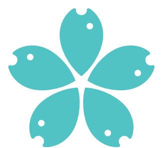

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Faculty of Fisheries
and Protection
of Waters

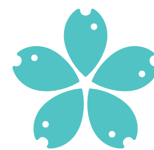
Jihočeská univerzita
v Českých Budějovicích
University of South Bohemia
in České Budějovice

ISBN 978-80-7514-081-4

2018

Polyspermy produces viable mosaics in sturgeon

Viktoriia Iegorova



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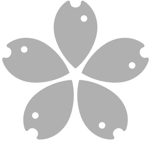

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Title of thesis:
Polyspermy produces viable mosaics in sturgeon
Polyspermie u jeseterů dává vzniknout životaschopným mozaikám

Ph.D. thesis, USB FFPW, RIFCH, Vodňany, Czech Republic, 2018, 71 pages, with the summary in English and Czech.
Graphic design & technical realisation: JENA Šumperk, www.jenasumperk.cz
ISBN 978-80-7514-081-4-

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CHAPTER 1

GENERAL INTRODUCTION

1.1. FAMILY ACIPENSERIDAE: CHARACTERISTICS AND CURRENT STATUS

Sturgeons are considered the most valuable fish in the world (Dettlaff and Vassetzky, 1991, Pikitch et al., 2005), famous for the quality of their meat and especially the black caviar, a luxury product and highly nutritious delicacy (Carmona et al., 2009). Sturgeons belong to Family Acipenseridae (Order Acipenseriformes), and are made up of four genera: *Acipenser, Huso, Pseudoscaphirhynchus* and *Scaphirhynchus* (Bemis et al., 1996; Rajkov et al., 2014). Together, these four genera comprise twenty-five species, namely *Acipenser dabryanus, A. gueldenstaedtii, A. mikadoi, A. naccarii, A. nudiventris, A. persicus, A. schrenckii, A. sinensis, A. stellatus, A. sturio, A. baerii, A. fulvescens, A. medirostris, A. oxyrinchus, A. transmontanus, A. brevirostrum, A. ruthenus, Huso dauricus, H. huso, Pseudoscaphirhynchus fedtschenkoi, P. hermanni, P. kaufmanni, Scaphirhynchus suttkusi, S. albus,* and *S. platorynchus* (Carmona et al., 2009).

Family Acipenseridae arose 200-250 million years ago and inhabit the coastal waters of the Atlantic and Pacific oceans, the Mediterranean and Black seas, along with rivers, lakes and estuaries of the Northern Hemisphere (Dettlaff and Vassetzky, 1991; Bemis et al., 1997; Pikitch et al., 2005). The highest concentration of these fishes is in the Caspian, Black and Azov seas (Dettlaff and Vassetzky, 1991). These 'living fossils' differ from modern bony fishes by possessing a cartilaginous skeleton (Chebanov and Galich, 2013). Sturgeons retain primitive traits including a heterocercal caudal fin (asymmetrical, generally with the continuation of the spinal cord), ganoid scales in longitudinal rows on the body, and spiracles (Parks, 1978; Gardiner, 1984; Doroshov, 1985). The general morphology of sturgeons is rather unique: an elongate body with a ventral flat base, rostrum and snout with sensory barbells (Gardiner, 1984; Billard and Lecointre, 2001). This ancient group of fishes possesses features that helped them survive until the present time. For example, spawning occurs in all seasons in a wide range of temperatures; gametes retain the ability for fertilization for up to one hour after being released into the water. Sturgeons are able to eat a wide variety of food items. They are also ecologically plastic, being both diadromous (migrations between marine and freshwater systems) and potamodromous (migrations within freshwater) (Cherr and Clark, 1985; Dettlaff et al., 1993; Bemis and Kynard, 1997). Acipenseriforms migrate mostly for two basic reasons: feeding and reproduction. Downstream migrations always associated with feeding. The interfaces between freshwater and saltwater or between rivers and large lakes can be nutrient rich, with abundant food (Khodorevskaya et al., 1997), while upstream migrations are characteristically for spawning, which happen on the hard substrates and varying depths of freshwaters (Krykhtin and Svirskii, 1997; Billard and Lecointre, 2001; Pikitch et al., 2005; Carmona et al. 2009).

Sturgeons are generally long-lived fishes with slow growth and maturation (Pikitch et al., 2005). Representatives of this family grow continuously with age, and some fishes (for example, *H. huso*) can live up to 100 years and weighing 1,000 kg (Billard and Lecointre, 2001; Chebanov et al., 2011). Reproductive age differs among species and between the sexes. Sturgeons do not reproduce annually, and can have long intervals between spawnings (Table 1) (Billard and Lecointre, 2001). Identifying the sex in sturgeons is challenging due to their late maturity (Falahatkar et al., 2013). Fecundity also varies depending on the egg size, with a range of 6,000–7,000 oocytes per kg of body weight (egg diameter 3.4–3.8 mm) in *H. huso*, to approximately 30,000 oocytes (egg diameter 2.5 mm) per kg in *A. ruthenus* (Holcik, 1989).

Table 1. *Age of maturation and time between spawnings in Acipenseridae.*

Species	Location	First maturation		Years between spawnings	
		Males	Females	Males	Females
A. sturio	Gironde	7–15	16–20	–	–
A. nudiventris	Kura	6–9	12–14	1–2	2–3
A. sinensis	Yangtze	9–17	14–26	–	–
A. brevirostrum	Georgia	2–3	4–6	2	3–5
	S. Carolina	3–5	7–10	–	–
	Saint John river	10–11	12–18	–	–
A. fulvescens	St. Lawrence	15–17	20–24	–	–
A. gueldenstaedtii	Volga/Danube	11–13	12–16	2–3	5
A. stellatus	Volga	7	9	2–3	3–4
A. ruthenus	Danube	3–5	4–7	0–1	1–2
A. persicus	Volga, Ural	8–15	12–18	2–4	2–4
A. baerii	Ob, Yenissei	11–13	17–18	1–4	3–6
	Farms	2–4	7–8	1–3	2–4
A. oxyrinchus	St. Lawrence River	22–24	27–28	1–5	3–5
A. schrenckii	Amur River	10–14	10–14	–	~4
A. dabryanus	Yangtze	4–7	6–8	4–6	6–8
H. dauricus	Amur	14–21	17–23	3–4	4–5
H. huso	Caspian Sea Basin	10–15	15–18	3–4	3–4
	Volga	14–16	19–22	3–4	5–6
Pseudoscaphirhynchus kaufmanni	Amu Darya	5–7	6–8	4–5	4–5
Scaphirhynchus albus	Mississippi River Basin	5–9	9–12 or 15–20	2–3	3–10

"–" there is no information
Data summarized from the following sources: Birstein 1997a; Doroshov et al., 1997; Krykhtin and Svirskii, 1997; Kynard, 1997; Mayden and Kuhajda, 1997; Zhuang et al., 1997; Billard and Lecointre, 2001; Chebanov et al., 2011, 2013.

Currently, all sturgeon species are in the Red List, with seventeen of them in the critically endangered list (The IUCN Red List). The decline of sturgeon populations is attributed to:

- Wild sturgeon females killed for caviar. Illegal fishing and poaching caused decreasing sturgeon populations around the world (Zhuang et al, 1997; Cohen, 1997; Birstein et al., 1997b);
- Long time to sexual maturity, making it extremely difficult to renew a population (Pikitch et al., 2005);
- Dam constructions impede sturgeon migrations (Birstein et al., 1997b; Pikitch et al., 2005); being diadromous, sturgeons are unable to reach their spawning grounds and lose the natural signals for spawning migrations (Speer et al., 2000; Billard and Lecointre, 2001);
- Pollution from urban and agricultural runoff and industrial discharge affect their reproduction and physiology. Mineral oil, diesel fuel, hexachlorocyclohexane, and raw

sewage inhibit spawning, and cause muscle atrophy and death of pre-larvae (Dettlaff and Vassetzky, 1991; Birstein et al., 1997b; Speer et al., 2000; Pikitch et al., 2005).

It has therefore become important and urgent to save the surviving members of this important but critically endangered family. It is necessary to develop strategies for their conservation in natural habitats and culture them in fish farms (ex situ conservation), not only for market production, but also for renewing populations in the wild (Birstein et al., 1997b; Billard and Lecointre, 2001; Pikitch et al., 2005; Carmona et al., 2009). In this regard, it is necessary to study fertilization aspects in sturgeon and to understand the reasons for embryonic abnormalities.

1.2. STURGEON GAMETES AND EMBRYO DEVELOPMENT

Sturgeon gametes differ from those of other fishes. The eggs are large (Debus et al., 2002) and encased in four envelopes (Cherr and Clark, 1982; Psenicka et al., 2010). The envelopes serve many purposes: fixing the egg to the substratum, attracting spermatozoa, preventing polyspermy (the penetration of the additional spermatozoa), and mechanically protecting the embryo (Cherr and Clark, 1982; Zelazowska, 2010). Sturgeon eggs contain multiple micropyles (canals through the egg envelopes that allow the penetration of spermatozoa into the egg), while holostean and teleostean eggs have only a single micropyle (Ginsburg, 1972; Cherr and Clark, 1982; Zelazowska, 2010) (Figure 1). The number of micropyles in sturgeons varies among species and among the eggs of individual females (Dettlaff et al., 1993). According to Ginsburg (1972), there is a wide range of the number of micropyles within *Acipenser stellatus, Huso huso* and *A. gueldenstadtii*. On average, *A. stellatus* eggs have fewer than five micropyles while those of *H. huso* and *A. gueldenstadtii* have 6-10 each. However, the number varies, with as many as 52 micropyles having been reported from individual eggs (Table 2).

Table 2. *Number of micropyles in sturgeons (Ginsburg, 1972).*

Species	Number of female from which eggs were taken	Number of eggs studied	Number of micropyles in egg			Distribution of eggs of different females, according to average number of micropyles						
			minimum	maximum	average	less than 5	5–10	10 -15	15–20	20 – 25	25–30	30–35
Acipencer stellatus	10	398	1	13	4.8	6	4					
Huso huso	8	140	1	17 (33*)	6.6	2	4	2				
Acipenser gueldenstadtii	24	972	1	43(52*)	9.7	3	15	2	2		1	1

* This number was found in only one egg

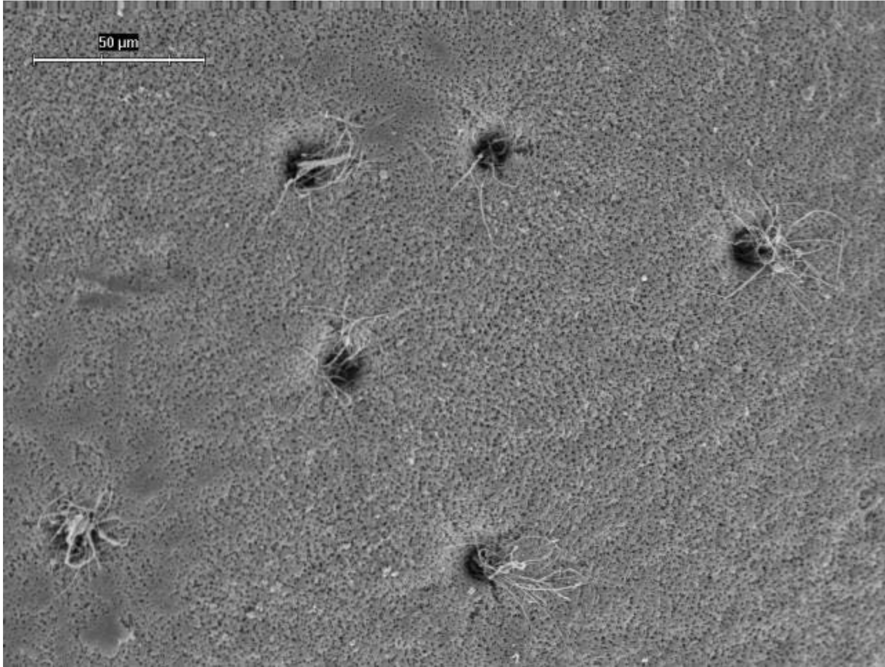

Figure 1. *Acipenser ruthenus micropyles (Psenicka et al., 2011), scale bar: 50 µm.*

Sturgeon males release enormous amounts of sperm into the water, measuring hundreds of cubic centimeters (up to 1000 cm^3 in *Huso dauricus*), where 1 cm^3 contains up to 10 billion spermatozoa. Sturgeon spermatozoa can remain motile for long periods after being released into the water: from several minutes to several hours (Dettlaff et al., 1981). Sturgeon sperm have an acrosomal reaction, as do the sperm of echinoderms, mollusks, enteropneusts and amphibians. Acrosomal reaction is a mechanism that allows the passage of the sperm through the egg's cell membrane, without the need for micropyles, to reach the cytoplasm. However, the cell membrane of sturgeon eggs is impermeable, and the sperm can penetrate the egg only through micropyles (Ginsburg, 1972). Therefore, the role or significance of the acrosomal reaction in sturgeons is not clear (Ginsburg, 1972; Psenicka et al., 2010). For example, teleost fishes have micropyllar channels and lost the acrosomal reaction during evolution, while amphibian eggs have no micropyles and yet retain this mechanism for fertilization (Ginsburg, 1972).

Sturgeon embryonic development is similar to that in amphibians, displaying holoblastic cleavage in the animal pole (Dettlaff and Vassetzky, 1991; Bolker, 1994; Park et al., 2013). However, the vegetal pole is also holoblastic, but with an uneven and asymmetric pattern (Colombo et al., 2007).

Embryonic development in sturgeons can be divided into several stages: 1) fertilization, 2) blastulation, 3) gastrulation, 4) neurulation 5) heart beating, and 6) hatching (Dettlaff and Vassetsky, 1991; Park et al., 2013). First cleavage is indicated by a limited furrow at the animal pole. Following this cleavage, the embryo is divided into four cells. The embryo then proceeds through further divisions (8-cell, 16-cell, etc.) until blastulation (Park et al., 2013) (Figure 2).

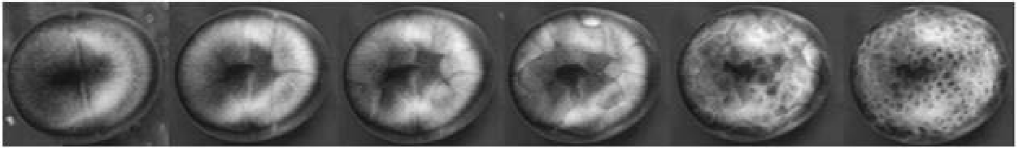

Figure 2. *Developmental pattern of a normally dividing A. ruthenus embryo, from 2-cell to 256-cell stage (Iegorova et al., 2018).*

However, embryos are sometimes known to show atypical division, with three and six cells during the 1st and 2nd divisions. These embryos develop further with higher number of blastomeres than in normally developed embryos. According to Dettlaff et al. (1981) atypically divided embryos die before hatching, or hatching can sometimes occur accompanied by malformations before death. Dettlaff et al. (1981) concluded that the reason for atypical cleavage is polyspermy, as several spermatozoa can penetrate through the multiple micropyles. It was found that temperature could also influence development and damage the cytoplasm, resulting in malformed embryos, or mosaic division where development stops in part of the embryo, leading to death.

The appearance of atypically divided embryos needs to be studied in detail, including the reason for the formation of extra blastomeres, their fate, survival rate, and their possible effect on the broodstock.

1.3. FERTILIZATION ASPECTS: ARE MONOSPERMY, PHYSIOLOGICAL POLYSPERMY OR PATHOLOGICAL POLYSPERMY CHARACTERISTIC OF STURGEONS?

Fertilization — the fusion of spermatozoa and egg nuclei to produce a diploid zygote — has long been of interest to embryologists (Ginsburg, 1972; Monroy, 2017). Successful fertilization is necessary for embryogenesis in animals and plants (Iwao, 2012). Numerous spermatozoa compete for the egg and surround it, but only one participates in karyogamy (fusion of male and female pronuclei), and other spermatozoa are excluded from the development (physiological monospermy). Some barriers like jelly layers stop the penetration of the multiple spermatozoa. However, multiple sperm do manage to reach the eggs. When that happens, the egg itself, as the last line of defence, blocks polyspermy immediately upon the successful passage of one sperm through the oocyte membrane (Iwao, 2012; Monroy, 2017). This is characteristic of urodeles (Hynobius), anurans (frogs) and most fishes, including primitive fishes (Iwao, 2014). However, under some circumstances, such as highly concentrated sperm suspensions or bad condition of the eggs, numerous spermatozoa may penetrate into the cytoplasm, thus provoking a syngamy with multiple sperm nuclei. This can lead to aneuploidy, abnormalities in development, malformations, and embryo death (pathological polyspermy) (Boveri, 1901; Ginsburg, 1972; Hunter, 1998; Wang et al., 2003; Snook et al., 2011; Iwao, 2012). Nevertheless, some animals (ctenophora, elasmobranchs, newts, salamanders, reptiles and birds) appear to 'tolerate' polyspermy without any pathological effects (physiological polyspermy); one male pronucleus participates in karyogamy and forms a synkaryon, while the other spermatozoa undergo degeneration and are excluded from the nuclei fusion, thus ensuring embryonic development with diploid configuration (Ginsburg, 1972; Snook et al., 2011; Iwao, 2012).

It is believed that physiological polyspermy evolved in association with large sized eggs in urodeles, reptiles and birds (Iwao, 2014). Levitan (1993, 1998, 2002) noted that the small *Strongylocentrotus purpuratus* eggs reduce the chance of sperm penetration, while the larger *S. droebachiensis* eggs are easier to fertilize, increasing the susceptibility to polyspermy.

The sperm head has a slightly bigger diameter than the micropylar channel, and as a result, only one sperm can penetrate through a single micropyle. Each channel is double-tapering, limiting the sperm's access to the oolemma or perivitelline space (Cherr and Clark, 1982). After fusion, the perivitelline space, filled with the material from cortical granules, becomes enlarged and other spermatozoa are not able to pass through the envelope (Psenicka et al., 2010). However, it is still not clear which type of fertilization is characteristic of sturgeons, as their eggs are built to allow monospermic and polyspermic fertilization.

There are several opinions regarding the manner of fertilization in sturgeons. Based on different insemination conditions, cytological investigations, and observations of embryo development, Ginsburg, (1972) concluded that physiological polyspermy is characteristic of sturgeons whereas, highly diluted sperm suspensions (as in natural conditions) produce monospermic embryos that cleaved into 2, 4, 8, 16, etc. blastomeres during division. Moreover, the creation of a cytoplasmic projection in the sturgeon eggs after fusion with a sperm, blocks entry for any additional sperm into other micropyles (Psenicka et al., 2010). Ginsburg (1972) also described pathological fertilization, where after numerous spermatozoa pass into the cytoplasm, embryos developed atypically with 3 or 6 blastomeres and die before hatching, due to malformations. Numerous spermatozoa were histologically observed in sturgeon eggs after insemination with highly concentrated (undiluted) sperm, and polyspermy was found to be likely due to the presence of multiple micropyles in the sturgeon eggs. Atypically dividing cells, such as those with 3 cells at the 2-cell stage, were frequently found in the embryos (up to 20%) after artificial insemination in the hatcheries (Dettlaff et al., 1981).

However, Cherr and Clark (1985) noted that the presence of multiple micropyles does not increase the chances of polyspermy. They fertilized *A. transmontanus* eggs with highly concentrated sperm suspension, and found only a small number of polyspermic embryos. Interestingly, they observed many more polyspermic embryos under conditions of normal fertilization.

In Chapter 2 we describe the types of fertilization in sturgeon, the frequency of atypically divided cells and the reason for their occurrence.

1.4. PLOIDY LEVEL IN STURGEONS

Acipenseriformes are well known for multiple levels of ploidy, making sturgeons a good model for molecular evolution studies in fishes (Ludwig et al., 2001). However, the karyotype of sturgeon species has still to be studied well (Vasil'ev et al., 2010). Techniques used for detection of chromosomes and microchromosomes (which were found later) still raise questions about the exact number of chromosomes (Ludwig et al., 2001; Fontana, 2002).

Despite several studies, there is no consensus even on the number of chromosomes in each sturgeon species, which depends on the method chosen for studying the ploidy level (Ludwig et al., 2001). Pioneering work using sturgeon karyotyping was done in the early 1960s by Serebryakova (1972) and Burtzev et al. (1973). They described 60 chromosomes in *A. ruthenus, H. huso* and *H. dauricus*, and considered them to be diploid. However, microchromosomes had not yet been identified at that time. Based on the hypothesis that sturgeon species containing 60 chromosomes are diploids, Ohno et al. (1969) described *S. platorhynchus* with 112 chromosomes (nearly a half of them were microchromosomes) and assigned a tetraploid status for this species.

The amount of nuclear DNA was used as a means of understanding the ploidy relationships among the groups (Fontana, 2002). Thus, compared to teleost fishes, the amount of nuclear DNA in *S. platorhynchus* is two times as high: 3.2 picograms per nucleus (pg/N) vs 1.7 pg/N in teleosts (Ohno et al., 1969), and it was therefore considered a tetraploid species.

Birstein et al. (1993) found that the Sakhalin sturgeon (*A. mikadoi*) has a very large genome size (13.93–14.73 pg/N), and considered it to be a 16-ploid species with 500 chromosomes, as the amount of DNA was two times larger than in the octoploid species, *A. gueldenstaedtii* and *A. baerii* (7.87–8.30 pg/N). Ludwig et al. (2001) concluded that *A. mikadoi* is octoploid based on microsatellite data obtained in his study and the number of chromosomes proposed by Birstein et al. (1993). Vasil'ev et al. (2009) clearly demonstrated that *A. mikadoi* is a tetraploid (268 ± 4 chromosomes) using karyotypic analysis. Zhou et al. (2013) used nuclear DNA contents, karyotyping and fluorescence *in situ* hybridization (FISH) with 5.8S + 28S rDNA as a probe for ploidy determination. They determined that the DNA content was 8.2–9.1 pg/N and the number of chromosomes was 268. The rDNA FISH detected 18 signals, and *A. mikadoi* was determined to be functional tetraploid.

Birstein et al. (1993) argued that it is more probable that *A. brevirostrum* is a 16-ploid fish with 500 chromosomes. However, microsatellite analysis by Ludwig et al. (2001) revealed 362–370 chromosomes. *A. brevirostrum* was considered a tetra- or octoploid species since it had loci with disomic, tetrasomic, and octosomic heritability. Kim et al. (2004) concluded that *A. brevirostrum* should be hexaploid based on the karyotype (354–372 chromosomes) and a large genome size (ca 13 pg/N).

Fontana et al. (1994) studied the number and chromosomal localization of the nucleolar organizing regions (NORs). NORs in *A. ruthenus* are localized on the telomeric regions of two morphologically different pairs of chromosomes, and the species contain 118 ± 4 chromosomes, suggesting that *A. ruthenus* is diploid. In *A. baerii* (250 ± 8), *A. transmontanus* (248 ± 8) and *A. naccarii* (246 ± 8) NORs are localized on eight chromosomes settled in two quadruplets, and Fontana et al. (1994) considered them tetraploid with 240–250 chromosomes.

Table 3. *Chromosome numbers and ploidy in different Acipenseriform species (modified after Vasil'ev et al., 2010).*

Species	Chromosome number	Ploidy level	
		"evolutionary scale"	"recent scale"
Scaphirhynchus platorynchus	~112	4	2
Acipenser sturio	116 ± 4	4	2
A. nudiventris	118 ± 2	4	2
A. ruthenus	118 ± 2	4	2
	118 ± 4	4	2
A. stellatus	118 ± 2	4	2
A. oxyrinchus	121 ± 3	4	2
H. huso	116 ± 4	4	2
A. gueldenstaedtii	250 ± 8	8	2
A. persicus	~258	8	4
A. baerii	249 ± 5	8	4
A. nacarii	239 ± 7	8	4
A. brevirostrum	~372	12	6
	372 ± 6	12	6
A. transmontanus	248 ± 8	8	4
	~271	8	4
A. sinensis	264 ± 4	8	4
A. fulvescens	262 ± 6	8	4
A. mikadoi	262 ± 4	8	4
A. medirostris	249 ± 8	8	4
A. schrenckii	238 ± 8	8	4
	266 ± 4	8	4
A. dauricus	268 ± 4	8	4

Microsatellite analysis indicated that the 120-chromosome species are functional diploids based on the loci studied (*A. sturio, A. stellatus, A. ruthenus, A. oxyrinchus, H. huso, H. dauricus, S. albus,* and *S. platorynchus*). Species with about 250 chromosomes were classified as functional tetraploids: *A. baerii, A. gueldenstaedtii, A. naccarii, A. persicus* and *A. transmontanus* (Ludwig et al., 2001).

In summary, all Acipenseriformes are divided into three groups depending on the number of chromosomes: 1) 120 chromosomes species (*H. huso, A. stellatus, A. sturio, A. nudiventris,* and *A. ruthenus*); 2) 240 – 270 chromosomes species (*A. gueldenstaedtii, A. persicus, A. baerii, A. naccarii, A. transmontanus, A. sinensis, A. schrenckii, A. fulvescens,* and *A. medirostris*); 3) species with approximately 370 chromosomes (*A. brevirostrum*) (Vasil'ev et al., 2009). There is evidence that 120-chromosome species have a tetraploid origin, and that their diploid ancestor is already extinct (Vasil'ev et al., 2009). Based on these data, two ploidy scales were proposed for Acipenseriformes: 1) the "evolutionary scale": diploid species (extinct), tetraploid (with 120 chromosomes), octaploid (with 250), and 12-ploid (370 chromosomes) species and 2) the "recent scale": diploid (120-chromosomes), tetraploid (250-chromosomes), and hexaploid (370-chromosomes) species (Vasil'ev et al., 2009, 2010) (Table 3).

1.5. PLOIDY MANIPULATION

Chromosome manipulation (production of polyploid fish) plays an important role in aquaculture and improves economically important pisciculture characteristics; polyploid organisms are known for rapid growth and disease resistance. Several naturally polyploid species (carp, gibel carp, crucian carp, salmon, and sturgeon) have been already chosen as important species for aquaculture (Zhou and Gui, 2017).

Different methods have been established for "chromosomal engineering":

- artificial tetraploidization;
- artificial triploidization;
- hormonal sex-reverse;
- artificial androgenesis;
- artificial gynogenesis.

1.5.1. Artificial tetraploidization

Production of tetraploid lines is important in aquaculture for several reasons: 1) It is believed that genome doubling (tetraploidization) helps to improve the growth rate, and fishes with higher levels of ploidy are bigger in size (Dunham, 2011). Tetraploidy can be achieved by inhibiting the first mitotic division, thus producing one tetraploid cell instead of two diploid cells. 2) Tetraploid organisms can be crossed with diploid individuals for mass production of sterile triploids (Makhrov, 2015). 3) Tetraploid fish are needed for production of other kinds of polyploids, such as triploids, tetraploids, pentaploids and hexaploids. For example, when induced tetraploid rainbow trout females were inseminated by UV-irradiated sperm from diploid rainbow trout males, most of the progeny were diploid, with a few gynogenetic tetraploids also obtained. However, fertilization of these eggs with a normal sperm produced triploids and pentaploids; fertilization by diploid normal sperm produced tetraploids and hexaploids (Chourrout et al., 1986; Chourrout and Nakayama, 1987). Yoshikawa et al. (2008) listed the ways by which polyploid loaches (*Misgurnus anguillicaudatus*) can be obtained using gametes from tetraploid fishes: triploid fish can be obtained after fertilization of diploid and haploid gametes; tetraploids can be produced after crossing diploid gametes; a different type of tetraploidy can be obtained after inhibiting the second meiosis after fertilization of haploid eggs with a haploid polar body and diploid sperm; and hexaploids can be produced by inhibition of the second meiosis of fertilized diploid eggs with a diploid polar body and a diploid spermatozoa.

1.5.2. Artificial triploidization

Induced triploidy is the most effective method of producing sterile fish for aquaculture (Benfey, 2010). Triploids can be obtained not only by fertilization of diploid and haploid gametes but also using cold/hot/pressure shock for blocking the 2nd polar body release just after fertilization with normal spermatozoa (Arai, 2001).

Interest in triploid fish production exists for several reasons: they are used for mass production of fish, for sport fishing (it is easy to control the number of fish produced), and for chimera production. Triploid fish generally exhibit sterility in males and females. However, males sometimes exhibit some gonadal development. Sterility can reduce energetic costs of reproduction and direct more of the body's resources towards growth. However, it has been reported that the growth of triploids until maturation is similar to that of diploids, if not

slower (Arai, 2001; Makhrov, 2015). After maturation, however, triploids demonstrate better growth and survival, and the meat quality is better than in diploids (Arai, 2001; Makhrov, 2015).

Interspecific triploid hybrids grow faster than diploids, accommodate better to changing water salinity, and can be used as a base for chimera production or restoring extinct species (Makhrov, 2015). However, production of interspecific triploids seems to induce considerable abnormality and mosaicism (Zou et al. 2004).

Although diploids and triploids are similar in morphology, triploids often demonstrate skeletal and anatomical malformations (Maxime, 2008). These malformations likely happen after pressure or heat shock is applied to the embryos (Makhrov, 2015). On the other hand, triploids have been seen to be less prone to cancer than diploids (Thorgaard et al. 1999).

1.5.3. Hormonal sex-reverse

Female progeny are generally preferred by fish farmers for caviar production (especially sex-reversed triploid females) (Makhrov, 2015). In trout farming, for example, there is no practical need in a less than a half of males for artificial insemination of females, and farmers try to have more females and to control the sex in trout (Okada et al., 1979). Therefore, a method of producing females by hormonal treatment was developed, in which fish are given micro doses of hormones in water or in food during early developmental stages. However, this method is best avoided if the fish are consumed for their meat, because of the direct exposure to hormones (Makhrov, 2015). The, sex-reversal method using hormones can be useful for another purpose: to obtain gametes for triploid clonal female production. Thus, females are converted into males using 17-alpha methyltestosterone. Upon maturing, these males produce diploid sperm, which are then fertilized with normal haploid eggs to produce triploid female progeny (Arai, 2001; Yosikawa et al., 2008).

1.5.4. Artificial androgenesis and gynogenesis

Androgenesis is a strategy for restoring critically endangered or extinct species. Induced androgenesis has several uses: production of inbred strains and clones, sex regulation, genetic mapping, and analysis of nucleo-cytoplasmic interaction (Grudinina et al., 2006; Makhrov, 2015). This technique can also be used to produce androgenic fish, with all-paternal inheritance. Viable androgenetic diploids can be generated by inhibiting the first mitotic division, to double the number of paternally derived chromosomes, after fertilization of genetically inactivated eggs by UV rays or radioactive irradiation of normal spermatozoa (Chourrout and Nakayama, 1987; Makhrov, 2015).

Gynogenesis is a form of all-female inheritance. The main intention of induced gynogenesis is the production of genetically identical populations (clonal lines). Clonal lines can be important for the improvement of fish stocks, and can be obtained by using denucleated sperm to activate the egg. Thus, there is no sharing of genetic material from the male, while diploidization of the maternal chromosomes is achieved by inhibiting either the second polar body extrusion or the first cleavage (Arai, 2001; Makhrov, 2015).

1.5.5. Chromosomal mosaicism

The methods described above can also result in mosaicism in the embryos (Mada et al., 2001; Tanaka et al., 2003; Chourrout et al., 1986). A mosaic is an individual with two or more distinct genotypes despite developing from a single fertilized egg or zygote (King and

Stansfield, 1990). Haplo-diploid, diplo-triploid mosaics have several kinds of cell populations with different ploidy levels within an individual. Although mosaicism is not very common among fish and other aquatic animals, flow cytometry techniques can help distinguish mosaics from normal individuals (Arai, 2001).

Yoshikawa et al. (2007) described diploid-triploid mosaicism in loach (*Misgurnus anguillicaudatus*) females, which laid haploid, diploid and triploid eggs in one spawning. Microsatellite genotyping of diploid-triploid mosaics has shown that triploid genotypes contain two alleles specific to the clonal diploid and one allele from the normal diploid male; diploid eggs from mosaic females had a genotype identical to that of the diploid male, and one of the three alleles of the mosaic female was transmitted to haploid eggs. Yamaki et al. (1999) applied pressure shock on inseminated amago salmon eggs and obtained diploid fish, which produced haploid and diploid eggs.

On the other hand, Morishima et al. (2004) reported that diploid-triploid loach (*Misgurnus anguillicaudatus*) males produced clonal unreduced diploid spermatozoa. When a normal diploid female was crossed with a mosaic diploid-triploid male, only triploid progeny resulted, which exhibited microsatellite genotypes with two alleles identical to the clonal diploid genotypes, and one allele derived from the female. After the eggs underwent UV irradiation and were fertilized with diploid sperm from the mosaic male, androgenetic diploids were produced that exhibited microsatellite genotypes and DNA fingerprints identical to those of natural clone. With these results, Morishima et al. (2004) concluded that diploid-triploid mosaic males generate clonal diploid spermatozoa with genetically identical genotypes. However, diploid-tetraploid males obtained by Yamaki et al. (1999) by inhibiting the first mitotic cleavage produced haploid sperm.

Induction of chromosomal mosaics can be a useful tool for the production of isogenic strains in sturgeons.

1.6. HYBRIDIZATION IN STURGEONS

Acipenseriformes is the only group among vertebrates that is characterized by a high ability for hybridization (Birstein et al., 1997c). Sturgeons can hybridize between species with the same and/or different ploidy level (Billard and Lecointre, 2001), and demonstrate interspecific and inter-generic hybridization in the wild and under artificial conditions (Havelka et al., 2011).

Sturgeon hybrids can be sterile or fertile. Usually, hybrids produced from parents with the same ploidy level are fertile (2n x 2n or 4n x 4n), which is unusual for vertebrate hybrids, while crosses between species of different ploidy (2n x 4n) produce triploids and they are commonly sterile (Williot et al., 1993; Billard and Lecointre, 2001).

Table 4. *Natural hybridization of sturgeon species and their ploidy levels (Birstein et al., 1997c).*

Interspecies hybrids	Intergenera hybrids
1. Caspian Sea basin[1]	
(a) Volga River	
A. ruthenus (4n) × *A. stellatus* (4n)	*H. huso* (4n) × *A. gueldenstaedtii* (8n)
A. stellatus (4n) × *A. ruthenus* (4n)	*H. huso* (4n) × *A. ruthenus* (4n)
A. nudiventris (4n) × *A. gueldenstaedtii* (8n)	
A. gueldenstaedtii (8n) × *A. ruthenus* (4n)[2]	
A. gueldenstaedtii (8n) × *A. stellatus* (4n)	
A. gueldenstaedtii (8n) × *A. persicus* (8n)[3]	
(b) Kama River	*Huso huso* (4n) × *A. nudiventris* (4n)
	H. huso (4n) × *A. gueldenstaedtii* (8n)
	H. huso (4n) × *A. stellatus* (4n)
	A. ruthenus (4n) × *H. huso* (4n)
(c) Ural River	
A. nudiventris (4n) × *A. stellatus* (4n)	*H. huso* (4n) × *A. stellatus* (4n)
A. stellatus (4n) × *A. nudiventris* (4n)	
(d) Kura River	
A. nudiventris (4n) × *A. stellatus* (4n)	*H. huso* (4n) × *A. nudiventris* (4n)
A. stellatus (4n) × *A. nudiventris* (4n)	
A. nudiventris (4n) × *A. gueldenstaedtii* (8n)	
(e) Sefir-Rud River	
A. nudiventris (4n) × *A. stellatus* (4n)	
A. nudiventris (4n) × *A. gueldenstaedtii* (8n)	
(f) Caspian Sea	*H. huso* (4n) × *A. persicus* (8n)
2. Sea of Azov basin[4]	
Don River	
A. ruthenus (4n) × *A. stellatus* (4n)	
3. Black Sea basin[5]	
(a) Danube	
A. ruthenus (4n) × *A. stellatus* (4n)	*A. gueldenstaedtii* (8n) *H. huso* (4n)
A. ruthenus (4n) × *A. nudiventris* (4n)	*A. stellatus* (4n) × *H. huso* (4n)
A. stellatus (4n) × *A. ruthenus* (4n)	*A. nudiventris* (4n) × *H. huso* (4n)
A. ruthenus (4n) × *A. gueldenstaedtii* (8n)	*H. huso* (4n) × *A. stellatus* (4n)
A. stellatus (4n) × *A. gueldenstaedtii* (8n)	
A. nudiventris (4n) × *A. gueldenstaedtii* (8n)	
A. sturio (4n) × *A. gueldenstaedtii* (8n)	
(b) Black Sea	
A. gueldenstaedtii (8n) × *A. sturio* (4n)	
A. gueldenstaedtii (8n) × *A. nudiventris* (4n)	
4. Siberian rivers	
Main rivers (Yenisey, Lena, Ob, Kolyma)[6]	Amur River[7]
A. baerii (8n) × *A. ruthenus* (4n)	*Huso dauricus* (4n) × *A. schrenckii* (8n?)
5. Central Asia[8]	
Amu-Darya River	
Pseudoscaphirhyncus kaufmanni (4n) × *P. hermanni*	
6. North America[9]	
Missouri and Mississippi Rivers	
Scaphirhynchus albus (?) × *S. platorynchus* (4n)	

The most well-known hybrid in the Acipenseriformes family is bester, which is an intergeneric hybrid between sterlet (*A. ruthenus*) and beluga (*H. huso*). Bester grows rapidly and reaches a large size, which is characteristic of beluga, and they have freshwater tolerance, which is a trait of sterlet (Burtzev, 1972; Carmona et al., 2009).

Many other hybridizations between various Acipenserid species have been created: bester × *H. huso*, bester × *A. ruthenus*, *A. ruthenus* × *A. nudiventris*, *H. huso* × *A. nudiventris*, *A. gueldenstaedtii* × *H. huso*, *A. gueldenstaedtii* × *A. ruthenus*, *H. huso* × *A. stellatus* and *A. stellatus* × *A. medirostris* (Nicolyukin, 1966, Krylova, 1999).

Aquaculturists have producing sturgeon hybrids generally with the intention of improving upon the characteristics of the pure species (Bronzi et al., 1999). This has led to rapid production of hybrids in sturgeon aquaculture (Krylova, 1999). Table 4 demontrates the large variability in cross-fertilization between sturgeon species and genera.

Sturgeon hybridization also frequently occurs in nature (for example, *H. dauricus* x *A. schrenckii* in the Amur river) (Krykhtin and Svirskii, 1997). This can be due to several reasons: conservation programs (Becker et. al., 2007), environmental changings (Freyhof et al., 2005), escape from hatcheries, and accumulation of several sturgeon species under dams and their spawning at the same time.

The deliberate production of hybrids became controversial due to the real risk of their escaping into open waters and the possibility of subsequent genetic contamination of wild sturgeon populations (Billard and Lecointre, 2001). Furthermore, the rapid growth of many hybrids can help replace native species and contribute to their extinction (Ludwig et al., 2009). Identification of sturgeon hybrids is very complicated, making it almost impossible to identify them (Ludwig, 2008). Suitable genetic markers appear to be the only way to distinguish the hybrids from pure sturgeon species (Fontana, 2002). In Chapter 3 we demonstrate a huge possibilities for sturgeon in hybridization and their great plasticity.

1.7. AIMS OF THE THESIS

The Acipenseridae family still needs to be well studied, especially with respect to fertilization, hybridization, and chromosome manipulation techniques.

The main aims of this study are as follows:
1) To determine the type of fertilization in sturgeons (monospermy/polyspermy).
2) To understand the reasons for the atypical division (AD) of cells during artificial breeding.
3) To investigate if AD embryos are able to develop beyond the feeding stage, and to analyze their ploidy and reasons for the abnormalities.
4) To classify the types of AD embryos and estimate their respective frequencies.
5) To investigate sturgeon hybridization plasticity, and produce the first interspecific hybrid from three parents.

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CHAPTER 2

POLYSPERMY PRODUCES VIABLE HAPLOID/DIPLOID MOSAICS IN STURGEON

Iegorova, V., Psenicka, M., Lebeda, I., Rodina, M., Saito, T., 2018. Polyspermy produces viable mosaics in sturgeon. Biology of Reproduction 99, 695–706.

https://academic.oup.com/biolreprod/article/99/4/695/4985831

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My share on this work was about 60%.

Biology of Reproduction, 2018, 99(4), 695–706
doi:10.1093/biolre/ioy092
Research Article
Advance Access Publication Date: 25 April 2018

OXFORD

Research Article

Polyspermy produces viable haploid/diploid mosaics in sturgeon†

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†**Grant Support:** This study was supported by the Ministry of Education, Youth and Sports of the Czech Republic, projects CENAKVA (CZ.1.05/2.1.00/01.0024), "CENAKVA II" (LO1205 under the NPU I program), the Grant Agency of the University of South Bohemia in Ceske Budejovice (125/2016/Z), and by the Czech Science Foundation (17-19714Y).
Edited by Dr. Myriam Hemberger, PhD, Babraham Institute

Received 28 June 2017; Revised 31 December 2017; Accepted 24 April 2018

Abstract

Most of sturgeon species (Acipenseridae) are currently critically endangered. Attempts to revive these populations include artificial breeding in hatcheries. However, under artificial reproduction, sturgeon embryos occasionally develop atypically, showing 3, 5, 6, 7, 9, or 10 cells at the 2- to 4-cell stage. This study was undertaken with the objective of understanding the mechanism of the atypical division (AD) in embryos during artificial breeding. Using several sturgeon species, we tested two hypotheses: (1) polyspermy and (2) retention of the second polar body. We found that (1) AD embryos survive similar to controls, (2) the ratio of AD embryos is positively correlated with the amount of sperm used for fertilization, (3) the number of micropyles and the area covered by them in AD embryos is significantly greater when compared to controls, (4) numerous spermatozoa nuclei are in the cytoplasm after fertilization, (5) all AD embryos are mosaics, and (6) AD fishes with n/2n ploidy contain diploid cells from maternal and paternal genetic markers, while the haploid cells contained only paternal ones. These results clearly indicate that AD embryos arise from plasmogamy where the accessory spermatozoon/spermatozoa entry the egg and develop jointly with zygotic cells. This suggests that a well-controlled fertilization procedure is needed to prevent the production of sturgeon with irregular ploidy, which can have detrimental genetic effects on sturgeon populations. On the other hand, if AD fish can produce haploid-derived clonal gametes, induction of multiple-sperm mosaicism might be a useful tool for the rapid production of isogenic strains of sturgeons.

Summary Sentence

We believe that findings of this study can help to avoid negative effect on sturgeon propagation programs and might be useful for breeding and genomic research.

Key words: sturgeon, polyspermy, mosaicism, haploidy.

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Introduction

Sturgeons (Family Acipenseridae, Order Acipenseriformes) are among the earliest freshwater fishes, having evolved around 200 million years ago [1–2]. Nowadays, mainly due to human activities, including overexploitation for the harvesting of caviar, habitat degradation, damming of rivers, and poaching, having brought the entire Acipenseriformes order to the brink of extinction [3–4]. Twenty-seven species of sturgeon are on the IUCN Red List, the majority of them considered "critically endangered," the highest category of threat in the Red List [5]. Therefore, artificial reproduction under controlled conditions has become an important strategy to renew valuable sturgeon stocks at least as a temporary measure until they can be restored, and concomitant favorable changes occur in their environment [6–7]. Furthermore, in order to produce healthy sturgeon seed for release into natural waters in the future, it is necessary to cull out genetically deficient fish that could disrupt the gene constitution of wild populations.

In normal development, sturgeon eggs divide into multiples of two cells until the blastula stage. However, atypically dividing (AD) cells, such as those with 3 cells at the 2-cell stage, are frequently found in up to 20% of the embryos that are artificially inseminated in hatcheries [8]. According to Ginsburg [9–10], AD embryos do not survive and most die before hatching due to malformations. It was speculated that atypical cleavage patterns were caused by polyspermy, since a numerous spermatozoa are histologically observed in sturgeon eggs after insemination using highly concentrated (undiluted) sperm and polyspermy was likely to occur due to the presence of multiple micropyles in the sturgeon egg, which varies among individuals and species [11–13].

It is known that unusual culture conditions, such as thermal or pressure shock to the developing eggs, can inhibit the second polar body extrusion in fish [14–15]. Therefore, although circumstantial evidences (such as considerable number of spermatozoa in AD sturgeon eggs and multiple number of micropyles) suggest polyspermy as a source of AD embryos appearance, it is difficult to exclude the possibility that the extra blastomeres in the AD embryos were produced by the retention of the second polar body.

Polyspermy tends to induce multipolar mitosis due to the presence of supernumerary centrioles, resulting in inviable aneuploid cells as the chromosomes segregate randomly among the multiple spindles [16]. Perhaps due to the technical limitations at that time, Ginsburg [9–10] made no mention about the genotype of the extra blastomeres in AD embryos, or the viability of AD embryos has not been thoroughly investigated. In general, there is little information about genetics of this anomaly and the polyspermy hypothesis has remained an untested speculation. However, if the embryos are viable and aneuploidy does not occur for some reason, it becomes impossible to determine whether AD embryos occur as a result of polyspermy or the retention of the second polar body in pure *A. ruthenus*, because AD embryos are expected to be 3n or n/2n mosaic in either case. To overcome this problem, the unique karyotype of the order Acipenseriformes can be brought to aid. Depending on the level of ploidy and the number of chromosomes, sturgeons can be divided into three groups: functional diploids (2n) with approximately 120 chromosomes (e.g., *Huso huso, Acipenser ruthenus, A. sturio,* and *Polydon spathula*), functional tetraploids (4n) with around 240 chromosomes (*A. gueldenstaedtii, A. naccarii, A. baerii*), and functional hexaploids (6n), with ~360 chromosomes (*Acipenser brevirostrum*) [17–23]. In this study, hybrids between *A. ruthenus* (2n) and *A. gueldenstaedtii* (4n) were used to elucidate the origin of the extra blastomere(s) in AD embryos. All normally developed hybrid embryos (*A. ruthenus* (2n) × *A. gueldenstaedtii* (4n)) are triploids (3n). If the second polar body is retained, the ploidy of the hybrid AD progeny is either n/3n mosaic or 4n, whereas hybrid embryos resulting from polyspermy are either 2n/3n mosaic or 5n. Also, hybrids, between *A. ruthenus* (2n) and *H. huso* (2n) were used for DNA marker-based species identification analysis after sorting AD cells to study the origin of the extra blastomere(s).

In any case, if even a small number of AD fish survive, they can disrupt the gene constitution of a population, as they may produce gametes derived from the blastomeres with abnormal ploidies. To address this possibility, it is necessary to determine the mechanism underlying the occurrence of AD embryos in sturgeon.

In this study, we first investigated if AD embryos are able to develop beyond the feeding stage. Next, we performed fertilization experiments using *A. ruthenus* eggs with different amount of sperm, in order to reproduce the results obtained by Ginsburg [10]: the number of AD embryos increased as the sperm concentration used for insemination was increased. In the course of the experiments, we classified the types of AD embryos and their frequency at the 4-cell stage, and studied the effect of micropyle topology on the occurrence of AD. We performed histological and cytological observations to find a numerous spermatozoa in the micropyles and the cytoplasm. We sorted n/2n mosaic cells and distinguished the origin of the haploid cells.

Materials and methods

Ethics

All experiments were completed in accordance with national (reference number: 2293/2015-MZE-17214) and institutional guidelines on animal experimentation and care and were approved by the Animal Research Committee of the Faculty of Fisheries and Protection of Waters in Vodnany, Czech Republic.

Gamete collection

Acipenser ruthenus males and females (0.9–2.1 kg, 3–5 years old), *A. baerii* males and females (5.4–8.4 kg, 13 years old), *A. gueldenstaedtii* male (11.5 kg, 22 years old), and *H. huso* male (39 kg, 22 years old) were kept outdoors in 4 m^3 tanks with water temperature 8–12°C in the hatchery of the Research Institute of Fish Culture and Hydrobiology in Vodnany, Czech Republic. Before spawning, fish were moved to a closed recirculation system with 15°C water temperature and hold 3–4 days without feeding. Males were given a single intramuscular injection of acetone-dried homogenized carp pituitary extract (CPE) at 4 mg/kg body weight (BW) to induce spermiation. At 42 h postinjection, sperm was collected from the urogenital papilla using a catheter, transferred to a 250 ml cell-culture container, and stored at 4°C until use. Ovulation in the females was stimulated by means of intramuscular injection of CPE in two doses, the first given 36 h before stripping (0.5 mg/kg BW) and the second 24 h before stripping (4.5 mg/kg BW) [8]. Ovulated eggs were sampled using the microsurgical incision of the oviducts as described by Podushka [24].

Sperm concentration and AD embryos

The relationship between the occurrence of AD and sperm concentration was studied by inseminating five groups of eggs (10 g each) with different concentrations of sperm, using three sturgeon breeding pairs (two *A. ruthenus* and one *A. baerii*) (Figure 1). Sperm concentration was estimated using a Burker cell hemocytometer (Meopta,

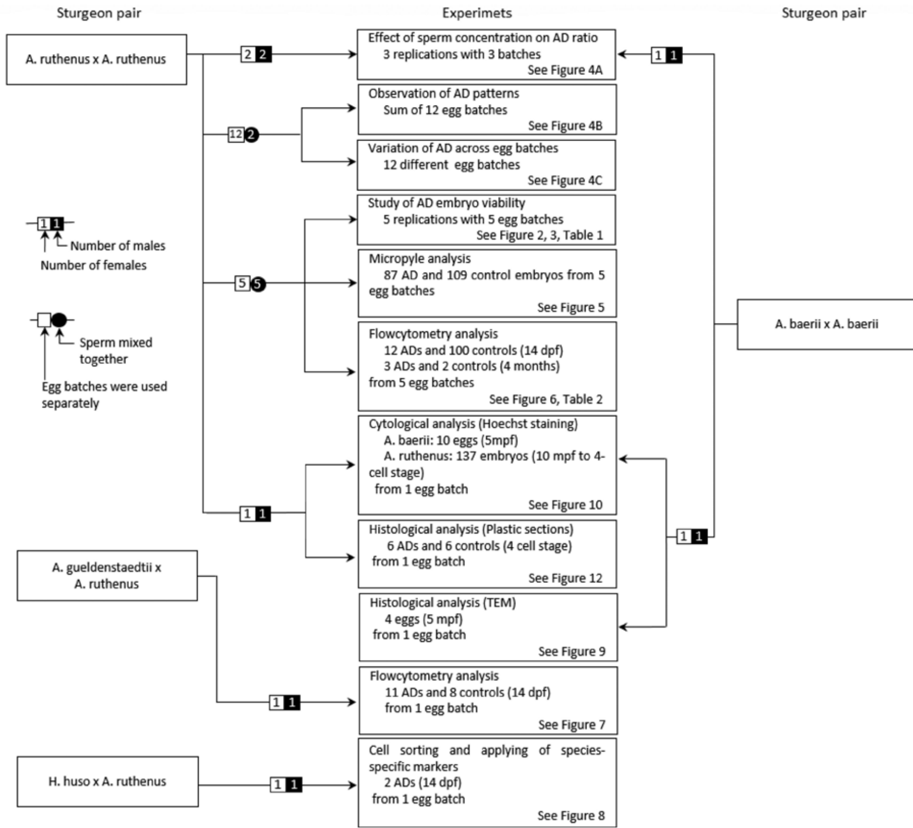

Figure 1. Flow diagram of the study. A different pair of sturgeons were used in each experiment/analysis.

Czech Republic).The approximate number of spermatozoa per egg was then calculated. The stickiness of the fertilized eggs was removed by treating with 0.1% tannic acid for 10 minutes. The incubating temperature was maintained at 15 ± 1°C. After fertilization, AD embryos (3, 5, 6, 7, 9, 10 cells) were collected and kept separately in dechlorinated water in 100 mm culture dishes. Embryos with normal cell division were used as controls.

Number of blastomeres in AD embryos

The occurrence of 3, 5, 6, 7, 9, and 10 blastomeres was studied using eggs from 12 *A. ruthenus* females fertilized with a mixture of sperm from two *A. ruthenus* males (10 g of eggs/1 ml sperm/30 ml water) (Figure 1). Stickiness was removed as described above. Embryos were fixed in 4% formalin at the 4-cell stage, and the number of blastomeres was counted.

Micropyle analysis

The number of micropyles was counted and their spread (distance between the two most distant micropyles) measured in 87 AD embryos and 109 embryos with normal cell division randomly selected from five egg batches (Figure 1). The chorion in sturgeon eggs comprises three layers [8,25–26]. After removing stickiness, the two outer layers were removed using forceps and stained with 0.1% methylene blue in distilled water for 30 min. The number of micropyles on each egg was determined by stereomicroscopy (Leica M165 imaging system) along with the distance between the two most widely separated micropyles.

Survival

To calculate the AD embryo survival rate, separate groups of eggs from five *A. ruthenus* females were fertilized with sperm from five *A. ruthenus* males. Stickiness was removed as described above. At the 2- to 4-cell stage, AD embryos were separated from normally divided, which were used as a control. Embryos were incubated 14 days in dechlorinated water at 15 ± 1°C (Figure 1).

Ploidy analysis of *A. ruthenus*

Atypically divided embryos were incubated until hatching at 8 days postfertilization (dpf). After sacrificing the larvae with an overdose of MS-222 (Sigma), they were dissected into three parts: head, trunk,

and tail. All three parts were used for ploidy analysis. Ploidy level was evaluated by flow cytometry (Paa Partec CCA I; Partec GmbH, Münster, Germany) using 4’ 6-diamidino-2-phenylindole (CyStain DNA 2 step kit; Partec GmbH). In total, 100 controls and 12 AD embryos were analyzed (Figure 1). Three fish (AD1, AD2, AD3) exhibiting atypical division were maintained to 4 months and then sacrificed for ploidy analysis of organs. Eight parts were selected for analysis: head, gill, heart, kidney, intestine, liver, gonad, and caudal fin.

Intraspecific hybridization

A. ruthenus (2n) eggs were fertilized with undiluted *A. gueldenstaedtii* (4n) sperm to test whether AD embryos were produced by polyspermy or second polar body retention. Ploidy levels of hatched hybrid larvae were analyzed by flow cytometry as described above. In total, 8 controls (*A. gueldenstaedtii* embryos) and 11 AD hybrids were analyzed (Figure 1).

Cell sorting and applying of species-specific markers

A. ruthenus (2n) eggs were fertilized with high concentrated *H. huso* (2n) sperm to test the possibility of AD embryos producing (Figure 1). Two atypically divided embryos were kept 2 weeks until sacrificing for cells sorting. Each larva was minced and treated with 0.3% trypsin for 1 h. One percent of bovine serum albumin was added into the homogenate and was filtrated through 50 μm mesh. After 10 min centrifugation at 400 g, supernatant was replaced by 1 ml of PBS containing 2 μl of Hoechst 33342 and 1 μl of propidium iodide. The live cells (propidium iodide negative) were sorted according their relative DNA content (Hoechst labeling) using BD Influx cell sorter (BD Biosciences, New Jersey, USA).

DNA from the sorted haploid and diploid cells was extracted using a DNA extraction kit according to manufacturer’s instructions (GenElute Mammalian Genomic DNA Miniprep Kit; Sigma-Aldrich). Species-specific nuclear markers ARp_247 + 247_uni (*A. ruthenus*) and HHp_153 + 153_uni (*H. huso*) were applied for identification of haploid/diploid cells occurrence by a presence/absence of a given band [27].

Histological analysis

To observe number of micropyles, four *A. baerii* embryos were fixed in 2.5% glutaraldehyde 5 min after fertilization. Samples for TEM and ultrathin sections were dehydrated by acetone and embedded in Spurr resin blocks. Series of semi-thin sections were cut using a Leica UCT ultramicrotome (Leica Microsystems), contrasted by uranyl acetate and lead citrate, and examined in a TEM JEOL 1010 operated at 80 kV (Figure 1).

Plastic sections of six *A. ruthenus* 4-cell stage embryo and six *A. ruthenus* 6-cell stage embryos were processed in order to keep structure of tissues with a lot of lipids. Embryos were fixed with bouin’s fixative for 24 h, and then replaced with 80% ethanol for long-term storage. Eighty percent EtOH was gradually replaced into 100% EtOH. After dehydration, embryos were infiltrated with Technovit 7100 on a shaker in the order as follows: 25% Technovit 7100 in EtOH for 12 h, 50% Technovit 7100 in EtOH for 12 h, 75% Technovit 7100 in EtOH for 12 h, 100% Technovit for 1 day two times. After adding Technovit 7100 Hardener I and II, embryos were incubated for 24 h in a mold at −30°C. Then, embryos were polymerized by increasing the temperature up to 60°C in an incubator for 24 h. The specimen was sectioned using Leica RM2235 as according to the manufactured protocols. The thickness of each section was 4 μm. Sections were stained with hematoxylin and eosin and observed under the microscope (Figure 1).

Cytological analysis

A. ruthenus embryos 10–60 min postfertilization (mpf) (50 embryos), 75–120 mpf (40 embryos), 135–180 mpf (39 embryos), 2-cells (5 embryos), 3-cells (3 embryos), and *A. baerii* 5 mpf (10 embryos) were fixed in 2.5% glutaraldehyde to analyze nuclei in embryos. Before observation, fixed embryos were washed three times in PBS, dechorionated by forceps and labeled by 0.2% Hoechst 33342. After 2 h incubation in the dark, embryos were observed using Olympus IX83 microscopy (Tokyo, Japan) (Figure 1).

Statistics

All statistical analyses were performed using R software (version 3.4.1). The frequency of occurrence of AD was tested using Fisher’s exact test, and the *P*-values were adjusted using the Benjamini-Hochberg method in order to reduce the false discovery rate for the multiple tests. The difference in number of micropyles and their spread was tested using Welch’s t-test, $P < 0.05$ for both tests. The number of embryo nuclei at each time point was compared using Steel-Dwass test by means of the Monte Carlo method. *P*-value less than 0.05 was considered statistically significant.

Results

Shape and survival of AD embryos

Atypically divided embryos were found at the 2- to 4-cell stage (Figure 2). Although the number of blastomeres was abnormal in these embryos, they continued division and formed normal blastomeres at the 256-cell stage (Figure 2). From 28.6 to 100% of AD embryos (44.3–86.5% in control groups) survived after hatching (7 dpf) (Figure 3A and B). Such fish grew normally for at least 4 months, until they were sacrificed for flow cytometer analysis. Some fish from AD embryos exhibited malformations (mainly abnormal body shape such as a bent body axis or partial dwarfism) but survived into the feeding stage too (Table 1).

Effect of sperm concentration on the frequency of AD embryos

Atypically divided embryos were observed in all experimental groups, even at the lowest amount of sperm (10 000 spermatozoa/egg) used for insemination in this study (Figure 4A). As the amount of sperm increased, the rate of AD embryos tended to increase too, although the peak did not correlate with the highest amount of sperm, which was characteristically for both *A. ruthenus* and *A. baerii* pairs (Figure 4A). The rate of fertilization was largely constant (around 70–80%) and independent of the amount of sperm used (Figure 4A). The exception was *A. ruthenus* Pair 2, where the fertilization rate was low (22.3%) and the amount of sperm was lowest, but the ratio of AD embryos was significantly higher (Figure 4A). In the repeated fertilization tests, we observed 3, 5, 6, 7, 9, and 10 cells embryos at the 4-cell stage (Figure 4B). The number of AD embryos showing 6 cells at the 4-cell stage was significantly higher than those showing other numbers of cells (Figure 4B). The frequency of occurrence of AD embryos varied (about 1–16%) among batches of eggs even when the amount of sperm and eggs remained the same (Figure 4C).

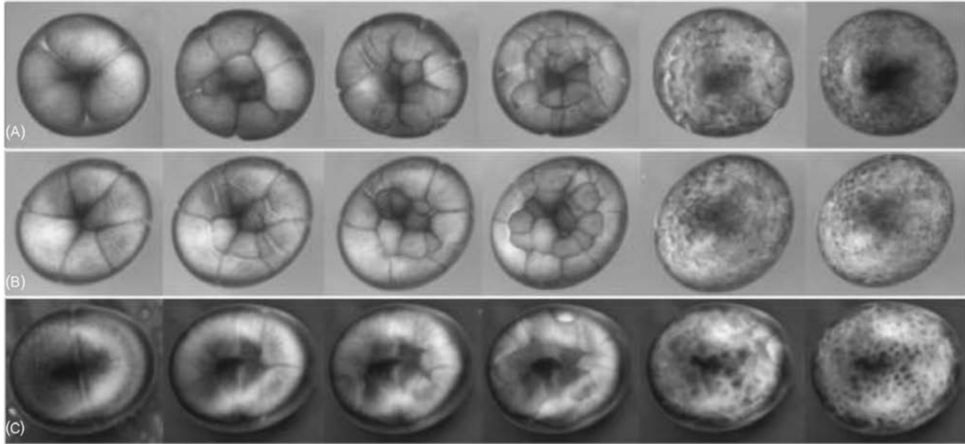

Figure 2. AD blastomeres of *A. ruthenus* embryo showed continuous division during 2- to 256-cell stage. (A) 3-cell AD embryo, (B) 6-cell AD embryo, and (C) normally divided embryo.

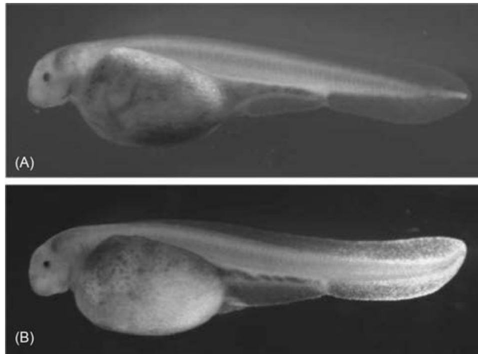

Figure 3. Normally developed *A. ruthenus* larva from AD embryo. (A) AD embryo developed from 3 cells at 2- to 4-cell stage, (B) control embryo.

The number of micropyles and their spread (area occupied) in AD embryos

The micropyles on the separated chorion were successfully visualized using methylene blue (Figure 5A). The number of micropyles varied among the eggs, and ranged from 2 to 14. Chorions of AD embryos had significantly greater numbers of micropyles ($P < 0.001$) when compared to the control (Figure 5B). The spread (area on the chorion occupied) of the micropyles was quantified by the distance between the two most distant micropyles, and also varied among the eggs, ranging from 108 to 675 μm. The average distance on the AD embryos was significantly higher from that normally divided ($P < 0.001$) (Figure 5C).

Ploidy of AD larvae

Flow cytometry analysis was conducted to study the ploidy levels of the AD embryos. All AD larvae at the hatching stage were composed of both haploid and diploid cells, while controls had a single diploid peak (Figure 6). In a single AD larva, the proportion of haploid cells ranged between 10 and 100% in the head, trunk and tail. Ploidy analysis of organs from 4-month AD fish demonstrated that each organ had a different ploidy (Table 2). Interestingly, some organs in AD1 and AD2 fish were comprised of haploid cells, including the germ line (Table 2).

Effect of the sperm-derived genotype on additional blastomeres

To test the polyspermy hypothesis vs. the second polar body retention hypothesis, ploidy of hybrid AD embryos was analyzed (Figure 7A). Hybrid AD embryos of *A. ruthenus* (2n) eggs × *A. gueldenstaedtii* (4n) sperm were successfully produced by insemination with highly concentrated sperm. As the amount of sperm increased, the number of AD embryos increased (data not shown). Flow cytometry analysis showed all hybrid AD larvae to be 2n/3n mosaic (Figure 7B).

Identification of *A. ruthenus* and *H. huso* using nuclear DNA markers

We applied *A. ruthenus* and *H. huso* specific oligonucleotide markers ARp_247 and HHp_153 in combination with 247_uni and 153_uni respectively on the obtained DNA from *A. ruthenus* × *H. huso* hybrids sorted cells. Both hybrid larvae were haploid/diploid mosaics. DNA from diploid cells were amplified by both *A. ruthenus* and *H. huso* specific oligonucleotides, while haploid cells were amplified only using oligonucleotides specific for *H. huso*, which proved the paternal origin of the haploid cells (Figure 8).

Histological and cytological analysis

We observed 12 spermatozoa in micropyles in the average from analyzed four *A. baerii* embryos (Figure 9). Numerous spermatozoa nuclei were located in *A. baerii and A. ruthenus* embryos cytoplasm (Figure 10). Analysis of 10 randomly taken *A. baerii* embryos 5 mpf

Table 1. Survival rate of the AD embryos until 14 days postfertilization.

Trial	Experiment group	Total number of embryos	4 dpf (%)	7 dpf (%)	14 dpf (%)	Shape of embryos at 14 dpf	
						Normal	Abnormal
1	AD*	8	8 (100)	8 (100)	8 (100)	7	1
	Control	549	516 (93.9)	516 (93.9)	475 (86.5)		
2	AD	10	7 (70.0)	7 (70.0)	7 (70.0)	7	0
	Control	474	367 (77.4)	366 (77.2)	365 (77.0)		
3	AD	42	16 (38.0)	15 (35.7)	12 (28.6)	7	5
	Control	545	370 (67.8)	367 (67.5)	342 (62.7)		
4	AD	35	26 (74.2)	25 (71.4)	16 (45.7)	11	5
	Control	314	269 (85.6)	258 (82.2)	139 (44.3)		
5	AD	16	9 (56.2)	6 (37.5)	6 (37.5)	5	1
	Control	507	474 (93.5)	442 (87.2)	348 (68.6)		

"AD" indicates "atypically divided" at the 2- to 4-cell stage.

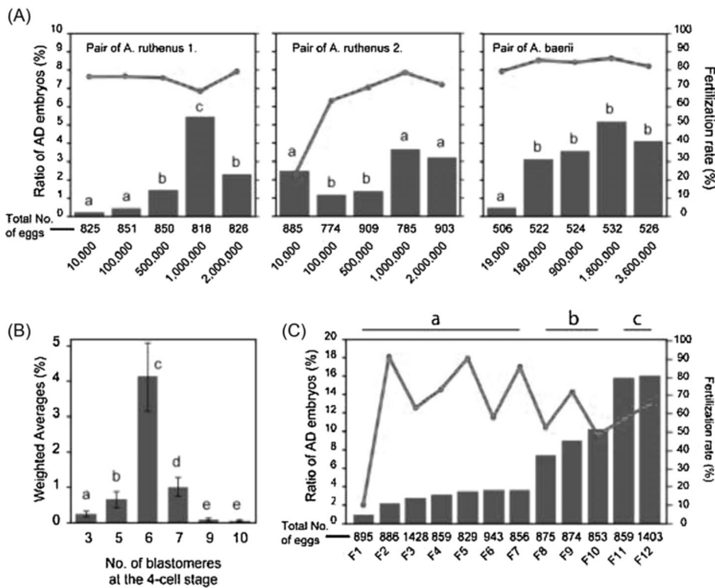

Figure 4. Sperm concentration and AD embryos appearance. (A) Ratio of AD embryos (%) with increasing amount of sperm used for insemination in two pairs of *A. ruthenus* and one pair of *A. baerii*. Oblique numbers under the columns are approximate number of spermatozoa per egg used in each treatment. Fertilization rate is shown by red lines. Statistically significant differences between treatments are indicated by a different alphabet letters on each column (Fisher exact test, *P*-value adjustment by Benjamini-Hochberg method, $P < 0.05$). (B) Embryos with a different number of blastomeres at the 4-cell stage. Columns show weighted average (%) of the occurrence of 3, 5, 6, 7, 9, and 10 blastomeres each in 12 replications. Error bars on the columns show standard deviations. In comparing two columns, a significant difference is indicated if the letter on top of the column is different (Fisher exact test, *P*-value adjustment by Benjamini-Hochberg method, $P < 0.05$). (C) Occurrences of AD embryos from 12 *A. ruthenus* females (F1-F12) in *A. ruthenus*. Red lines show the fertilization rate. In comparing two columns, a significant difference is indicated if the letter on top of the column is different (Fisher exact test, *P*-value adjustment by Benjamini-Hochberg method, $P < 0.05$).

demonstrated that each embryo contained 6 nuclei in the average in the cytoplasm.

Fixed *A. ruthenus* embryos demonstrate a significant difference in number of nuclei between groups 10–60 min with the remaining groups: 75–120 min, 135–180 min, 2–4 cell stage [Figure 11]. In group 10–60 min, embryos contained from 0 to 10 nuclei. This number has trend for decreasing, and at the 2- to 4-cell stage it was not possible to find any nuclei.

Histologically observed *A. ruthenus* embryos 4- and 6-cell stage demonstrate that normally and abnormally divided embryos contain a nucleus into each blastomere (Figure 12).

Discussion

This study was undertaken with the main objective of understanding the genesis of AD in sturgeons under artificial fertilization. There are

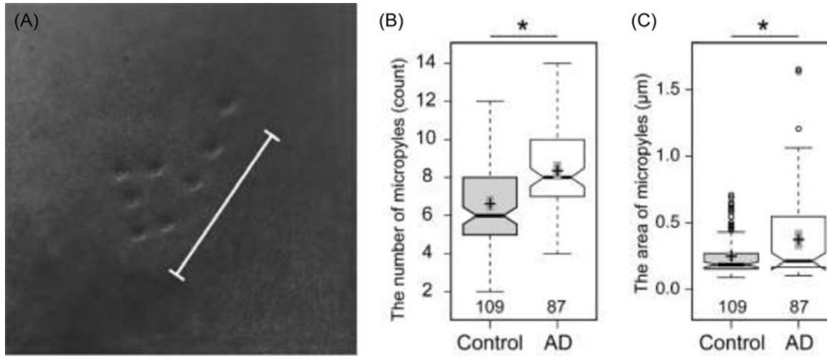

Figure 5. The AD embryos have their origin from eggs with significantly larger numbers of micropyles that also had larger coverage, when compared to normal eggs. (A) Methylene blue stained micropyles of AD *A. ruthenus* embryo. Eight micropyles are visible. The coverage of the micropyles was calculated as the linear distance between the two most distant micropyles. (B) Box plot comparing the average number of micropyles between the normal and the AD embryos, which was significantly different at $P < 0.001$ (Welch *t*-test). (C) Box plot comparing the micropyle coverage, measured as the linear distance between the two most distant micropyles, between the normal and the AD embryos. The average coverage was significantly different at $P < 0.001$ (Welch *t*-test). In the box plots, the center lines show the medians, box limits indicate the 25th and 75th percentiles, whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by open circles, crosses represent sample means, and bars indicate 95% confidence intervals of the means.

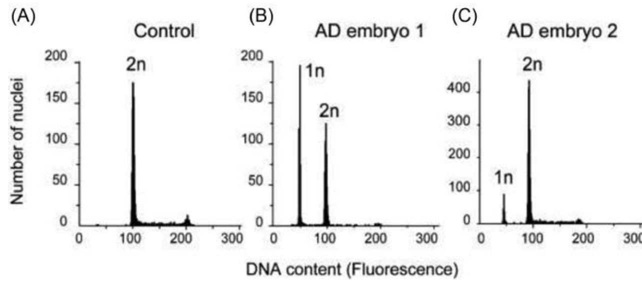

Figure 6. Flow cytometry analysis showed *A. ruthenus* AD embryos to be haploid/diploid mosaics. (A) A normally dividing embryo at the hatching stage with a single diploid (2n) peak. (B) AD embryo 1 with one haploid (1n) and one diploid (2n) peak. In this embryo, the haploid peak is higher than the diploid peak. (C) AD embryo 2 is a 1n/2n mosaic as well but with a lower haploid peak.

Table 2. Ploidy of *Acipenser ruthenus* organs.

	Mainly ectoderm			Mainly mesoderm		Mainly endoderm		Germ line	
	Head	Gill	Caudal fin	Heart	Kidney	Intestine	Liver	Gonad	Notes
Control-1	2n	2n	2n	2n	2n	2n	2n	*ND	All tissues comprise 2n cells.
Control-2	2n	2n	2n	2n	2n	2n	2n	2n	All tissues comprise 2n cells.
AD1	n/2n	n	n/2n	n/2n	n/2n	n	n/2n	n/2n	Gills and intestine only comprise n cells
AD2	n/2n	n/2n	n	n/2n	n/2n	n	n/2n	n	Gonad comprise n cells
AD3	n/2n	n/2n	2n	2n	2n	2n	n/2n	2n	2n cells are dominant in some tissues

Note: Only functional ploidy levels are shown.
*ND: no data.

no reports of AD embryos in the wild. There were two possible hypotheses to be considered: (1) polyspermy, and (2) retention of the second polar body. Our experiments yielded the following results: (1) AD embryos survival was similar to control, (2) the frequency of AD embryos tended to correlate positively with the amount of sperm, as shown in previous report [10], (3) the number of micropyles in AD embryos and the area occupied by them were both significantly greater than the respective values in the controls, (4) numerous spermatozoa were located in the cytoplasm after fertilization, (5) all AD embryos are mosaics, and (6) the diploid cells from *AD embryos* contained maternal and paternal genetic markers, while the haploid cells contained only paternal ones.

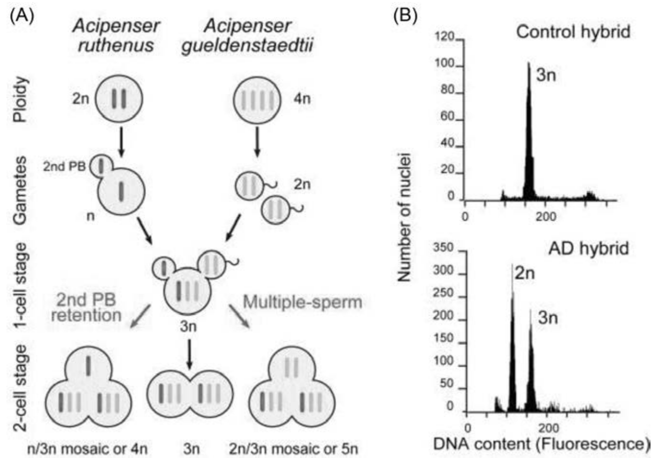

Figure 7. Intraspecific fertilization indicated AD embryos appearance after numerous spermatozoa penetration. (A) Experimental design to test the second polar body (PB) retention and polyspermy hypotheses. The functional ploidy levels of each fish are 2n (*A. ruthenus*) and 4n (*A. gueldenstaedtii*). Hybrid zygote cells show 3n ploidy. Additional blastomeres formed by second polar body retention are expected to show haploid DNA from *A. ruthenus* and to be n/3n mosaics or 4n, while embryos, produced by polyspermy are expected to be *A. gueldenstaedtii*-derived diploid blastomeres and to show 2n/3n mosaic or 5n. Hybrid zygote cells must be triploids (3n). (B) The control hybrid embryo at the hatching stage had a single triploid (3n) peak. The AD hybrid embryo showed diploid (2n) and triploid (3n) peaks.

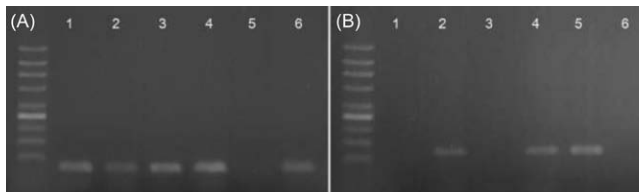

Figure 8. Identification of *H. huso* and *A. ruthenus* by presence/absence of a band. (A) Amplification of species-specific primer 153_HHp with combination 153_uni (*H. huso*). (B) Amplification of species-specific primer 247_ARp with combination 247_uni (*A. ruthenus*). 1 = haploid cells from sample AD1; 2 = diploid cells from sample AD1; 3 = haploid cells from sample AD2; 4 = diploid cells from sample AD2; 5 = control *A. ruthenus*; 6 = control *H. huso*.

"Multiple-sperm mosaics"

Cleavage abnormality, called "mosaic cleavage," in which some region of the egg remains undivided, perhaps due to damaged cytoplasm, has been observed in sturgeons [8] and may lead to atypical number of blastomeres and AD embryos. However, we found that the cells originating from abnormally divided blastomeres in AD embryos contained a nuclei and (Figure 12) and continued to divide during development, indicating that the abnormality manifested was not a mosaic cleavage (Figure 2).

All sterlet AD embryos that contained 3, 5, 6, 7, 9, and 10 blastomeres were n/2n mosaics, whereas *Acipenser ruthenus* × *A. gueldenstaedtii* AD hybrids showed 2n/3n mosaicism. This indicates that AD embryos are created by supernumerary sperm, as a retention of the second polar body would result in n/3n mosaicism or 5n. Identification of haploid/diploid cells by species-specific markers demonstrated that haploid cells were amplified only by oligonucleotides specific for *H. huso* male, which proved the paternal origin of the haploid cells.

Unfortunately, we could not determine the mechanism by which AD or abnormal numbers of blastomeres occur in this study. Generally, two spindles derived from the two sperm centrioles form 4 cells and induce aneuploidy in each blastomere as the chromosomes segregate randomly among multiple spindles [16]. However in our experiments, all AD embryos showed n/2n (or 2n/3n in hybrids) mosaicism and we did not observe any aneuploid embryos, either among the ADs or the controls. Strictly speaking, polyspermy is used to define a situation whereby "karyogamy" occurs between three or more gametes (often one female and two male gametes). However, what we observed was an additional "plasmogamy" apart from normal fusion of female and male pronucleus. A sperm nucleus or nuclei destined to form an additional blastomere began development independently and probably one cycle later than zygote. In fact, we frequently observed that six cells were formed directly after the 2-cell stage, a phenomenon observed previously as well [8]. If a single supernumeral sperm nucleus in the egg is unpacked to form the pronucleus and starts division at the 2-cell stage, the 4-cell stage embryo will have six cells. The fact that most of AD embryos had six cells at the 2- to 4-cell stage suggests that they are vulnerable to this type of AD. However, this does not explain how the other types of AD (3, 5, 7, 9, 10 cells) happen. Perhaps it is the difference

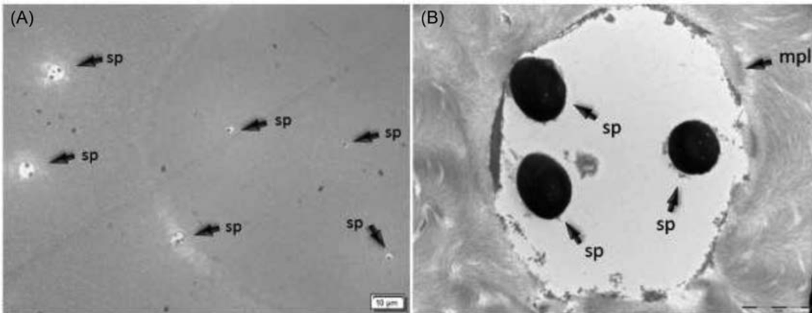

Figure 9. Penetration of numerous spermatozoa into *A. baerii* egg. (A) Multiple spermatozoa in the micropyles. Transmission electron microscope (TEM). Scale bar: 10 μm. (B) TEM demonstrates three spermatozoa inside of one micropyle. Scale bar: 2 μm. Sp = spermatozoa, mpl = micropyle.

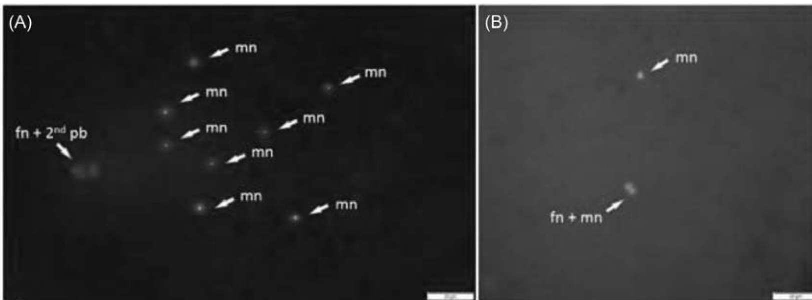

Figure 10. Hoechst-stained nuclei. (A) *A. baerii* embryo 5 min after fertilization. (B) *A. ruthenus* embryo 30 min after fertilization. Mn = male nucleus, fn = female nucleus, 2nd pb = second polar body. Scale bar: 50 μm.

in timing of the pronucleus formation and/or the number of additional sperm in the blastodisc of the oocyte. In any case, although we have used the term "polyspermy" to explain the occurrence of AD, it might be more appropriate to term the AD embryos "multiple-sperm mosaics" to avoid any misunderstanding of the phenomenon.

In addition to this, even though the ratio of AD embryos appears to be positively correlated with the amount of sperm, there seems to be a point where the association is no longer true, for as yet unknown reasons.

AD embryo survival

We produced AD embryos in a fairly straightforward manner, even in hybrids, by inseminating the eggs with a large amount of sperm. For more than a half of century, it has been believed that AD embryos develop atypically, and that most show malformations and die prior to hatching [8,10]. Our results demonstrated that many AD embryos (7 dpf: 35.7–100%, 14 dpf: 28.6–100%) survived (Table 1), and most of them developed into feeding fry with normal morphology. Some malformed fish also survived into the feeding stage. The longest living AD fish was an *A. ruthenus* × *A. gueldenstaedtii* hybrid that survived to 9 months and was sacrificed for examination (data not shown). Viable n/2n mosaics have also been observed in the other animals. Luttikhuizen and Pijnacker [28] found that the tellinid bivalve, *Macoma balthica*, embryos demonstrated mosaicism comprising haploid and diploid cells, perhaps as a result of an additional spermatozoon, and developed normally. In some salmonid species, normal and viable haploid-diploid mosaics have been reported, although the mechanism of mosaicism has not been studied so far [14,29–30]. Generally, an embryo that only comprised haploid cells demonstrates "haploid syndrome" and cannot develop further than the embryonic stage. However, our results suggest that haploid cells can be viable and functional in a diploid environment. This has been shown before in other fish. Tanaka et al. [31] tested this hypothesis in goldfish, *Carassius auratus,* by means of haploid-diploid chimeras produced by transplanting haploid blastomeres into a diploid blastula embryo, and found that about 14% of the haploid-diploid chimeras survived beyond 1 year.

Sperm-derived haploid cells distributed unevenly in the body

Analysis of the ploidy level of *A. ruthenus* AD larvae and 4-month-old fish revealed that different parts of the body or organs had haploid cells in different ratios. Furthermore, some organs of the older fish showed apparently normal ploidy (2n), while others contained mainly haploid cells, and vice versa. Such an uneven distribution of haploid cells has also been observed in mosaic charr [30]. This result

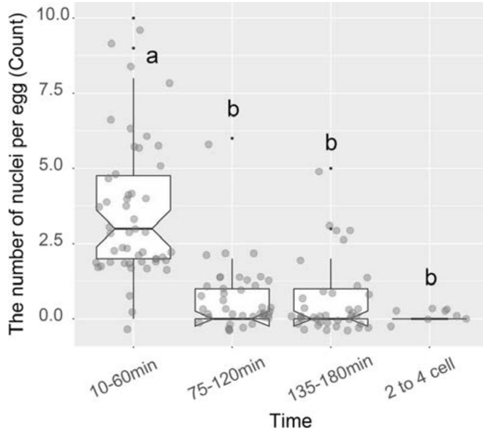

Figure 11. The box plots demonstrate the number of nuclei in *A. ruthenus* eggs in groups 10–60 min, 75–120 min, 135–180 min, and 2- to 4-cell stage. Box plots: center lines show the medians; box limits indicate the interquartile range (between the 25th and 75th percentiles); whiskers extend 1.5 times past the interquartile range, outliers are represented by black dots; notch indicate median with 95% confidence interval; the data points were randomly jittered to separate overlapping points slightly. n = 50, 40, 39, 8. Boxes with different letters signify significant difference ($P < 0.05$).

indicates that analyzing just one part of body (blood, tail, etc.) would not be representative of the ploidy level of the individual.

Dettlaff and Ginsburg [8,10] have already shown that polyspermy can be avoided by lowering the concentration of sperm during fertilization. Such a dilution is strongly recommended for sturgeon reproduction, as an evaluation of the ploidy level of the broodstock by sampling from multiple parts of the body.

Topology of micropyles affects the frequency of polyspermy

Ginsburg [10] hypothesized that polyspermic fertilization is more likely in sturgeon when compared to other fish due to the presence of multiple micropyles in the eggs. Our observation of the relationship between the number/density of micropyles and the frequency of AD showed that *A. ruthenus* eggs appear to be particularly vulnerable, with eggs containing greater numbers of micropyles generating significantly higher numbers of AD embryos. In fact, we observed many spermatozoa in each micropyles of *A. baerii* embryos (Figure 9). On the other hand, Psenicka et al. [26] concluded that a cytoplasmic projection in the sturgeon egg created after fusion with the spermatozoon rapidly expands concentrically from the point of first spermatozoon penetration toward the vegetal pole, blocking entry by additional spermatozoa into other micropyles. It is generally believed that a large number of micropyles on eggs increase the chance of fertilization in nature, because sturgeons broadcast eggs over a wide area, diluting spermatozoon and egg densities immediately after spawning [10]. This balance of spermatozoa concentration and the number of micropyles is disrupted in artificial conditions, and the system blocking polyspermy is counteracted by insemination with highly concentrated sperm.

Do AD embryos possess haploid germ cells?

We found haploid cells distributed among all germ layer derivatives, including gonads. Although we could not confirm whether germ cells are generated from the haploid cells, because gonads also contain large numbers of somatic cells, there is no reason to assume that haploid cells cannot differentiate into germ cells. Saito et al. [32–33] have shown that primordial germ cells (PGC), precursors to germ cells, are specified by inheritance of germplasm in sturgeon. Thus, it can be presumed that haploid nuclei can reach the region in which germplasm is rich enough to specify and form PGCs as normal nuclei do. Hypothetically, additional blastomeres produced by supernumerary sperm exhibit the paternal genome exclusively. Thus, if these haploid-derived germ cells produce gametes, the gametes must carry only the paternal genome, and all haploid-derived gametes will be clonal, although they should recover diploid status during gametogenesis before undergoing meiosis to generate gametes. Yoshikawa et al. [34] have shown that, in the generation of the natural clonal loach *Misgurnus anguillicaudatus*, type A spermatogonia undergo additional chromosome duplication and produce clonal diploid spermatozoa, although the mechanism of this phenomenon is still unknown. To avoid genetic contamination caused by haploid cells in artificial reproduction, it is important to know whether the haploid cells can produce PGCs and, if so, whether they differentiate into gametes. On the other hand, if AD fish can produce haploid-derived clonal gametes, induction of multiple-sperm mosaicism might be a useful tool for production of isogenic strains in sturgeons in a short time frame.

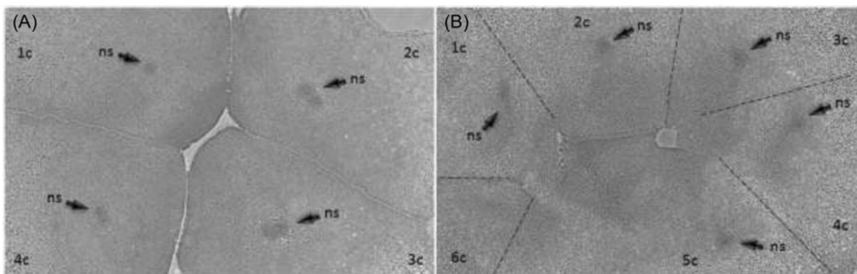

Figure 12. Histological section of *A. ruthenus* embryos. (A) Control 4-cell stage embryo. (B) Abnormal 6-cells embryo. Ns = nucleus, c = cell.

Comparative polyspermy in sturgeon compared to other animal taxa

In the animal kingdom, polyspermic fertilization is generally considered a pathological event leading to death of the embryo [35–36]. Although some taxa display the ability to overcome polyspermy (typical for invertebrates) or benefit from it (typical for urodeles), only one spermatozoon nuclei is involved in ongoing development of the animal, with others eliminated before the first mitotic division [16]. In the present research, whereas the ratio of AD embryo was no more than a little above 16% after highly concentrated sperm fertilizations, the cytological analysis demonstrated that almost all randomly taken *A. ruthenus* and *A. baerii* embryos contained multiple nuclei in the cytoplasm just after fertilization. The number of supernumerary sperm showed significant decrease as embryos develop. Previous research from our laboratory revealed some similarities in the early embryonic development of sturgeon and amphibians [32]. Our findings indicate that the sturgeon shares with urodeles the ability to survive supernumerary spermatozoon fertilization. Namely, sturgeons utilize physiological polyspermy: multiple spermatozoa penetrate inside the egg but do not participate in the development. Probably, the number of sperm in the cytoplasm exceeded the capacity of this mechanism in artificial conditions in sturgeon.

To the best of our knowledge, the sturgeon presents a developmental pattern unique in the animal kingdom, in which supernumerary spermatozoa give rise to blastomeres that can develop independently along with the zygote-derived cells and result in a viable haploid/diploid mosaics.

Acknowledgments

We thank all members of the Laboratory of Germ Cells, Faculty of Fisheries and Protection of Waters, University of South Bohemia in Ceske Budejovice for their support. We are grateful to Dr. Dmytro Bytyutskyy for his help during experiments.

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CHAPTER 3

FIRST EVIDENCE OF VIABLE PROGENY FROM THREE INTERSPECIFIC PARENTS IN STURGEON

Iegorova, V., Havelka, M., Psenicka, M., Saito, T., 2018. First evidence of viable progeny from three interspecific parents in sturgeon. Fish Physiology and Biochemistry. doi: 10.1007/s10695-018-0553-6.

It was allowed by publisher Springer Nature on 20th of September, 2018 to include the manuscript in this Ph.D. thesis.

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FIRST EVIDENCE OF VIABLE PROGENY FROM THREE INTERSPECIFIC PARENTS IN STURGEON

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ABSTRACT

Polyspermy is the most commonly observed cause of embryonic abnormalities in fertilization, often resulting in death. In sterlet (*Acipenser ruthenus*), however, polyspermic embryos have high survival (similar to a control group) and morphological development is similar to monospermic larvae. Ploidy of these individuals is n/2n mosaic (whereas the normal state for *A. ruthenus* is a functional diploid). This study was undertaken to test whether sturgeon eggs can be fertilized by several spermatozoa from different species to produce viable offspring from three interspecific parents: *A. ruthenus* (2n), *A. gueldenstaedtii* (4n) and *A. baerii* (4n). Four trials were performed: (1) and (2) *A. baerii* eggs were fertilized with a mixture of *A. ruthenus* and *A. gueldenstaedtii* sperm; (3) *A. gueldenstaedtii* eggs were fertilized with a mixture of *A. baerii* and *A. ruthenus* sperm; and (4) *A. gueldenstaedtii* eggs were fertilized with a mixture of *A. gueldenstaedtii* and *A. ruthenus* sperm. Fertilized embryos with abnormal cleavage (3, 5, 6, 7, 9, and 10 cells) were collected and kept separately until 14 days post-fertilization. Ploidy level of 25 larvae (hatched from abnormal cleaved embryos) was evaluated by flow cytometry. Forty-four percent of observed hybrids had a mosaic 2n/3n ploidy. Five larva were processed further with microsatellite analysis and demonstrated that three specimens were heterospecific polyspermic larvae, containing alleles from three parents, and two specimens were conspesific polyspermic larvae from two parents. This astonishing phenomenon was emphasized by the fact that it was generated without any significant intervention.

Keywords: *Sturgeon · Polyspermy · Mosaicism · Hybridization*

INTRODUCTION

Sturgeons (Acipenseridae) are an ancient family (Bemis and Grande 1997) with unique biological characteristics that can be challenging for research (Carmona et al. 2009). Sturgeon eggs have multiple micropyles, and the number varies among females of different species and between eggs of individual females (Dettlaff et al. 1993): *Acipenser ruthenus* eggs contain five to 13 micropyles (Zalenskii 1878); up to 52 micropyles in *A. gueldenstaedtii* eggs (Dettlaff and Vassetzky 1991); and *A. baerii* eggs have two to 16 micropyles (Debus et al. 2002).

Ploidy in sturgeon fishes also varies and can be categorized into three classes depending on chromosome number: functional diploids (2n) have ~120 chromosomes (*Huso huso*, *A. sturio*, *A. ruthenus*); functional tetraploids (4n) have ~240 chromosomes (*A. baerii*,

A. gueldenstaedtii, A. naccarii, A. mikadoi); and functional hexaploids (6n) have ~360 chromosomes (*A. brevirostrum*) (Ludwig et al. 2001; Fontana and Colombo 1974; Fontana 1994; Kim et al. 2005; Birstein and Vasiliev 1987; Vasil'ev 2010). However, sturgeons hybridize more easily than other vertebrates (Birstein et al. 1997) between species with the same and/or different ploidy level (Billard and Lecointre 2001) and demonstrate interspecific and intergeneric hybridization in the wild and under artificial conditions (Havelka et al. 2011).

Multiple micropyles, which are typical for sturgeon eggs, and fertilization with high concentrated sperm suspensions are associated with the occurrence of polyspermy (Dettlaff et al. 1981). It is believed that polyspermy is the most commonly observed reason for embryonic abnormalities in fertilization, often resulting in death (Boveri 1901; Wang et al. 2003). However, recent research by Iegorova et al. (2018) demonstrates that sturgeon polyspermic embryos are unique in their ability to survive and to develop into morphologically normal fry after abnormal cleavage as 3, 5, 6, 7, 9 and 10 blastomeres at the 2- to 4-cell stage. It was described that ploidy level of *A. ruthenus* larvae from abnormally divided embryos (abnormally divided embryos = AD embryos) was n/2n and *A. gueldenstaedtii* x *A. ruthenus* hybrids had 2n/3n mosaic ploidy. That was the reason of fusion of one sperm pronucleus with the egg pronucleus generating a zygote, and the accessory sperm pronucleus or pronuclei developed separately from the zygote (Iegorova et al. 2018).

Based on these characteristics of sturgeon, we tested the possibility of producing a polyspermic interspecific hybrid by using gametes from three parents.

Broodfish and gamete collection

Two *A. baerii* females, two *A. gueldenstaedtii* females, four *A. ruthenus* males, three *A. gueldenstaedtii* males, and one *A. baerii* male were held in 4 m^3 tanks at 15 °C in the hatchery of the Research Institute of Fish Culture and Hydrobiology in Vodnany, Czech Republic. To induce spermiation, sturgeon males were injected with acetone-dried homogenized carp pituitary extract (CPE) at a dose of 4 mg/kg body weight (BW). Using a catheter, sperm was collected from the urogenital papilla at 42 h post-injection, transferred to a 250 ml cell-culture container, and stored at 4 °C until fertilization. Sperm concentration was estimated using a Burker cell hemocytometer (Meopta, Czech Republic) at 20x magnification on a Nikon ECLIPSE Ci-S phase contrast microscope (Nikon, Japan). Ovulation in sturgeon females was induced with two doses of CPE by intramuscular injection: the first was given 36 h before stripping (0.5 mg/kg BW) and the second 24 h before stripping (4.5 mg/kg BW) (Dettlaff et al. 1981). Ovulated eggs were collected by microsurgical incision of oviducts as described by Podushka (1999).

Fertilization and identification of AD embryos

Four separate fertilizations were completed: (1) Eggs from one *A. baerii* (4n) female were fertilized with a mixture of sperm from one *A. ruthenus* (2n) and one *A. gueldenstaedtii* (4n) male; (2) Eggs from one *A. baerii* (4n) female were fertilized with a mixture of sperm from one *A. ruthenus* (2n) and one *A. gueldenstaedtii* (4n) male; (3) Eggs from one *A. gueldenstaedtii* (4n) female were fertilized with a mixture of sperm from one *A. baerii* (4n) and one *A. ruthenus* (2n) male; and (4) Eggs from one *A. gueldenstaedtii* (4n) female were fertilized with a mixture of sperm from one *A. gueldenstaedtii* (4n) and one *A. ruthenus* (2n) male. Approximately 450 eggs (10 g) were fertilized for each group, using 2.3312 10^9 spz x 1 ml. To remove the stickiness of the outer layer, fertilized eggs were treated with 0.1% tannic acid solution (three times in 10 min). Embryos with 3, 5, 6, 7, 9, and 10 blastomeres at the 2- to

4-cell stage were considered as AD embryos. AD embryos and normally dividing embryos were separated and counted.

Survival and ploidy analysis

Embryos were incubated in dechlorinated water at 15 ± 1 °C. After 14 days, survival was noted and ploidy level of 25 larvae, developed from ADs, were randomly chosen from all four groups in total and evaluated by flow cytometry (Paa Partec CCA I; Partec GmbH, Münster, Germany) using 4′, 6-diamidino-2-phenylindole (CyStain DNA 2step kit; Partec GmbH) according to manufacturer's instructions. Normally developed hybrids were used as a controls, and their expected ploidy was 3n and 4n.

Microsatellite genotyping

To prove an existence of several interspecific spermatozoa in the egg, five randomly chosen AD larvae were processed for microsatellite genotyping: two larvae from *A. baerii* x *A. ruthenus* x *A. gueldenstaedtii*: AD1 and AD2; two larvae from *A. gueldenstaedtii* x *A. baerii* x *A. ruthenus* fertilizations: AD3 and AD4; and one larva from *A. gueldenstaedtii* x *A. gueldenstaedtii* x *A. ruthenus* group: AD5. Genomic DNA was extracted using a DNA extraction kit (GenElute Mammalian Genomic DNA Miniprep Kit; Sigma-Aldrich®) according to manufacturer's instructions. Presence of multiple spermatozoa in AD larvae was estimated by parentage-like assignment using seven informative microsatellite markers, i.e., *AfuG_135* (Welsh et al. 2003), *Aox_27, Aox_45* (King et al. 2001), *Spl_101, Spl_107, Spl_163* and *Spl_173* (McQuown et al. 2000). Amplification was carried out according to the protocol described by Havelka et al. (2013). Microsatellite fragment analysis was performed on an Applied Biosystems SeqStudio Genetic Analyzer using a GeneScan LIZ 600 size standard (Applied Biosystems), and genotypes were scored in GENEIOUS 8.1.9, using Microsatellite Plugin 1.4.4. The complexity of the duplicated sturgeon genome and the nature of current microsatellite genotyping make it impossible to reliably determine allele dosage behind a specific peak. Hence, peak patterns were treated as dominant data and interpreted as "allele phenotypes" (Rodzen et al. 2004). In several cases, we were able to estimate a real genotype at a fully heterozygote loci. Alleles that were not mutually shared by males, and by males and female were identified private alleles and tracked in allele phenotypes of AD larvae.

RESULTS

Survival rate of three species hybrids

AD embryos were detected in all groups. The percentage of abnormally divided embryos ranged from 5 to 10% in each group. Hatching was observed at 10 dpf (days post-fertilization). Survival of abnormally cleaved embryos was up to 49% at 4 dpf; 15–42% at 7 dpf, and 15–27% of AD survived to 14 dpf. Survival of normally cleaved embryos was 77–90% at 4 dpf; 76–88% at 7 dpf; and 76–81% by 14 dpf (Table 1, Fig. 1).

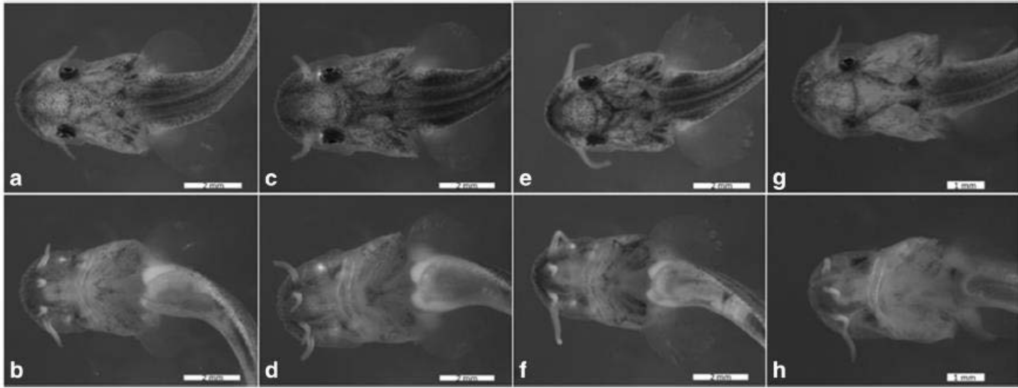

Figure 1. *Demonstration of 22-day post-fertilization larvae, (dorsal and ventral view) from the combination: A. baerii eggs x mix of sperm from A. ruthenus and A. gueldenstaedtii. a, b Produced three species larvae. Scale bar, 2 mm. c, d A. gueldenstaedtii larva. Scale bar, 2 mm. e, f A. baerii larva. Scale bar, 2 mm. g, h A. ruthenus larva. Scale bar, 1 mm.*

Ploidy level of obtained AD embryos

From 25 analyzed larvae, 88% of AD embryos were mosaic: 44% had 2n/3n ploidy (three-species progenies) (Fig. 2a), 40% had 2n/4n ploidy (polyspermic *A. baerii* x *A. gueldenstaedtii* larvae) and 4% had 2n/5n ploidy (unexpected ploidy). The remaining 12% of AD embryos consisted from: 4% of diploids, 4% of triploids, 4% of tetraploids (Table 1). Controls contained triploids and tetraploids, as it was expected (Fig. 2b).

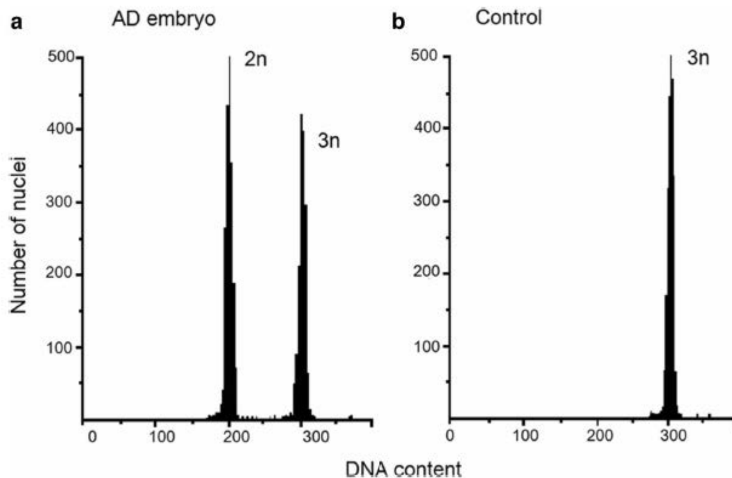

Figure 2. *Flow cytometry analysis showed three-parent AD embryos to be diploid/triploid mosaics. a The AD embryo had a diploid (2n) and a triploid (3n) peak. b A normally divided embryo at the hatching stage had a single triploid (3n) peak (A. baerii x A. ruthenus).*

Microsatellite genotyping

Fertilization *A. baerii* x *A. ruthenus* x *A. gueldenstaedtii*. Allele phenotypes of sample AD1 showed the presence of private alleles of both *A. gueldenstaedtii* male and *A. ruthenus* male at five informative loci *AfuG_135, Aox_27, Aox_45, Spl_101,* and *Spl_107*. At the most informative locus *Aox_45*, the specimen had five alleles clearly showing that the specimen originated by five genomes; two from diploid oocyte (with genotype 164/167) of tetraploid *A. baerii* female, two from diploid spermatozoon (with genotype 134/158) of tetraploid *A. gueldenstaedtii* male, and one from haploid spermatozoon (with genotype 155) of diploid *A. ruthenus* male. It clearly shows that specimen AD1 originated from heterospecific polyspermy (Table 2).

Allele phenotypes of the second specimen in this group (AD2) had only private alleles transmitted from *A. gueldenstaedtii* male with no private alleles from *A. ruthenus* male. At two informative loci *AfuG_135* and *Aox_27*, the specimen presented three phenotypic alleles transmitted from *A. gueldenstaedtii* male (Table 2). It shows that the specimen originated from conspecific polyspermy.

Fertilization *A. gueldenstaedtii* x *A. baerii* x *A. ruthenus*. Allele phenotypes of specimen AD3 showed the presence of private alleles of both *A. baerii* male and *A. ruthenus* male at four informative loci, i.e., *Aox_45, Spl_101, Spl_163,* and Spl_173. On contrary, allele phenotypes of the second specimen in this group had only private alleles derived from *A. ruthenus* male with no private alleles from *A. baerii* male. At the most informative locus *Spl_173*, the specimen AD3 inherited phenotypic allele 254 from diploid oocyte (genotype 254/254) of tetraploid *A. gueldenstaedtii* female; alleles 266 and 270 from diploid spermatozoon of tetraploid *A. baerii* male, and one allele 236 from haploid spermatozoon of diploid *A. ruthenus* male (Table 3). Specimen AD4 inherited two alleles 254 and 258 from diploid oocyte of tetraploid *A. gueldenstaedtii* female and two alleles 236 and 266 from two haploid spermatozoa of diploid *A. ruthenus* male (Table 3). It shows that the specimen AD3 originated from heterospecific polyspermy while the specimen AD4 from conspecific polyspermy.

Fertilization *A. gueldenstaedtii* x *A. gueldenstaedtii* x *A. ruthenus*. Allele phenotypes of the specimen AD5 showed the presence of private alleles of both *A. gueldenstaedtii* male and *A. ruthenus* male at three informative loci, i.e., *AfuG_135, Aox_45,* and *Spl 163*. The most informative was locus *Aox_45*. At this locus, the specimen AD5 had five alleles clearly showing that the specimen originated from five genomes, i.e., two from diploid oocyte (with genotype 140/158) of tetraploid *A. gueldenstaedtii* female, two from diploid spermatozoon (with genotype 134/176) of tetraploid *A. gueldenstaedtii* male, and one from haploid spermatozoon (with genotype 146) of diploid *A. ruthenus* male (Table 4). It clearly showed that specimen AD5 originated from heterospecific polyspermy. Other loci were not fully informative for the estimation of polyspermy; however, no locus was contradictory to the conclusion.

Discussion

This study is a first report about three interspecific parent fertilization in the animal kingdom. Here, we demonstrate sturgeon fertilization characteristics, great plasticity for hybridization between species and survival.

Our previous research described physiological polyspermy in sturgeons. Cytologically analyzed *A. ruthenus* and *A. baerii* embryos demonstrated high number of spermatozoa in the cytoplasm right after fertilization and that number of supernumerary spermatozoa is significantly decreasing before the first cleavage. At the same time, sometimes additional spermatozoa were participating in the development in a way "karyogamy with additional

plasmogamy". A sperm nucleus or nuclei destined to form an additional blastomere began developing independently and probably 1 cycle later than zygote (Iegorova et al. 2018). Our present work demonstrates that no significant intervention was needed to generate interspecific three-parent hybrids, where two species gave rise to a hybrid zygote and the third species contributed supplementary sperm-derived cells.

In Fig. 3 we list all possible variations that could be obtained using gametes from three species (as an example fertilization of *A. baerii* (4n) eggs with a mixture of sperm from *A. ruthenus* (2n) and *A. gueldenstaedtii* (4n)). Monospermic fertilization of *A. baerii* egg by a single *A. ruthenus* spermatozoon would produce a normally divided triploid embryo. If *A. baerii* egg was fertilized by a single *A. gueldenstaedtii* spermatozoa, the embryo should be a normally divided tetraploid. Due to multiple micropyles, *A. baerii* egg could be fertilized by several spermatozoa. Polyspermic fertilization of *A. baerii* eggs with several *A. ruthenus* spermatozoa should result in abnormally divided mosaics with ploidy level 1n/3n. Polyspermic fertilization of *A. baerii* egg by several *A. gueldenstaedtii* spermatozoa would produce an abnormally divided embryo with a mosaic ploidy of 2n/4n. Using gametes from three parents, and fertilizing *A. baerii* egg with *A. gueldenstaedtii* spermatozoa and *A. ruthenus* spermatozoa, would produce either 2n/3n or 1n/4n mosaic hybrid. The 2n/3n mosaics would be created if *A. ruthenus* sperm pronucleus fused with *A. baerii* egg pronucleus (3n) and the accessory *A. gueldenstaedtii* sperm pronucleus developed singly as 2n. However, 1n/4n mosaics would appear if *A. gueldenstaedtii* sperm pronucleus fused with *A. baerii* egg pronucleus (4n) and the accessory *A. ruthenus* sperm pronucleus developed singly as 1n.

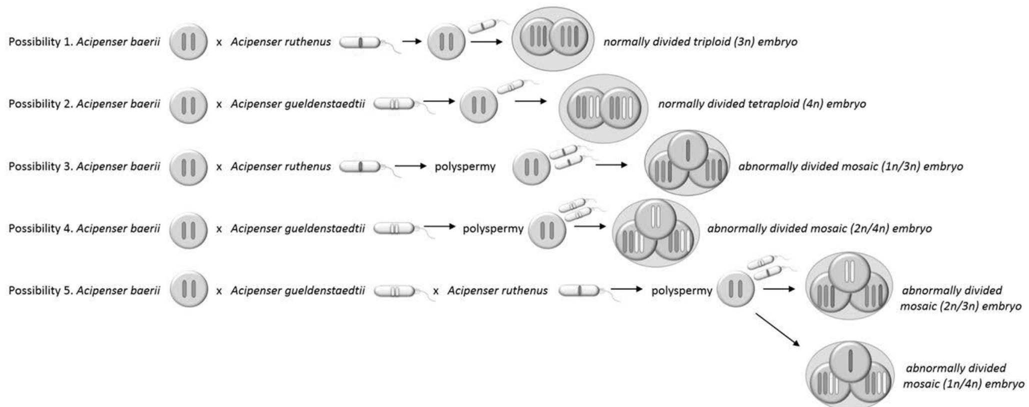

Figure 3. *A schematic illustration of all possible variations during fertilization of gametes from three parents using an example of A. baerii insemination by A. gueldenstaedtii and A. ruthenus spermatozoa.*

In our study, 88% of AD embryos displayed mosaic ploidy: 44% of polyspermic embryos from three species: *A. baerii* x *A. gueldenstaedtii* x *A. ruthenus*, 40% of polyspermic embryos from two species: *A. baerii* x *A. gueldenstaedtii*, and 4% of 2n/5n embryos, which could be an unexplained fusion of pronuclei. Triploids and tetraploids could be obtained due to asymmetrical division of blastomeres, as described by Dettlaff et al. (1993), which we could consider as AD embryos. Their ploidy suggests that they were monospermic progeny from two species. Besides mosaics, triploids and tetraploids, was detected one diploid fish. Probably it was occurred due to androgenotes: one decondensed sperm nuclei from *A. baerii* or *A. gueldenstaedtii* became a male pronuclei and continued development without fusion with female pronucleus and produced a diploid larva. Successful amplification of DNA fragments

from three sturgeon species proved the heterospecific polyspermy: three larvae contained alleles from three parents.

Survival of AD three parents' hybrid embryos

During the last few decades, it was believed to be fatal if more than one spermatozoa fused with the oocyte: polyspermic embryos develop abnormally and perish before hatching or can develop into abnormal larvae that subsequently die. However, Iegorova et al. (2018) showed that polyspermic embryos had almost the same survival rate as controls. Our present work demonstrates that three-parent hybrids still have high survival, although the survival rate was lower than the control (27 vs 81% at 14 dpf), which indicated that probably some combinations of interspecific pronuclei can be lethal. Interestingly, we did not find any 1n/4n mosaics (*A. baerii* x *A. gueldenstaedti* hybrid with *A. ruthenus* accessory sperm pronuclei) in our trials, which could represent a lethal combination.

Do AD embryos produce clonal gametes?

Morishima et al. (2004) have shown a diploid/triploid loach (*Misgurnus anguillicaudatus*) males produce clonal unreduced diploid spermatozoa. When normal diploid female was crossed with mosaic diploid/triploid male, only triploid progeny appeared and exhibited microsatellite genotypes with two alleles identical to the clonal genotype and one allele derived from female. When UV-irradiated eggs were fertilized by diploid spermatozoa from the mosaic male, they gave rise to the occurrence of androgenetic diploids and they exhibited microsatellite genotypes and DNA fingerprints, absolutely identical to those the natural clones. These results of Morishima et al. (2004) clearly concluded that the diploid-triploid mosaic male generate clonal diploid spermatozoa, with genetically identical genotypes.

In our previous research (Iegorova et al. 2018), we produced mosaic 1n/2n sterlet (*A. ruthenus*). We found that haploid cells were distributed among all germ layer derivatives, including gonads. Hypothetically, additional blastomeres produced by supernumerary spermatozoa exhibit the paternal genome exclusively. Thus, if these haploid-derived germ cells produce gametes, the gametes must carry only the paternal genome, and all haploid gametes be will clonal. If AD fish can produce clonal gametes, induction of multiple-sperm mosaicism might be a useful tool for production of isogenic strains in sturgeons in a short time.

CONCLUSION

Our findings indicated that sturgeons present a developmental pattern unique in the animal kingdom. This study clearly refutes statements about fatality of organisms if more than one spermatozoa fertilizes the oocyte, especially when gametes belong to interspecific animals. Here, we demonstrate a great plasticity in sturgeon hybridization, and easy ploidy manipulations, which could be an important strategy in aquaculture for mass production of clonal gametes.

ACKNOWLEDGMENTS

We thank all members of the Laboratory of Germ Cells, Faculty of Fisheries and Protection of Waters, University of South Bohemia in Ceske Budejovice for their support.

FUNDING INFORMATION

This study was supported by the Ministry of Education, Youth and Sports of the Czech Republic, projects CENAKVA (CZ.1.05/2.1.00/01.0024), "CENAKVA II" (LO1205 under the NPU I program), the Grant Agency of the University of South Bohemia in Ceske Budejovice (125/2016/Z), by the Czech Science Foundation (17-19714Y) and project Biodiversity (CZ.02.1.01/0.0/0.0/16_025/0007370).

COMPLIANCE WITH ETHICAL STANDARDS

All experimental procedures were performed in accordance with national and institutional guidelines on animal experimentation and care and were approved by the Animal Research Committee of the Faculty of Fisheries and Protection of Waters in Vodnany, Czech Republic.

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Table 1*. Survival rate of the AD embryos and ploidy level of 25 analyzed larvae that developed from AD embryos.*

Trial	Status of embryo	Total number of embryos	4 dpf	7 dpf	14 dpf	Ploidy level of AD larvae					
						2n	3n	4n	2n/3n	2n/4n	2n/5n
1	AD*	45	22 (49%)	19 (42%)	10 (22%)	1	1	1	0	3	0
	Normal	110	85 (77%)	84 (76%)	84 (76%)						
2	AD	156	34 (22%)	33 (21%)	33 (21%)	0	0	0	9	5	1
	Normal	410	330 (80%)	328 (80%)	323 (79%)						
3	AD	22	7 (32%)	6 (27%)	6 (27%)	0	0	0	1	0	0
	Normal	412	364 (88%)	364 (88%)	333 (81%)						
4	AD	20	9 (45%)	3 (15%)	3 (15%)	0	0	0	1	2	0
	Normal	424	380 (90%)	350 (82%)	332 (78%)						

AD means ‘atypically divided’ at the 2- to 4-cell stage.

Table 2. *Fertilization A. baerii ♀ x A. ruthenus ♂ x A. gueldenstaedtii ♂.*

Sample	Marker	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5	Allele 6	Allele 7	Allele 8
A. baerii	AfuG135	200				216			
A. ruthenus	AfuG135			208	212				
A. gueldenstaedtii	AfuG135		204		212		232		
AD1	AfuG135	200	204		212	216	232		
AD2	AfuG135		204		212	216	232		
A. baerii	Aox27			150		158			
A. ruthenus	Aox27		134						
A. gueldenstaedtii	Aox27	130		150	154		162		
AD1	Aox27	130	134	150			162		
AD2	Aox27	130		150	154		162		
A. baerii	Aox45		146				164	167	
A. ruthenus	Aox45		146		155				
A. gueldenstaedtii	Aox45	134		152		158			
AD1	Aox45	134			155	158	164	167	
AD2	Aox45	134				158	164		
A. baerii	Spl101			316	336	340			358
A. ruthenus	Spl101		300					352	
A. gueldenstaedtii	Spl101	296					350		
AD1	Spl101		300		336	340	350		
AD2	Spl101	296			336	340			
A. baerii	Spl107				308				
A. ruthenus	Spl107	296	300						
A. gueldenstaedtii	Spl107		300	304	308	312			
AD1	Spl107	296			308	312			
AD2	Spl107		300	304	308	312			
A. baerii	Spl163				224	264			
A. ruthenus	Spl163		212	220					
A. gueldenstaedtii	Spl163	200	212	220					
AD1	Spl163		212	220	224	264			
AD2	Spl163	200	212	220	224	264			
A. baerii	Spl173	236		254		262			
A. ruthenus	Spl173	236					266		
A. gueldenstaedtii	Spl173	236	246	254	260				
AD1	Spl173	236	246	254		262			
AD2	Spl173	236	246		260				

Table 3. *Fertilization A. gueldenstaedtii ♀ x A. baerii ♂ x A. ruthenus ♂.*

Sample	Marker	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5	Allele 6	Allele 7	Allele 8
A. gueldenstaedtii	AfuG135			216	224	228			
A. baerii	AfuG135	208		216					
A. ruthenus	AfuG135	208	212						
AD3	AfuG135	208		216	224				
AD4	AfuG135	208	212		224				
A. gueldenstaedtii	Aox27	130	134	162					
A. baerii	Aox27	130		162					
A. ruthenus	Aox27		134						
AD3	Aox27	130	134						
AD4	Aox27	130	134						
A. gueldenstaedtii	Aox45	134	140				158		173
A. baerii	Aox45				149	155		164	
A. ruthenus	Aox45			146		155			
AD3	Aox45				149	155	158		173
AD4	Aox45		140	146		155			173
A. gueldenstaedtii	Spl101			320	324	328			
A. baerii	Spl101	316					332		360
A. ruthenus	Spl101		300					352	
AD3	Spl101	316		320				352	360
AD4	Spl101			320	324			352	
A. gueldenstaedtii	Spl107			296	300	304	320		
A. baerii	Spl107	264	272		300				
A. ruthenus	Spl107			296	300				
AD3	Spl107	264	272	296	300		320		
AD4	Spl107			296	300	304			
A. gueldenstaedtii	Spl163	204			224	228	236		
A. baerii	Spl163					228		264	
A. ruthenus	Spl163		212	220					
AD3	Spl163			220	224	228	236	264	
AD4	Spl163	204	212				236		
A. gueldenstaedtii	Spl173			254	258	264			
A. baerii	Spl173		250				262	266	270
A. ruthenus	Spl173	236						266	
AD3	Spl173	236		254				266	270
AD4	Spl173	236		254	258			266	

Table 4. *Fertilization A. gueldenstaedtii ♀ x A. gueldenstaedtii ♂ x A. ruthenus ♂.*

Sample	Marker	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5	Allele 6	Allele 7	Allele 8
A. gueldenstaedtii	AfuG135			216	224	228			
A. gueldenstaedtii	AfuG135				224		240		
A. ruthenus	AfuG135	208	212						
AD5	AfuG135	208			224	228	240		
A. gueldenstaedtii	Aox27	130	134	162					
A. gueldenstaedtii	Aox27	130							
A. ruthenus	Aox27		134						
AD5	Aox27	130	134						
A. gueldenstaedtii	Aox45	134	140			158		173	
A. gueldenstaedtii	Aox45	134				158	170		176
A. ruthenus	Aox45			146	155				
AD5	Aox45	134	140	146		158			176
A. gueldenstaedtii	Spl101		320	324	328				
A. gueldenstaedtii	Spl101		320		328	332			
A. ruthenus	Spl101	300					352		
AD5	Spl101		320	324	328		352		
A. gueldenstaedtii	Spl107	296	300	304	320				
A. gueldenstaedtii	Spl107		300	304		368			
A. ruthenus	Spl107	296	300						
AD5	Spl107	296	300	304		368			
A. gueldenstaedtii	Spl163		204			224	228	236	
A. gueldenstaedtii	Spl163	188				224	228	236	
A. ruthenus	Spl163			212	220				
AD5	Spl163	188	204		220			236	
A. gueldenstaedtii	Spl173		254	258	264				
A. gueldenstaedtii	Spl173		254	258					
A. ruthenus	Spl173	236				266			
AD5	Spl173		254	258	264	266			

CHAPTER 4

GENERAL DISCUSSION
ENGLISH SUMMARY
CZECH SUMMARY
ACKNOWLEDGMENTS
LIST OF PUBLICATIONS
TRAINING AND SUPERVISION PLAN DURING THE STUDY
CURRICULUM VITAE

GENERAL DISCUSSION

Sturgeons produce one of the most valuable wildlife resources (Carmona et al., 2009). Unfortunately, they are currently listed as critically endangered (IUCN Red List). In order to renew the populations and to release healthy fishes into the wild, it is important to understand and control the fertilization process, and thus avoid detrimental genetic effects in sturgeon populations.

In this study (Chapter 2) we report on investigations into the types of fertilization in sturgeons. We determined the cause of abnormal embryo development and analyzed the ploidy and survival rate of the embryos. We classified the types of atypical embryos and estimated their frequencies. In addition, we discovered a novel probable method of ploidy manipulation for mass production of clones by generating AD embryos. Investigating hybridization potential in sturgeons, we produced a first hybrid from three interspecific parents, which were hybrids obtained among *A. gueldenstaedtii, A. baerii,* and *A. ruthenus.* Each descendant had one mother and two fathers from different species. The sperm pronucleus of one species was fused with the egg pronucleus of a second species, resulting in a diploid line, and the sperm pronucleus of the third species was then fused with the egg cytoplasm, which gave rise to a haploid line (Chapter 3).

1.1. TYPES OF FERTILIZATION IN STURGEON

Most animals display monospermy, where only one sperm penetrates the egg and joins the female gamete to form the zygote, thus becoming integral to its development. Several mechanisms have evolved to block polyspermy, the penetration of additional sperm into the oocyte. For example, sturgeon eggs are covered by multilayered envelopes: a jelly coat and a three-structured layer (Cherr and Clark, 1985). Jelly layers do not allow mass penetration of the spermatozoa into the egg; immediately upon the entry of the first spermatozoa, the egg's plasma membrane quickly blocks any additional sperm from penetrating the egg. The sperm that participates in fertilization releases a signal in the egg cytoplasm that disseminates rapidly through the entire egg cytoplasm ("fast block"). After fusion, the envelope (chorion) is separated from the egg by enlargement of the perivitelline space, and filling up with the material from cortical granules, thus blocking the entrance to other spermatozoa ("slow block") (Psenicka et al., 2010; Snook et al., 2011; Iwao, 2012).

However, polyspermy can still occur (Snook et al., 2011). In physiologically polyspermic eggs (such as in ctenophora, elasmobranchs, urodele amphibians, reptiles and birds) supernumerary spermatozoa always penetrate the egg, but syngamy happens with only one sperm nucleus, and a diploid organism is formed (Iwao, 2012). In taxa exhibiting pathological polyspermy, each sperm penetrates the egg, produces multiple microtubule-organizing centers, which can lead to developmental problems (this can happen in vertebrates and invertebrates as well) (Snook et al., 2011).

In contrast with teleost fishes, which have the only one micropyle in the egg (Kudo, 1991), sturgeon eggs have multiple micropyles. The presence of multiple micropyles or poor egg quality can result in the penetration of several spermatozoa into the egg (Dettlaff et al., 1993). In Chapter 2 we show that the number of micropyles in atypically divided embryos and the area occupied by them are significantly higher than the corresponding values in the controls. This means that embryos with high numbers of micropyles have greater chances of becoming polyspermic.

Histological observations on the micropyles of four randomly selected *A. baerii* (5 min pf) fixed embryos revealed an average of 12 spermatozoa within each egg. Analyzing the

cytoplasm, we detected an average of 6 sperm nuclei in 10 randomly selected *A. baerii* (5 min pf) embryos.

However, in fixed *A. ruthenus* embryos (from 10 min pf until 4-cell stage), we found that multiple sperm nuclei have a tendency to degenerate, such that embryos 10–60 min pf contained 0 to 10 nuclei, while at the 2- to 4-cell stage, no nuclei were found. Thus, although sturgeons eggs are capable of allowing multiple spermatozoa to penetrate because of the presence of numerous micropyles, additional spermatozoa are eliminated as the embryo develops. Sturgeon eggs thus demonstrate characteristics of physiological polyspermy. Similar results were obtained in *A. ruthenus* by Persov (Ginsburg et al., 1972), although numerous spermatozoa were found at various depths of the ooplasm of several eggs (from 2 to 28).

Ginsburg (1972) stated that monospermic fertilization is characteristic of sturgeons, after tests with different insemination volumes and observations of the developing eggs. It was concluded that while polyspermy may be obtained experimentally, it was distinctly pathological. In order to investigate this further, we performed fertilization tests with different sperm concentrations. Our results demonstrated that the number of atypically divided (AD) embryos tended to correlate positively with the sperm concentration used for insemination. However, increasing the sperm concentration beyond 200x did not result in any further increase in AD embryos, as there appears to be a limit to the amount of sperm that can enter the egg. Survival and morphological development of the embryos studied were similar to those of controls (described below).

During embryogenesis we observed atypically divided embryos, with 3, 5, 6, 7, 9, 10 cells at the 2–4 cell stage (normal cell division for sturgeon embryos is 2, 4, 8, 16, etc. cells). These AD cells were produced using highly concentrated spermatozoa for fertilization, although it is possible to produce AD embryos using low concentrations of spermatozoa also. We observed up to 20% of the AD embryos (similar to Dettlaff and Vassetzky, 1991), and found that the most frequent number of cells was six.

Ploidy analysis of the atypically divided *A. ruthenus* embryos revealed that they are n/2n mosaics (functional ploidy of normally developed *A. ruthenus* embryos is 2n). Investigating the origin of the haploid cells, we conclude that mosaicism in sturgeons has a paternal origin:

1) Atypically divided embryos, produced by fertilization of *A. ruthenus* (2n) ♀ with *A. gueldenstaedtii* (4n) ♂, had 2n/3n ploidy, which indicates that diploid cells had a paternal origin and appeared from the additional diploid spermatozoa; in case of second polar body retention, the ploidy would be n/3n or 4n, and 3n in case of normal development.
2) We produced AD embryos from a cross between *A. ruthenus* (2n) ♀ x *H. huso* (2n) ♂ fertilization, and obtained n/2n mosaics and their sorted haploid and diploid cells. We applied species-specific markers for identification of *A. ruthenus* and *H. huso* by the presence/absence of a specific band. The results demonstrated that the additional haploid set of chromosomes originated from the male (*H. huso*).

Based on these results we suggest that physiological polyspermy is characteristic in sturgeons, because:

- multiple spermatozoa can penetrate the egg due to the presence of numerous micropyles;
- high densities of sperm provoke the penetration of additional spermatozoa into the egg;
- the additional spermatozoa that have penetrated into the egg degenerate.

However, atypically developed embryos did not necessarily result from polyspermy (fusion of three or more gametes). Our observations indicated that plasmogamy could occur

simultaneously with the normal fusion of female and male pronuclei (karyogamy): a single sperm nucleus or multiple nuclei formed an additional blastomere and developed independently (probably one cycle later than that of the zygote). We use the term "polyspermy" to refer to the occurrence of AD embryos. However, embryos displaying "karyogamy with an additional plasmogamy" are multiple-sperm mosaics (Figure 1).

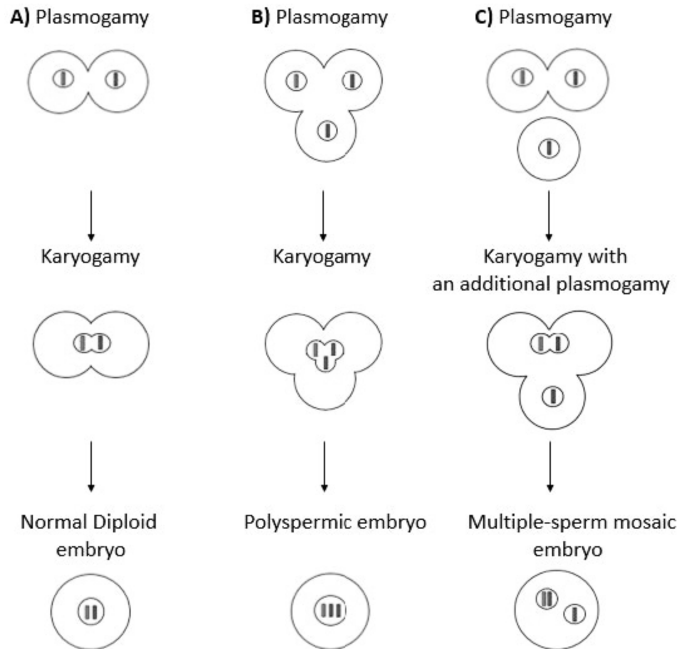

Figure 1. *Types of fertilization in sturgeons (example with haploid gametes). A). Normal fusion of sturgeon gametes, producing a diploid organism. B). Polyspermic embryo appearance (3n). C). An additional plasmogamy occurs at the same time as the karyogamy, producing multiple-sperm mosaic embryos (1n/2n ploidy).*

1.2. HYBRIDIZATION PLASTICITY IN STURGEONS

Sturgeons are the only group among vertebrates with interspecific and intergeneric hybrids displaying homogeneous or heterogeneous ploidy levels. Similarities in the ploidy level, karyotype and genome similarity among different species of the Order Acipenseriformes engender opportunities for relatively easy hybridization in the wild and under artificial conditions (Birstein et al., 1997; Billard and Lecointre, 2001; Havelka et al., 2011).

Our results in Chapter 2 demonstrate that multiple spermatozoa can penetrate the sturgeon egg. This situation can happen during conspecific fertilization or interspecific hybridization. Therefore, we were interested to investigate the plasticity in hybridization and the potential of sturgeon eggs to accept several interspecies spermatozoa with different ploidies, and the ability to develop further. We performed fertilization using three interspecific parents simultaneously: *A. ruthenus* (2n), *A. baerii* (4n), *A. gueldenstaedtii* (4n), in order to produce progeny that would develop after the fusion of the oocyte of one female and several spermatozoa from two males. In each group, up to 10% of the atypically developed embryos were obtained with ploidy levels of 2n/3n, 2n/4n, 2n/5n, 4n, 3n, and 2n. The majority (44% of all ADs) exhibited 2n/3n ploidy, which implies that they were produced from three

interspecific parents. We also analyzed five larvae, using microsatellite genotyping, to detect the presence of multiple spermatozoa in AD larvae. We found that three specimens were heterospecific polyspermic larvae, containing alleles from three parents, and two specimens were conspecific polyspermic larvae from two parents, which confirms the fertilization of eggs by several interspecific spermatozoa.

Hybridization has evidently played an important role in sturgeon evolution. Sturgeons are polyploid organisms and there is a high occurrence of polyploidy in sturgeon hybridization (Leggatt and Iwama, 2003). Considering chromosome formation and their composition, both possible mechanisms of polyploidy can be inferred: autopolyploidy and allopolyploidy (Zhou and Gui, 2017). Autopolyploids appear from a single diploid species, when multiple chromosome sets occur due to polyspermy or gynogenesis. Environmental changes, such as abnormally low temperatures in nature or cold shock in laboratory conditions, can also result in polyploidy, as shown by the occurrence of diploid gametes (Leggatt and Iwama, 2003). Interspecific or intergeneric hybridization may be the cause of allopolyploidy (Leggatt and Iwama, 2003; Zhou and Gui, 2017). Allopolyploidy can happen in nature due to anthropogenic factors: dam constructions, diversion of river water for irrigation (Speer et al., 2000), etc. Dams can block migration routes and disrupt natural signals, which lead to the accumulation of different sturgeon species and their spawning in the limited area at the same time (Havelka et al., 2011). Several evolutionary scenarios have been proposed to understand the origin of polyploidy in sturgeons, from several independent polyploid events in different lineages to numerous hybridization events in auto- and allopolyploid combinations (Rajkov et al., 2014). Perhaps physiological polyspermy, multiple-sperm mosaics (Chapter 2) and high levels of hybridization plasticity in sturgeons (Chapter 3) are some of the reasons for evolutionary ploidy changes, which need to be the subjects of future studies.

1.3. SURVIVAL RATE OF AD EMBRYOS

It has been believed for several decades that atypically dividing embryos die before hatching, or exhibit malformations, soon leading to death (Dettlaff and Vassetzky, 1991). We produced up to 20% of AD embryos by inseminating the eggs with highly concentrated sperm suspensions. Our results demonstrated that AD embryos have a survival rate that is similar to that of control fish (7 dpf: 35.7–100%, 14 dpf: 28.6–100% for ADs and 7dpf: 67.5–93.9%, 14 dpf: 62.7–86.5% for controls), and most of them developed into hatching larvae with normal morphology. Some malformed fish even survived into the feeding stage.

Hybrids produced from three interspecific parents also showed good survival, although it was lower than in the control fish: up to 42% at 7 dpf and up to 27% at the 14 dpf survival rate for the ADs and 88% at 7 dpf; 81% by 14 dpf for controls. Generation of the three-parent AD progeny was straightforward and did not need any significant intervention.

In our study we obtained viable mosaics, which contained haploid cells distributed in different parts of the body, including organs. Some organs of 4 month old fish demonstrated apparently normal ploidy (2n), while others contained mainly haploid cells (1n). It is expected that haploid organisms die during embryonic development. However Yamaki et al. (1999) described surviving haploid-diploid mosaic charr (*Salvelinus leucomaenis*) that showed no visible differences in morphology from normal charr. While there were no external abnormalities, the mosaics did include large populations of small erythrocytes (haploid) along with those of normal size (diploid). Furthermore, while the cells from the liver, spleen, and blood were haploid, the brain cells were diploid. These results demonstrate that haploid cells can be viable in a diploid environment.

1.4. FUTURE PROSPECTS FOR AD EMBRYOS AND UTILITY OF MULTIPLE-SPERM INDUCED MOSAICISM AS A CHROMOSOME MANIPULATION TECHNIQUE FOR MASS CLONING OF STURGEON

In Chapter 1 we described the importance of chromosome manipulation techniques in aquaculture. One of main goals of chromosomal manipulation is the production of clones. However, the survival rate of cloned fish is very low due to the side effects of the treatment (Arai, 2001).

Gynogenesis is a technique that can be used to obtain all-maternal inheritance in progeny, with a rapid generation of inbred lines. Conversely, androgenesis can be used to obtain exclusively paternal inheritance. These techniques can be used for rapid generation of inbred lines and in recovery programs for endangered species using cryopreserved sperm (Thorgaard, 1986).

Unreduced gametes are expected to be clonal and can be used to produce gynogenetic triploid progeny, using triploid female gametes and fertilizing them by UV-irradiated heterospecific sperm (Arai and Mukaino, 1997). Yamaki et al. (1999) reported that haploid-diploid amago salmon laid haploid and unreduced diploid eggs, and produced both diploid and triploid progeny when its oocytes were fertilized with haploid spermatozoa of a normal diploid male.

In the above, we have described *A. ruthenus* AD embryos and 4-month old fish with a mix of haploid and diploid cells. Haploid cells were distributed among all germ layers, including gonads. Although we could not confirm whether germ cells were generated from the haploid cells, there is no reason to assume that haploid cells cannot differentiate into germ cells. Saito and Psenicka (2014, 2015) have shown that primordial germ cells (PGC) are specified by inheritance of germplasm in sturgeon. Thus, it can be assumed that haploid nuclei can reach the region in which germplasm is rich enough to specify and form PGCs, as normal nuclei do. Additional blastomeres produced by supernumerary sperm are expected to contain the paternal genome exclusively. Thus, if these haploid-derived germ cells produce gametes, the gametes must carry the paternal genome only, and thus all haploid-derived gametes will be clonal (Iegorova et al., 2018).

To avoid genetic contamination caused by haploid cells in artificial reproduction, it is important to know if haploid cells can produce PGCs and if so, whether they differentiate into gametes. On the other hand, if AD fish can produce haploid-derived clonal gametes, induction of multiple-sperm mosaicism might be a useful tool for production of isogenic strains in sturgeons in a short time frame (Iegorova et al., 2018).

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ENGLISH SUMMARY

Polyspermy produces viable mosaics in sturgeon

Iegorova Viktoriia

Sturgeons (Acipenseridae) are an ancient family with unique biological characteristics. A long evolutionary history, polyploidy, hybridization between species and genera, and the presence of multiple micropyles, make this family unique.

Sturgeons are the source of black caviar, the most expensive luxury roe. They reach reproductive age rather late, with as many as twenty-eight years before first spawning in some species, and spawning does not happen annually for the same individual. Multiple human activities such as overfishing, dam construction, and pollution, coupled with long restoration times, have made much of this family critically endangered, and their conservation now depends on artificial reproduction in hatcheries.

Not much is known about the mechanism of fertilization in sturgeons. This has been a topic of debate for a long time, and different fertilization mechanisms have been proposed for sturgeon fertilization: monospermy, physiological and pathological polyspermy. Here, we determined in our study that physiological polyspermy is the correct type of fertilization in sturgeons. Multiple spermatozoa are able to penetrate the egg, but they tend to degenerate over time. However, when the sperm concentration is high, fertilization can occur forming multiple-sperm mosaics, which are distinguished by the atypical cleavage at the 2–4 cell stage as 3, 5, 6, 7, 9, 10 cells, or after hatching using flow cytometry. Apart from normal fusion of the female and male pronuclei, these mosaics occur as a result of additional "plasmogamy," fusion of sperm and egg cytoplasm without fusion of their nuclei. All parts of the body (head, body, tail) of these fishes have different ploidies. Organs of four-month old fish were found to exhibit ploidy variation: haploid, diploid, haploid-diploid mosaic. These individuals showed a high survival rate, and morphological development was similar to that of control fish. These results indicate that uncontrolled fertilization and release of multiple-sperm mosaics into the wild under restoration programs can result in sturgeons with irregular ploidy, and induce detrimental genetic effects on sturgeon populations. Ploidy variation in mosaic fishes indicates that ploidy analysis of just one part of body (blood, tail, etc.) is not representative of the ploidy level of the individual.

Investigation of ploidy of the atypically divided hybrid embryos produced from *A. ruthenus* (2n) female and *A. gueldenstaedtii* (4n) male, and application of species-specific markers on the AD hybrid produced from *A. ruthenus* (2n) female and *H. huso* (2n) male, clearly showed that additional sets of chromosomes have a paternal origin. These mosaic fishes are expected to produce gametes with the paternal genome exclusively. This means that AD fish can produce clonal gametes and the induction of multiple-sperm mosaicism might be a useful tool for the rapid production of isogenic strains of sturgeons.

We found that sturgeons exhibit great plasticity in hybridization. The presence of numerous micropyles allows the penetration of several spermatozoa into the egg and their contribution to the developing embryo even when this fertilization occurs among three interspecific parents. Such individuals exhibit a mosaic ploidy, but have a good survival rate, and can be produced without significant intervention.

Our findings indicated that sturgeons present a developmental pattern unique in the animal kingdom. This study clearly refutes statements about mortality in sturgeons if more than one sperm fertilizes the oocyte, especially when gametes belong to interspecific fish.

We believe that findings of this study can help avoid negative effects of current sturgeon propagation programs, and will be useful in breeding and genomics research.

CZECH SUMMARY

Polyspermie u jeseterů dává vzniknout životaschopným mozaikám

Iegorova Viktoriia

Jeseteři (Acipenseridae) jsou starobylá skupina živočichů s unikátními biologickými charakteristikami, jako je dlouhá evoluční historie, polyploidie, možná hybridizace mezi druhy i rody a jikry s mnohočetnými mikropylárními otvory – to vše dělá tuto skupinu jedinečnou.

Jeseteři jsou zdrojem černého kaviáru, nejdražších luxusních jiker. Reprodukčního věku dosahují poměrně pozdě, u některých druhů dochází k prvnímu tření okolo 28 let s tím, že tření není u všech jedinců pravidelné. Dlouhá doba pro obnovu spojená s mnohými lidskými činnostmi jako je: nadměrný rybolov, výstavba přehrad a znečištěné prostředí činí tuto skupinu kriticky ohroženou a jejich ochrana je nyní závislá na umělé reprodukci.

Mechanizmus oplození u jeseterů není moc probádaný a je již dlouhou dobu předmětem mnoha zkoumání. Na toto téma byly vedeny dlouholeté diskuse, přičemž jeseterům byly přisuzovány různé mechanizmy oplození monospermie, fyziologická a patologická polyspermie. V naší práci jsme zjistili, že jeseteři se rozmnožují pomocí tzv. fyziologické polyspermie. Při oplození do vajíčka proniká více spermií, které postupně degradují. Ovšem při použití více koncentrovaného spermatu může dojít ke vzniku polyspermní mozaiky, kterou lze rozlišit díky atypickému rýhování ve stadiu 2.–4. buňky jako 3-, 5-, 6-, 7-, 9-, 10- buněk nebo po vykulení pomocí průtokové cytometrie. Kromě normálního splynutí samičího a samčího prvojádra dochází k tzv. plazmogamii, splynutí cytoplazmy spermie a vajíčka bez splynutí jader. Všechny části těla (hlava, tělo, ocas) těchto ryb mají rozdílnou ploidii. Orgány studovaných čtyřměsíčních ryb vykazují ploidní variance: haploidní, diploidní nebo haploidní-diploidní mozaiky. Tito jedinci mají vysokou míru přežití a morfologický vývoj je podobný kontrole. Z toho vyplývá, že nekontrolované oplození a vypouštění těchto mozaik do volné přírody v rámci reintrodukce může způsobit výskyt jeseterů s nepravidelnou ploidií a indukovat škodlivé genetické účinky na populace jeseterů. Různé ploidie u mozaik naznačují, že analýza ploidie pouze z jedné části těla (krev, ocas atd.) není reprezentativní pro celkovou ploidní úroveň jedince.

Zkoumání ploidie atypicky se rýhujících hybridních embryí, produkovaných křížením mezi *A. ruthenus* (2n) samicí a *A. gueldenstaedtii* (4n) samcem, a aplikace druhově specifických markerů na AD hybrida, vzniklými křížením mezi *A. ruthenus* (2n) samicí a *H. huso* (2n) samcem, prokazuje, že dodatečný set chromozomů je paternálního původu. Hypoteticky by tyto mozaiky měly produkovat gamety pouze s otcovským genomem, neboť hybridní zárodečné buňky v tomto případě nejsou schopny projít meiózou. To znamená, že AD hybridi mohou produkovat klonální gamety a jejich cílená indukce může být užitečným nástrojem pro rychlou produkci isogenních linií jeseterů.

Zjistili jsme, že jeseteři nabízejí vysokou míru plasticity, co se týče hybridizace. Četné mikropylární otvory umožňují penetraci mnoha spermií do vajíčka a podílejí se na vývoji, i pokud oplození nastává mezi třemi rodiči různých druhů. Tito jedinci vykazují také ploidní mozaiku, přežívají a mohou být snadno produkováni bez jakéhokoli významného zásahu.

Naše zjištění ukázaly, že jeseteři představují vývojový vzorec jedinečný v živočišné říši. Tato studie jednoznačně odmítá výroky o neživotaschopnosti jeseterů, jestliže více spermií oplodní oocyt, zvláště pokud gamety patří jedincům odlišných druhů.

Věříme, že zjištění z této studie mohou pomoci vyhnout se negativnímu vlivu polyspermních mozaik v reintrodukčních programech jeseterů a mohou být užitečné pro chovný a genomický výzkum.

ACKNOWLEDGMENTS

I would like to express my gratitude to my supervisor Taiju Saito, Ph.D. for his kind help during my study, support and patience. His creativeness in science teached me that nothing is impossible. It was a big honor for me to be his student.

I am also grateful to my consultant Assoc. Prof. Martin Pšenička for his advises and help during my work. He was available to help in all coming tasks any time. His managing of our laboratory was excellent. Thank you, for a good atmosphere and a nice sense of humor, which also was needed, especially during spawning seasons.

I am grateful for a kind help to all members of our Laboratory of Germ Cells.

And I would like to express a big gratitude to Martin Kahanec for his help during spawning.

I was glad to make Ph.D. study in the University of South Bohemia in České Budějovice.

This study was financially supported by

- Ministry of Education, Youth and Sports of the Czech Republic – projects "CENAKVA" (No. CZ.1.05/2.1.00/01.0024), "CENAKVA II" (No. LO1205 under the NPU I program), project Biodiversity (CZ.02.1.01/0.0/0.0/16_025/0007370), by the Czech Science Foundation (project No. 17-19714Y), Grant Agency of the University of South Bohemia in České Budějovice (projects No.125/2016/Z).

LIST OF PUBLICATIONS

PEER-REVIEWED JOURNALS WITH IF

Iegorova, V., Psenicka, M., Lebeda, I., Rodina, M., Saito, T., 2018. Polyspermy produces viable haploid/diploid mosaics in sturgeon. Biology of Reproduction 99: 695–706. (IF 2017 = 3.184)

Iegorova, V., Psenicka, M., Saito, T., 2018. First evidence of viable progeny from three interspecific parents in sturgeon. Fish Physiology and Biochemistry. (doi: 10.1007/s10695-018-0553-6.) (IF 2017 = 1.735)

Fatira, E., Havelka, M., Labbe, C., Depince, A., **Iegorova, V.**, Pšenička, M., Saito T., 2018. Application of interspecific Somatic Cell Nuclear Transfer (iSCNT) in sturgeons and an unexpectedly produced gynogenetic sterlet with homozygous quadruple haploid. Scientific Reports 8: 5997. (IF 2017 = 4.122)

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ABSTRACTS AND CONFERENCE PROCEEDING

Iegorova V., Psenicka M., Saito T., 2015. Abnormal cleavage patterns produce haploid/diploid mosaicism in sturgeon (*Acipenser ruthenus*). In: Book of Abstracts. 5th International Workshop on the Biology of Fish Gametes. 7–11 September, 2015, Ancona, Italy (oral presentation).

Iegorova, V., Pšenička, M., Saito, T., 2016. Polyspermy produces haploid/diploid viable mosaics in sterlet (*Acipenser ruthenus*). The 22nd International Congress of Zoology. November 14–19, 2016, Okinawa, Japan (poster).

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Iegorova, V., Psenicka, M., Saito, T., 2017. Sturgeon phenomenon: first evidence of progeny from three parents. 6th International Workshop on the Biology of Fish Gametes. September 4–7, 2017, Vodnany, Czech Republic (oral presentation).

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Iegorova, V., Psenicka, M., Saito, T. Physiological polyspermy in sturgeons. 11th International symposium on Reproductive Physiology of fish. June 3–8, 2018, Manaus, Brazil (poster).

Iegorova, V., Havelka, M., Psenicka, M., Saito, T., 2018. Heterospecific polyspermy in sturgeons. Sustaining iconic diadromous fishes. June 18–20, 2018, Arendal, Norway (poster).

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Iegorova, V., Pšenička, M., Saito, T., 2016. Polyspermy produces haploid/diploid viable mosaics in sterlet (*Acipenser ruthenus*). The 22nd International Congress of Zoology. November 14–19, 2016, Okinawa, Japan (poster).	2016
Iegorova, V., Psenicka, M., Saito, T., 2017. Sturgeon phenomenon: first evidence of progeny from three parents. 6th International Workshop on the Biology of Fish Gametes. September 4–7, 2017, Vodnany, Czech Republic (oral presentation).	2017
Iegorova, V., Psenicka, M., Saito, T., 2018. Physiological polyspermy in sturgeons. 11th International Symposium on Reproductive Physiology of fish. June 3–8, 2018, Manaus, Brazil (poster).	2018
Iegorova, V., Havelka, M., Psenicka, M., Saito, T., 2018. Heterospecific polyspermy in sturgeons. Sustaining iconic diadromous fishes. June 18–20, 2018, Arendal, Norway (poster).	2018

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