby enabling them to cope with a broader range of conditions and thus result in broader ecological tolerance than in diploids (Liu & Adams, 2007; Otto & Whitton, 2000). Polyploids may also benefit from their gene redundancy, thereby offering them protection against deleterious recessive mutations *via* the masking of recessive alleles (Comai, 2005) or, in the long term, enabling the gene duplicates to assume new or slightly varied functions (Adams, 2007; Cheng *et al*., 2018). On the other hand, the changes in cell architecture caused by polyploidy may imply certain disadvantages. Increasing the genomic content usually increases cell volume and may alter the surface-area-to-volume ratio (SA:V) and consequently the rate of metabolic processes, especially those in which membranes are involved (Cavalier-Smith, 1978; Levin, 1983). A further disadvantage of polyploidy may be related to problems with the mechanics of the pairing and separation of chromosomal homologues during mitosis and meiosis, potentially causing aneuploidy (Komen & Thorgaard, 2007; McCombie *et al*., 2005). Finally, polyploidy alters optimized patterns of gene expression and epigenetic modifications (Hegarty & Hiscock, 2008) and may change the regulatory networks that are established and optimized before polyploidization occurs (Comai, 2005; Wertheim *et al*., 2013).

The river systems in which sturgeon live and/or reproduce have been severely modified by human activity that has resulted in a number of environmental changes, for example in water temperatures (Huang & Wang, 2018; Van Eenennaam *et al*., 2005; Zhang *et al*., 2019), the crucial factor affecting the physiological function of ectothermic fishes (Rombough, 1997; Zhang & Kieffer, 2017). Sturgeon migration to natural spawning grounds has been blocked by the construction of hydroelectric dams that have altered the annual and/or seasonal magnitude of the thermal regime of rivers and modified the frequency, duration, timing and rate of change in water temperatures (Olden & Naiman, 2010; Rivers-Moore *et al*., 2013). In addition, other anthropogenic activities such as the release of thermal effluent from power stations (Edinger *et al*., 1968; Webb & Nobilis, 2007) and land-use changes including increased urbanization (Nelson & Palmer, 2007) have had both a direct and indirect impact on water temperature in rivers. The fact that climate change has increased global surface temperatures and is predicted to continue to do so in the future (IPCC, 2021) cannot be ignored either. Projected increases in air temperatures, combined with poorer accumulation of the winter snowpack, the earlier onset of peak spring flows and lower summer baseflows, may all have direct implications for the thermal regimes of streams and rivers (Van Vliet *et al*., 2011). Fish sensitivity to thermal fluctuations varies in terms of the window of thermal tolerance of each species and each life stage (Dahlke *et al*., 2020; Pörtner & Farrell, 2008) and the most sensitive stages to thermal stress – spawning and early life – tend to have the narrowest thermal windows (Pörtner & Peck, 2010). In human-modified habitats, the probability that these life stages will face temperature fluctuations or shifts is increasing, and acute temperature changes produced by anthropogenic activities are typically more severe than the routine changes of temperature that occur in nature (Mandal *et al*., 2016). If the early developmental stages of different ploidy levels are exposed to temperature conditions beyond their optimal thermal range, differences in performance may intensify as the result of ploidy-related differences at both genetic and physiological levels. Furthermore, as hypothesized by Shivaramu *et al*. (2020), the exposure of sturgeon gametes to fluctuating temperature stresses could cause meiotic or fertilization disorders and result in an increased rate of autopolyploidization, which thus makes this issue even more topical.

Artificial induction of polyploidy in sturgeons has been conducted using different types of physical shocks that have been proven useful for creating polyploid sturgeon populations for future research (Beyea *et al*., 2005; Rożyński *et al*., 2015). In the past, the artificial induction of triploidy has been successful in sturgeon species from all chromosome classes (A, B, C) (*e.g*., Flajšhans *et al*., 2020; Flynn *et al*., 2006; Van Eenennaam *et al*., 1996; Vassetzky, 1967), as has the induction of tetraploidy in sturgeon species belonging to classes A and B (Lebeda *et al*., 2020; Lebeda & Flajšhans, 2015). Moreover, a combination of artificial treatments causing the second polar body retention (SPBR) and the first mitotic division suppression (FMDS) has led to the obtaining of hexaploid individuals of Siberian sturgeon *A. baerii* Brandt 1869, a species belonging to chromosome class B (Lebeda *et al*., 2020). Optimized polyploidy induction protocols provide us with the possibility of studying the effects of environmental factors, including the impact of temperature on early development and the fitness of different ploidy levels in sturgeons.

Temperature changes are reflected in enzyme activity, metabolic rate, growth, development and even locomotory functions (Fry, 1971). Temperatures beyond both the high and low limits of the thermal optimum that do not reach lethal limits are called suboptimal and reduce performance because tissue demands for oxygen exceed the oxygen delivery capacity (Pörtner *et al*., 2000; Pörtner & Zielinski, 1998; Sommer *et al*., 1997). This phenomenon is caused by the slowing of oxygen circulation and ventilation in the cold, or by an insufficient increase under warm conditions (Pörtner, 2001). Since the presence of polyploidy affects organisms at both genetic and physiological levels, differences in ploidy levels between individuals may be exaggerated under suboptimal temperature conditions (Hansen *et al*., 2015; Hyndman *et al*., 2003; Sambraus *et al*., 2017). The identification of the differences between diploid and polyploid sturgeons reared simultaneously at optimal and suboptimal temperatures could contribute to current knowledge of the physiology, fitness and viability of polyploids, and may help predict their performance in both human-modified and natural habitats, as well as on farms where bad hatchery practices or technological errors can shift temperatures into suboptimal levels. Yet, if the polyploids can survive and take advantage of the changing temperature conditions, they can theoretically reach maturation, participate in spawning and contaminate the future generation with progeny with abnormal ploidy levels (Schreier *et al*., 2011, 2021). The reproduction of polyploids with diploids may decrease breeding success in natural populations due to the production of progeny with reduced viability. Thus, knowledge of polyploid performance provides additional information regarding the likelihood of such a scenario.

To date, no research has investigated the performance of early developmental stages (embryonal, prelarval or larval periods) of autopolyploid sturgeon at suboptimal temperatures. Nevertheless, the most critical phase of fish rearing occurs in these immature stages and it is generally held that these stages are more sensitive to harmful environmental effects than older individuals (Blaxter, 1992; Wang *et al*., 1987) and are, moreover, essential for the recruitment of new fish into the population (Blaxter, 1992). Therefore, we focused on these early stages using *A. ruthenus* as the model species of sturgeon. In *A. ruthenus*, the optimal temperature for the incubation of embryos is 13–16°C (Chebanov & Galich, 2011), although commercial hatcheries insist on 15–16°C for incubating embryos and rearing prelarvae and larvae (Gela *et al*., 2012). The exact limits of thermal tolerance in these developmental stages have not been reported for this species. Our goal was to induce SPBR, FMDS and SPBR+FMDS to obtain biologically triploid (3*n*), tetraploid (4*n*) and hexaploid (6*n*) individuals of *A. ruthenus*, the model sturgeon species used in our experiments, and to assess the occurrence of abnormal ploidy levels in the progeny obtained from artificial inductions. Furthermore, we incubated and reared diploid (2*n*) and polyploid embryos, prelarvae and larvae at zoptimal (16°C) and suboptimal temperatures (10 and 20°C) assessed in the preliminary test and evaluated the effect of polyploidy on basic performance parameters, survival and growth to examine whether or not young *A. ruthenus* polyploid cope better with suboptimal conditions than their 2*n* siblings.

2 | MATERIALS AND METHODS

Artificial reproduction, polyploidy induction and the rearing and sampling of experimental animals were carried out at the Genetic Fisheries Centre, Faculty of Fisheries and Protection of Waters (FFPW), the University of South Bohemia in České Budějovice, Czech Republic. The storage and cytometric analysis of samples were conducted in the University's laboratories in Vodňany.

2.1 | Ethical statement

All experiments were carried out in accordance with the Animal Research Committee of the FFPW. Fish were kept according to principles based on the EU harmonized animal welfare act of the Czech Republic and to principles of laboratory animal care that complied with national law (Act No. 246/1992 on the protection of animals against cruelty).

2.2 | Experimental fish

2.2.1 | Reproduction

The artificial spawning of *A. ruthenus* was conducted according to the methodology described by Gela *et al*. (2008). For reproduction, six females and five males aged 6–8 years were used. From each female, 190 g of oocytes were obtained and mixed together. From each selected male, 6 ml of sperm with spermatozoa motility of at least 70% were placed into a single container and stored on ice. Simultaneously, albino *A. ruthenus* were bred to obtain a visually distinguishable internal control group for the larval test in Experiment 2 (see Section 2.4). Albino *A. ruthenus* were bred using the same methods as in the *A. ruthenus* of normal coloration: chosen fish were aged

6–8 years, 50 g of oocytes were obtained from each of two albino females and 1.5 ml of sperm was taken from each of two albino males.

2.2.2 | Gamete activation and polyploidy induction

Individual sperm batches were activated with water at 16°C and the resulting suspensions were immediately mixed with the oocytes. The amounts of the components used for the individual gamete activations were as follows: 100 g of oocytes + 0.5 ml of sperm from each of the five males + 400 ml of water for normal fertilization (untreated group); 200 g of oocytes + 1 ml of sperm from each of the five males + 800 ml of water for SPBR induction; 400 g of oocytes + 2 ml of sperm from each of the five males + 1600 ml of water for FMDS and SPBR+FMDS inductions (separated and processed in two batches of 200 g of oocytes); and 100 g of oocytes + 1.5 ml of sperm from each of the two males + 400 ml of water for the albino *A. ruthenus*. Three minutes after gamete activation, the treatment for removal of egg stickiness was performed by oocyte exposure to a 0.04% tannic acid bath, first for 40 s, then for 30 and 20 s, with rinsing in hatchery water after each exposure. When this treatment was complete, the incubation of the untreated and albino groups started. The individuals intended to be polyploids were kept in a controlled environment of 16°C until treatments were induced. The SPBR, FMDS and SPBR+FMDS inductions were conducted to obtain 3*n*, 4*n* or 6*n* individuals, respectively. For SPBR induction, the eggs were immersed in a water bath for 2 min at 34°C, 18 min after gamete activation, the shock parameters being chosen according to our previous experience with *A. ruthenus* (Lebeda *et al*., 2015, 2018). The FMDS was induced 60 min post-activation using a shock of 37°C for 2 min (Lebeda & Flajšhans, 2015). The SPBR+FMDS induction was achieved by the combination of the treatments used for both SPBR and FMDS inductions with incubation at 16°C.

2.2.3 | Incubation and rearing of prelarvae and larvae

Incubation was carried out using Kannengieter jars supplied with recirculated dechlorinated tap water at 16°C, according to the methodology used by Gela *et al*. (2012). To calculate the fertilization and neurulation rates, approximately 100 embryos were randomly sampled in triplicate from each experimental group (untreated, SPBR-induced, FMDS-induced and SPBR+FMDS-induced) 4 h and also 3 days post-fertilization (in the four-cell stage and after the neurulation had finished, respectively), and living and dead embryos were counted by observation under a stereomicroscope. After hatching, the prelarvae were reared in tanks using the methods commonly employed in sturgeon hatcheries (Gela *et al*., 2012); all individual groups were maintained separately. The fish that survived the prelarval period were fed *ad libitum* with freshly hatched *Artemia nauplii*.

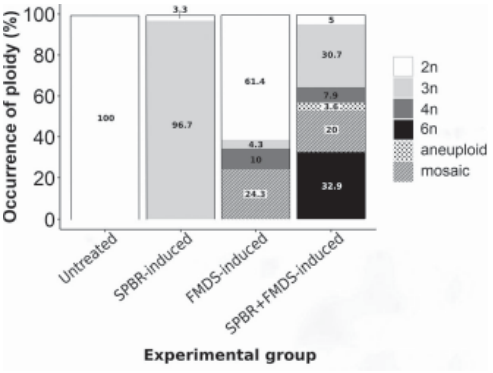

FIGURE 1 The occurrence of ploidy levels in *Acipenser ruthenus* prelarvae obtained from the different treatments (untreated, SPBR-induced, FMDS-induced and SPBR+FMDS-induced) and incubated at 16°C. Results were obtained from the analysis of 30 individuals belonging to the untreated and SPBR-induced groups, and from 140 individuals from the FMDS-induced and SPBR+FMDS-induced groups. The occurrence of mosaic prelarvae is shown as the sum of the percentages of all types of mosaics observed for a particular group. The percentage of individual mosaic types corresponded to 23.6% and 0.7% of 2*n*/4*n* and 2*n*/3*n*, respectively, for the FMDS-induced group, and 12.1%, 1.4%, 0.7%, 0.7%, 0.7%, 0.7%, 0.7% and 2.9% of 3*n*/6*n*, 2*n*/4*n*, 1*n*/2*n*, 1*n*/5*n*, 2*n*/6*n*, 2*n*/3*n*/4*n*, 4*n*/8*n* and aneuploid mosaics, respectively, for the SPBR+FMDS-induced group. (□) 2*n*; (■) 3*n*; (■) 4*n*; (■) 6*n*; (■) aneuploid; (■) mosaic

2.2.4 | Ploidy analysis

When hatching had finished, 30 prelarvae from the untreated and SPBR-induced groups, as well as all 140 prelarvae from the SPBR+FMDS-induced group and an equal number from the FMDS-induced group, which had previously been kept separately at 16°C, were used for the preliminary ploidy investigation. This step was included to evaluate the efficiency of the polyploidy induction in all treated groups and the sampling strategy in Experiments 1 and/or 2 (see Sections 2.3 and 2.4). The prelarvae were euthanized with CO_2 and processed for flow cytometric analysis (FCA) following Lecommandeur *et al.* (1994). The cell nuclei from prelarval tail tissue were analysed using a CCA I flow cytometer (Partec GmbH, Münster, Germany) as per Lebeda and Flajšhans (2015), and the DNA content was estimated relative to diploid cells from *A. ruthenus* prelarval tail tissue, which served as a standard.

Since none of the polyploid levels was induced with 100% efficiency (Figure 1), after terminating the experiments we measured the ploidy levels in all prelarvae obtained from the SPBR, FMDS and SPBR+FMDS inductions included in Experiments 1 and/or 2. These prelarvae were frozen in a DMSO-citrate buffer (Vindelov *et al*., 1983) and subsequently stored, processed and analysed as described by Hubálek and Flajšhans (2020). The individuals from the untreated group were regarded as 2*n* since the FCA confirmed a 100% occurrence of this ploidy (Figure 1).

TABLE 1 The design of Experiment 1 and Experiment 2

	Start	Groups	Kept at (°C)	Experimental treatment (°C)	Number of boxes per group per treatment	Individuals per box	Duration	Internal control (albino sterlet)
Experiment 1	Neurula stage	Untreated, SPBR-induced, FMDS-induced, SPBR+FMDS-induced	16	10, 16, 20	3	Approx. 100 (1.4 g of oocytes)	Until the end of endogenous feeding	No
Experiment 2	5 days of exogenous feeding	Untreated, SPBR-induced	16	10, 16, 20	5	33	3 weeks	22 larvae per box

2.3 | Experiment 1: Suboptimal temperatures for embryos and prelarvae

This experiment covered the period beginning in the neurula stage and ending at the end of endogenous feeding (EF). The putative 2*n*, 3*n*, 4*n* and 6*n* individuals were reared at 10, 16 and/or 20°C. At the end of the trial, the mortality in the neurula-to-hatching period (abbreviated as hatching period, HP), survival in the prelarval period (PP), prelarval body mass and the cumulative survival were evaluated for each ploidy level and statistically compared. The suboptimal temperature conditions and the optimal time for beginning the experiment were selected based on preliminary tests (Supporting Information Text S1). The design of Experiment 1 is summarized in Table 1.

The experiment started 3 days post-fertilization. Just before the trial began, 36 3.75 l rearing boxes, each equipped with a 10 cm silicone aquarium hose with an adapter and air stone, were filled with 3 l of fresh water at 16°C. Then, 1.4 g of embryos (approx. 100 individuals) from the untreated group were transferred from the incubation jar into each of the nine boxes; the same process was repeated for the embryos belonging to the SPBR-, FMDS- and SPBR+FMDS-induced groups. The boxes were distributed equally in three incubators kept at temperatures of 10, 16, and 20°C, with three boxes from each group in each incubator. The incubators were equipped with lighting, and a 16 h:8 h light/dark photoperiod was guaranteed by LED strip lights with timers. Each of the incubators had an air pump incorporated, with an air splitter supplying tubes attached to the adapters of each rearing box to ensure permanent aeration. The aeration tubes were detached from the adapters only when the individual boxes were manipulated.

Manipulation took place once per day. The dead embryos and prelarvae were counted, recorded and removed from rearing boxes, and the prelarvae obtained from polyploidy inductions were sampled for subsequent FCA. Next, the mechanical impurities were eliminated using a silicone tube and one-third of the water was gently replaced by fresh water at the same temperature. The experiment ended when the prelarvae reached the end of the endogenous feeding period, which was detected by the presence of numerous melanin (faecal) plugs, expelled from the fishes' anal openings, on the bottom of the rearing boxes. When these plugs appeared, the prelarvae were counted and up to 40 individuals per box from the untreated and SPBR-induced groups were weighed. All individuals obtained from the polyploidy inductions were sampled for FCA.

2.4 | Experiment 2: Suboptimal temperatures for larvae and juveniles

In this experiment, the 2*n* and putative 3*n* larvae hatched at 16°C were transferred to boxes at 10, 16 and/or 20°C once exogenous feeding had begun. Larvae were transferred together with the internal control albino *A. ruthenus* larvae. This albino control enabled us to assess environmental variation between rearing structures and express standardized performances for the tested groups

(Kirpichnikov, 1987; Vandeputte *et al*., 2002). This was because the change in the number of larvae in individual rearing boxes resulting from different survival rates could have had an impact on the performance of the surviving larvae and possibly mask the effects of ploidy. To avoid this, the internal control of easily distinguishable albino specimens allowed us to calculate corrected survival and corrected body masses of 2*n* and 3*n* individuals following Kocour (2013). The experimental treatment lasted for 3 weeks. The corrected survival and body masses of 2*n* and 3*n* individuals in the larval period (LP) were evaluated and statistically compared at the end of the trial. The putative tetraploid and hexaploid larvae were not used for this experiment because of their scarcity. The design of Experiment 2 is summarized in Table 1.

The experiment began after 5 days of exogenous feeding. The rearing boxes from Experiment 1 were used and, in all, 30 boxes were filled with 3 l of water at 16°C. Subsequently, 22 albino larvae were placed in each of the boxes. Then, 33 diploids were added to each of the 15 boxes and 33 putative triploids to each of the remaining 15 boxes. The boxes with both the albino larvae and 2*n* larvae of normal coloration were distributed equally between three incubators kept at temperatures of 10, 16, and 20°C in five boxes per temperature. The same process was performed for the boxes holding both the albino larvae and putative 3*n* larvae of normal coloration. All incubators were equipped with the same system for aeration and lighting as in Experiment 1, and permanent aeration and a 16 h:8 h lighting regime were provided as before.

The feeding of experimental larvae started the following day and continued until the end of the trial. Approximately 0.3 ml of freshly prepared *Artemia* was supplied using a Pasteur pipette to each rearing box three times per day. Sediments were siphoned from the boxes twice a day and 1.5 l of water was changed daily.

During the first 3 days of rearing in boxes, dead individuals were replaced by fresh ones belonging to the same group (the losses during this period were considered to be the side effect of manipulation that occurred when placing the larvae into the rearing boxes). After this acclimation period, the dead larvae were counted and removed from rearing boxes every day but no longer replaced. Dead larvae obtained from the SPBR induction were saved for subsequent FCA. After 3 weeks, the surviving larvae were counted and individually weighed, and SPBR-induced individuals were sampled for FCA. Corrected survival was calculated for each rearing box and corrected body mass was calculated for each individual using the following formulae (Kocour, 2013):

$C_{si} = S_i{}^* c_1/c_2$

C_{si} = corrected survival of group *i*

S_i = observed survival of group *i*

c_1 = mean survival of the albino group in all rearing boxes

c_2 = mean survival of the albino group in a particular rearing box

$W_{ci} = w_i{}^* c_1/c_2$

W_{ci} = corrected body mass of individual *i*

w_i = observed body mass of individual *i*

c_1 = total mean body mass of the albino group

c_2 = mean body mass of control group reared in the same box as individual *i*

2.5 | Statistical analysis

All datasets were tested for homoscedasticity and normal distribution using Levene's test and a Shapiro–Wilk test, respectively. An arcsine transformation was used prior to analyses for all percentage data (embryonic mortality, prelarval survival and corrected survival of larvae) and for corrected body masses of larvae since this helped to meet the criterion of residual normality in this case. The effect of treatment on fertilization, neurulation and hatching rates was evaluated using a one-way ANOVA with a *post hoc* Tukey's HSD test (Table 2). The analysis of embryonic mortality was conducted using a factorial two-way ANOVA, where treatment and temperature were treated as fixed effects; for prelarval survival and corrected survival of larvae, the effect of treatment was replaced by the effect of ploidy. The body masses at the end of endogenous feeding and the corrected body masses of larvae were analysed using a three-way mixed effects ANOVA with ploidy and temperature set as fixed effects and the rearing box set as a random effect. Significant differences between groups were identified by *post hoc* Tukey's HSD test; the effect of treatment or ploidy within temperature, the effect of temperature within ploidy and the effect of temperature alone were all evaluated. Statistical tests were performed using R programming language software version 4.0.1, with an α of 0.05 set as the significance level.

3 | RESULTS

3.1 | Fertilization, neurulation and hatching rate

The fertilization, neurulation and hatching rates differed significantly between the individual treated groups (ANOVA, $F_{(3,8)} = 19.66$, 52.58, 288.81, respectively, $P < 0.001$; Table 2). The fertilization rate in the SPBR+FMDS-induced group was lower than in other groups, while the neurulation and hatching rates of all treated groups were lower than in the untreated group (Table 2 and Supporting Information Table S1).

3.2 | The efficiency of polyploidy induction

The occurrence of various ploidy levels in hatched individuals obtained from different treatments and incubated under optimal temperature conditions (16°C) is summarized in Figure 1. In the untreated group, fertilization obtained 100% of 2*n* individuals. The efficiency of the polyploidy induction was also very high in the case of SPBR induction, with 96.7% of prelarvae being 3*n* ($n = 30$) but significantly lower in the FMDS and SPBR+FMDS inductions, with 10% and 32.9% of

TABLE 2 The fertilization, neurulation and hatching rates observed in differently treated embryos of *Acipenser ruthenus* incubated at 16°C

Group	Fertilization rate (%)	Neurulation rate (%)	Hatching rate (%)
Untreated	82.00 ± 1.53^{b}	69.11 ± 6.85^{c}	64.8 ± 0.95^{d}
SPBR-induced	80.49 ± 2.70^{b}	39.60 ± 2.54^{b}	36.32 ± 2.34^{c}
FMDS-induced	75.76 ± 1.50^{b}	30.22 ± 6.01^{ab}	20.19 ± 2.57^{b}
SPBR+FMDS-induced	62.18 ± 6.36^{a}	19.85 ± 2.39^{a}	6.92 ± 2.07^{a}

Note: Values are expressed as mean ± SD, different alphabetic superscripts denote significant differences among differently treated groups ($\alpha = 0.05$).

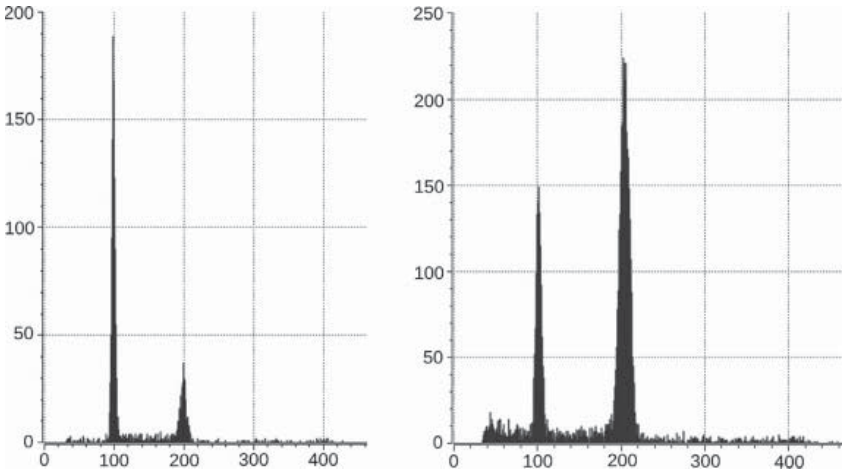

FIGURE 2 Examples of nuclear DNA content histograms obtained after flow cytometric analysis of two 2*n*/4*n* mosaics of *Acipenser ruthenus* with different proportions of 2*n* and 4*n* cells. In the first individual (left), the 2*n* cells (~channel 100) are more numerous than the 4*n* cells (~channel 200), while in the second individual (right), the 4*n* cells predominate over the 2*n* cells

analysed individuals ($n = 140$) being 4*n* and 6*n*, respectively. A significant amount of hatched prelarvae from the FMDS induction were mosaics, 23.6% of which were 2*n*/4*n* and 0.7% 2*n*/3*n*. The occurrence of mosaics in the SPBR+FMDS-induced group was high as well, with 12.1% of prelarvae being 3*n*/6*n*, 1.4% 2*n*/4*n*, 0.7% 1*n*/2*n*, 0.7% 1*n*/5*n*, 0.7% 2*n*/6*n*, 0.7% 2*n*/3*n*/4*n*, 0.7% 4*n*/8*n* and 2.9% aneuploid mosaics. The proportion of cells having different ploidy in individual mosaics varied considerably (Figure 2).

3.3 | Experiment 1

The mean embryonic mortalities at 10, 16 and 20°C were, respectively (mean ± SD), 6.14 ± 1.66% ($n = 260$), 2.74 ± 1.16% ($n = 291$) and 15.23 ± 13.17% ($n = 302$) in the untreated group, 9.55 ± 5.71% ($n = 296$), 4.31 ± 2.98% ($n = 300$) and 11.13 ± 4.29% ($n = 266$) in the SPBR-induced group, 43.95 ± 9.52% ($n = 275$), 43.95 ± 1.28% ($n = 265$) and 67.08 ± 22.96% ($n = 282$) in the FMDS-induced group and, finally, 74.22 ± 2.77% ($n = 283$), 79.93 ± 7.59% ($n = 300$) and 81.04 ± 3.57% ($n = 305$) in the SPBR+FMDS-induced group. Embryonic mortality (evaluated for HP) was significantly affected by the

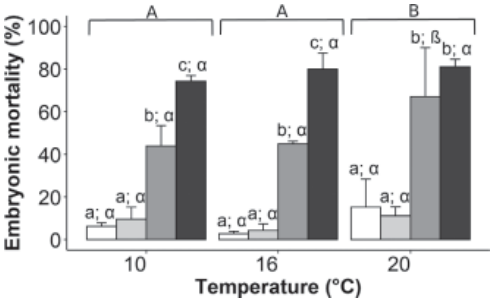

FIGURE 3 Mortality of *Acipenser ruthenus* in the neurula stage to hatching period observed in Experiment 1 in untreated, SPBR-induced, FMDS-induced and SPBR+FMDS-induced groups incubated at 10, 16 and 20°C. Capitals denote significant differences between incubation temperatures ($\alpha = 0.05$). Lowercase letters denote significant differences between differently treated groups at particular temperatures ($\alpha = 0.05$). Greek letters denote significant differences between incubation temperatures in specifically treated groups ($\alpha = 0.05$). (□) untreated; (▨) SPBR induced; (▩) FMDS induced; (■) SPBR+FMDS-induced

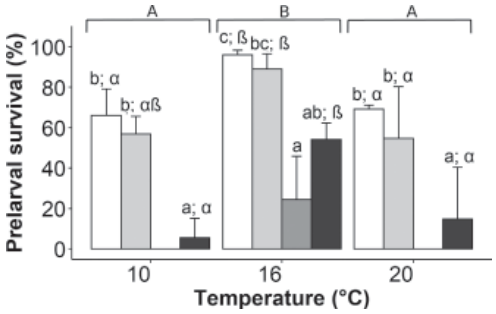

FIGURE 4 The survival of 2*n*, 3*n*, 4*n* and 2*n*/4*n* prelarvae of *Acipenser ruthenus* reared at 10, 16 and 20°C. Capitals denote significant differences between rearing temperatures ($\alpha = 0.05$). Lowercase letters denote significant differences between ploidies at particular temperatures ($\alpha = 0.05$). Greek letters denote significant differences between rearing temperatures in particular ploidies ($\alpha = 0.05$). The survival of 6*n* and 3*n*/6*n* prelarvae is not included since none survived the prelarval period. (□) 2*n*; (▥) 3*n*; (▦) 4*n*; (■) 2*n*/4*n*

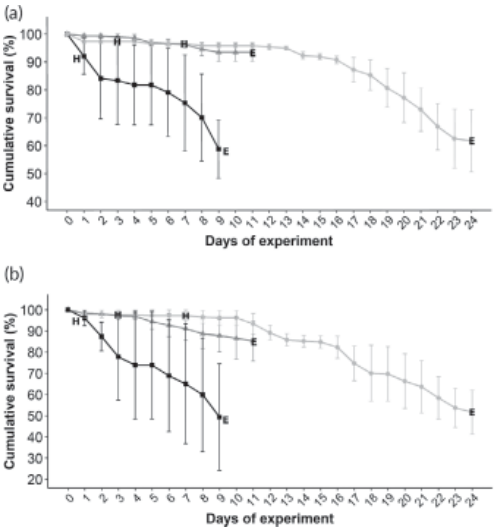

FIGURE 5 The cumulative survival of *Acipenser ruthenus* 2*n* (a) and 3*n* (b) individuals at 10, 16 and 20°C. Incubation lasted from the neurula stage until the end of endogenous feeding; the cumulative survival is expressed as percentage values. H, hatching; E, end of endogenous feeding. (a) Temperature: (–) 10°C; (–) 16°C; (–) 20°C. (b) Temperature: (–) 10°C; (–) 16°C; (–) 20°C

treatment (ANOVA, $F_{(3,24)} = 125.29$, $P < 0.001$) and temperature (ANOVA, $F_{(2,24)} = 6.58$, $P = 0.005$), and the interaction of treatment and temperature was nonsignificant (ANOVA, $F_{(6,24)} = 1.10$, $P = 0.39$). The incubation of embryos at 20°C led to greater mortality than at 10 and 16°C, which was caused mainly by a statistically significant increase in mortality in the FMDS-induced group (Figure 3 and Supporting Information Table S2). Embryonic mortality in the untreated and SPBR-induced groups did not differ at any temperature, while mortality in the FMDS-induced group was always higher. The highest mortalities were observed in the SPBR+FMDS-induced group (Figure 3 and Supporting Information Table S2).

Since the ploidy screening in hatched prelarvae showed a high occurrence of 2*n*/4*n* and 3*n*/6*n* mosaics, it was considered reasonable to calculate prelarval survival not only for 2*n*, 3*n*, 4*n* and 6*n* individuals but also for the mosaics. All the 6*n* and 3*n*/6*n* individuals (at 10, 16 and 20°C, $n = 26$, 18, 21 and 6, 7, 4, respectively) died during the prelarval period, as did the 4*n* individuals at suboptimal temperatures (at 10 and 20°C, $n = 11$ and 3, respectively), and thus they were not included in the statistical analysis. The prelarval survival rates at 10, 16 and 20°C were, respectively (mean ± SD), 65.99 ± 13.15% ($n = 244$), 96.11 ± 2.22% ($n = 283$) and 69.15 ± 2.05% ($n = 258$) in 2*n* individuals, 56.85 ± 8.78% ($n = 268$), 88.97 ± 7.45% ($n = 287$) and 54.65 ± 25.72% ($n = 237$) in 3*n* individuals, and 5.56 ± 9.62% ($n = 35$), 54.18 ± 8.13% ($n = 33$) and 14.81 ± 25.66% ($n = 17$) in 2*n*/4 mosaics, while the survival of 4*n* prelarvae at 16°C was 24.44 ± 21.43% ($n = 11$). The effects of ploidy and temperature on prelarval survival were significant (ANOVA, $F_{(3,24)} = 44.95$, $P < 0.001$; ANOVA, $F_{(2,24)} = 20.95$, $P < 0.001$, respectively), while the interaction between the factors was nonsignificant (ANOVA, $F_{(6,24)} = 0.35$, $P = 0.903$). The prelarvae from all experimental groups reared at 16°C survived better than those at 10 and 20°C. The survival of 2*n* individuals did not differ from that of 3*n* individuals at any temperature, while the survival of 4*n* was significantly lower. The mosaic survival rate was lower than in 2*n* and 3*n* individuals at 10 and 20°C but did not differ from 4*n* and 3*n* individuals at 16°C (Figure 4 and Supporting Information Table S3). The cumulative survival at different ploidy levels and temperatures is shown in Figures 5 and 6.

The body masses of individuals at the end of the prelarval period after rearing at 10, 16 and 20°C were, respectively, 23 ± 1.19 ($n = 120$), 20.73 ± 0.63 ($n = 120$) and 19.41 ± 0.52 ($n = 120$) mg in 2*n*, and 19.53 ± 0.52 ($n = 120$), 19.49 ± 0.62 ($n = 120$) and 20.82 ± 0.79 ($n = 100$) mg in 3*n*. Body mass did not differ significantly between ploidies (ANOVA, $F_{(1,12)} = 0.47$, $P = 0.506$) or between rearing temperatures (ANOVA, $F_{(2,12)} = 1.42$, $P = 0.279$), with the interaction between ploidy and temperature also being nonsignificant (ANOVA, $F_{(2,12)} = 1.05$, $P = 0.379$) (Supporting Information Figure S1 and Table S4).

3.4 | Experiment 2

The corrected survival rates of larvae reared for 3 weeks at 10, 16 and 20°C were, respectively (mean ± SD), 77.88 ± 4.34%, 84.89 ± 14.99% and 57.25 ± 4.64% in 2*n* and 73.41 ± 16.37%, 76.23 ± 19.41% and 44.74 ± 6.03% in 3*n* ($n = 165$ per temperature and ploidy). The corrected survival of larvae was affected by temperature

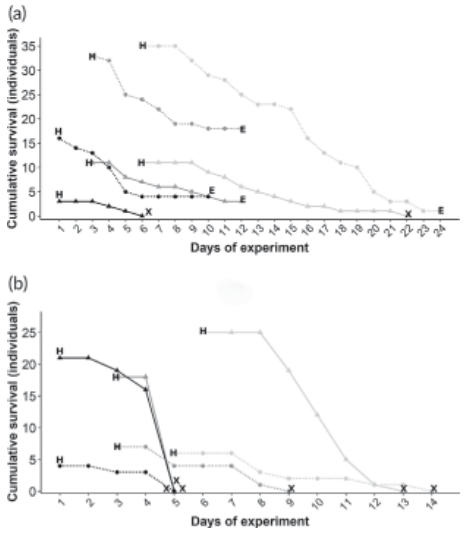

FIGURE 6 The cumulative survival of *Acipenser ruthenus* tetraploids and 2*n*/4*n* mosaics from the FMDS-induced group (a), and hexaploids and 3*n*/6*n* mosaics from the SPBR+FMDS-induced group (b) at 10, 16 and 20°C. Incubation lasted from the neurula stage until the end of endogenous feeding; cumulative survival is expressed as the absolute numbers of individuals. The graphs begin at hatching since the ploidy was determined in the prelarval period.
H, hatching; E, end of endogenous feeding; X, death of all individuals before reaching the end of endogenous feeding. (a) Ploidy: (◦) 2*n*/4*n*; (▪) 4*n*. Temperature: (◦) 10°C; (▫) 16°C; (▪) 20°C. (b) Ploidy: (▫) 3*n*/6*n*; (▪) 6*n*. Temperature: (◦) 10°C; (▫) 16°C; (▪) 20°C

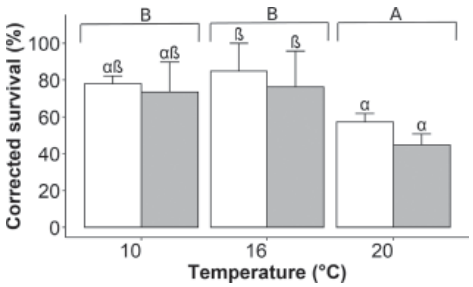

FIGURE 7 Corrected survival of 2*n* and 3*n* larvae of *Acipenser ruthenus* reared for 3 weeks at 10, 16 and 20°C. Capitals denote significant differences between rearing temperatures ($\alpha = 0.05$). Greek letters denote significant differences between rearing temperatures in particular ploidies ($\alpha = 0.05$). (□) 2*n*; (▥) 3*n*

(ANOVA, $F_{(2,19)} = 9.27$, $P = 0.002$) but unaffected by ploidy (ANOVA, $F_{(1,19)} = 3.51$, $P = 0.077$), and the interaction between factors was nonsignificant (ANOVA, $F_{(2,19)} = 0.004$, $P = 0.996$). The corrected survival at 20°C was lower than at 10 and 16°C (Figure 7 and Supporting Information Table S5).

The corrected body masses of larvae reared for 3 weeks at 10, 16 and 20°C were, respectively (mean ± SD), 191.26 ± 43.83 ($n = 157$), 165.71 ± 63.8 ($n = 138$) and 140.98 ± 67.78 ($n = 41$) mg in 2*n*, and 232.11 ± 67.7 ($n = 136$), 187.06 ± 81.98 ($n = 140$) and 150.09 ± 60.2 ($n = 30$) mg in 3*n*. Temperature had significant effect on the corrected body mass of larvae (ANOVA, $F_{(2,20)} = 3.89$, $P = 0.037$), while both the ploidy effect (ANOVA, $F_{(1,20)} = 2.84$, $P = 0.108$) and the effect of interaction (ANOVA, $F_{(2,20)} = 0.19$, $P = 0.825$) were nonsignificant. The greatest corrected body masses were observed in larvae reared at 10°C and the smallest in individuals at 20°C, while the body mass of larvae reared at 16°C did not differ from other temperatures (Supporting Information Figure S2 and Table S6).

4 | DISCUSSION

4.1 | The effect of temperature on polyploids

In this study, it was crucial to define suboptimal temperatures – above all in *A. ruthenus* – because Acipenseridae members have different temperature sensitivities (Andrei *et al*., 2018). The use of 10 and 20°C lowered the survival of diploids reared from neurula until the onset of the endogenous period by 30% and 32% (compared to optimal temperature conditions). These results suggest that the appropriate suboptimal temperatures were selected for use in this study. The effect of temperature on the rate of early development was obvious from the length of EF, which lasted for 11 days at 16°C but was reduced by 2 days at 20°C and prolonged by 13 days at 10°C in both diploids and triploids. The considerable alterations in the rate of development have also been observed in other studies dealing with the impact of suboptimal temperatures on sturgeon embryos and prelarvae, for example in green sturgeon *A. medirostris* Ayres 1854 (Van Eenennaam *et al*., 2005), lake sturgeon *A. fulvescens* Rafinesque 1817 (Zubair *et al*., 2012) and white sturgeon *A. transmontanus* Richardson 1836 (Wang *et al*., 1987). However, these studies focused solely on the performance of the individuals of common ploidy.

4.1.1 | Triploids

In our study, HP mortality, prelarval survival and corrected survival of larvae did not differ significantly between 2*n* and putative or diagnosed 3*n* individuals at any incubation or rearing temperature, although it is of note that the mean survival of 3*n* prelarvae and larvae were always slightly lower. In the available literature, the relationship between the viability of 2*n* and 3*n* in early developmental stages of sturgeons is not consistent and previous studies have only ever been carried out using physiologically optimal temperature conditions for incubation and rearing. For example, no apparent differences in neurulation and hatching rates were observed in 2*n* and 3*n* bester (Fopp-

Bayat *et al.* 2007b; Omoto *et al.*, 2005); pressure-shock-induced triploids of *A. baerii* had the same hatching rate as diploids (Flajšhans *et al.*, 2020), while the embryos of 3*n A. transmontanus* showed increased mortality in the period between the neurula stage and hatching (Van Eenennaam *et al.*, 1996). Fopp-Bayat *et al.* 2007a dealt with the viability of untreated hybrids of *A. baerii* × bester (*Huso huso* × *A. ruthenus*) with ∼180 chromosomes and triploidized hybrids of the same species with ∼300 chromosomes, and found that the mortality of 3*n* hybrids before the onset of exogenous feeding was approximately twice as high as the mortality of 2*n* hybrids. In these studies, these researchers evaluated gastrulation, neurulation and hatching rates and/or survival at the beginning of exogenous feeding but none noted different timing in developmental stages between 2*n* and putative or diagnosed 3*n* individuals. In our study, the putative 2*n* and 3*n* individuals reached neurula stage at approximately the same time. The hatching and the end of the endogenous period occurred in 2*n* and 3*n* individuals reared at individual temperatures synchronously, assuming a similar rate of early development at both ploidy levels. The body masses of 3*n* individuals at the end of prelarval period and corrected body masses of 3*n* larvae reared for 3 weeks in different temperatures did not differ from their 2*n* counterparts. Overall, we believe that the performance of these two ploidy levels at optimal and suboptimal temperatures was similar. On the other hand, some differences may become more evident as fish become older. For example, Leal *et al.* (2021) observed lower body mass and growth rates in 3*n A. transmontanus* in a 15-week growth trial with 2-month-old fish kept under optimal temperature conditions, and after a 6-week trial of 12-month-old fish at both optimal and suboptimal temperatures (Leal *et al.*, 2019).

4.1.2 | Higher ploidy levels

Unfortunately, few tetraploid individuals could be obtained for this study, which hindered any attempt at drawing conclusions from their performance. Furthermore, it is difficult to calculate to what extent the mortality of the FMDS-induced group was caused by shock or by tetraploidy. On the other hand, it is vital to highlight that the mortality of FMDS-induced embryos was significantly higher at 20°C and none of the 4*n* prelarvae was able to survive the prelarval period at both suboptimal temperatures. This could indicate that temperature tolerance in tetraploids is not as wide as it is in diploids and triploids. Tetraploids may have altered genomic networks leading to dosage imbalances and abnormal expressions (Yin *et al.*, 2018), and the effect of this limitation could theoretically be even greater at suboptimal temperatures, where the level of expression could change (Politis *et al.*, 2017). Another possible explanation may be related to the changes in the SA:V ratio in nuclei and erythrocytes (Bytyutskyy *et al.*, 2014), or reduced haemoglobin, as observed, for example, in triploids of *A. transmontanus* (Leal *et al.*, 2020). However, to the best of our knowledge, no study dealing with the haematology of induced tetraploids in sturgeon exists. The survival of 2*n*/4*n* mosaics was significantly lower than that of diploids and it was significantly lower in suboptimal temperatures. We suppose that the cause of this trend was the same as in tetraploids but was less obvious due to the presence of 2*n* cells in these mosaics. The performance of prelarvae identified as mosaics by the flow cytometry of tail tissue could also be affected by nonidentical ploidy levels in different tissues of these individuals. For example, Iegorova *et al.* (2018) demonstrated that mosaicism was not present in all organs of their 4-month-old fish derived from multiple-sperm fertilization. The performance could also theoretically vary between mosaics due to differing proportions of 2*n* and 4*n* cells, since these ratios differ between mosaic individuals, as was the case in the study by Iegorova *et al.* (2018).

The SPBR+FMDS-induced embryos had the highest mortality in HP of all the groups examined and all their hexaploid and 3*n*/6*n* prelarvae died before the end of endogenous feeding. Lebeda *et al.* (2020) similarly noted that the induced hexaploids of *A. baerii* did not survive the yolk stage. The presence of a hexaploid genotype could be lethal in sturgeons, although the double heat shock might be the cause of the total mortality of hexaploids and 3*n*/6*n* mosaics.

4.2 | The impact of polyploidy induction on mortality in early developmental stages

SPBR induction was successfully conducted in this experiment, with 96.7% of analysed prelarvae being 3*n*. The loss of embryos during the period between fertilization and neurulation was higher than in the untreated group, although this phenomenon was probably the direct result of the shock and the mortality in HP was considerably lower.

The artificial FMDS in *A. ruthenus* was optimized by Lebeda and Flajšhans (2015), who observed a hatching rate of 12% with 67% tetraploid prelarvae when working with 100 g of unstuck eggs. In this study, the implementation of the authors' findings led to a hatching rate of 20.2%, although only 10% of prelarvae were found to be tetraploid. The lower efficiency of our induction could be explained by the greater number of eggs treated by heat shock (400 g of oocytes were separated and treated in two batches of 200 g of oocytes) or by a female effect potentially affecting the optimal timing for the application of the inducing treatment (Lebeda *et al.*, 2020). Although only a few tetraploids were obtained in our experiment, some material for Experiment 1 was still generated despite the general difficulty in provoking tetraploidy in fish (Piferrer *et al.*, 2009). Lebeda and Flajšhans (2015) believed that their heat FMDS-inducing shock was responsible for the high mortality observed in the first 2 months after induction. On the other hand, Sakao *et al.* (2006) used pressure-induced FMDS for both gynogenetic rediploidization and tetraploidization in masu salmon *Oncorhynchus masou* (Brevoort 1856) and observed that all tetraploid embryos began to die simultaneously around the hatching period (34 days post-fertilization), while the gynogenotes had normal morphology and survived beyond 50 days post-fertilization. Based on this finding, Sakao *et al.* (2006) suggested that the mortality of tetraploids may be the result of tetraploidy itself rather than simply a side effect of the inducing treatment. As is obvious from the above, it is difficult to gauge to what extent mortality in

young stages is caused by shock or by the ploidy level itself. We thus began Experiment 1 in the neurula stage and considered the inducing treatment to be responsible only for the losses in earlier developmental stages.

There is little available information about the artificial production of hexaploidy fish in the literature. In *A. baerii*, hexaploidy was achieved by SPBR followed by FMDS in an experiment conducted using a small number of eggs on Petri dishes, which gave a hatching rate of 3.1%, of which 66.7% of individuals had hexaploidy (Lebeda *et al.*, 2020). In our study, an analogical principle was applied in *A. ruthenus* for mass induction and yielded a 6.9% hatching rate and 32.9% prelarval exaploidy. Taking into account the number of successfully induced polyploids and their percentage of the total number of prelarvae obtained, the effectivity of SPBR+FMDS induction was, surprisingly, higher than the effectivity of the FMDS induction. One possible explanation is that SPBR delayed the very early development, as observed, for example, by Hassan *et al.* (2018), and thus changed the optimal timing for FMDS. To the best of our knowledge, the induction of hexaploidy *via* a combination of SPBR and FMDS has only ever been achieved by the present study and by Lebeda *et al.* (2020). In other fish species, the hexaploidy lines were created by SPBR after fertilization of diploid oocytes by diploid sperm in, for example, loach *Misgurnus anguillicaudatus* (Cantor 1842) (Kijima *et al.*, 1996) and rainbow trout *Oncorhynchus mykiss* (Walbaum 1792) (Chourrout & Nakayama, 1987).

The occasional, random occurrence of mosaics – that is, individuals possessing two or more distinct genotypes despite developing from a single fertilized egg or zygote (King & Stansfield, 1990) – has previously been reported in sturgeons (Fopp-Bayat *et al.*, 2017; Van Eenennaam *et al.*, 1996, 2020). Iegorova *et al.* (2018) observed atypically developing embryos in *A. ruthenus* with an odd number of cells at the two- to four-cell stage, and found that these embryos are 1*n*/2*n* mosaics arising from plasmogamy where the accessory spermatozoon/spermatozoa enter the egg and develop jointly with zygotic cells. However, little is known about the occurrence of mosaics in progeny developing in heat- or pressure-shocked eggs after polyploidy induction in sturgeons. Lebeda *et al.* (2020), who worked with a functional ploidy scale, observed 2*n*/6*n* mosaics in *A. baerii* after heat shock for SPBR, and these individuals could arise due to the phenomenon mentioned above and successful SPBR. The 2*n*/4*n* individuals, the most numerous mosaics in our experiment, could also theoretically emerge from 1*n*/2*n* 'multiple-sperm mosaics', in which the shock for FMDS could result in a duplication of genetic material in both genotypes. However, the origin of some mosaics observed in our experiment remains unclear. For example, the predominant mosaics arising from the hexaploidy induction might be 2*n*/6*n* since SPBR in 1*n*/2*n* mosaics should create 1*n*/3*n*, and subsequent FMDS should duplicate the genetic material in both genotypes. Nevertheless, we observed only one prelarva possessing the 2*n*/6*n* ploidy level, while the majority of mosaics were 3*n*/6*n*. Another unexplained observation is the absence of mosaics in SPBR-induced and untreated groups. This could indicate that the 1*n*/2*n* and 1*n*/3*n* embryos recorded in these groups died during the embryonal period, while the higher mosaics in the FMDS and SPBR+FMDS induction were able to reach hatching.

4.3 | The effect of temperature on ploidy composition in sturgeon populations

The impact of human activities markedly affects the temperature regime in sturgeon habitats. Depending on future greenhouse gas emission scenarios, a rise in global temperatures from 1.0–1.8 (best estimate 1.4) to 3.3–5.7 (best estimate 4.4) °C is expected in the years leading up to 2100 (IPCC, 2021). River temperatures are also expected to rise and Van Vliet *et al.* (2011), for example, investigated the impact of changes in both air temperature and river discharge on daily water temperatures in rivers worldwide. Their sensitivity analysis revealed increases in annual mean river temperatures of +1.3, +2.6 and +3.8°C under air temperature increases of +2, +4 and +6°C, respectively. In addition to the impact of global climate change on the temperature of river waters, river fragmentation by dams and other installations, the use of rivers for the disposal of industrial and municipal effluent, the deforestation and urbanization of watersheds, and other anthropogenic modifications may all block access to and/or alter sturgeon spawning habitats and may have direct effects on temperature regimes during and after breeding (Birstein *et al.*, 1997; Lenhardt *et al.*, 2006; Rochard *et al.*, 1990).

The spawning sites of many sturgeon populations now lie below hydroelectric dams (Chebanov & Savelyeva, 1999; Deguo *et al.*, 2007; Duncan *et al.*, 2004) that provoke downstream temperature fluctuations due to hypolimnetic (bottom layer) water release (Poff *et al.*, 2007) and pulsed reservoir discharges associated with on-demand hydroelectric power generation (Bunt *et al.*, 1999). In *A. ruthenus*, spawning extends from April to June (Hochleithner & Gessner, 1999) and optimal spawning temperatures range between 12 and 17°C; below 9°C and above 21°C spawning ceases (Sokolov & Vasil'ev, 1989). The suboptimal temperatures used in this study lie within the temperature range for reproduction and, moreover, it is becoming increasingly likely that temperature fluctuations will occur during or soon after breeding in human-modified habitats. In addition, human intervention and global warming affect the seasonal temperature profiles of the rivers where sturgeon live and/or reproduce (Lassalle *et al.*, 2010; Zhang *et al.*, 2019). Seasonal shifts in the optimal temperature window for *A. ruthenus* spawning may affect its timing since it is known that elevated temperatures can truncate spring spawning in fish (Pankhurst & Munday, 2011). However, although the spawning cues in *A. ruthenus* have only ever been poorly studied, water temperature is very likely not the only factor affecting reproduction since the timing of spawning migration and/or spawning itself might also be correlated with water levels, turbidity (Kalmykov *et al.*, 2010) and the photoperiod (Kubala *et al.*, 2019) in this species. The latter factor was identified as the most crucial factor affecting spawning timing in shortnose sturgeon *A. brevirostrum* Lesueur 1818 (Kieffer & Kynard, 2012). On the other hand, insufficient knowledge of the environmental drivers of reproduction in *A. ruthenus* and the considerable level of uncertainty regarding the magnitude of alterations in temperature profiles both complicate drawing conclusions about the probability of future spawning or the exposure of early developmental stages to suboptimal temperature conditions.

Finally, alterations in seasonal temperature profiles in rivers or streams used as the water supplies for fish hatcheries might increase demands for water temperature control in order to meet the requirements of the early developmental stages (Hanson & Ostrand, 2011). If insufficient attention is given to water temperature management or in the event of technological errors, the probability of occurrence of suboptimal temperature conditions will rise. If trends like those described in our study occur in other sturgeon species, suboptimal temperatures may have a direct impact on the recruitment of autopolyploid sturgeons into new populations. In this study we found that the probability of this type of recruitment is unaffected in autotriploid sturgeons but is significantly lower in sturgeon autopolyploids with higher ploidy levels, even under optimal temperature conditions and even more so under suboptimal temperatures.

5 | CONCLUSION

Based on our findings, we report that the embryos, prelarvae and larvae of triploid *A. ruthenus* do not differ from diploids in their ability to survive, grow and develop under suboptimal temperature conditions. The embryos obtained from FMDS induction and the tetraploid prelarvae of *A. ruthenus* were less viable even at the optimal temperature and even less so at the suboptimal temperatures we tested, thereby indicating that the limits of their temperature tolerance are closer to the optimum than in diploid individuals. The high mortality of embryos arising from the SPBR+FMDS induction and absence of hexaploid and *3n*/*6n* individuals surviving the prelarval period at both the tested suboptimal and optimal temperatures show that the autohexaploid genome state may be lethal. If our observations are correct and the same trends are present in other sturgeon species, suboptimal temperature conditions may impact the ploidy composition of wild living sturgeon populations. As the result of human-induced changes in sturgeon habitats, the probability that early developmental stages of sturgeons will experience shifts in temperatures to near suboptimal values and/or temperature fluctuations is increasing. Farmed fish may be exposed to these temperature shifts and fluctuations as a result of technological errors or bad hatchery practices. Based on our findings, we assume that the autotriploid genome state in young sturgeons does not affect the probability of recruitment into the population, while the recruitment of autopolyploids with higher ploidy is significantly less probable even under optimal temperature conditions and all the more so in suboptimal temperatures.

AUTHOR CONTRIBUTIONS

M.H., M.F. and V.K. conceptualized and designed the experiments. M.R. was responsible for broodstock management and artificial propagation. M.H., M.F. and V.K. conducted the artificial fertilization and polyploidy inductions. M.H. and V.K. performed the rearing experiments. M.H. analysed the ploidy levels of collected samples using flow cytometry. T.T. and M.H. were responsible for data analysis and the presentation of the results. M.H. wrote the manuscript, and M.F. and V.K. edited and reviewed it. All co-authors revised the text and agreed on the final version. Funding to support this research and study supervision was secured by M.F. The authors declare no conflict of interests.

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ORCID

Martin Hubálek https://orcid.org/0000-0001-7243-6944
Vojtěch Kašpar https://orcid.org/0000-0001-6099-6709
Tomáš Tichopád https://orcid.org/0000-0002-9154-2969
Marek Rodina https://orcid.org/0000-0003-1145-3083
Martin Flajšhans https://orcid.org/0000-0002-0357-5788

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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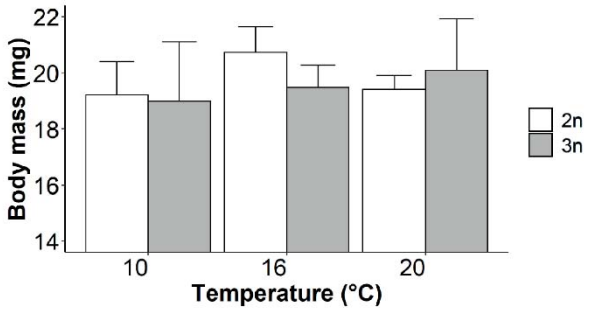
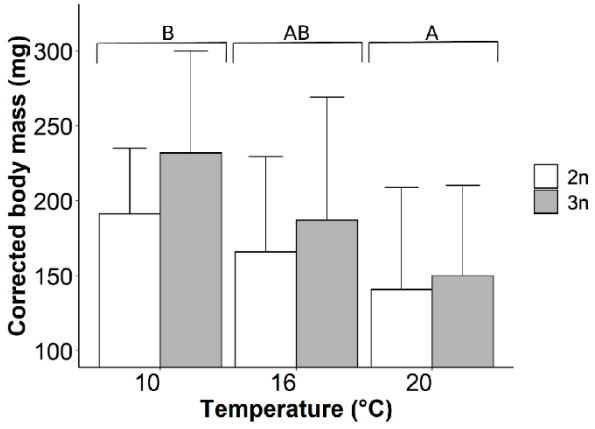
Supporting information: How do suboptimal temperatures affect polyploid sterlet *Acipenser ruthenus* during early development?

Supporting Information Text 1: Preliminary tests of proper transition time and suboptimal temperatures

The goal of the first preliminary test was to determine the optimal time for the transition of embryos incubated at the optimal temperature of 16 °C (Gela et al., 2012) to significantly different temperature conditions. Since the manipulation of the embryos soon after fertilization led to high mortality (even in the case of a transition to a temperature close to the optimum), we decided to start the first experiment at the neurula stage, which appeared to be more resistant to temperature changes. Moreover, the use of neurula-stage individuals enabled us to start this experiment with the same number of living embryos, and so the initial survival was unaffected by differences in fertilization efficiency or by the side effects of artificial treatments in polyploidized groups. The second preliminary test specified the higher and lower suboptimal limits for the rearing of the early developmental stages of *A. ruthenus*. The neurula-stage untreated embryos (putative 2n) were kept at 6, 8, 10, 12, 14, 16, 18, 20, 22, 24 and 26 °C until the end of endogenous feeding. The initial temperatures of 10 and 20 °C, which resulted in 50 % less survival than at 16 °C, are thus defined as 'suboptimal'.



Supporting Information Figure 1: The body mass of diploids and triploids of *Acipenser ruthenus* reared at 10, 16 and 20 °C evaluated at the end of prelarval period. The differences between ploidies and rearing temperatures were nonsignificant (α=0.05).



Supporting Information Figure 2: The corrected body masses of 2n and 3n larvae of *Acipenser ruthenus* reared for three weeks at 10, 16 and 20 °C. Capitals denote significant differences between rearing temperatures (α=0.05).

Supporting Information Table 1: Pairwise comparisons of fertilization, neurulation and hatching rates under different treatments using HSD-Tukey post hoc analyses.

	Fertilization rate			Neurulation rate			Hatching rate		
Contrast	df	t-ratio	P-value	df	t-ratio	P-value	df	t-ratio	P-value
Untreated vs. SPBR-induced	8	0.585	0.9339	8	6.948	0.0005	8	12.183	<.0001
Untreated vs. FMDS-induced	8	2.373	0.1603	8	9.243	0.0001	8	19.825	<.0001
Untreated vs. SPBR+FMDS-induced	8	6.923	0.0006	8	11.985	<.0001	8	28.305	<.0001
SPBR-induced vs. FMDS-induced	8	1.788	0.3447	8	2.295	0.1783	8	7.642	0.0003
SPBR-induced vs. SPBR+FMDS-induced	8	6.338	0.001	8	5.037	0.0044	8	16.122	<.0001
FMDS-induced vs. SPBR+FMDS-induced	8	4.55	0.0081	8	2.742	0.0958	8	8.48	0.0001

Supporting Information Table 2: Pairwise comparisons of neurula stage-to-hatching mortalities under different treatments and at different temperatures using HSD-Tukey post hoc analyses.

Factor	Contrast	df	t-ratio	P-value
Temperature	10 °C vs. 16 °C	24	0.578	0.8328
	10 °C vs. 20 °C	24	-2.812	0.0252
	16 °C vs. 20 °C	24	-3.39	0.0066
Treatment|Temperature	Temperature = 10 °C:			
	untreated vs. SPBR-induced	24	-0.596	0.9324
	untreated vs. FMDS-induced	24	-5.323	0.0001
	untreated vs. SPBR+FMDS-induced	24	-8.844	<.0001
	SPBR-induced vs. FMDS-induced	24	-4.727	0.0005
	SPBR-induced vs. SPBR+FMDS-induced	24	-8.249	<.0001
	FMDS-induced vs. SPBR+FMDS-induced	24	-3.522	0.0088
	Temperature = 16 °C:			
	untreated vs. SPBR-induced	24	-0.414	0.9755
	untreated vs. FMDS-induced	24	-6.389	<.0001
	untreated vs. SPBR+FMDS-induced	24	-10.599	<.0001
	SPBR-induced vs. FMDS-induced	24	-5.975	<.0001
	SPBR-induced vs. SPBR+FMDS-induced	24	-10.184	<.0001
	FMDS-induced vs. SPBR+FMDS-induced	24	-4.209	0.0017
	Temperature = 20 °C			
	untreated vs. SPBR-induced	24	0.504	0.9755
	untreated vs. FMDS-induced	24	-6.672	<.0001
	untreated vs. SPBR+FMDS-induced	24	-8.287	<.0001
	SPBR-induced vs. FMDS-induced	24	-7.176	<.0001
	SPBR-induced vs. SPBR+FMDS-induced	24	-8.791	<.0001
	FMDS-induced vs. SPBR+FMDS-induced	24	-1.615	0.3897
Temperature|Treatment	Treatment: untreated			
	10 °C vs. 16 °C	24	0.949	0.6152
	10 °C vs. 20 °C	24	-1.483	0.3167
	16 °C vs. 20 °C	24	-2.432	0.0573
	Treatment: SPBR-induced			
	10 °C vs. 16 °C	24	1.13	0.5053
	10 °C vs. 20 °C	24	-0.383	0.9225
	16 °C vs. 20 °C	24	-1.514	0.3025
	Treatment: FMDS-induced			
	10 °C vs. 16 °C	24	-0.118	0.9924
	10 °C vs. 20 °C	24	-2.832	0.0241
	16 °C vs. 20 °C	24	-2.715	0.0313
	Treatment: SPBR+FMDS-induced			
	10 °C vs. 16 °C	24	-0.805	0.7035
	10 °C vs. 20 °C	24	-0.925	0.63
	16 °C vs. 20 °C	24	-0.12	0.9921

Supporting Information Table 3: Pairwise comparisons of survival of 2n, 3n, 4n and 2n/4n prelarvae at different temperatures using HSD-Tukey post hoc analyses.

Factor	Contrast	df	t-ratio	P-value
Temperature	10 °C vs. 16 °C	24	-5.771	<.0001
	10 °C vs. 20 °C	24	-0.345	0.9367
	16 °C vs. 20 °C	24	5.426	<.0001
Ploidy|Temperature	Temperature: 10 °C			
	2n vs. 3n	24	0.589	0.9343
	2n vs. 4n	24	5.692	<.0001
	2n vs. 2n/4n	24	4.855	0.0003
	3n vs. 4n	24	5.103	0.0002
	3n vs. 2n/4n	24	4.266	0.0014
	4n vs. 2n/4n	24	-0.837	0.8364
	Temperature: 16 °C			
	2n vs. 3n	24	0.802	0.853
	2n vs. 4n	24	5.636	<.0001
	2n vs. 2n/4n	24	3.282	0.0155
	3n vs. 4n	24	4.834	0.0003
	3n vs. 2n/4n	24	2.48	0.0889
	4n vs. 2n/4n	24	-2.354	0.1139
	Temperature: 20 °C			
	2n vs. 3n	24	0.858	0.8261
	2n vs. 4n	24	5.864	<.0001
	2n vs. 2n/4n	24	4.412	0.001
	3n vs. 4n	24	5.006	0.0002
	3n vs. 2n/4n	24	3.554	0.0082
	4n vs. 2n/4n	24	-1.452	0.4806
Temperature|Ploidy	Ploidy: 2n			
	10 °C vs. 16 °C	24	-2.531	0.0466
	10 °C vs. 20 °C	24	-0.172	0.9839
	16 °C vs. 20 °C	24	2.359	0.0666
	Ploidy: 3n			
	10 °C vs. 16 °C	24	-2.319	0.0723
	10 °C vs. 20 °C	24	0.097	0.9948
	16 °C vs. 20 °C	24	2.416	0.0593
	Ploidy: 4n			
	10 °C vs. 16 °C	24	-2.588	0.0412
	10 °C vs. 20 °C	24	0	1
	16 °C vs. 20 °C	24	2.588	0.0412
	Ploidy: 2n/4n			
	10 °C vs. 16 °C	24	-4.104	0.0011
	10 °C vs. 20 °C	24	-0.615	0.8132
	16 °C vs. 20 °C	24	3.489	0.0052

Supporting Information Table 4: Pairwise comparisons of the body mass of 2n and 3n individuals at the end of the prelarval period at different temperatures using HSD-Tukey post hoc analyses.

Factor	Contrast	df	t-ratio	P-value
Temperature	10 °C vs. 16 °C	12	-1.686	0.2502
	10 °C vs. 20 °C	12	-0.907	0.6462
	16 °C vs. 20 °C	12	0.777	0.7237
Ploidy|Temperature	Temperature: 10 °C			
	2n vs. 3n	12	0.279	0.7853
	Temperature: 16 °C			
	2n vs. 3n	12	1.475	0.1659
	Temperature: 20 °C			
	2n vs. 3n	12	-0.568	0.5806
Temperature|Ploidy	Ploidy: 2n			
	10 °C vs. 16 °C	12	-1.79	0.2142
	10 °C vs. 20 °C	12	-0.218	0.9741
	16 °C vs. 20 °C	12	1.572	0.2945
	Ploidy: 3n			
	10 °C vs. 16 °C	12	-0.594	0.826
	10 °C vs. 20 °C	12	-1.064	0.5531
	16 °C vs. 20 °C	12	-0.471	0.8859

Supporting Information Table 5: Pairwise comparisons of the survival of 2n and 3n individuals after three weeks rearing in the larval period at different temperatures using HSD-Tukey post hoc analyses.

Factor	Contrast	df	t-ratio	P-value
Temperature	10 °C vs. 16 °C	19	-1.809	0.1935
	10 °C vs. 20 °C	19	2.629	0.0418
	16 °C vs. 20 °C	19	4.305	0.0011
Ploidy|Temperature	Temperature: 10 °C			
	2n vs. 3n	19	1.161	0.2602
	Temperature: 16 °C			
	2n vs. 3n	19	1.116	0.2783
	Temperature: 20 °C			
	2n vs. 3n	19	0.955	0.3515
Temperature|Ploidy	Ploidy: 2n			
	10 °C vs. 16 °C	19	-1.261	0.4335
	10 °C vs. 20 °C	19	1.901	0.1655
	16 °C vs. 20 °C	19	2.994	0.0195
	Ploidy: 3n			
	10 °C vs. 16 °C	19	-1.297	0.4139
	10 °C vs. 20 °C	19	1.82	0.19
	16 °C vs. 20 °C	19	3.095	0.0157

Supporting Information Table 6: Pairwise comparisons of the body mass of 2n and 3n individuals after three weeks rearing during the larval period at different temperatures using HSD-Tukey post hoc analyses.

Factor	Contrast	df	t-ratio	P-value
Temperature	10 °C vs. 16 °C	20	1.95	0.151
	10 °C vs. 20 °C	20	2.624	0.0412
	16 °C vs. 20 °C	20	1.003	0.5838
Ploidy|Temperature	Temperature: 10 °C			
	2n vs. 3n	20	-1.48	0.1543
	Temperature: 16 °C			
	2n vs. 3n	20	-0.592	0.5605
	Temperature: 20 °C			
	2n vs. 3n	20	-0.736	0.4701
Temperature|Ploidy	Ploidy: 2n			
	10 °C vs. 16 °C	20	0.939	0.6228
	10 °C vs. 20 °C	20	1.699	0.2302
	16 °C vs. 20 °C	20	0.901	0.6456
	Ploidy: 3n			
	10 °C vs. 16 °C	20	1.817	0.1895
	10 °C vs. 20 °C	20	2.007	0.1365
	16 °C vs. 20 °C	20	0.524	0.8607

CHAPTER 5

HOW DOES INDUCED POLYPLOIDY AFFECT THE SWIMMING AND PHYSIOLOGICAL PERFORMANCE OF STERLET (*Acipenser ruthenus*) AND SIBERIAN STURGEON (*Acipenser baerii*) AND THEIR RECIPROCAL HYBRIDS?

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My share on this work was about 55%.

How does induced polyploidy affect the swimming and physiological performance of sterlet (*Acipenser ruthenus*) and Siberian sturgeon (*Acipenser baerii*) and their reciprocal hybrids?

Martin Hubálek*, Vojtěch Kašpar, Hung Quang Tran, Vlastimil Stejskal, Tomáš Tichopád, Kateřina Grabicová, Martin Flajšhans

University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Centre of Aquaculture and Biodiversity of Hydrocenoses, Zátiší 728/II, 389 25 Vodňany, Czech Republic; * Email: mhubalek@frov.jcu.cz

Abstract

Spontaneous triploidization has been widely documented in cultured populations of many sturgeon species. Any evidence of this phenomenon in the wild should not be ignored given future perspectives of climate changes that could increase the risk of polyploidization in nature. Answers to the questions regarding the fitness and physiology of newly formed sturgeon polyploids will shed light on how these fish perform in aquaculture and nature, and improve scientific knowledge regarding the consequences of polyploidy in fishes. In this study, we compared the critical swimming speed (U_{crit}) of nine-month-old normoploid and triploidized individuals (obtained from normal fertilization and the induction of second polar body retention, respectively) of two Eurasian sturgeon species, sterlet (*Acipenser ruthenus*) and Siberian sturgeon (*Acipenser baerii*), and their reciprocal hybrids (sterlet × Siberian sturgeon and Siberian sturgeon × sterlet). We assessed the primary haematological indices (blood haemoglobin concentration = Hb, haematocrit = PCV and erythrocyte count = RBC count), secondary haematological indices (mean erythrocyte volume = MEV, mean erythrocyte haemoglobin = MEH and mean erythrocytic haemoglobin concentration = MEHC) and plasma/serum chemistry (cortisol, osmolality and glucose) of both non-exercised and exercised sturgeons to investigate the ploidy-related differences in resting and post-exercised individuals. We found that in both purebreds and hybrids, triploidized sturgeons exhibited the same absolute and corrected U_{crit} as normoploids. As expected, there were some differences between ploidies in haematological parameters, as triploidized sturgeons had lower RBC counts and larger erythrocytes with more haemoglobin than normoploids. However, the increase in ploidy did not affect Hb, PCV, MEHC, plasma cortisol, plasma osmolality or serum glucose, except for the greater PCV observed in triploidized sterlet × Siberian sturgeon and higher levels of serum glucose in triploidized Siberian sturgeon × sterlet than in normoploids of the same hybrids. After exhaustive exercise, changes in Hb, PCV, MEH, MEHC, plasma cortisol, plasma osmolality and serum glucose were observed whose magnitudes were consistent between normoploid and triploidized populations. We thus conclude that triploidized sterlet, Siberian sturgeon, sterlet × Siberian sturgeon and Siberian sturgeon × sterlet are able to fully compensate for having fewer erythrocytes in their Hb and PCV and have the same oxygen carrying capacity as normoploids. Moreover, the magnitude of haematological and blood chemistry responses to exhaustive exercise did not differ between normoploid and triploidized sturgeons, and the second polar body retention-induced polyploidy did not negatively affect swimming performance.

Keywords: chromosomal manipulations, haematology, hybridization, second polar body retention, stress response, triploidization

1. Introduction

Polyploidization, the addition of one or multiple complete sets of chromosomes, is a highly dynamic process that is thought to be a fundamental driver of evolution and speciation in fishes (Ohno, 1970; Leggatt and Iwama, 2003; Le Comber and Smith, 2004). As a result of at least three whole-genome duplications (Rajkov et al., 2014), the widest range of chromosome numbers in any vertebrate is found in extant sturgeons (Acipenseriformes) (Fontana, 2002). Karyotype data distinguish three discrete groups in these paleopolyploid, non-teleost fishes: species with ~120 (Group A), ~240 (Group B) and ~360 (Group C) chromosomes in their somatic cells (Ludwig et al., 2001; Kim et al., 2005). However, the increase in the chromosome count resulting from the inheritance of an additional chromosome set or sets can occur *de novo* in each generation and lead to neopolyploidy (Ramsey and Schemske, 2002; Hardie and Hebert, 2004). In fishes, neopolyploidy can arise spontaneously as the result of the second polar body retention (SPBR) (Flajšhans et al., 1993; Piferrer et al., 2009; Glover et al., 2015), which hereafter is referred as triploidization. Spontaneous triploidization has been widely reported in cultured sturgeons from Group A and B, and there is evidence that it occurs in the wild (for a review, see Havelka and Arai, 2019; Schreier et al., 2021). Moreover, SPBR in sturgeons can be artificially induced by applying physical shocks to fertilized eggs. Although induced SPBR is not a common practice in sturgeon aquaculture (unlike groups such as salmonids, in which this practice increases the profitability of fish farms), it has been successfully performed in sturgeon species of all chromosome groups (A, B, C) (e.g. Vassetzky, 1967; Van Eenennaam et al., 1996; Flynn et al., 2006; Flajšhans et al., 2020).

Unlike induced polyploidy, interspecific hybridization has recently become a popular tool in sturgeon aquaculture since certain hybrids are more viable, grow quicker and reach sexual maturation earlier than their parent species (Bronzi et al., 1999; Zhang et al., 2013; Boscari et al., 2014). Sturgeons are highly prone to hybridizing both in captivity (Zhang et al., 2013) and in the wild (Ludwig et al., 2009), and so farm escapees or accidental/deliberate introductions of sturgeon hybrids into natural waters could pose a threat to native populations (Jenneckens et al., 2000; Maury-Brachet et al., 2008). Sturgeon hybrids usually possess chromosome numbers that are intermediate to their parents' (Omoto et al., 2005), although evidence of both spontaneous (Omoto et al., 2005; Zhou et al., 2011) and artificially induced (Omoto et al., 2005; Fopp-Bayat et al., 2007) triploidization suggests that their inclination to SPBR – which leads to an increase in the chromosome count – is the same as in pure species.

The persistence of sturgeons for creating polyploid genome states underlines the unique genome plasticity of these archaic fishes and raises questions concerning the consequences of triploidization for the fitness and physiology of both pure species and interspecific hybrids. The importance of this issue is demonstrated by the number of cultured sturgeons with unusual ploidy levels. Furthermore, as hypothesized by Shivaramu et al. (2020a), climate change and global warming may expose sturgeon gametes to rapidly fluctuating temperature stresses and lead to meiotic or fertilization disorders, thereby possibly increasing rates of polyploidization in natural habitats. Optimized SPBR-induction protocols provide us with the possibility to investigate the impact of polyploidy on sturgeons, which will help fill gaps in scientific knowledge regarding the consequences of a polyploid genome state in fishes.

Triploidization can alter the performance of fish in several ways. Cells of triploid fish are bigger than in diploids since they carry 50% more DNA due to their extra set of chromosomes; however, this increase in cell size is compensated for by a decrease in number (Benfey, 1999; Piferrer et al., 2009). The increase in cell and nuclei size may result in a lower surface-area-to-volume ratio, which could directly impair gas exchange and the rate of other cell membrane functions, and so harm the physiological performance of triploids (Leal et al., 2020; Riseth

et al., 2020). Triploidy may also alter gene interactions, and interrupt regulatory factor stoichiometry and epigenetic gene expression patterns, causing as a consequence a reduction in fitness or performance (Shrimpton et al., 2007). Furthermore, triploidy may have an impact on chromosome segregation during meiosis and lead to partial or complete sterility. On the other hand, in many species adult triploid fish clearly grow faster than individuals with normal ploidy because of the redirection of energy from gametogenesis to somatic growth (Benfey, 1999; Tiwary, 2004; Piferrer et al., 2009) – although this does not seem to be the case at least in triploidized sturgeons belonging to Group B that are probably fertile (Schreier et al., 2013; Havelka et al., 2016). Triploid fish may also exhibit higher levels of heterozygosity with, potentially, associated fitness benefits due to overdominance and reduced inbreeding depression (Leary et al., 1985; Coltman and Slate, 2003).

Swimming performance can be used as a direct indicator of physical fitness in fishes (Rajotte and Couture, 2002; Plaut, 2001; Massé et al., 2013). Of all the methods employed to measure swimming performance, the critical swimming speed (U_{crit}) test performed by forced swimming against an incrementally increasing velocity of water until exhaustion is still the most widely used by researchers (Hammer, 1995; Plaut, 2001; Kieffer, 2010). Critical swimming speeds have previously been successfully used to evaluate the effects of various biotic or abiotic factors on the swimming ability of fish (Plaut, 2001; Farrell, 2008; Kieffer and Cooke, 2009). It is thought to serve as a proxy for whole-organism performance and condition (Cooke and Connor, 2010; Brennan et al., 2016; Botha et al., 2019), which also makes it a suitable tool for comparative studies with polyploids. The vast majority of studies exploring the U_{crit} of triploidized fishes have been conducted on salmonids, the results of which indicate that triploids do not differ from diploids in their swimming performance (Small and Randall, 1989; Stillwell and Benfey, 1997; Bernier et al., 2004; Lijalad and Powell, 2009; Scott et al., 2015; Riseth et al., 2020). U_{crit} studies of sturgeons have increased in recent years (summarized in Downie and Kieffer, 2017), presumably due to the general awareness of the conservation implications of dam construction and the migratory challenges they imply for these threatened fishes (Peake et al., 1997; Geist et al., 2005; Cheong et al., 2006). However, no study of the impact of triploidization on sturgeons' swimming performance has been published to date. Such a study is of particular interest because sturgeons have markedly lower swimming performances than salmonids (Peake et al., 1997; McKenzie et al., 2001; Downie and Kieffer, 2017) and differ in their metabolic demands (Kieffer et al., 2001).

The acute stress of exhaustive exercise induces severe metabolic, acid-base and ionic changes in fishes (Kieffer, 2010). The primary stress response in fishes is generally characterized by the release of stress hormones, cortisol and catecholamines (adrenaline and epinephrine) into the bloodstream by the endocrine system (Randall and Ferry, 1992). Secondary stress responses occur as the consequence of the release of stress hormones, which provoke alterations in haematological parameters (e.g. in haemoglobin and haematocrit) and changes in blood and tissue chemistry (e.g. in glucose, lactate and osmolality) (reviewed, for example, in Wendelaar-Bonga, 1997 and Seibel et al., 2021). The investigation of changes in haematological parameters and blood chemistry associated with swimming to exhaustion provides useful information about the effects of various intrinsic and/or extrinsic factors on the nature, magnitude and capacity of stress responses (Kennedy and Farrell, 2006; Ralph et al., 2012; Aride et al., 2018), and can be used to evaluate the physiological limitations of triploidized sturgeons. To date, the stress response of triploidized sturgeons has only ever been investigated in two North American species, the shortnose sturgeon (*Acipenser brevirostrum*; Beyea et al., 2005) and white sturgeon (*Acipenser transmontanus*; Leal et al., 2019, 2020), and in neither study was the U_{crit} test used to induce this response. After acute stress, some differences between sturgeons of

normal ploidy and triploidized individuals have been observed, including greater plasma osmolality and chloride ion concentrations (Beyea et al., 2005), lower haematocrit and haemoglobin response (Leal et al., 2020), and a poorer capacity for modification in cellular metabolic enzyme activity in triploidized sturgeons (Leal et al., 2019).

The aim of this study was thus to evaluate the effects of SPBR-induced polyploidy on swimming performance, haematological parameters, blood chemistry and physiological stress response in two Eurasian sturgeon species, sterlet (*Acipenser ruthenus*) and Siberian sturgeon (*Acipenser baerii*), and their reciprocal hybrids (sterlet × Siberian sturgeon and Siberian sturgeon × sterlet). A U_{crit} test was used to estimate the swimming performance in both the purebred and hybrid sturgeons arising from normal fertilization (normoploid fish; resultant ploidy status referred to as 'normoploidy' in the text) or triploidization via SPBR-induction (triploidized fish; resultant ploidy status referred to as 'SPBR-induced polyploidy' in the text). Furthermore, primary haematological indices (haemoglobin, haematocrit and erythrocyte count), secondary haematological indices (mean erythrocyte volume, mean erythrocyte haemoglobin and mean erythrocytic haemoglobin concentrations), cortisol, glucose and osmolality levels were assessed in the blood of resting and exercised normoploid and triploidized purebred and hybrid sturgeons to explore the impact of SPBR-induced polyploidy on haematology and blood chemistry when at rest, and how they change as a response to acute stress.

2. Material and methods

Artificial reproduction, polyploidy induction, rearing, swimming trials and blood sampling of experimental fish were carried out at the Genetic Fisheries Centre, Faculty of Fisheries and Protection of Waters (FFPW), the University of South Bohemia in České Budějovice, Czech Republic. The haematological and chemical analyses were conducted in the FFPW laboratories in Vodňany, and in the accredited private biochemical laboratory STAFILA (AeskuLab Ltd.) in České Budějovice, Czech Republic.

2.1. Ethical statement

All experiments were carried out in accordance with the Animal Research Committee of the FFPW. Fish were kept according to principles based on harmonized EU animal welfare legislation and on laboratory animal care complying with Czech Republic national law (Act No. 246/1992 on the Protection of Animals against Cruelty).

2.2. Experimental fish

The broodstock for breeding was obtained from the Genetic Fisheries Centre of FFPW. Three females and three males of sterlet (St) aged 6–8 years, and three females and three males of Siberian sturgeon (Sib) aged 8–10 years, were bred following the methodology used by Gela et al. (2008). Two populations of purebreds (St, Sib) and two populations of reciprocal hybrids (St × Sib, Sib × St) were produced. For each cross, 65 g of oocytes from each of the three females and 1 ml of sperm from each of the five males were used for fertilization. The activation of the gametes was conducted as described by Gela et al. (2008) using 800-ml water at 16 °C for each cross. The subsequent treatment for the removal of egg stickiness

using tannic acid was performed following Flajšhans et al. (2020). Once these procedures were completed, the eggs from each cross were separated into two equal parts and kept in a controlled environment at 16 °C. One half was left untreated, while the second half was triploidized via SPBR-induction. In St and St × Sib, a treatment of 34 °C was applied for 2 minutes 18 minutes post-activation (Hubálek et al., 2022), while in Sib and Sib × St, the same treatment was applied for 2.5 minutes 18 minutes post-activation (Flajšhans et al., unpublished data).

The incubation of untreated and triploidized embryos from each cross was carried out using Zuger jars supplied with hatchery water at 16 °C (Gela et al., 2012). Subsequent rearing, feeding, stocking densities and growth conditions followed Shivaramu et al. (2020b). After hatching, fish were initially reared in 0.3-m^3 rectangular indoor tanks and then transferred to 3.5-m^3 indoor circular tanks 100 days post-hatching (dph). One rearing tank was used for each combination of cross and treatment. Fish were individually tagged by inserting Passive Integrated Transponder (PIT) tags (134.2 kHz; AEG Comp., Germany) into the dorsal muscle at 250 dph. In order to determine ploidy, a 0.5-cm^2 sample of caudal fin tissue was obtained from each tagged fish, frozen in a DMSO-citrate buffer (Vindelov et al., 1983) and subsequently stored, processed and analyzed using a CCA I flow cytometer (Partec GmbH, Germany) (Hubálek and Flajšhans, 2021). After the ploidy analysis, fish without the expected ploidy status (normoploidy, SPBR-induced polyploidy) were removed. The functional ploidy levels and theoretical chromosome counts corresponding to normoploidy and SPBR-induced polyploidy in different crosses are summarized in Table 1.

The swimming trials started nine months post-hatching. In all, 18 randomly selected fish per ploidy per cross (144 fish in total) were used in the individual swimming tests (hereafter referred to as 'exercised'). Each fish was fasted for approximately 24 hours before their swimming capacity was tested. Finally, 20 fish of the same age that did not undergo the swimming trial were randomly selected from each combination of ploidy and cross (200 fish in total) and were also fasted for 24 hours. These fish served as a non-swimming control (hereafter referred to as 'non-exercised'). The lengths and masses of both exercised and non-exercised normoploid and triploidized sturgeons from individual crosses are listed in Table 1.

2.3. Swimming trial

Swimming performances were tested in a 30-l Steffensen-type swim tunnel (Loligo systems Inc., Tjele, Denmark), which is designed to exercise fish at controlled speeds in non-turbulent water with a uniform velocity profile. AutoResp© software (Loligo systems Inc., Tjele, Denmark) was used to control and record water velocity in the rectangular swimming chamber (dimensions: 46×14×14 cm). Flow calibrations were carried out using a multifunctional handheld flowtherm NT.2 unit (Höntzsch GmbH, Waiblingen, Germany). The tunnel was submerged in a buffer tank equipped with a heating immersion circulator and two airstones. The tunnel and the buffer tank were filled with fresh dechlorinated tap water, which was replaced daily. Water temperature in the tunnel was maintained at 20 °C and the oxygen content at ≥ 80% saturation. In order to eliminate any disturbance from the tank's surroundings, a piece of black textile material was used to cover the swimming tunnel during each trial.

On each testing day, fish were chosen in rotation from the ploidy and crosses, gently removed from their holding tanks, individually identified, and placed into the swimming chamber. Only one fish was used at a time. The habituation period lasted for 20 min: 10 min with no velocity followed by 10 min at 5 cm s^{-1} to allow the fish to adapt and orientate themselves in the

current. Thereafter, the fish were subjected to a stepped velocity test until exhaustion. Water velocity was set at 20 cm s^{-1} and subsequently increased by 10 cm s^{-1} at 5-min intervals. Fatigue was determined by sustained contact by fish for 10 s with a grid placed downstream. After the first period of fatigue, the water velocity was decreased to the initial test level (20 cm s^{-1}) for < 20 s until the fish left the grid. If the fish did not come off the grid within 20 s, the trial was terminated. However, if the fish did leave the grid, the water velocity was increased back to the speed the first fatigue occurred, and fish was allowed to swim until a second fatigue period. The duration of the trial and velocity of the final completed swimming period were recorded. Immediately after the trial was terminated, fish were measured (body length = BL, width, depth) and weighed; as well, a blood sample was taken for further analysis, and then the fish were returned to the holding tanks to recover. Every fish was tested only once to avoid any training effect. The fish that did not swim against the current at the initial test velocity (20 cm s^{-1}) were classified as 'nonswimmers' and returned to the holding tanks; they were then replaced with a new fish of the same ploidy and cross, which were then habituated and tested as described above. The overall ratio of nonswimmers of all fish exposed to the trial was less than 5%; no blood samples were taken from these fish, which were not included in the critical swimming speed (U_{crit}) calculations.

The absolute U_{crit} was calculated using the formula employed by Brett (1964): U_{crit} (cm s^{-1}) = Vf + T1/t × dv, where Vf is the velocity (cm s^{-1}) of the last completed swimming period, T1 is the time fish swam at the final velocity before fatigue (min), t is the time increment (5 min) and dv is the velocity increment (10 cm s^{-1}). For the calculation of the corrected U_{crit}, the absolute U_{crit} was converted to BL s^{-1}. Since the cross-sectional area of the largest fish was < 10% of the cross-sectional area of the swimming chamber, no correction for a solid blocking effect was used (Bell and Terhune, 1970).

2.4. Blood sampling and analyses

Like the exercised fish, non-exercised fish had blood samples taken, and were measured (BL) and weighed before being returned to the holding tanks. From each exercised and non-exercised fish, a 1.5-ml blood sample was obtained. Blood was drawn by caudal venipuncture using 20 gauge needles and nonheparinized 2-ml syringes. Immediately after collection, 0.5 ml of the sample was placed in 1.5-ml Safe-Lock Eppendorf tube, and the remaining blood (1 ml) was transferred into a heparinized glass container (100 IU sodium heparin per 1 ml blood) to prevent coagulation. Subsequently, 0.7 ml of heparin-treated blood was placed in a 1.5-ml Safe-Lock Eppendorf tube, the remaining blood in the container being used for the immediate assay of primary blood indices, that is, blood haemoglobin concentration (Hb), erythrocyte count (RBC count) and haematocrit (PCV). Both the nonheparinized and heparinized blood samples in the Eppendorf tubes were centrifuged at 3,000 rpm for 10 minutes at 4 °C (Fresco 21, Thermo Scientific, Czech Republic); the samples of serum and plasma were collected and stored at -80 °C until analysed for cortisol, osmolality and glucose.

The analyses of the primary blood indices were conducted following Svobodová et al. (2012). Each of these parameters were measured in triplicate and averaged. The photometrical cyanmethaemoglobin method was used to determine Hb (g l^{-1}) at a wavelength of 540 nm. The RBC count (10^{-12} l^{-1}) was assessed in a 1:20 dilution of the blood sample in the Hayem solution with a Bürker haemocytometer, with cells always counted in 20 rectangles. The PCV (%) was analysed in microhaematocrit capillaries after centrifugation at 14,000 rpm for 3 min. Secondary blood indices including mean erythrocyte volume (MEV), mean erythrocyte heamoglobin (MEH) and mean erythrocytic haemoglobin concentrations (MEHC) were calculated from averaged primary blood indices for every individual following Svobodová et al. (2012).

Glucose concentrations were assessed from serum samples using an Abbott Architect c8000 clinical chemistry analyser (Abbott, Chicago, IL, USA) and assay kit B3L8X7 G3-5375/R02 (Abbott, USA) following the manufacturer's instructions. Cortisol was initially analysed from serum samples with the Immulite 2000Xpi Siemens (Siemens Healthcare GmbH, Erlangen, Germany) immunochemistry analyser and a cortisol assay kit (L2KCO2); nevertheless, given that the cortisol levels of all the samples originating from the non-exercised groups were below the limit of quantification (LOQ) which was 10 ng ml^{-1}, the results of this analysis were not used in the present study. The cortisol levels were thus analysed in plasma samples using liquid chromatography with mass spectrometry (LC-HRMS; LC pump Vanquish, autosampler Pal RSI, QExactive HF mass spectrometer, Thermo Fisher Scientific). Plasma samples were prepared according to Grabicová et al. (2018), briefly, 2.5 ng of isotopically labelled cortisol-D_4 (Sigma Aldrich) and 100 L of extraction solvent (acetonitrile acidified by formic acid, both LC-MS grade, Sigma Aldrich) were added to 100 L of plasma. The samples were vortexed and placed to -20 °C for 24 hrs. Before the analysis, samples were centrifuged (10,000 rpm, 6 min, Mini spin, Eppendorf, Germany) and aliquot of 100 L was taken to vials for analysis. The description of the LC-HRMS analysis is given in Supplementary material (SM 1).

Plasma osmolarity was measured using a freezing point osmometer Osmomat 3000 (Gonotec GmbH, Germany) following the manufacturer´s instruction. All measurements were conducted in triplicate for each specimen and averaged.

2.5. Statistical analyses

The assumption of normality was tested using Shapiro test, and if needed, log transformation was applied to the response variable. To assess whether the body mass and BL had any effect on the absolute and corrected U_{crit} of different ploidies (normoploidy, SPBR-induced polyploidy) originating from the same cross (St, Sib, St × Sib, or Sib × St), an ANCOVA with body mass and BL as covariates and ploidy as a fixed factor was performed. Since the effect of covariates on absolute U_{crit} was not significant in any of the crosses (Supplementary material, SM 2), the differences between ploidies were determined using a one-way ANOVA. The effect of BL on corrected U_{crit} was significant in some crosses (Supplementary material, SM 2) and when this was a case, an ANCOVA with BL as a covariate and ploidy as a fixed factor was run. In order to compare the absolute and corrected U_{crit} of different crosses, an ANCOVA with body mass and BL as covariates and cross as a fixed factor was applied to evaluate the effect of fish size. Since the covariates have had no significant effect on the absolute U_{crit} (Supplementary material, SM 3), a one-way ANOVA with cross as a fixed factor was run. Given that BL significantly affected the corrected U_{crit} (Supplementary material, SM 3), the effect of cross on U_{crit} was analysed with an ANCOVA using BL as a covariate. The analysis of haematological and blood chemistry data was conducted for each cross using a factorial two-way ANOVA, where ploidy and exercise were treated as fixed factors. Since the cortisol levels below LOQ were observed in non-exercised groups, one half of LOQ value was used for each sample not reaching LOQ to enable statistical analysis. Significant differences between groups were identified by a post-hoc Tukey's HSD test. Validity of tests was visually evaluated using a Q-Q plot and a fitted values against the residual values. All statistical analyses were performed using R statistical software (4.0.1), with an α of 0.05 predetermined as the significance level.

3. Results

3.1. Swimming performance

The absolute critical swimming speeds in normoploid and triploidized sturgeons were, respectively, (mean ± SD) 71.92 ± 9.22 and 71.71 ± 11.72 cm s^{-1} in St, 63.21 ± 9.63 and 64.16 ± 13.56 cm s^{-1} in Sib, 69.81 ± 9.71 and 69.61 ± 8.07 cm s^{-1} in St × Sib, and, finally, 63.72 ± 10.93 and 64.68 ± 10.80 cm s^{-1} in Sib × St. The absolute U_{crit} was not significantly affected by ploidy in either St ($F_{1,34}$ = 0.004, p = 0.95), Sib ($F_{1,34}$ = 0.06, p = 0.81), St × Sib ($F_{1,34}$ = 0.004, p = 0.95), or Sib × St ($F_{1,34}$ = 0.07, p = 0.79). The corrected swimming speeds in normoploid and triploidized sturgeons were, respectively, (mean ± SD) 2.60 ± 0.51 and 2.26 ± 0.56 BL s^{-1} in St, 1.73 ± 0.27 and 1.83 ± 0.49 BL s^{-1} in Sib, 1.99 ± 0.28 and 1.98 ± 0.39 BL s^{-1} in St × Sib, and 1.81 ± 0.34 and 1.80 ± 0.41 BL s^{-1} in Sib × St. The effects of ploidy on corrected U_{crit} were not significant in St ($F_{1,33}$ = 0.51, p = 0.48), Sib ($F_{1,34}$ = 0.59, p = 0.45), St × Sib ($F_{1,33}$ = 0.11, p = 0.74), or Sib × St ($F_{1,33}$ = 0.25, p = 0.62). Both absolute and corrected U_{crit} differed significantly between crosses ($F_{3,140}$ = 5.39, p = 0.002 and $F_{3,139}$ = 4.68, p = 0.004, respectively); the results of the individual comparisons are shown in Figures 1 and 2.

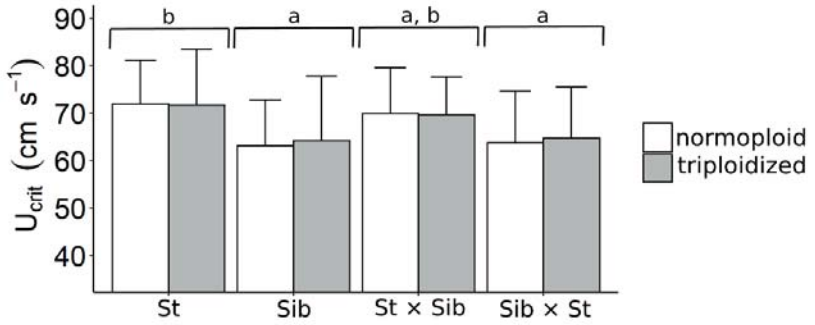

Figure 1. *Absolute critical swimming speed (Ucrit) of normoploid and triploidized purebreds and hybrids of sturgeon. Lowercase denotes significant differences between individual crosses (α = 0.05). Abbreviations: U_{crit}=critical swimming speed, St = sterlet, Sib = Siberian sturgeon, St × Sib = sterlet × Siberian sturgeon, Sib × St = Siberian sturgeon × sterlet*

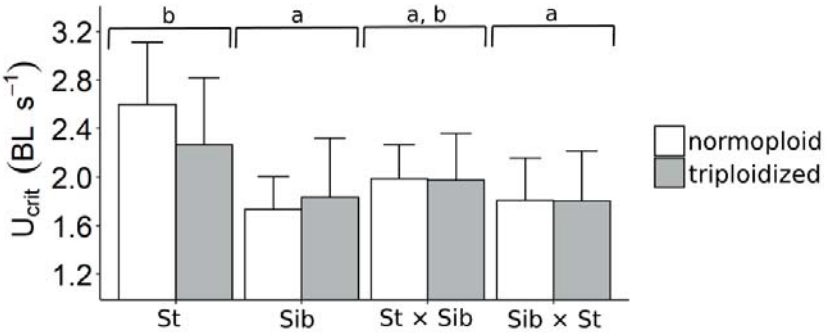

Figure 2. *Corrected critical swimming speed (Ucrit) of normoploid and triploidized purebreds and hybrids of sturgeon. Lowercase denotes significant differences between individual crosses (α = 0.05). Abbreviations: U_{crit}=critical swimming speed, St = sterlet, Sib = Siberian sturgeon, St × Sib = sterlet × Siberian sturgeon, Sib × St = Siberian sturgeon × sterlet.*

3.2. Haematology and blood chemistry

3.2.1. Sterlet

The results of the haematological and blood chemistry analyses of St are summarized in Table 2. Triploidized St had lower RBC counts but higher MEV and MEH when compared to normoploids of the same species. Ploidy had no impact on the other haematological parameters (Hb, PCV and MEHC) or on any of the blood chemistry parameters (plasma cortisol, plasma osmolality and serum glucose). Fish from the exercised group had significantly higher levels of PCV, plasma cortisol, plasma osmolality and serum glucose but lower MEHC than non-exercised fish; exercise did not affect either Hb, the RBC count, MEV or MEH. Both normoploid and triploidized St responded to exercise with similar changes in haematology and blood chemistry, as shown by the non-significant ploidy-to-exercise interactions for all investigated parameters.

3.2.2. Siberian sturgeon

The results of the haematological and blood chemistry analyses of Sib are summarized in Table 3. Triploidized individuals of Sib had lower RBC counts and higher MEV and MEH than their normoploid counterparts. Normoploids did not differ from SPBR-induced polyploids in the other haematological parameters (Hb, PCV and MEHC) or in their blood chemistry (plasma cortisol, plasma osmolality and serum glucose). Hb, MEH and MEHC were lower in the exercised than in the non-exercised group; however, the opposite effect was observed for plasma cortisol, plasma osmolality and serum glucose. Exercise did not induce significant changes in either PCV, the RBC count or MEV. Both normoploid and triploidized Sib had similar responses to exercise in their haematological and blood chemistry parameters, as shown by the non-significant ploidy-to-exercise interactions.

3.2.3. Sterlet × Siberian sturgeon

The results of the haematological and blood chemistry analyses of St × Sib are summarized in Table 4. Statistical analysis revealed that triploidized St × Sib had lower RBC counts but higher PCV, MEV and MEH than their normoploid counterparts. The ploidies did not differ in either of the other haematological parameters (Hb, MEHC) or blood chemistry parameters (plasma cortisol, plasma osmolality and serum glucose). Exercised hybrids had significantly lower Hb, MEH and MEHC but higher plasma cortisol, plasma osmolality and serum glucose compared to non-exercised hybrids; exercise had no impact on PCV, the RBC count or MEV. The ploidy-to-exercise interaction was non-significant for all examined parameters, assuming the same haematological and blood chemistry response to exercise in both normoploid and triploidized St × Sib.

3.2.4. Siberian sturgeon × sterlet

The results of haematological and blood chemistry analyses of Sib × St are summarized in Table 5. Triploidized Sib × St had lower RBC counts but higher MEV, MEH and serum glucose compared to normoploids; ploidy had no effect on Hb, PCV, MEHC, plasma cortisol or plasma

osmolality. The exercised group had lower Hb and MEHC but higher plasma cortisol and serum glucose than the non-exercised group; however, no differences were observed in PCV, the RBC count, MEV, MEH or plasma osmolality. The magnitude of the haematological and blood chemistry response to exercise did not differ between ploidies given that the effect of ploidy-to-exercise interaction was non-significant for all the analysed parameters.

Table 1. *Ploidy status, functional ploidy level, theoretical chromosome counts, and body masses (mean ± SD) and lengths (mean ± SD) of normoploid and triploidized purebreds and sturgeon hybrids. Normoploid = arisen through normal fertilization; Triploidized = arisen through SPBR-induction. Abbreviations: St = sterlet, Sib = Siberian sturgeon, St × Sib = sterlet × Siberian sturgeon, Sib × St = Siberian sturgeon × sterlet.*

Experimental group	Ploidy status	Functional ploidy	Theoretical chromosome count	Non-exercised body mass (g)	Non-exercised body length (cm)	Exercised body mass (g)	Exercised body length (cm)
normoploid St	normoploidy	2n	120	68 ± 18	26.3 ± 2.4	67 ± 17	26.1 ± 2.1
triploidized St	SPBR-induced polyploidy	3n	180	86 ± 26	29.0 ± 2.7	88 ± 24	29.9 ± 3.0
normoploid Sib	normoploidy	4n	240	113 ± 30	33.7 ± 2.9	114 ± 26	33.7 ± 3.1
triploidized Sib	SPBR-induced polyploidy	6n	360	92 ± 15	31.7 ± 2.0	94 ± 20	32.0 ± 2.1
normoploid St × Sib	normoploidy	3n	180	96 ± 30	32.3 ± 3.4	100 ± 34	32.9 ± 3.8
triploidized St × Sib	SPBR-induced polyploidy	4n	240	102 ± 27	32.1 ± 2.6	102 ± 20	32.3 ± 1.8
normoploid Sib × St	normoploidy	3n	180	99 ± 27	31.7 ± 2.6	107 ± 26	32.3 ± 2.6
triploidized Sib × St	SPBR-induced polyploidy	5n	300	116 ± 18	34.2 ± 1.8	113 ± 31	33.6 ± 3.3

Table 2. *Haematological and blood chemistry parameters measured in non-exercised and exercised normoploid and SPBR-induced polyploid sterlet, and the results of the statistical analyses. Significant results (α = 0.05) are highlighted in bold font. Cortisol levels are expressed as minimum – maximum values, other parameters are expressed as mean ± SD. Limits of quantification (LOQ) for cortisol analysis ranged from 0.30 to 0.76 ng ml^{-1} in normoploid sterlet, and from 0.24 to 0.64 ng ml^{-1} in SPBR-induced sterlet. Abbreviations: Hb = blood haemoglobin concentration, PCV = haematocrit, RBC count = erythrocyte count, MEV = mean erythrocyte volume, MEH = mean erythrocyte haemoglobin, MEHC = mean erythrocytic haemoglobin concentration.*

Parameter	Normoploid		SPBR-induced polyploid		Statistical analyses		
	Non-exercised	Exercised	Non-exercised	Exercised	Ploidy	Exercise	Ploidy: Exercise
Hb (g l^{-1})	65.9 ± 8.0	64.2 ± 7.5	63.7 ± 9.8	66.9 ± 14.6	$F_{1,72}$ = 1.21, p = 0.27	$F_{1,72}$ = 0.09, p = 0.76	$F_{1,72}$ = 0.77, p = 0.38
PCV (%)	22 ± 3	24 ± 3	21 ± 3	25 ± 3	$F_{1,72}$ = 0.03, p = 0.87	**$F_{1,72}$ = 17.41, p = 9×10^{-5}**	$F_{1,72}$ = 2.98, p = 0.09
RBC count (10^{-12} l^{-1})	0.85 ± 0.13	0.92 ± 0.21	0.69 ± 0.16	0.71 ± 0.16	**$F_{1,72}$ = 41.88, p = 1×10^{-8}**	$F_{1,72}$ = 3.43, p = 0.07	$F_{1,72}$ = 0.79, p = 0.38
MEV (fl cell^{-1})	261 ± 49	271 ± 70	320 ± 66	373 ± 111	$F_{1,72}$ = 30.36, p = 6×10^{-7}	$F_{1,72}$ = 1.72, p = 0.19	$F_{1,72}$ = 2.15, p = 0.15
MEH (pg cell^{-1})	80 ± 20	73 ± 20	96 ± 21	98 ± 24	**$F_{1,72}$ = 41.54, p = 1×10^{-8}**	$F_{1,72}$ = 1.11, p = 0.29	$F_{1,72}$ = 0.86, p = 0.36
MEHC (g Hb mm^{-3})	0.31 ± 0.06	0.27 ± 0.02	0.30 ± 0.03	0.27 ± 0.08	$F_{1,72}$ = 2.30, p = 0.13	**$F_{1,72}$ = 40.45, p = 2×10^{-8}**	$F_{1,72}$ = 4.76, p = 0.06
Cortisol (ng ml^{-1})	< LOQ–18	18 ± 12	< LOQ–8.1	16 ± 12	$F_{1,72}$ = 0.01, p = 0.94	**$F_{1,72}$ = 247.23, p < 2×10^{-16}**	$F_{1,72}$ = 0.01, p = 0.94
Osmolality (mOsm kg^{-1})	255 ± 11	263 ± 9	255 ± 7	260 ± 10	$F_{1,72}$ = 7.36, p = 0.34	**$F_{1,72}$ = 0.92, p = 0.008**	$F_{1,72}$ = 0.33, p = 0.57
Glucose (mmol l^{-1})	3.1 ± 0.9	3.5 ± 1.3	3.1 ± 0.8	3.6 ± 1.1	$F_{1,72}$ = 0.20, p = 0.66	**$F_{1,72}$ = 4.32, p = 0.01**	$F_{1,72}$ = 1.15, p = 0.29

Table 3. *Haematological and blood chemistry parameters measured in non-exercised and exercised normoploid and SPBR-induced polyploid Siberian sturgeon, and the results of the statistical analyses. Significant results (α = 0.05) are highlighted in bold font. Cortisol levels are expressed as minimum – maximum values, other parameters are expressed as mean ± SD. Limits of quantification (LOQ) for cortisol analysis ranged from 0.25 to 0.60 ng ml-1 in normoploid Siberian sturgeon, and from 0.31 to 0.64 ng ml-1 in SPBR-induced Siberian sturgeon. Abbreviations: Hb = blood haemoglobin concentration, PCV = haematocrit, RBC count = erythrocyte count, MEV = mean erythrocyte volume, MEH = mean erythrocyte haemoglobin, MEHC = mean erythrocytic haemoglobin concentration.*

Parameter	Normoploid		SPBR-induced polyploid		Statistical analyses		
	Non-exercised	Exercised	Non-exercised	Exercised	Ploidy	Exercise	Ploidy: Exercise
Hb (g l^{-1})	65.7±13.0	55.0 ± 7.5	59.8 ± 9.7	53.3 ± 10.4	$F_{1,72}$ = 2.54, p = 0.12	**$F_{1,72}$ = 11.43, p = 0.001**	$F_{1,72}$ = 1.39, p = 0.24
PCV (%)	25 ± 3	25 ± 3	24 ± 3	24 ± 3	$F_{1,72}$ = 1.90, p = 0.17	$F_{1,72}$ = 0.86, p = 0.36	$F_{1,72}$ = 0.3, p = 0.59
RBC count (10^{-12} l^{-1})	0.59 ± 0.08	0.60 ± 0.10	0.43 ± 0.11	0.42 ± 0.08	**$F_{1,72}$ = 133.54, p < 2×10^{-16}**	$F_{1,72}$ = 0.66, p = 0.42	$F_{1,72}$ = 0.08, p = 0.78
MEV (fl cell^{-1})	427 ± 73	437 ± 144	580 ± 108	607 ± 115	**$F_{1,72}$ = 126.33, p < 2×10^{-16}**	$F_{1,72}$ = 0.003, p = 0.96	$F_{1,72}$ = 1.88, p = 0.18
MEH (pg cell^{-1})	112 ± 27	95 ± 33	147 ± 41	133 ± 39	**$F_{1,72}$ = 40.87, p = 2×10^{-8}**	**$F_{1,72}$ = 16.62, p = 1×10^{-4}**	$F_{1,72}$ = 0.28, p = 0.60
MEHC (g Hb mm^{-3})	0.26 ± 0.05	0.22 ± 0.03	0.25 ± 0.06	0.22 ± 0.04	$F_{1,72}$ = 0.26, p = 0.61	**$F_{1,72}$ = 20.14, p = 3×10^{-5}**	$F_{1,72}$ = 0.11, p = 0.74
Cortisol (ng ml^{-1})	< LOQ–3.4	21 ± 10	< LOQ–2.1	22 ± 11	$F_{1,72}$ = 0.05, p = 0.82	**$F_{1,72}$ = 513.78, p < 2×10^{-16}**	$F_{1,72}$ = 0.38, p = 0.54
Osmolality (mOsm kg^{-1})	250 ± 7	257 ± 8	249 ± 6	257 ± 10	$F_{1,72}$ = 0.96, p = 0.33	**$F_{1,72}$ = 14.71, p = 3×10^{-4}**	$F_{1,72}$ = 0.002, p = 0.96
Glucose (mmol l^{-1})	2.5 ± 0.7	3.0 ± 0.8	2.4 ± 0.7	3.2 ± 0.9	$F_{1,72}$ = 0.27, p = 0.61	**$F_{1,72}$ = 13.67, p = 4×10^{-4}**	$F_{1,72}$ = 3.17, p = 0.08

Table 4. *Haematological and blood chemistry parameters measured in non-exercised and exercised normoploid and SPBR-induced polyploid sterlet × Siberian sturgeon, and the results of the statistical analyses. Significant results (α = 0.05) are highlighted in bold font. Cortisol levels are expressed as minimum – maximum values, other parameters are expressed as mean ± SD. Limits of quantification (LOQ) for cortisol analysis ranged from 0.40 to 0.78 ng ml-1 in normoploid sterlet × Siberian sturgeon, and from 0.29 to 0.75 ng ml-1 in SPBR-induced sterlet × Siberian sturgeon. Abbreviations: Hb = blood haemoglobin concentration, PCV = haematocrit, RBC count = erythrocyte count, MEV = mean erythrocyte volume, MEH = mean erythrocyte haemoglobin, MEHC = mean erythrocytic haemoglobin concentration.*

Parameter	Normoploid		SPBR-induced polyploid		Statistical analyses		
	Non-exercised	Exercised	Non-exercised	Exercised	Ploidy	Exercise	Ploidy: Exercise
Hb (g l^{-1})	70.1 ± 11.0	60.8 ± 9.1	72.3 ± 10.0	62.0 ± 8.9	$F_{1,72}$ = 2.01, p = 0.16	**$F_{1,72}$ = 16.63, p = 1×10^{-4}**	$F_{1,72}$ = 0.02, p = 0.88
PCV (%)	24 ± 3	24 ± 3	26 ± 3	26 ± 3	**$F_{1,72}$ = 7.75, p = 0.01**	$F_{1,72}$ = 0.22, p = 0.64	$F_{1,72}$ = 0.02, p = 0.89
RBC count (10^{-12} l^{-1})	0.72 ± 0.09	0.71 ± 0.13	0.57 ± 0.14	0.65 ± 0.14	**$F_{1,72}$ = 19.56, p = 4×10^{-5}**	$F_{1,72}$ = 0.78, p = 0.38	$F_{1,72}$ = 2.6, p = 0.11
MEV (fl $cell^{-1}$)	344 ± 53	351 ± 65	486 ± 123	410 ± 88	**$F_{1,72}$ = 38.2, p = 4×10^{-8}**	$F_{1,72}$ = 3.51, p = 0.07	$F_{1,72}$ = 3.85, p = 0.054
MEH (pg $cell^{-1}$)	99 ± 18	88 ± 14	134 ± 42	97 ± 20	**$F_{1,72}$ = 13.95, p = 4×10^{-4}**	**$F_{1,72}$ = 16.46, p = 1×10^{-4}**	$F_{1,72}$ = 2.09, p = 0.15
MEHC (g Hb mm^{-3})	0.29 ± 0.05	0.25 ± 0.03	0.28 ± 0.04	0.24 ± 0.02	$F_{1,72}$ = 0.43, p = 0.51	**$F_{1,72}$ = 26.95, p = 2×10^{-6}**	$F_{1,72}$ = 0.22, p = 0.64
Cortisol (ng ml^{-1})	< LOQ–1.4	15 ± 7	< LOQ–5.8	10 ± 5	$F_{1,72}$ = 0.01, p = 0.91	**$F_{1,72}$ = 451.39, p < 2×10^{-16}**	$F_{1,72}$ = 4.32, p = 0.07
Osmolality (mOsm kg^{-1})	249 ± 5	255 ± 16	251 ± 7	259 ± 13	$F_{1,72}$ = 3.53, p = 0.06	**$F_{1,72}$ = 18.2, p = 6×10^{-5}**	$F_{1,72}$ = 1.25, p = 0.27
Glucose (mmol l^{-1})	3.0 ± 0.9	3.3 ± 0.8	2.8 ± 0.7	3.3 ± 0.8	$F_{1,72}$ = 4.63, p = 0.23	**$F_{1,72}$ = 6.14, p = 0.02**	$F_{1,72}$ = 2.79, p = 0.10

Table 5. *Haematological and blood chemistry parameters measured in non-exercised and exercised normoploid and SPBR-induced polyploid Siberian sturgeon × sterlet, and the results of the statistical analyses. Significant results (α = 0.05) are highlighted in bold font. Cortisol levels are expressed as minimum – maximum values, other parameters are expressed as mean ± SD. Limits of quantification (LOQ) for cortisol analysis ranged from 0.36 to 0.65 ng ml-1 in normoploid Siberian sturgeon × sterlet, and from 0.29 to 0.65 ng ml-1 in SPBR-induced Siberian sturgeon × sterlet. Abbreviations: Hb = blood haemoglobin concentration, PCV = haematocrit, RBC count = erythrocyte count, MEV = mean erythrocyte volume, MEH = mean erythrocyte haemoglobin, MEHC = mean erythrocytic haemoglobin concentration.*

Parameter	Normoploid		SPBR-induced polyploid		Statistical analyses		
	Non-exercised	Exercised	Non-exercised	Exercised	Ploidy	Exercise	Ploidy: Exercise
Hb (g l^{-1})	70.1 ± 17.2	62.4 ± 9.3	66.9 ± 9.3	60.2 ± 6.7	$F_{1,72}$ = 0.31, p = 0.58	**$F_{1,72}$ = 8.59, p = 0.005**	$F_{1,72}$ = 2.28, p = 0.14
PCV (%)	26 ± 5	27 ± 2	26 ± 3	28 ± 3	$F_{1,72}$ = 1.54, p = 0.22	$F_{1,72}$ = 2.97, p = 0.09	$F_{1,72}$ = 0.10, p = 0.75
RBC count (10^{-12} l^{-1})	0.80 ± 0.11	0.80 ± 0.13	0.58 ± 0.12	0.56 ± 0.13	**$F_{1,72}$ = 77.21, p = 7×10^{-13}**	$F_{1,72}$ = 0.09, p = 0.76	$F_{1,72}$ = 0.31, p = 0.58
MEV (fl $cell^{-1}$)	326 ± 74	347 ± 64	464 ± 78	520 ± 144	**$F_{1,72}$ = 75.92, p = 1×10^{-12}**	$F_{1,72}$ = 1.93, p = 0.17	$F_{1,72}$ = 0.47, p = 0.5
MEH (pg $cell^{-1}$)	89 ± 24	80 ± 17	118 ± 24	114 ± 35	**$F_{1,72}$ = 40.34, p = 2×10^{-8}**	$F_{1,72}$ = 2.15, p = 0.15	$F_{1,72}$ = 0.35, p = 0.55
MEHC (g Hb mm^{-3})	0.28 ± 0.06	0.23 ± 0.03	0.25 ± 0.02	0.22 ± 0.04	$F_{1,72}$ = 2.67, p = 0.11	**$F_{1,72}$ = 26.27, p = 3×10^{-6}**	$F_{1,72}$ = 0.76, p = 0.39
Cortisol (ng ml^{-1})	< LOQ–3	22 ± 9	< LOQ–1.1	16 ± 7	$F_{1,72}$ = 1.64, p = 0.20	**$F_{1,72}$ = 856.84, p < 2×10^{-16}**	$F_{1,72}$ = 2.39, p = 0.13
Osmolality (mOsm kg^{-1})	249 ± 5	250 ± 8	248 ± 9	251 ± 11	$F_{1,72}$ = 0.15, p = 0.70	$F_{1,72}$ = 1.14, p = 0.29	$F_{1,72}$ = 0.002, p = 0.97
Glucose (mmol l^{-1})	2.4 ± 0.6	3.1 ± 0.7	2.8 ± 0.8	3.7 ± 1.1	$F_{1,72}$ = 5.49, p = 0.02	$F_{1,72}$ = 12.2, p = 8×10^{-4}	$F_{1,72}$ = 0.03, p = 0.87

4. Discussion

4.1. Swimming performance

4.1.1. The effect of triploidization on swimming performance

In our study, no differences were observed between the absolute or corrected U_{crit} in either the nine-month-old normoploid and triploidized populations of the two sturgeon purebreds (St and Sib) or the two hybrids (St × Sib and Sib × St). We thus cannot provide any evidence for SPBR-induced polyploidy altering whole-organism performance in sturgeons. Previously, no research had ever investigated the impact of increased ploidy on swimming performance in sturgeons and so ours is the first study to tackle this subject. The effect of polyploidy on U_{crit} has been thoroughly studied in salmonids and in this group normoploid and triploidized fish also performed similarly, e.g. Atlantic salmon (*Salmo salar*) (Lijalad and Powell, 2009; Riseth et al., 2020), brook trout (*Salvelinus fontinalis*) (Stillwell and Benfey, 1997), coho salmon (*Oncorhynchus kisutch*) (Small and Randall, 1989), chinook salmon (*Oncorhynchus tshawytscha*) (Bernier et al., 2004) and rainbow trout (*Oncorhynchus mykiss*) (Scott et al., 2015). However, it is worth noting that Lijalad and Powell (2009) and Bernier et al. (2004) all reported significantly lower aerobic scope in triploidized Atlantic salmon and, simultaneously, a slight reduction in their mean corrected U_{crit} that did not reach statistical significance. In our work, the measurement of swimming performance was not linked to oxygen consumption and, overall, we were unable to observe any obvious trends in the reduction in critical swimming speeds in triploidized sturgeons that could be attributed to a poorer aerobic scope. Nevertheless, Leal et al. (2020, 2021), in a comparison of the aerobic metabolism of normoploid and triploidized white sturgeon exposed to exhaustive exercise (manual chasing), reported lower aerobic scope in three-month-old triploidized sturgeon in both ambient and elevated water temperatures (Leal et al., 2020), as well as in approximately 5.5-month-old sturgeons kept at ambient temperature (Leal et al., 2021). With this in mind, it may be of interest to investigate whether or not triploidized sturgeons tested for U_{crit} have lower aerobic scopes than their normoploid counterparts.

4.1.2. Swimming performance of purebreds

The mean corrected U_{crit} of the Sib (1.78 BL s^{-1}, irrespective of ploidy) agreed with the study by Qu et al. (2013, U_{crit} = 1.72 BL s^{-1}) but was lower than reported in the studies by Cai et al. (2015, U_{crit} = 3.3 BL s^{-1}) and Yuan et al. (2016, U_{crit} = 2.28 BL s^{-1}). Similarly, the mean corrected U_{crit} of the St (2.43 BL s^{-1}, irrespective of ploidy) was lower than in other work including the studies by Mandal et al. (2016) and Cai et al. (2017) (3.4 and 3.5 BL s^{-1}, respectively), except for the study of Shivaramu et al. (2018) conducted on intraspecific hybrids and purebreds of two geographically distant populations (1.63–2.4 BL s^{-1}). These differences could reflect discrepancies in the swimming protocols, the most notable of which were the velocity and time increments. The impact of these variables on the resulting U_{crit} was investigated by Downie and Kieffer (2017) in the shortnose sturgeon. Unfortunately, the authors who reported higher U_{crit} in Sib and St adjusted the velocity increments individually to length of each tested specimen; in our study and in that by Downie and Kieffer (2017), on the other hand, fixed velocity increments were used, which makes it impossible to draw conclusions about the impact of this parameter on differences in U_{crit}. As well, the time increments in our study (5 min) were shorter than those used in the studies that reported higher U_{crit} in Sib and St (10–30 min). Given that Downie and Kieffer (2017) found that 5-min intervals generated the same or higher U_{crit} than longer intervals (i.e. 15 or 30

min) taking into account the velocity increments with which it was combined, we do not believe that the time interval was responsible for the lower corrected swimming speeds in our study. We assume that the differences were due to fish size. Our Sib were longer than both those used by Cai et al. (2015) and Yuan et al. (2016) (mean BL: 33.0 cm vs. 13.9 cm and BL range: 16.5–21.2 cm, respectively), and our St were longer than those used in the studies by Mandal et al. (2016) and Cai et al. (2017) mean BL: 27.0 cm vs. 13.6 cm and 13.7 cm, respectively). Peake et al. (1995) examined the swimming performance of lake sturgeon (*Acipenser fulvescens*) vs. ontogeny using three size classes (BL range: small fish: 12–22 cm, intermediate fish: 23–55 cm and large fish: 106–132 cm) and found that the corrected U_{crit} was considerably higher in small as opposed to larger size classes (mean BL s^{-1}: 2.07 vs. 1.02 and 0.94, respectively). He et al. (2013), who investigated the changes in U_{crit} with ontogeny using 2.5–12.5-month-old Chinese sturgeon (*Acipenser sinensis*), also observed that the corrected U_{crit} decreased with length. Peake et al. (1995) hypothesize that this trend could be due to the fact that small fish spend more energy on growing lengthwise than on girth (muscle) but that as fish grow their rate of length-growth decreases. Larger fish develop muscle mass to reduce drag but their U_{crit} decreases with length since sturgeon do not increase enough muscle mass to overcome the drag (Peake et al., 1995).

4.1.3. Swimming performance of hybrids

Only a few number of studies have ever investigated the U_{crit} of interspecific hybrids and none has ever addressed this topic in sturgeons. In our study, the mean U_{crit} of both sturgeon hybrids lay in between the values of their parental species, as reported by Hawkins and Quinn (1996) and Seiler and Keeley (2007) in salmonids. The hybrids of both Yellowstone cutthroat (*Oncorhynchus clarkii bouvieri*) and rainbow trout had higher U_{crit} than their parent species with the poorest swimming abilities (Seiler and Keeley, 2007); on the other hand, hybrids of cutthroat (*O. clarkii*) and steelhead (*Oncorhynchus mykiss*) trout were found not to differ from their parents (Hawkins and Quinn, 1996). Our results show that the U_{crit} of St × Sib did not differ significantly from Sib × St but that, unlike St × Sib, Sib × St had significantly lower U_{crit} than their parent species with the highest U_{crit} (St). Shivaramu et al. (2019b) compared growth and survival traits in St, Sib and their interspecific hybrids during a 862-day rearing period and reported seemingly higher values of mid-parent heterosis for mean BW in St × Sib than in Sib × St, as well as positive heterosis for cumulative survival in St × Sib but negative heterosis in Sib × St. Slight differences in U_{crit} between these two hybrids might thus reflect the greater fitness of St × Sib. However, the differences in the morphology of sturgeon hybrids mentioned above could also play a role since morphology was found to be a parameter that noticeably affects swimming abilities in sturgeons (Qu et al., 2013). As Chebanov et al. (2018) summarize, the vast majority of morphological characters are inherited matroclinally in hybrids of St and Sib; St × Sib are thus 'morphologically closer' to St, which performed better than Sib in our swimming trials, while the morphology of Sib × St is closer to Sib.

4.2. Haematology and stress physiology in normoploid and triploidized sturgeons

4.2.1. The effect of SPBR-induced polyploidy on primary and secondary blood indices

As expected, triploidized St, Sib, St × Sib and Sib × St had lower RBC counts than normoploids, but their erythrocytes were greater. Given that polyploid cells carry more genomic DNA than normoploid cells, cell volume increases to accommodate the larger cell nuclei, and this increase

in cell volume is accompanied by a decrease in cell number (Benfey, 1999). This decrease could be help prevent high blood pressure due to the size of the larger blood cells (Houston, 1997). The larger erythrocytes of the triploidized purebreds and hybrids contained more haemoglobin (as was obvious from MEH) and had the same haemoglobin concentrations (MEHC) as the erythrocytes of normoploids. However, due to the compensation in erythrocyte numbers, Hb and PCV did not differ between ploidies other than in St × Sib, which had higher PCV in the triploidized population. Although all the authors who have compared the haematology of normoploid and triploidized sturgeons report increased MEH in triploidized individuals and – with the exception of the study by Rożyński et al. (2015) – no differences in MEHC between ploidies, information about Hb and PCV levels is contradictory. For example, Rożyński et al. (2015) observed elevated Hb in triploidized Sib and similar PCV in ploidies, while Beyea et al. (2005) mentioned similar Hb in unstressed normoploid and triploidized shortnose sturgeon and in sturgeon sampled immediately or 2-h post-stress (15 min chasing and handling), and higher Hb in normoploids at 6 h post-stress. Non-stressed SPBR-induced polyploids had lower PCV but no differences were noticeable after stress (Beyea et al., 2005). Leal et al. (2019, 2020) reported overall lower Hb and PCV in triploidized specimens after examining the physiological responses of normoploid and triploidized white sturgeon acclimatized to or kept at optimal and elevated temperatures, and non-stressed or stressed by water level reduction or manual chasing. Inconsistent results regarding the impact of triploidization on Hb and PCV were also obtained in teleost fishes (see the reviews by Benfey, 1999 and Tiwary et al., 2004). These haematological indices are important indicators of the oxygen carrying capacity of fish and based on its decrease, several authors mentioned that triploidized fishes have poorer oxygen carrying capacities than normoploids (Dorafshan et al., 2008; Leal et al., 2019, 2020). This was not the case in our study since our results show that SPBR-induced polyploids of St, Sib, St × Sib and Sib × St were able to fully compensate for having fewer erythrocytes in their Hb and PCV levels, and thus we found no evidence for poorer oxygen capacity in triploidized sturgeons.

4.2.2. Exercise-induced changes in primary and secondary blood indices

Interestingly, the testing of the swimming performances led to a significant decrease in Hb and no change in PCV in Sib, St × Sib and Sib × St, and had no impact on Hb in St, probably due to its increased PCV after the trial. The decrease in Hb was probably unrelated to changes either in the RBC count, which was unaffected by exercise in any species or hybrid used in this study, or in changes in the erythrocyte volume, since MEV did not change despite the drop in MEHC. Unlike our results, Brown and Kieffer (2019) reported increased Hb and unchanged PCV in shortnose sturgeon following exhaustive exercise, while Leal et al. (2019) reported an increase in both parameters immediately after exhaustive exercise in white sturgeon. These authors used five- and three-min manual chasing to exhaust fish, a type of exercise whose nature and length thus obviously differed from our progressive increase in the water current velocity, which could explain the different physiological responses. Patterns of stress-induced changes in Hb and PCV may also vary between sturgeon species, as demonstrated by Baker et al. (2005), who used five-min manual chasing to exhaustion in shortnose sturgeon and Atlantic sturgeon (*Acipenser oxyrinchus*) and observed a significant decrease in Hb and PCV in Atlantic sturgeon 1 h after forced activity compared to unchanged Hb and PCV in shortnose sturgeon at any sampling time post-exercise (0, 1 and 2 h). Overall, in sturgeons, the patterns of physiological responses to exhaustive exercise may be species-specific and so warrant further study. Despite this, the goal of our study was to examine whether or not responses differ between normoploids and SPBR-induced polyploids. We conclude that the

testing of U_{crit} induced similar patterns of changes in primary and secondary blood indices in both normoploid and triploidized St, Sib, St × Sib and Sib × St, and that the magnitude of changes did not differ between ploidies. This result contradicts the study by Leal et al. (2020), who reported lower haematological responses (Hb and PCV) to exhaustive exercise in triploidized white sturgeon.

4.2.3. Cortisol, osmolality and glucose levels

The concentrations of serum glucose observed in both non-exercised and exercised normoploid and triploidized St (Table 1) fall within the wide range of values reported for this species (Lee et al., 2012; Delafkar et al., 2019; Abdollahpour et al., 2021), as was the case for Sib (Table 2) (Feng et al., 2011; Hasanalipour et al., 2013; Duman, 2019). Plasma osmolality levels in our normoploid and triploidized populations of St (Table 1) and Sib (Table 2) also agreed with previously published research on these species (Rodriguez et al., 2002; Bayrami et al., 2017); likewise, plasma osmolality and serum glucose in the sturgeon hybrids tested in this study (Tables 3 and 4) were in the range of values of their parent species. However, the low values of plasma cortisol observed in our non-exercised purebreds and hybrids are worth mentioning. As reviewed by Pankhurst (2011), sturgeons generally have lower baseline cortisol values than teleosts, and plasma cortisol under 1 ng ml^{-1} has been reported in some sturgeon species such as in resting pallid sturgeon (*Scaphirhynchus albus*) (mean 0.67 ng ml^{-1}, Webb et al., 2007) and green sturgeon (*Acipenser medirostris*) (mean 0.4 ng ml^{-1}, Lankford et al., 2003). Nonetheless, while the lowest cortisol values in our non-exercised normoploid and triploidized St were below LOQ, and the highest values were 18 and 8.1 ng ml^{-1}, respectively, the lowest mean cortisol concentrations reported by Krayushkina et al. (2006), Bayrami et al. (2017) and Abdollahpour et al. (2021) ranged from 16.3 to 21.95 ng ml^{-1} in resting St. In comparison to the above-mentioned studies of St, some researchers indicate notably lower cortisol levels in Sib, for example Eagderi et al. (2020; mean 4.7 ng ml^{-1}), which are nevertheless still higher than in our non-exercised Sib (range < LOQ–3.4 and < LOQ–2.1 ng ml^{-1} in normoploid and triploidized populations, respectively). Cortisol secretion in fishes can be affected by a variety of intrinsic and extrinsic factors that are not regarded as direct stressors and may cause further great variability in results between studies of the same fish species (see the reviews by Martínez-Porchas et al., 2009; Ellis et al., 2012). Finally, Lankford et al. (2003) observed mean plasma cortisol concentrations of 0.4 ng ml^{-1} and, three months later, 8.9 ng ml^{-1} in resting green sturgeon, which demonstrates the great magnitude of cortisol fluctuations that occur even within the same sturgeon population.

4.2.4. Exercise-induced changes in cortisol, osmolality and glucose

In our study, all three blood chemistry parameters were consistently higher in exercised St, Sib, St × Sib and Sib × St than in their non-exercised counterparts, although not significantly so for plasma osmolality in Sib × St. The increase in plasma cortisol and glucose following acute stress occurs in both sturgeons and teleosts (reviewed in Pankhurst, 2011). Stress-induced rises in plasma osmolality have been reported in many sturgeon studies (e.g. Zuccarelli et al., 2008; Allen et al., 2009; Cocherell et al., 2011), although this trend is untypical in freshwater fishes because catecholamines causes changes in gill permeability and branchial circulation that can improve gas transfer and oxygen uptake, which leads to increased ion losses and decreased osmolality (reviewed in Wendelaar-Bonga, 1997; Seibel et al., 2021). Allen et al.

(2009) hypothesized that the rise in plasma osmolality in stressed sturgeons could be the result of less plasma volume due to water moving out of circulation and into tissues (Okimoto et al., 1994), or haemoconcentrations caused by increased urine flow rates (Wood and Randall, 1973; Tervonen et al., 2006).

Although the St, Sib, St × Sib and Sib × St used in the present study responded to exhaustive exercise with significant changes in plasma cortisol, plasma osmolality and serum glucose, the magnitudes of the stress responses were consistent between normoploid and triploidized individuals of given purebreds and hybrids; the resting and post-stress values of the tested parameters likewise did not differ between the analysed ploidies. The only exception was the constantly higher glucose in the triploidized Sib × St (see Table 4) than in their normoploid counterparts, which may indicate that the triploidized population of this hybrid responded to the rearing conditions by increasing its glucose levels and was thus chronically stressed. However, as emphasized by some authors (Mommsen et al., 1999; Flodmark et al., 2001), care has to be taken when using glucose as the only stress indicator. The hypothesis of triploidized Sib × St being stressed more than normoploids was not supported by the other stress indicators investigated in this study (haematological parameters, cortisol, osmolality). Also, the level of liver glycogen stores which may directly affect blood glucose level often varies greatly even between the closely related experimental specimens (Mommsen et al., 1999; Polakof et al., 2012). Therefore, the possibility of a random selection of individuals with higher levels of these parameters from triploidized group, e.g. fish that fed more intensively before the fasting period, cannot be excluded from consideration. Taking all this into account, we assumed that the effect of certain undesirable confounding factors could be responsible for differences in glucose between normoploid and triploidized populations of Sib × St as they may alter glucose levels in blood and so distort the conclusions in any study, as mentioned in 4.2.3. for cortisol. Other studies – for example Beyea et al. (2005) in shortnose sturgeon and Leal et al. (2020) in white sturgeon – comparing the physiology of normoploid and triploidized sturgeon have reported similar cortisol and glucose concentrations immediately after exhaustive exercise. Moreover, in both these studies, the kinetics of post-stress recovery did not differ between ploidies, assuming that triploidized sturgeons have a similar capacity to their normoploid counterparts in terms of their cortisol and glucose response and recovery. Nonetheless, it is worth noting that Beyea et al. (2005) reported consistently higher plasma osmolality and chloride ion concentrations in triploidized sturgeons following exhaustive exercise and so hypothesized that triploidized sturgeons might require longer period than normoploids to recover from acute exercise, and/or that osmolality and chloride ion concentrations were constitutively higher in triploidized individuals. Other studies have failed to document any differences in plasma osmolality between normoploid and triploidized sturgeons, either when resting or acutely stressed (Leal et al., 2019; presented study). Overall, the primary and secondary stress responses of normoploid and triploidized sturgeon appear to be quite similar, although some minor differences have been documented that may be related to species specificity. The effect of polyploidy on stress physiology has been most thoroughly investigated in salmonids and several studies of this group of fishes have failed to find any differences in primary and secondary responses between normoploid and triploidized fish exposed to various acute stressors (e.g. Sadler et al., 2000; Leggatt et al., 2006; Thompson et al., 2016; Chalmers et al., 2018). Some differences between ploidies have been documented, although they tend to be generally minor (Biron and Benfey, 1994; Benfey and Biron, 2000; Taylor et al., 2007).

5. Conclusion

Triploidized St, Sib, St × Sib and Sib × St exhibited similar swimming performances to their normoploid counterparts. The increase in ploidy resulted in lower RBC counts but did not lower the oxygen carrying potential for aerobic processes, as was obvious from Hb and PCV levels. Neither did the ploidies differ in terms of the blood chemistry parameters that were investigated. The only exception – constantly elevated serum glucose in triploidized Sib × St compared to normoploids of the same hybrid – was not considered a ploidy-derived physiological difference. Although exhaustive exercise elicited changes in the levels of certain haematological parameters (Hb, PCV, MEH and MEHC) and blood chemistry parameters (plasma cortisol, plasma osmolality and serum glucose), the magnitudes of these stress responses were consistent between normoploid and triploidized populations from the same crosses. Based on our observations, we thus conclude that the presence of SPBR-induced polyploidy does not pose a physiological limitation for St, Sib, St × Sib or Sib × St, and does not affect their swimming performance. The absence of physiological and performance differences between normoploid and triploidized sturgeons may result from the fact that the history of these fishes is inherently associated with polyploidization events. This may pose a threat for both cultured and wild populations, since triploidized individuals of at least some sturgeon species are fertile and their engagement in reproduction may contaminate future generations with progeny with abnormal ploidy levels.

6. Author contributions

Martin Hubálek: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – Original draft. Vojtěch Kašpar: Conceptualization, Investigation, Methodology, Validation, Writing – Review & Editing. Hung Quang Tran: Investigation, Methodology, Writing – Review & Editing. Vlastimil Stejskal: Methodology, Project administration, Writing – Review & Editing. Tomáš Tichopád: Formal analysis, Validation, Writing – Review & Editing. Kateřina Grabicová: Investigation, Methodology, Writing – Review & Editing. Martin Flajšhans: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – Review & Editing.

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Supplementary material

SM 1. *Parameters of LC-HRMS method for determination of plasma cortisol level.*

Analytical column: Hypersil Gold aQ, 50×2.1 mm, 5 μm particles (Thermo Fisher Scientific)
LC gradient of mobile phases:

Time (min)	Ultra-pure water + 0.1 % formic acid (%)	Acetonitrile + 0.1 % formic acid (%)	Flow (μl min^{-1})
0	100	0	350
1	100	0	350
4	75	25	350
8	40	60	450
10	0	100	450
12	0	100	450
12.05	100	0	350
15	100	0	350

Operating mode: full scan in positive mode, range 100–750 m/z
HRMS parameters:

	Target peak m/z	Confirming peak m/z	Retention time (min)
Cortisol	363.2160	364.2194	5.4
Cortisol-D4	367.2411	368.2449	5.4

Method duration: 15 minutes
Calibration curve was linear in range: 0.5–125 ng ml^{-1}
Limits of quantification (LOQ): 0.24–0.78 ng ml^{-1}
Recoveries of fortified plasma samples: Samples for recovery calculation were fortified with native cortisol at 3 concentration levels (10, 50 and 100 ng ml^{-1}) and processed in triplicates for each combination of ploidy and cross.

Concentration level	Average recovery	SD	RSD
10 ng ml^{-1}	92%	0.28	31%
50 ng ml^{-1}	88%	0.12	13%
100 ng ml^{-1}	84%	0.22	26%

SM 2. *Statistical analyses of the effects of body mass and body length on absolute and corrected critical swimming speed (U_{crit}) within individual crosses. Significant results (α = 0.05) are highlighted in bold font. Abbreviations: St = sterlet, Sib = Siberian sturgeon, St × Sib = sterlet × Siberian sturgeon, Sib × St = Siberian sturgeon × sterlet.*

Parameter	Cross	Body mass	Body length
Absolute U_{crit}	St	$F_{1,32}$ = 0.01, p = 0.93	$F_{1,32}$ = 0.91, p = 0.35
	Sib	$F_{1,32}$ = 0.30, p = 0.59	$F_{1,32}$ = 0.28, p = 0.60
	St × Sib	$F_{1,32}$ = 0.18, p = 0.68	$F_{1,32}$ = 2.00, p = 0.17
	Sib × St	$F_{1,32}$ = 0.54, p = 0.47	$F_{1,32}$ = 0.001, p = 0.97
Corrected U_{crit}	St	$F_{1,32}$ = 0.003, p = 0.95	$F_{1,32}$ = 22.42, p = 4×10^{-5}
	Sib	$F_{1,32}$ = 0.08, p = 0.79	$F_{1,32}$ = 2.54, p = 0.12
	St × Sib	$F_{1,32}$ = 0.004, p = 0.95	$F_{1,32}$ = 4.45, p = 0.04
	Sib × St	$F_{1,32}$ = 1.10, p = 0.30	$F_{1,32}$ = 5.60, p = 0.02

SM 3. *Statistical analyses of the effects of body mass and body length on absolute and corrected critical swimming speed (U_{crit}) in all crosses. Significant results ($\alpha = 0.05$) are highlighted in bold font.*

Parameter	Body mass	Body length
Absolute U_{crit}	$F_{1,138} = 0.68$, $p = 0.41$	$F_{1,138} = 2.24$, $p = 0.14$
Corrected U_{crit}	$F_{1,138} = 1.92$, $p = 0.17$	$F_{1,138} = 85.72$, $p = 4 \times 10^{-16}$

CHAPTER 6

General discussion

6.1. Induced polyploidy and polyploidization capacity

Artificially induced polyploidy, namely triploidy, has been found an effective tool to improve commercial value or increase the efficacy of production of multiple fish species. The elevated potential for utilization of triploid fishes in aquaculture is mainly due to the benefits resulting from their sterility (Benfey, 1999; Piferrer et al., 2009; Flajšhans et al., 2013). This is not the case in sturgeons, where targeted polyploidization is not a common practice in commercial production (Schreier et al., 2013, 2021). On the other hand, induced polyploidy still has some potential to improve the profitability of sturgeons breeding in the future, especially in terms of caviar industry. Since the polyploid gametes contain more genetic material than gametes of normal ploidy, the females of elevated ploidy may produce caviar of higher granularity (Gille et al., 2015; Fiske et al., 2019; Kašpar, personal communication). Moreover, the artificial triploidization is usually achieved via induction of the second polar body (SPBR) and optimization of this process is essential for the successful induction of meiotic gynogenesis, a technique that has been found to considerably increase the proportion of females in newly formed sturgeon progeny (Chapter 2, Keyvanshokooh and Gharaei, 2010; Fopp-Bayat et al., 2018). Apart from its potential for commercial applications, artificial induction of polyploidy plays a pivotal role in the research of polyploid fishes (Leggatt and Iwama, 2003; Le Comber and Smith, 2004). It enables obtaining a sufficient amount of polyploid material for further examination, which has been the case in the studies presented in Chapter 4 and Chapter 5, among many others.

Successful optimization of the polyploidization protocol is a critical step for intentional creation of a polyploid population (Pandian and Koteeswaran, 1998; Zhou and Gui, 2017). The first study of this thesis (Chapter 2) was conducted on sterlet and introduced an optimized protocol for hydrostatic pressure shock-induced SPBR causing triploidization when untreated sperm was used for fertilization, or restoration of diploidy in meiotic gynogenesis after inactivated spermatozoa have been used to initiate embryogenesis. Apart from this study, the application of hydrostatic pressure for SPBR-induction in sturgeons has been reported only by Flynn et al. (2006) and Beyea et al. (2005) in single species – shortnose sturgeon, while most authors working with sturgeons chose temperature shocks to provoke SPBR. Among temperature treatments, heat shock is usually preferred to cold shocks (heat shock: e.g. Van Eenennaam et al., 1996; Mims and Shelton, 1998; Omoto et al., 2005; Wlasow and Fopp-Bayat, 2011; Lebeda et al., 2018; cold shock: Saber et al., 2008; Saber and Hallajian, 2014). Therefore, the efficiency of hydrostatic pressure and heat shock for SPBR was compared in Chapter 2. When used for triploidization, the optimized hydrostatic pressure shock of 55 MPa for 3 min at 18 min post gamete activation resulted in a similar fertilization rate but higher hatching rate than conventional heat shock performed according to the previous experience in sterlet (35 °C for 3 min duration 18 min post-activation) (Lebeda et al., 2014, 2018). Moreover, all the sampled prelarvae from the pressure-treated group were found to be biologically triploid, while triploidy was confirmed in only 80% of prelarvae sampled from the heat-shocked group (Chapter 2). This observation is consistent with general conclusion of Benfey (2009) that heat shock does not routinely result in 100% triploidization success due to uneven distribution of temperature to all treated eggs. Moreover, Nagler (2019) pointed out that hydrostatic pressure shock is more reliable at producing 100% triploids since it enables to treat the eggs more equally. The findings of the presented study confirmed the suitability of hydrostatic pressure shock for SPBR-induction in sterlet and prove that the optimization of this type of physical shock for other sturgeon species may improve the efficiency in obtaining their triploidized or gynogenetic progeny.

Chapter 4 provided additional information regarding artificial induction of polyploidy in sterlet. Apart from SPBR-induced triploidy, Chapter 4 included the induction of biological tetraploidy via the first mitotic division suppression (FMDS) and the induction of biological hexaploidy via the combination of treatments causing both SPBR and FMDS. In order to avoid inconsistency in the type of inducing treatment, all artificial inductions in Chapter 4 were conducted with heat shock, whose use in sturgeons was previously described for both SPBR and FMDS (Fopp-Bayat et al., 2007; Lebeda and Flajšhans, 2015).

In Chapter 4, the parameters of heat shock for SPBR slightly differed from the experiment presented in Chapter 2 – both shocks were applied 18 min post-activation of gametes, but the eggs in Chapter 4 were incubated at 16 °C instead of 15 °C, and immersed in a water bath of 34 °C for 2 min (Fopp-Bayat et al., 2007), while 35 °C treatment for 3 min was used in Chapter 2. When calculated relative to control, the protocol in Chapter 4 resulted in lower mortality before hatching and, despite its lower intensity and shorter duration, also higher triploidization success (96.7% vs. 80%). These observations suggest that the heat shock used in Chapter 4 may be more reliable for SPBR-induction in sterlet, however, it is worth mentioning that the experiments were conducted on eggs from different females and the variation in egg quality and genetic background may affect the efficiency and optimal timing of polyploidization treatment, as previously hypothesized also by Lebeda et al. (2020) in case of FMDS induction.

In comparison to SPBR, FMDS is generally more difficult to achieve in fishes, and its induction often results in high losses during the early development (Pandian and Koteeswaran, 1998; Piferrer et al., 2009). On the other hand, if induced successfully, it enables to study effects of polyploidy arising directly from WGD (Lebeda et al., 2020). Moreover, the use of biologically diploid gametes obtained from FMDS-induced sturgeons may help to eliminate rediploidization treatment during androgenesis or mitotic gynogenesis, the genome manipulations with potential for improving the conservation of endangered sturgeon species and/or revealing the sex determination system of sturgeons (Grunina et al., 2011; Lebeda and Flajšhans, 2015) unknown until recently. Artificial tetraploidization via FMDS-induction has so far been successfully achieved in several sturgeon species – sterlet (Lebeda and Flajšhans, 2015), Siberian sturgeon (Lebeda and Flajšhans, 2015; Lebeda et al., 2020) and Russian sturgeon (Lebeda et al., 2020). The protocol for FMDS-induction in sterlet has been optimized by Lebeda and Flajšhans (2015) and their findings were implemented in Chapter 4. The application of 37 °C shock for 2 min 60 min after gamete activation resulted in a higher hatching rate (20.2%) but considerably lower tetraploidization efficiency (10%) (Chapter 4) than reported by Lebeda and Flajšhans (2015) for mass induction (12 and 67%, respectively). Apart from the female effect possibly affecting optimal timing for the induction (Lebeda et al., 2020), the lower tetraploidization success in Chapter 4 might be the consequence of a greater amount of eggs treated by heat shock (200 g vs. 100 g). Few authors (Harris et al., 1989; Komen and Thorgaard, 2007) hypothesized that the depolymerization tolerance limit of the protein complexes involved in the mitotic division might be probably higher than in the case of meiotic division, and the uneven distribution of temperature during artificial FMDS might thus theoretically lower the efficiency of the treatment even more than in SPBR-induction. Based on this theory, the polyploidization success could be improved by the separation of greater amounts of sturgeon eggs into smaller batches before heat shock treatment. Moreover, the optimization of hydrostatic pressure protocol for FMDS induction may possibly increase the yields of tetraploid sturgeons in the future.

The combination of SPBR and FMDS-inducing treatments (SPBR + FMDS) in Chapter 4 has led to obtaining of biologically hexaploid sterlet. This double shock causing genome triplication has been previously applied only in Siberian sturgeon, where probably yielded the highest

chromosome number among all vertebrates karyotyped so far (Lebeda et al., 2020). Although the individuals obtained from SPBR + FMDS induction did not survive the prelarval period in both the presented study and in the study of Lebeda et al. (2020), the possibility of direct hexaploidization documents enormous ploidy plasticity in sturgeons as compared to other fishes or, more precisely, vertebrates in general.

6.2. Extended storage of samples for ploidy determination

A reliable and robust method of ploidy determination is the essential prerequisite for polyploid research (Pandian and Koteeswaran, 1998; Jankun et al., 2007). Due to their evolutionary ploidy status, well-preserved genome plasticity and high propensity to hybridization, sturgeons can possess complete zoological garden of ploidy levels, which increases the demands on the distinguishing ability of the investigating method (Havelka et al., 2011). Flow cytometric analysis of relative DNA content in cell nuclei is considered the gold standard technique for the measurement of ploidy level in fishes (Krasznai and Goda, 2021) and together with impedance-based measurement of cell volume (Coulter counter) represents the most preferred approach in sturgeon research (e.g. Van Eenennaam et al., 1996; Zhou et al., 2011; Schreier et al., 2011; Havelka et al., 2016; Lebeda et al., 2018; Fiske et al., 2019; Leal et al., 2019, 2020, 2021). Fiske et al. (2019) worked with two populations of white sturgeons of various ploidy levels and found 100% agreement between Coulter counter and flow cytometry results in ploidy assignment, while the image analysis of whole erythrocyte long axis lengths exhibited considerably lower accuracy in ploidy determination. As demonstrated by Bytyustkyy et al. (2014), measuring the surface area of erythrocyte nuclei from blood smears, especially in ploidy levels higher than 6n, may also lead to misinterpretation of ploidy due to non-linear increase in nuclear volume with increasing ploidy level. However, although the flow cytometry enables accurate and time-efficient determination of ploidy in sturgeons, its application for this purpose has been so far limited mainly to the examination of fresh samples (e.g. Zhou et al., 2011; Schreier et al., 2011; Havelka et al., 2016; Lebeda et al., 2018; Iegorova et al., 2018), and the only authors who reported measurement of DNA content from fixed tissues of sturgeons mentioned notably higher coefficients of variation after keeping samples in 45% ethanol at 4 °C for 2–3 days (Birstein et al., 1993). Since it is not always possible to access fresh material for flow cytometric analysis, Chapter 3 investigated the potential for extended storage of the three common sample types using sterlet as a model species of sturgeon. In order to improve the impact of the study, tench (*Tinca tinca*) representing Teleostei was included as the second model species. From 13 different procedures tested for up to ten days of storage of sterlet samples, the best results were achieved with fixation in 15% ethanol for blood, freezing at -80 °C in DMSO-citrate buffer (Vindelov et al., 1983) for fin tissue and freezing at -80 °C in 1% ethanol or physiological saline for larva tail tissue. The use of ice and dry ice for cooling and freezing makes the procedures feasible under field conditions, and the absence of centrifugation steps contributes to the simplicity and time efficiency of sample preparation, while the majority of other protocols developed for fixation/preservation of fish samples included several centrifugations (Allen, 1983; Brown et al., 2000; Lamatsch et al., 2000). Based on additional experience with fixation/preservation of blood, fin and larva tail tissue samples from various sturgeon species, the freezing at -80 °C in DMSO-citrate buffer was selected as the most appropriate method for long term storage, since it enabled successful analysis of relative DNA content in samples stored for more than 8 months with the absence of noticeable changes in their fluorescence or variation coefficients (Hubálek and Flajšhans, unpublished data). The application of the protocols proposed allows obtaining

sturgeon samples in field conditions far from laboratory facilities without the necessity of immediate analysis. It decreases the demand for rapid transport of samples to the laboratory and may help to provide a consistent supply of standard for ploidy examination. Last but not least, it enables the analysis of large numbers of specimens obtained at the same time (see Chapters 4 and 5), which is of particular importance especially when there is a need to examine sturgeons at the same stage of development, as was the case in Chapter 4.

6.3. The effects of autopolyploidy on performance and physiology

Numerous records of spontaneously occurring autopolyploidy in sturgeons have stimulated the research focused on its causes and consequences. Current knowledge in sturgeons suggests that spontaneous autopolyploids arise mostly from SPBR, and the incidence of this phenomenon may be linked to hatchery practices (Omoto et al., 2005; Gille et al., 2015; Van Eenennaam et al., 2020). On the other hand, the first evidence of wild spontaneous autopolyploids proved that the occurrence of spontaneous SPBR does not have to be limited solely to aquaculture facilities (Schreier et al., 2021). The studies dealing with the impact of SPBR on reproduction suggested that autopolyploids of some sturgeon species are fertile and the reproductive performance of their progeny obtained from backcrossing is questionable (Schreier et al., 2011, 2013; Havelka et al., 2014, 2016), while in other sturgeon species, autopolyploids arising from SPBR may exhibit sterility or delayed sexual maturity (Omoto et al., 2002, 2005; Schreier et al., 2021). However, the effects of autopolyploidy on physiology and physical performance in sturgeons have so far attracted little attention except for a single sturgeon species (Leal et al., 2018, 2019, 2020, 2021). The studies dealing with this issue are of particular interest since they may help to estimate the consequences of spontaneous polyploidy for both cultured and wild sturgeon populations. Moreover, taking into account that the evolution of sturgeons is associated with many polyploidization events and their capacity for polyploidization is well preserved, these studies may address the question of whether contemporary polyploidizations provide sturgeons with some unique advantages or rather lead to an evolutionary dead end.

As indicated in the past, ploidy related differences in cellular and organismal physiology may intensify the differences in performance under challenging environmental conditions, such as temperatures beyond the thermal optimum (Ojolick et al., 1995; Altimiras et al., 2002; Hyndman et al., 2003; Atkins and Benfey, 2008). Moreover, global warming and human-induced alterations to natural habitats are increasing the risk of the occurrence of temperatures that would be unfavourable for sturgeons (Lassalle et al., 2010; Zhang et al., 2019), as well as the theoretical risk of spontaneous autopolyploidization in nature (Shivaramu et al., 2020), the studies using suboptimal temperatures are thus highly topical. Recent works dealt with the impact of chronically elevated temperatures on the growth, physiology and metabolism of polyploid white sturgeon (Leal et al., 2018, 2019) and one of them reported that warm acclimated biological triploids exhibited considerably reduced growth rates and limitations in metabolic enzyme activity when compared to biological diploids (Leal et al., 2019). However, these studies were conducted solely on sturgeon juveniles aged 10–14.5 months, while the highest vulnerability to thermal stress and narrowest windows of thermal tolerance are presented in earlier developmental stages (Pörtner and Peck, 2010). Therefore, the study in Chapter 4 investigated survival and growth of early developmental stages of autopolyploid sturgeon at suboptimal temperature conditions. The study included two experiments – the experiment with biologically diploid, triploid, tetraploid and hexaploid sterlet kept at optimal (16 °C) and two suboptimal (10 and 20 °C) temperatures from the neurula stage until the end

of endogenous feeding, and the experiment with biologically diploid and triploid sterlet larvae reared at 10, 16 and 20 °C for three weeks. The results of Chapter 4 suggested that triploid embryos, prelarvae and larvae did not differ from diploids in their viability and developmental rate/growth at both optimal and suboptimal temperature conditions. These findings assume that autopolyploidy arising from SPBR may not pose a limitation for the early development of sturgeons and may not affect the probability of recruitment into a new population. If these autopolyploids reach the adult stage, they could decrease the reproduction success of a given population or contaminate the future generation with the progeny of abnormal ploidy, which may have negative implications on both sturgeon culture and conservation (reviewed in Schreier et al., 2021). Unlike the SPBR-induced group, the embryos obtained from FMDS induction exhibited considerably higher mortality than embryos of normal ploidy at both optimal and suboptimal temperatures, and the viability of successfully induced tetraploid prelarvae at optimal temperature was found reduced when compared to diploids. Moreover, none of the tetraploid individuals at suboptimal temperature conditions survived the prelarval period, thereby indicating lower thermal tolerance than in diploids. High mortality of FMDS-induced sturgeon seems to be a general characteristic of this specific population based on the findings of other studies that reported high mortality in early developmental stages not only in sterlet (Lebeda and Flajšhans, 2015) but also in Siberian sturgeon (Lebeda and Flajšhans, 2015; Lebeda et al., 2020) and Russian sturgeon (Lebeda et al., 2020). The authors of previously mentioned studies hypothesized that this mortality could have been due to the heat shock of higher temperature than commonly used for SPBR-induction (37 °C vs. 34–35 °C). However, it is noteworthy that tetraploidization can lead to certain instabilities in cell architecture and genomic regulatory networks, possibly causing deleterious chaos displayed as gene dosage imbalances and abnormal expressions (Yin et al., 2018). Moreover, lowering the surface-area-to-volume ratio resulting from increased cell/nuclei size may alter the rate of metabolic processes, as well as the capacity for their adjustments at temperatures out of optimal range (Hermaniuk et al., 2021). Although these alterations could result also from triploidization (Christensen et al., 2019; Riseth et al., 2020), they may have been more severe in tetraploids with more additional genetic material. Last but not least, the challenges mentioned might have been unbeatable for sterlet hexaploids, which were unable to survive the prelarval period (Chapter 4), as was the case with biological hexaploids of Siberian sturgeon in the study of Lebeda et al. (2020)

As obvious from what was mentioned above, autotriploidization is artificially more easily achievable than autotetraploidization and autohexaploidization and generates more viable progeny. Moreover, spontaneously arisen biological autotriploids of sturgeons have been reported much more frequently than autotetraploids (e.g. Blacklidge and Bidwell, 1993; Schreier et al., 2011, 2021; Zhou et al., 2011, 2013; Havelka et al., 2017; Van Eenenaam et al., 2020). For these reasons, Chapter 5 investigated the effects of SPBR-induced triploidization on nine-month-old juveniles of two pure sturgeon species, sterlet and Siberian sturgeon, and their reciprocal hybrids (Siberian sturgeon × sterlet, sterlet × Siberian sturgeon). Critical swimming speed (U_{crit}) served as a proxy of whole-organism performance in sturgeons arising from normal fertilization (normoploid) and SPBR-induced specimens, and several haematological and blood chemistry parameters were assessed from non-exercised and exercised fishes of both ploidies to examine how SPBR-induced polyploidy affects haematology and physiology. The U_{crit} is generally considered a valuable measure of swimming performance, the direct indicator of physical fitness (Plaut, 2001; Rajotte and Couture, 2002; Massé et al., 2013), and its testing is performed by forced swimming of fish against an incrementally increasing velocity of water until fatigue (Hammer, 1995; Plaut, 2001; Kieffer, 2010). The effect of SPBR-induced polyploidy on U_{crit} has been most thoroughly studied in salmonids (Small and Randall,

1989; Stillwell and Benfey, 1997; Bernier et al., 2004; Lijalad and Powell, 2009; Scott et al., 2015; Riseth et al., 2020), while analogical research on sturgeons has so far been missing. The results of Chapter 5 showed that in both sturgeon purebreds and hybrids, SPBR-induced polyploids exhibited similar U_{crit} as their normoploid counterparts. Although this conclusion was consistent with the research on salmonids, there is evidence for SPBR-induced polyploidy decreasing aerobic scope in sturgeons (Leal et al., 2020, 2021), therefore, U_{crit} of SPRB-induced sturgeons should have been reduced. However, it is worth noting that the studies reporting lower aerobic scope in sturgeons of elevated ploidy were conducted on white sturgeon, where SPBR-induced polyploidy was accompanied by a reduction of oxygen-carrying capacity as obvious from total blood haemoglobin (Hb) and haematocrit (PCV) (Leal et al., 2019, 2020). In our study, SPBR-induced polyploidy did not lower the levels of these haematological parameters in any of the purebreds or hybrids examined, and since inconsistent conclusion regarding the effects of SPRB-induced polyploidy on Hb and PCV has also been drawn by other authors dealing with sturgeons, these effects appear to be species- or population-specific (Beyea et al., 2005; Rożyński et al., 2015).

Apart from previously listed inconsistency in Hb and PCV data, the statements about the consequences of polyploidy for other haematological parameters of sturgeons are quite identical and, also, in good agreement with literature on teleostean fishes (Benfey, 1999; Piferrer et al., 2009). The SPBR-induced polyploidy reduces erythrocyte count, but increases mean erythrocyte volume and mean erythrocyte haemoglobin, and usually does not affect mean erythrocytic haemoglobin concentration (Chapter 5; Beyea et al., 2005; Rożyński et al., 2015; Leal et al., 2019). In sturgeons, the exposure to acute stressors sometimes elicits changes in some of the previously mentioned primary and secondary blood indices, but the patterns and magnitudes of these changes generally appear to be similar between SPBR-induced polyploids and normoploids (Chapter 5; Beyea et al., 2005; Leal et al., 2019), except for reduced Hb and PCV response to exhaustive exercise reported by Leal et al. (2020) in SPBR-induced white sturgeon.

In addition to its effects on haematology, acute stress provokes changes in sturgeons' blood chemistry, which often include increases in the levels of plasma cortisol, glucose, osmolality and lactate (Zuccarelli et al., 2008; Allen et al., 2009; Cocherell et al., 2011; Pankhurst, 2011). Previously published studies dealing with the physiology of SPBR-induced sturgeons reported that polyploidy did not affect cortisol response (Beyea et al., 2005; Leal et al., 2019), and the results of Chapter 5 were in agreement with this conclusion. The effect of SPBR-induced polyploidy on the levels of resting/post-stress glucose seems to be negligible as well (Chapter 5; Beyea et al., 2005; Leal et al., 2019, 2020). The only exception was constantly elevated glucose concentration in SPBR-induced Siberian sturgeon × sterlet compared to normoploids of the same hybrid, however, this discrepancy might also have been caused by an undesirable extrinsic or intrinsic factor (Chapter 5). Remarkable differences between SPBR-induced polyploids and normoploids were reported by Beyea et al. (2005), who examined post-stress blood chemistry of shortnose sturgeon. The observation of elevated plasma osmolalities and lactate levels (although non-significantly for lactate) in SPBR-induced polyploids led the authors to the conclusion that these polyploids were chronically stressed. On the other hand, the significant effect of SPBR-induced polyploidy on plasma osmolality was not documented in other sturgeon species (Chapter 5; Leal et al., 2019) as was the case for plasma lactate (Leal et al., 2019, 2020). Overall, the SPBR-induced polyploids of sturgeons appear to be equal to their normoploid counterparts in primary and secondary stress responses, although some minor differences have been reported that may be species-specific.

6.4. Conclusion

Although the polyploidization events played a significant role in the evolution of sturgeons, the contemporary autopolyploidizations do not seem to provide these fishes with any specific fitness advantage. This thesis confirmed that the early developmental stages of sturgeon polyploids originating from artificial FMDS perform poorly, and the probability of their recruitment into a new generation is low, especially under suboptimal temperature conditions. On the other hand, the obtained results indicate that polyploids arising from triploidization via SPBR may not suffer from any substantial physiological impairment and are as fit as their counterparts of normal ploidy. This finding underlines concerns regarding the possible negative impact of spontaneous autopolyploids on farm productivity or wild population recovery, taking into account that sturgeon autopolyploids can exhibit reproductive abnormalities or successfully mate with normal adults and produce progeny of altered ploidy. Apart from the insights into the effects of autopolyploidy on performance, this work broadens the knowledge regarding the artificial induction of polyploidy in sturgeons. It suggested hydrostatic pressure shock as an efficient tool for SPBR-induction, whose use for triploidization or restoration of normal ploidy during meiotic gynogenesis may be of particular interest for sturgeon research or caviar production. Last but not least, in this work, the novel ways for extended storage of various sturgeon samples for subsequent flow cytometric analysis have been introduced and verified. The protocols proposed are appropriate for both laboratory and field conditions and allow rapid and accurate measurement of relative DNA content for successful determination of ploidy level in the examined specimens. The specific conclusions of this thesis are as follows:

- Hydrostatic pressure shock of 55 MPa for 3 min duration applied to eggs 18 min post gamete activation is a highly efficient tool for SPBR in sterlet and can result in better yields of triploidized progeny than conventionally used heat shock.

- The most reliable procedures for 10-day storage of sturgeon samples for flow cytometric determination of ploidy level are: fixation at 0–4 °C in 15% ethanol for blood, freezing at -80 °C in DMSO-citrate buffer for fin tissue, and freezing at -80 °C in 1% ethanol or in physiological saline for larva tail tissue. Freezing at -80 °C in DMSO-citrate buffer is an appropriate method for long-term storage of all these sample types.

- Early developmental stages of SPBR-induced sterlet do not differ from their normoploid counterparts in the ability to survive, grow and develop under both optimal and suboptimal temperatures. The viability of embryos and prelarvae obtained from FMDS-induction is considerably lowered at optimal temperature, and even more so at the temperature conditions, which are suboptimal to normoploids. Biologically hexaploid sterlet arisen from SPBR followed by FMDS appeared to be unable to survive prelarval period.

- SPBR-induced sterlet, Siberian sturgeon and their reciprocal hybrids are able to fully compensate for having fewer erythrocytes than normoploids in their Hb and PCV levels, and their primary and secondary stress response to exhaustive exercise is not altered by the presence of polyploidy.

- SPBR-induced polyploidy does not impair the swimming performance of juvenile sterlet, Siberian sturgeon and their reciprocal hybrids.

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English summary

Sturgeons (order Acipenseriformes) are considered one of the most ancient groups of fishes still present on Earth. These 'living fossils' have diverged from teleost lineage approximately 200 million years ago, and their evolution is inherently associated with polyploidization events. As a result of at least three independent whole-genome duplications, the current representatives of this group exhibit karyotype diversity that is unique among vertebrates. Moreover, it becomes clear that the susceptibility of sturgeons to polyploidization is not limited to the past, since spontaneously arisen polyploidy has been widely reported in farmed populations of many sturgeon species, and there is also evidence that it randomly occurs in the wild. The persistence of sturgeons for creating polyploid genomes underlines the genome plasticity of these archaic fishes and makes them suitable candidates for polyploid research. However, only a few studies have ever investigated the effects of newly formed polyploidy on fitness or physiology of sturgeons, and these were conducted on a limited number of species. Broadening the scope of research is of particular interest, since it may shed light on how sturgeon neopolyploids perform in aquaculture and nature, uncover the impact of contemporary polyploidization on sturgeon farming and conservation, and contribute to scientific knowledge regarding the consequences of polyploidy on fishes. This thesis brings a new insight into the artificial induction of polyploidy in sturgeons, the extended storage of sturgeon tissue samples for subsequent determination of ploidy level, and the effects of newly formed polyploidy on some fitness parameters.

Artificial induction of polyploidy based on physical shocks has been found an efficient tool for the intentional production of autopolyploid sturgeons for further examination. Moreover, this genome manipulation has the potential to improve the profitability of sturgeon breeding, since the polyploid females are expected to yield caviar of higher granularity than the females of normal ploidy (normoploidy). The second chapter of this thesis introduced an optimized protocol for the creation of biologically triploid populations of sterlet (*Acipenser ruthenus*) via the second polar body retention (SPBR) achieved by hydrostatic pressure shock. Apart from the presented study, the application of hydrostatic pressure shock in sturgeons has only ever been reported in shortnose sturgeon (*Acipenser brevirostrum*), while the vast majority of researchers chose heat shock to provoke SPBR in sturgeons. The findings of our work suggested that hydrostatic pressure shock can be more reliable at producing 100% triploids and yield better hatching rates than conventional heat shock. The utilization of hydrostatic pressure shock may thus improve the efficiency in obtaining triploidized progeny of sturgeons.

Flow cytometric analysis of relative DNA content in cell nuclei is among the most preferred techniques for the measurement of ploidy levels in sturgeons. However, due to the absence of the procedures for extended storage of sturgeon samples, the application of flow cytometry has so far been limited to the examination of fresh material. The third chapter of this thesis provided the verified methods for 10-day storage of various types of sturgeon samples – blood, finclip and larva tail tissue. The protocols proposed are easy to use, do not contain centrifugation steps and allow field sampling. The range of their applications includes e.g. obtaining samples far from laboratory facilities without the necessity of immediate analysis, providing a consistent supply of standard for ploidy determination, or examination of a large number of specimens obtained at the same time.

Ploidy related differences at both genetic and physiological levels can intensify the differences in performance under challenging conditions and, consequently, affect the ploidy composition in sturgeon populations. Global warming and human-induced alterations to natural habitats are increasing the probability that early developmental stages will experience temperatures out of the optimal range, as well as the theoretical risk of spontaneous

autopolyploidization in nature. Therefore, the viability and growth of early developmental stages of autopolyploid sterlet kept at both optimal (16 °C) and suboptimal temperatures (10 and 20 °C) were investigated in fourth chapter of this thesis. The results suggested that irrespective of temperature, biologically triploid sterlet arising from SPBR do not differ from their normoploid counterparts in terms of survival and growth. Unlike triploids, the viability of embryos obtained from tetraploidization via the first mitotic division suppression (FMDS) was considerably reduced at optimal temperature and even more so at suboptimal conditions. The biologically tetraploid prelarvae were not able to survive at suboptimal temperatures, assuming their lower thermal tolerance when compared to normoploids or biological triploids. Last but not least, biological hexaploidy is probably lethal in sturgeons, since the individuals obtained from the combined treatment causing both SPBR and FMDS were unable to survive the prelarval period at any temperature.

In the fifth chapter of this thesis, the effects of SPBR-induced polyploidy on fitness, physiology and haematology were investigated using nine-month-old juveniles of two pure sturgeon species, sterlet and Siberian sturgeon (*Acipenser baerii*), and their reciprocal hybrids. As indicated by the measurement of the critical swimming speed, SPBR-induced polyploids of both purebreds and hybrids did not differ from their normoploid counterparts in swimming performance. The haematological analysis revealed that SPBR induced polyploids were able to fully compensate for having fewer erythrocytes than normoploids in blood haemoglobin and haematocrit, their potential to carry oxygen for aerobic processes was thus not altered by the presence of polyploidy. Moreover, the haematological and blood chemistry analysis of non-exercised and exercised sturgeons did not provide any evidence for SPBR-induced polyploidy affecting stress response. Together with the conclusions of the fourth chapter, these results may indicate that sturgeon polyploids arising from SPBR do not suffer from any substantial physiological limitation and are as fit as their counterparts of normal ploidy. Considering that sturgeon autopolyploids can exhibit reproductive abnormalities or successfully mate with normoploids and contaminate future generations with the progeny of abnormal ploidy levels, these findings underline concerns regarding the negative impact of spontaneous autopolyploidy on productivity of aquaculture or wild population recovery.

Czech summary

Jeseteři (řád Acipenseriformes) jsou považováni za jedny z nejstarobylejších ryb dosud žijících na planetě Zemi. Tyto „živoucí fosilie" se od kostnatých ryb oddělily přibližně před 200 miliony let a jejich evoluce je neodmyslitelně spjata s polyploidizačními událostmi. V důsledku nejméně třech nezávislých celogenomových duplikací se u dnešních zástupců jeseterů setkáváme s takovou úrovní karyotypové diverzity, která je mezi obratlovci unikátní. U chovaných populací mnoha jeseteřích druhů byla navíc pozorována spontánně vznikající polyploidie, což spolu s důkazy jejího náhodného výskytu ve volné přírodě dává tušit, že náchylnost jeseterů k polyploidizaci zůstala zachována dodnes. Přetrvávající sklony jeseterů vytvářet polyploidní genomy zdůrazňují genomovou plasticitu těchto archaických ryb a činí z nich vhodné kandidáty pro výzkum polyploidie. Vlivem indukované nebo spontánně vznikající polyploidie na fitness a fyziologii jeseterů se však doposud zabývalo pouze pár studií, které byly navíc realizovány jen na několika málo druzích. Rozšiřování výzkumu v této oblasti má zvláštní význam, neboť může pomoci objasnit, jak neopolyploidní jeseteři prosperují v akvakultuře nebo v přírodě, odhalit vliv současných polyploidizací na chov a konzervaci jeseterů a prohloubit vědecké poznání důsledků polyploidie pro ryby. Tato práce přináší nové poznatky ohledně umělé indukce polyploidie u jeseterů, prodloužení uchovatelnosti jeseteřích tkání pro následnou determinaci ploidní úrovně a vlivů nově vzniklé polyploidie na některé parametry fitness.

Umělá indukce polyploidie fyzikálními šoky je účinným nástrojem k cílené produkci autopolyploidních jeseterů pro další výzkum. Tato genomová manipulace má navíc potenciál zvýšit rentabilitu jejich chovu, polyploidní samice by totiž ve srovnání se samicemi běžné ploidie (normoploidie) mohly produkovat kaviár o větší zrnitosti. Druhá kapitola předkládané práce přináší optimalizovaný protokol pro vytváření biologicky triploidních populací jesetera malého (*Acipenser ruthenus*) pomocí retence druhého pólového tělíska (RDPT) hydrostatickým tlakem. S výjimkou této práce byla doposud aplikace šoku hydrostatickým tlakem u jeseterů publikována pouze u jesetera krátkorypého (*Acipenser brevirostrum*), zatímco drtivá většina autorů volila k indukci RDPT u jeseterů teplý šok. Na základě zjištění naší studie lze usoudit, že šok hydrostatickým tlakem může být pro produkci 100 % triploidů vhodnější než konvenčně používaný teplý šok, a zároveň vést k dosažení vyšší líhnivosti. S použitím šoku hydrostatickým tlakem by tedy mohlo být triploidizované potomstvo jeseterů získáváno efektivněji.

Měření relativního obsahu DNA v buněčných jádrech průtokovou cytometrií patří mezi nejběžnější metody určování ploidní úrovně jeseterů. Z důvodu absence postupů vedoucích k prodloužení uchovatelnosti jeseteřích vzorků bylo však využití průtokové cytometrie doposud omezeno na analýzu nativního materiálu. Výsledkem práce, popsané ve třetí kapitole, jsou ověřené metody pro desetidenní uchování různých typů jeseteřích vzorků. Navrhované protokoly jsou jednoduše využitelné, neobsahují centrifugace a umožňují terénní vzorkování. Rozsah možných aplikací skýtá například získávání vzorků daleko od laboratorního zázemí bez nutnosti bezprostřední analýzy, zajišťování konzistentního zdroje standardu pro determinaci ploidie nebo zkoumání velkého množství vzorků získaných v krátkém časovém úseku.

Rozdíly v genetice a fyziologii jedinců o různé ploidii mohou umocňovat rozdíly ve výkonnosti v náročných podmínkách a v důsledku toho ovlivňovat ploidní složení jeseteřích populací. Globální oteplování a člověkem způsobené alterace přirozených habitatů zvyšují pravděpodobnost vystavení raných vývojových stadií teplotám mimo optimální rozsah, a stejně tak teoretické riziko spontánní autopolyploidizace v přírodě. Ve čtvrté kapitole této práce byla tedy zkoumána životaschopnost a růst raných vývojových stadií autopolyploidních jeseterů malých v optimální (16 °C) teplotě a teplotách suboptimálních (10 a 20 °C). Výsledky výzkumu naznačují, že biologicky triploidní jeseteři malí se od svých normoploidních sourozenců neliší v přežití ani růstu, a to bez ohledu na teplotu. Životaschopnost embryí získaných tetraploidizací

prostřednictvím zadržení prvního mitotického dělení (ZPMD) byla naproti tomu výrazně snížena už při inkubaci v optimální teplotě, a ještě výrazněji v teplotách suboptimálních. Biologicky tetraploidní prelarvy navíc nebyly v suboptimálních teplotách schopné přežít, což poukazuje na jejich nižší teplotní toleranci v porovnání s normoploidy a biologickými triploidy. V neposlední řadě, biologická hexaploidie je u jeseterů zřejmě letální, neboť jedinci získaní z kombinované indukce RDPT a ZPMD nepřežili prelarvální periodu v žádné z testovaných teplot.

V páté kapitole této práce byl popsán zkoumaný vliv polyploidie indukované prostřednictvím RDPT na fitness, fyziologii a hematologii devítiměsíčních juvenilů dvou čistých jeseteřích druhů, jesetera malého a jesetera sibiřského (*Acipenser baerii*) a jejich reciprokých hybridů. Z výsledků měření kritické rychlosti plavání vyplynulo, že se indukovaní polyploidi obou čistých druhů a hybridů ve své výkonnosti nelišili od normoploidů. Hematologická analýza odhalila, že polyploidi získaní indukcí RDPT byli z hlediska úrovní krevního hemoglobinu a hematokritu schopni plně kompenzovat nižší počet erytrocytů v porovnání s normoploidy, jejich kapacita zadržovat kyslík pro aerobní procesy tedy nebyla přítomností polyploidie pozměněna. Z hematologické ani chemické analýzy krve jeseterů, kteří nefigurovali v plavacím testu, a jedinců použitých v plavacím testu, nevyplynul žádný důkaz, že by polyploidie ovlivňovala stresovou odpověď. Tyto výsledky spolu se závěry čtvrté kapitoly naznačují, že polyploidi jeseterů vzniklí z RDPT netrpí žádnou významnou fyziologickou limitací, a po stránce fyzické zdatnosti jsou srovnatelní s jejich protějšky běžné ploidní úrovně. S přihlédnutím ke skutečnosti, že autopolyploidi jeseterů mohou vykazovat reprodukční abnormality nebo se úspěšně pářit s normoploidy, a kontaminovat tak budoucí generace potomstvem o abnormálních ploidních úrovních, zdůrazňují zjištění této práce obavy z negativních důsledků spontánní polyploidie pro akvakulturní produkci nebo návratnost volně žijících populací.

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List of publications

Peer-reviewed journals with IF

Hubálek, M., Kašpar, V., Tichopád, T., Rodina, M., Flajšhans, M., 2022. How do suboptimal temperatures affect polyploid sterlet *Acipenser ruthenus* during early development? J. Fish Biol. 101(1), 77–91. (IF 2021 = 2.504)

Kašpar, V., **Hubálek, M.**, Pšenička, M., Arai, K., Taggart, J., Franěk, R., 2022. Cold-shock androgenesis in common carp (*Cyprinus carpio*). Aquaculture 548, 737610. (IF 2021 = 5.135)

Hubálek, M., Flajšhans, M., 2021. Simple field storage of fish samples for measurement of DNA content by flow cytometry. Cytometry A 99(7), 743–752. (IF 2021 = 4.714)

Flajšhans, M., Havelka, M., Lebeda, I., Rodina, M., Gela, D., **Hubálek, M.**, 2020. Application of hydrostatic pressure shock for retention of the second polar body in sterlet (*Acipenser ruthenus*). Aquaculture 520, 734947. (IF 2020 = 4.242)

Manuscripts prepared for submission to peer-reviewed journals with IF

Hubálek, M., Kašpar, V., Tran, H.Q., Stejskal, V., Tichopád, T., Grabicová, K., Flajšhans, M., 2022. How does induced polyploidy affect the swimming and physiological performance of sterlet (*Acipenser ruthenus*) and Siberian sturgeon (*Acipenser baerii*) and their reciprocal hybrids? (Manuscript)

International conferences

Hubálek, M., Flajšhans, M., 2021. Simple field storage of fish samples for measurement of DNA content by flow cytometry. Aquaculture Europe 2021, 4th–7th October 2021, Madeira, Portugal, Book of abstracts, p. 583. (poster presentation)

Flajšhans, M., Lebeda, I., **Hubálek, M.**, 2019. The use of flow cytometry for study of polyploidy in freshwater fish. In: Lucretti, S., Giorgi, D. (Eds), ExoFlowMetry 2019, The First International Workshop on Exotic Flow CytoMetry, Rome, 13th–15th November 2019, ENEA Casaccia Res. Centre. (invited lecture)

Training and supervision plan during study

Name	Martin Hubálek
Research department	2018–2022: Laboratory of Molecular, Cellular and Quantitative Genetics
Supervisor	Prof. Martin Flajšhans
Period	1st October 2018 until 14th September 2022

Ph.D. courses	**Year**
Pond aquaculture	2019
Basic of scientific communication	2019
Biostatistics	2019
Fish genetics	2019
Fish reproduction	2020
Ichthyology and fish taxonomy	2020
English language	2021

Scientific seminars	**Year**
Seminar days of RIFCH and FFPW	2019 2020 2021
USB conference of doctoral students	2021

International conferences	**Year**
Flajšhans, M., Lebeda, I., **Hubálek, M.**, 2019. The use of flow cytometry for study of polyploidy in freshwater fish. In: Lucretti, S., Giorgi, D. (Eds), ExoFlowMetry 2019, The First International Workshop on Exotic Flow CytoMetry, Rome, 13th–15th November 2019, ENEA Casaccia Res. Centre. (invited lecture)	2019
Hubálek, M., Flajšhans, M., 2021. Simple field storage of fish samples for measurement of DNA content by flow cytometry. Aquaculture Europe 2021, 4th–7th October 2021, Madeira, Portugal, Book of abstracts, p. 583. (poster presentation)	2021

Foreign stays during Ph.D. study at RIFCH and FFPW	**Year**
Andrea Schreier, Ph.D., Genomic Variation Lab, University of California, Davis, USA (80 days; microsatellite genotyping of the Kootenay River population of white sturgeon)	2019

Pedagogical activities	**Year**
Supervising a block of practice exercise for students of master study programme within the subject 'Breeding and Genetics of Fish' (24 hours)	2019, 2022
Training of students in measuring the relative DNA content for ploidy level determination by means of flow cytometry (34 hours)	2019–2022
Presentation of the activities of Laboratory of Molecular, Cellular and Quantitative Genetics – Open days FFPW, 'Tour de FROV' (30 hours)	2019, 2021, 2022
Two presentations for summer school students – topics: the activities of Laboratory of Molecular, Cellular and Quantitative Genetics; polyploidy in stugeons (8 hours)	2019, 2021
Consultant of Bachelor theses of Markéta Blahoutová, Radoslav Káčerik and Vladimír Novotný – expected year of defenses: 2023	

Curriculum vitae

PERSONAL INFORMATION

Name: Martin
Surname: Hubálek
Title: Dipl.-Ing.
Born: 17th October 1993, Plzeň, Czech Republic
Nationality: Czech
Languages: Czech (native speaker), English (B2 level – FCE certificate)
Contact: mhubalek@frov.jcu.cz

EDUCATION

2018 – present Ph.D. student in Fishery, Faculty of Fisheries and Protection of Waters, University of South Bohemia, České Budějovice, Czech Republic

2016–2018 Dipl.-Ing. in Fishery and Protection of Waters, Faculty of Fisheries and Protection of Waters, University of South Bohemia, České Budějovice, Czech Republic

2013–2016 B.Sc. in Fishery, Faculty of Fisheries and Protection of Waters, University of South Bohemia, České Budějovice, Czech Republic

RESEARCH INTEREST

- Spontaneous and induced polyploidy in fishes
- Uniparental inheritance in fishes
- Flow cytometry and other techniques of analytical cytology
- Reproductive biology in fishes, artificial propagation

COMPLETED COURSES

Basic of scientific communication, Biostatistics, Fish genetics, Fish reproduction, Ichthyology and fish taxonomy, English language

TRAINING

12/4 – 16/4 2021 Training course for acquiring qualification and professional competence (designing experiments and experimental projects) in the field of experimental animal use according to Act No. 246/1992 On the Protection of Animals Against Cruelty

RESEARCH STAY

1/10 – 19/12 2019 Andrea Schreier, Ph.D., Genomic Variation Lab, University of California, Davis, USA