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Research Article

Polyspermy produces viable haploid/diploid mosaics in sturgeon[†]

Viktoriia Iegorova[1],[*], Martin Psenicka[1], Ievgen Lebeda[1], Marek Rodina[1] and Taiju Saito[1],[2]

[1]Faculty of Fisheries and Protection of Waters, University of South Bohemia in Ceske Budejovice, Zátiší 728/II, Vodnany, Czech Republic and [2]Nishiura Station, South Ehime Fisheries Research Center, Ehime University, Uchidomari, Ainan, Ehime, Japan

[*]**Correspondence:** Faculty of Fisheries and Protection of Waters, University of South Bohemia in Ceske Budejovice, Zátiší 728/II, 389 25 Vodnany, Czech Republic. Tel: +420 776 587 948; E-mail: iegorova@frov.jcu.cz

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.

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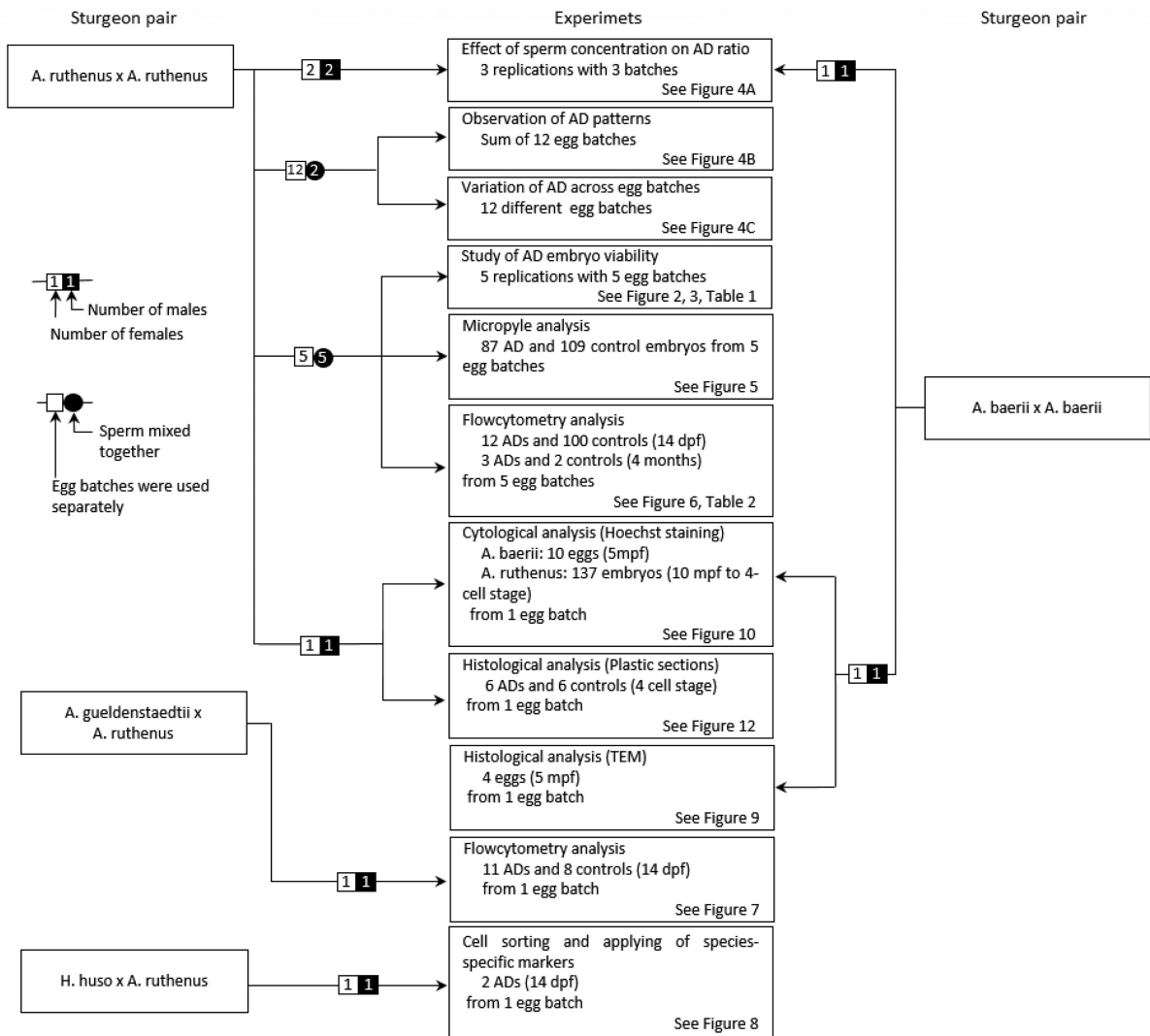
Figure 1. Flow diagram of the study. A different pair of sturgeons were used in each experiment/analysis.

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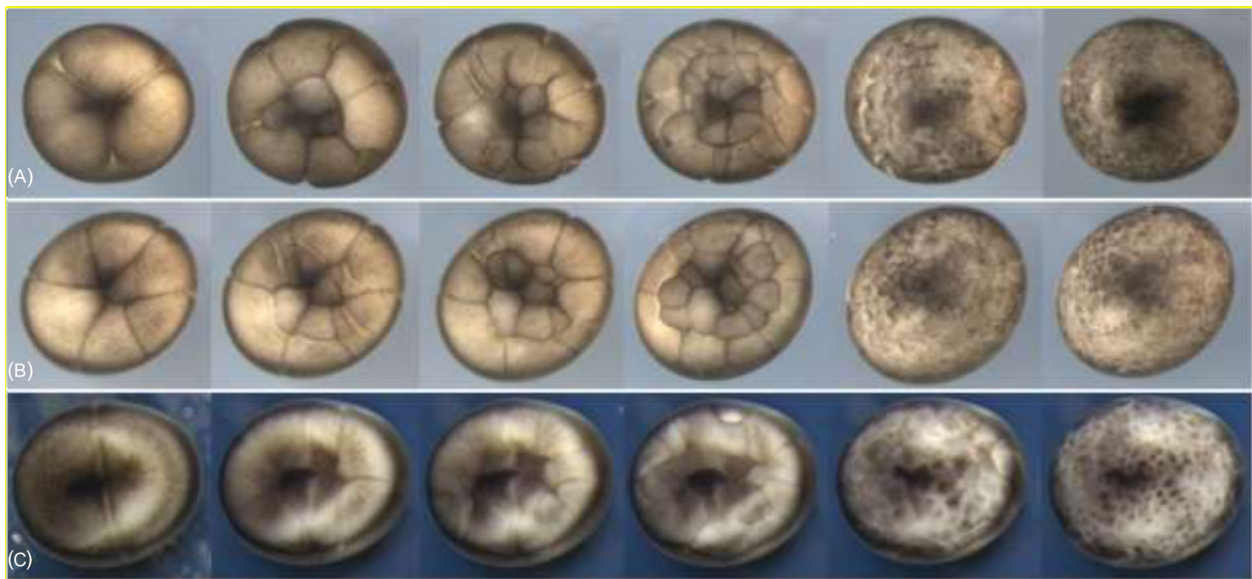
Figure 2. AD blastomeres of *A. ruthenus* embryo showed continuous division during 2- to 256-cell stage. (A) 3-cell AD embryo, (B) 6-cell AD embryo, and (C) normally divided embryo.

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)		

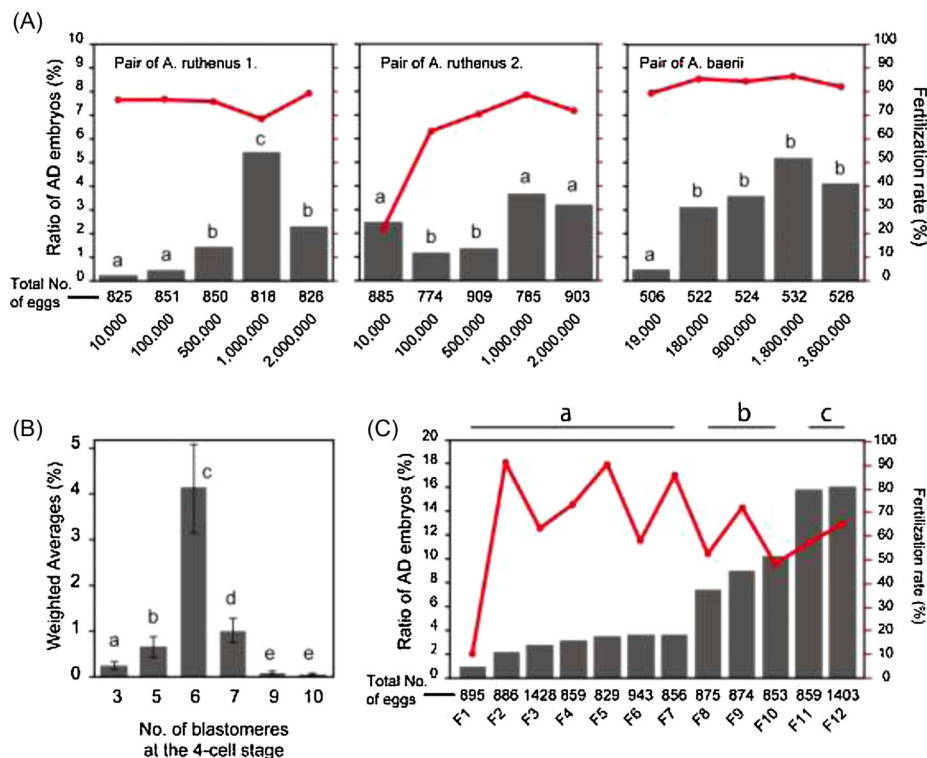"AD" indicates "atypically divided" at the 2- to 4-cell stage.

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
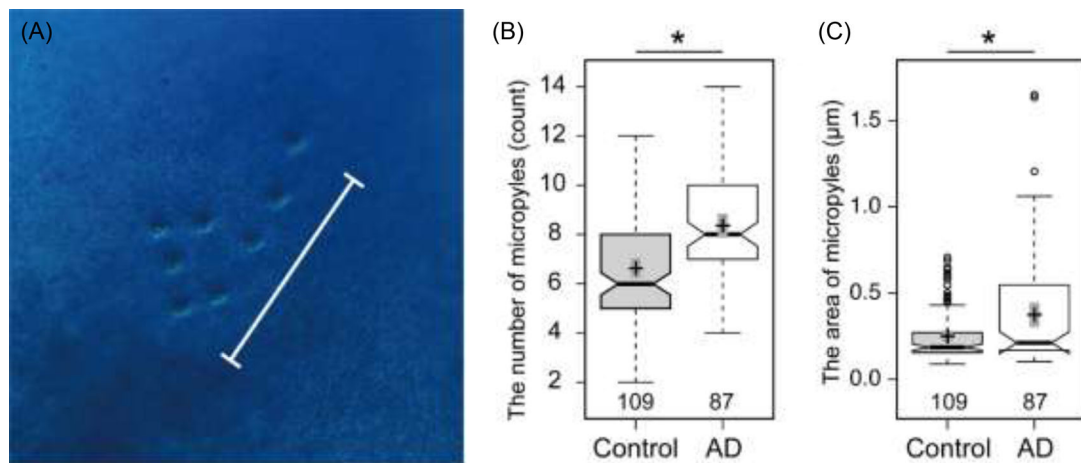
Figure 5. The AD embryos have their origin from eggs with significantly larger numbers of micropyles that also had larger coverage, when compared to normal eggs. (A) Methylene blue stained micropyles of AD *A. ruthenus* embryo. Eight micropyles are visible. The coverage of the micropyles was calculated 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.
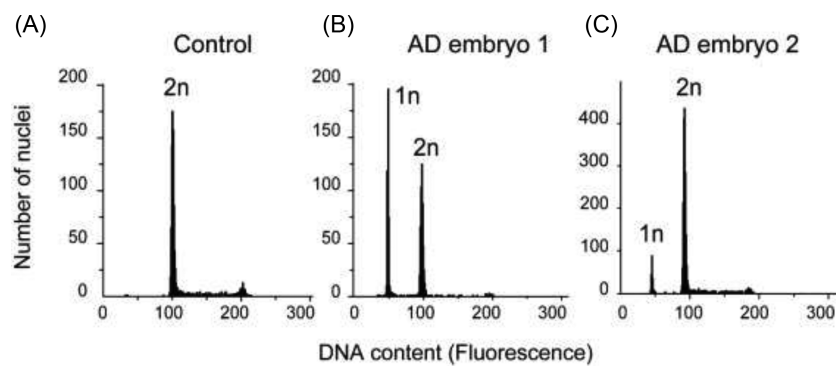

Figure 6. Flow cytometry analysis showed *A. ruthenus* AD embryos to be haploid/diploid mosaics. (A) A normally dividing embryo at the hatching stage with a single diploid (2n) peak. (B) AD embryo 1 with one haploid (1n) and one diploid (2n) peak. In this embryo, the haploid peak is higher than the diploid 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.
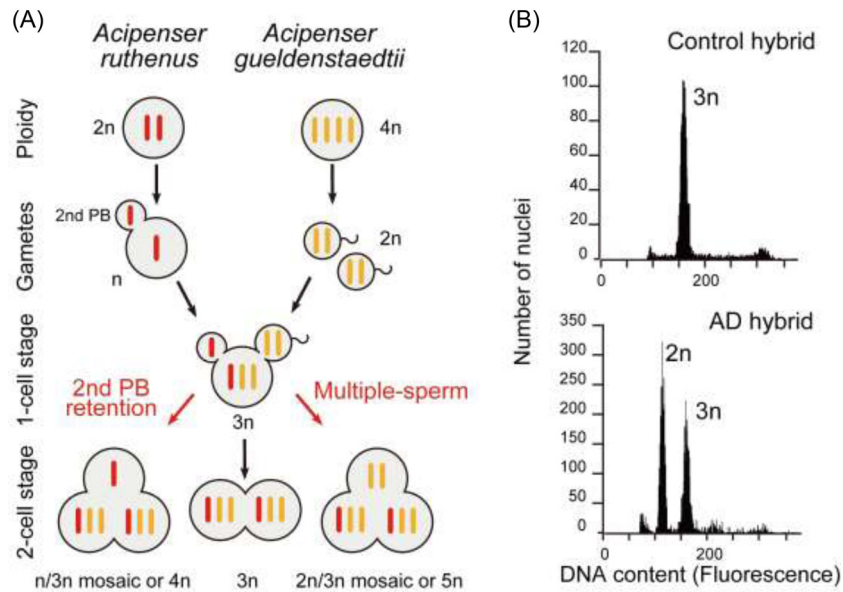
Figure 7. Intraspecific fertilization indicated AD embryos appearance after numerous spermatozoa penetration. (A) Experimental design to test the second polar body (PB) retention and polyspermy hypotheses. The functional ploidy levels of each fish are 2n (*A. ruthenus*) and 4n (*A. gueldenstaedtii*). 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.
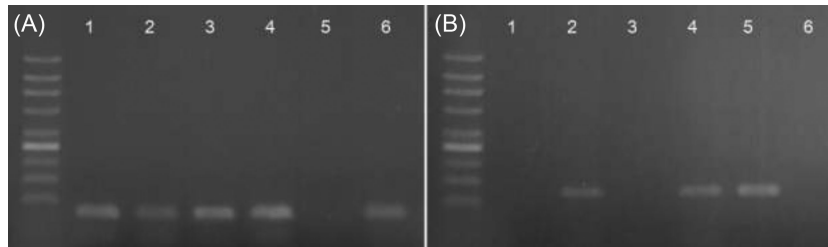

Figure 8. Identification of *H. huso* and *A. ruthenus* by presence/absence of a band. (A) Amplification of species-specific primer 153_HHp with combination 153_uni (*H. huso*). (B) Amplification of species-specific primer 247_ARp with combination 247_uni (*A. ruthenus*). 1 = haploid cells from sample 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
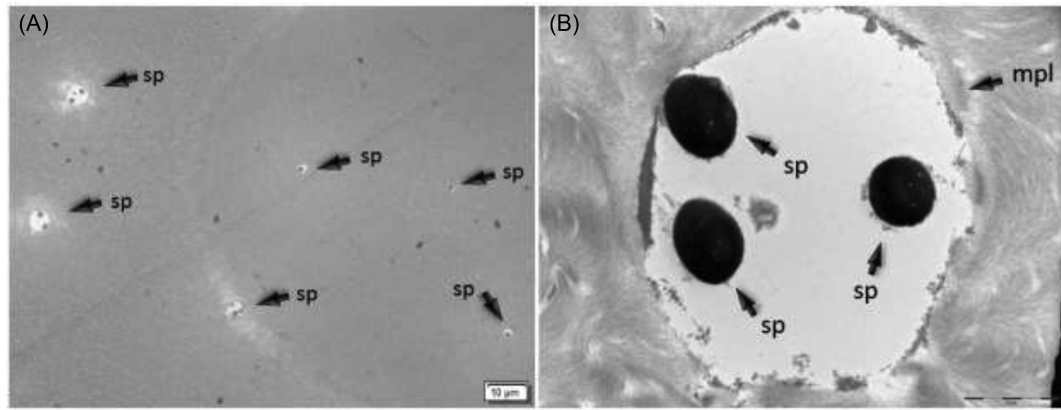
Figure 9. Penetration of numerous spermatozoa into *A. baerii* egg. (A) Multiple spermatozoa in the micropyles. Transmission electron microscope (TEM). Scale bar: 10 μm. (B) TEM demonstrates three spermatozoa inside of one micropyle. Scale bar: 2 μm. Sp = spermatozoa, mpl = micropyle.
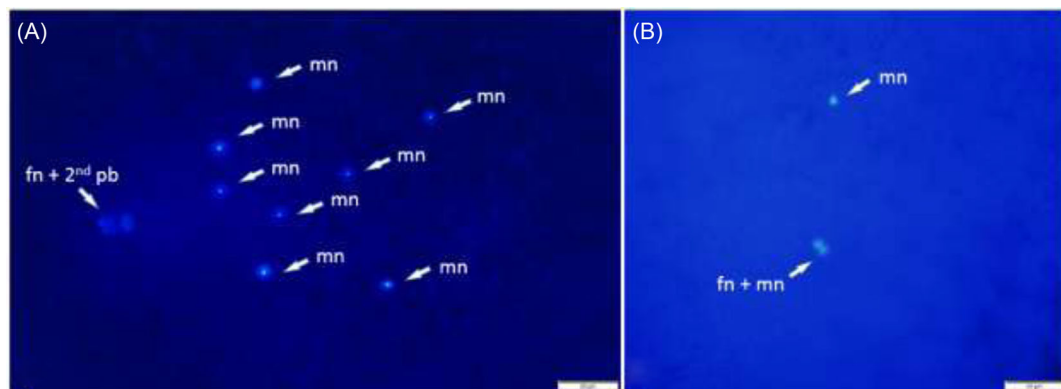

Figure 10. Hoechst-stained nuclei. (A) *A. baerii* embryo 5 min after fertilization. (B) *A. ruthenus* embryo 30 min after fertilization. Mn = male nucleus, fn = female nucleus, 2nd pb = second polar body. Scale bar: 50 μm.

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
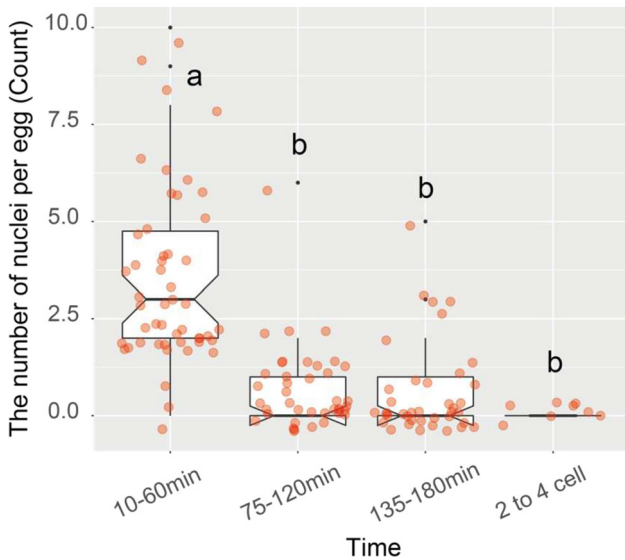
Figure 11. The box plots demonstrate the number of nuclei in *A. ruthenus* eggs in groups 10–60 min, 75–120 min, 135–180 min, and 2- to 4-cell stage. Box plots: center lines show the medians; box limits indicate the interquartile range (between the 25th and 75th percentiles); whiskers extend 1.5 times 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.
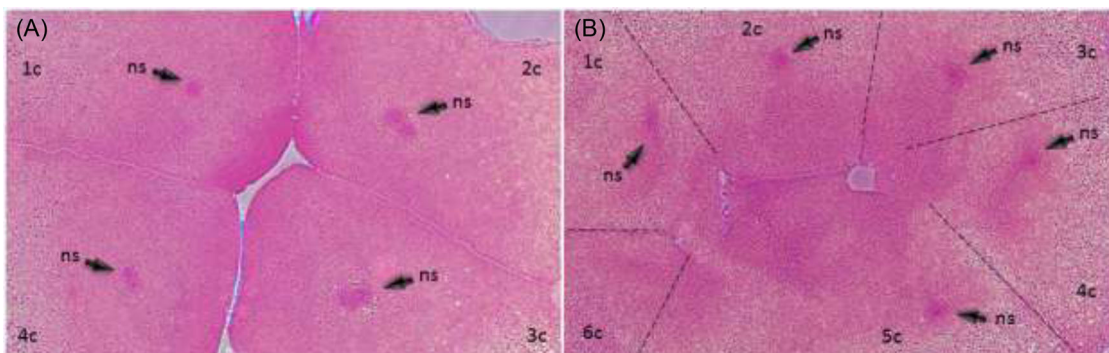

Figure 12. Histological section of *A. ruthenus* embryos. (A) Control 4-cell stage embryo. (B) Abnormal 6-cells embryo. Ns = nucleus, c = cell.

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

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