

**INDUCTION OF MEIOTIC GYNOGENESIS AND POLYPLOIDY IN
WHITE STURGEON (*ACIPENSER TRANSMONTANUS* RICHARDSON)**

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Abstract

White sturgeon (*Acipenser transmontanus*) eggs fertilized with untreated or UV-irradiated sperm (180-270 s, 1200 $\mu\text{W}/\text{cm}^2$) were subjected to either an early heat shock (32-34°C, 12-15 minutes post fertilization, 1-10 min duration), or an early cold shock (3°C, 12-15 minutes post fertilization, 15-180 min duration) to produce polyploids and meiotic gynogens. Control hatch rates ranged from 72-84%, compared with <1-21% for the gynogens. Ploidy analysis was performed on 2401 individuals using a Coulter Counter to analyze the particle size distribution of erythrocyte nuclei. Ploidy was confirmed for a subsample of 162 of these fish using flow cytometry. Efficiency of second polar body retention for the different temperature shocks ranged from 1-100%. In addition to the expected diploid and triploid fish, ploidy analysis of erythrocytes also revealed the existence of three tetraploid, six predominantly-haploid mosaic, and one diploid-tetraploid mosaic fish. The parental origin of gynogenetic, predominantly-haploid mosaic, and tetraploid fish was examined using sire and dam-specific Random Amplified Polymorphic DNA (RAPD) markers.

Introduction

Artificial induction of gynogenesis and polyploidy in sturgeon species is of interest to both commercial aquaculturists and researchers investigating the developmental biology and genetics of chondrosteian fish. The specific mechanisms of sex determination in chondrosteians are not known, and the effects of ploidy manipulation on sex segregation ratios in sturgeon have not been described. The potential production of all female populations would be of obvious interest to sturgeon caviar producers. The objective of this investigation was to produce gynogenetic, triploid and diploid control white sturgeon to allow for the eventual examination of sex differentiation and segregation ratios in these groups of fish. The earliest work on the use of artificial techniques for the production of meiotic gynogenetic sturgeon was reported by Romashov et al. (1963) but in this study none of the gynogenetic larvae survived beyond 192 days post-hatch. Preliminary experiments on the induction of gynogenesis in white sturgeon failed to produce any viable gynogens (Kowtal, 1987).

Material and Methods

Gamete collection

Experiments were conducted at the U.C. Davis Aquatic Center in the Spring of 1995, using as a broodstock the first hatchery generation of white sturgeon raised in captivity. Spawning induction and gamete collection was as described by Conte et al. (1988). Each of four females (experiments 1 through 4, respectively) were induced to spawn by injections of 40 µg/kg body weight of the gonadotrophin-releasing hormone analogue [D-Ala⁶, Pro⁹-NET]-GnRH. Males were induced to spermiate by a single injection of 1.5 mg/kg common carp pituitary extracts.

Experimental Design

Eggs were subjected to an early temperature shock (32-34°C, 1-10 min duration; or 3°C, 15-180 min duration) after fertilization with either untreated or UV-irradiated sperm to produce polyploids and meiotic gynogens, respectively (see Table 2). Temperature shocks were applied at 15 min post-fertilization in experiments 1 and 4, and at 12 min post-fertilization in experiments 2 and 3. All experiments included a diploid control group, a haploid control group to determine the efficacy of UV inactivation of the sperm (untreated eggs and UV-irradiated sperm), a triploid control group to determine the efficacy of second polar body retention (temperature shocked eggs and untreated sperm), and a treatment designed to induce gynogenesis (temperature shocked eggs and UV-irradiated sperm).

Sperm Treatment

For UV-irradiation, 2 ml of sperm stored in an atmosphere of pure oxygen at 4°C was diluted with 18 ml of seminal fluid (supernatant from surplus semen centrifuged at 6000 rpm for 20 min) and put into a rectangular pyrex dish (17 x 12 cm) to a depth of approximately 1 mm. This dish was placed on a gently rotating platform (90 rpm) 26-28 cm below two 15 W UVC bulbs (NIS G15T8) which provided an incident light intensity of 1200 µW/cm² as measured by the UVC probe of a UVX radiometer. The duration of UV-irradiation was adjusted for each batch of sperm such that the motility of the activated sperm was reduced to approximately 50%. Sperm was treated with UV irradiation for 180 to 270 seconds. At the completion of irradiation, 380 ml of 16°C water from the incubation system was added to the irradiated sperm suspension, and this mixture was immediately added to ova. The addition of the sperm suspension to the ova was considered to be the time of fertilization. Procedures following the UV-irradiation of the sperm were performed in the absence of visible light to prevent the possible photoreactivation of the sperm's DNA. A portion of each batch of untreated sperm was used to fertilize ova in the control diploid and polyploid treatment groups. The fertilization technique for these groups was as described except that 2 ml of undiluted sperm was added to 398 ml of incubation water immediately prior to fertilization.

Ova Treatment

Ova in coelomic fluid were maintained in a 4 l glass beaker at 16°C. For each treatment group 10-25 ml of ova were poured into a 190 mm diameter crystallizing dish and 400 ml of the appropriate sperm suspension was immediately added and the mixture was slowly stirred for three minutes. At this time the eggs were rinsed with 800 ml of fresh water four times to ensure that no viable sperm entered the incubation system. The eggs became sticky upon activation and they were quickly dispersed with a plastic transfer pipette to form a single layer across the bottom of the crystallizing dish. All dishes were kept at 16°C until the temperature shock. Heat shock treatments involved pouring the 16°C water off the eggs adhered to the bottom of the bowl, and immediately immersing the bowl in an oxygenated water bath set at the appropriate temperature. At the termination of the temperature shock the bowl was immediately immersed in a 15 l round fiberglass tank located within a semi-recirculating hatching system (16°C ± 0.5°C). Eggs that received a cold temperature shock were treated as described, using prechilled oxygenated water and a 3°C incubator for the duration of the cold shock treatment.

Verification of Gynogenesis

Gynogenesis was verified by the analysis of sire and dam-specific random amplified polymorphic DNA (RAPD) markers generated by the polymerase chain reaction (PCR) (Van Eenennaam et al., submitted). DNA was obtained from a single barbel which was rinsed in 200 µl sterile H₂O, and placed in a 500 µl eppendorf tube with 200 µl of 5% chelex, and 20 µg Proteinase K. The tube was then vortexed, incubated at 55°C for 2-3 hours, heated to 95°C for 12 minutes, and spun at 14,000 rpm in a microcentrifuge for 10 minutes. One hundred microliters of the clear supernatant was then removed and diluted 1:50 in 0.1X TE prior to use in the RAPD PCR reaction.

Verification of Polyploidy

Blood was collected from the caudal vein when the experimental animals were approximately 4 months old. The ploidy of blood cells was determined by nuclear volume analysis using a Coulter Counter Model ZM and 256 Channelyzer. In a subset of 162 fish, ploidy was determined by both blood cell nuclear volume and DNA content measurements to verify the accuracy of the Coulter Counter ploidy determination for this species.

Results

A total of 2401 samples were analyzed with the Coulter Counter and of these 2400 gave a reading which allowed for an unambiguous ploidy determination (Table 1). There was no overlap between the median channel data values observed for haploid, diploid, triploid, and tetraploid blood cell nuclei. Flow cytometry DNA content analysis of a random subset of 162 blood samples agreed with the Coulter Counter ploidy determination. Mosaic individuals with both predominantly-haploid (less than 35% diploid) and diploid-tetraploid blood cells were included in this concordantly classified subset. The mean flow cytometer channel data values for haploid, diploid, triploid and tetraploid erythrocyte nuclei corresponded to estimated nuclear DNA contents of 5.5, 10.7, 15.6, and 20.2 pg, respectively.

Table 1. Mean channel data from Coulter Counter and flow cytometry analysis of erythrocyte nuclei derived from diploid, triploid, tetraploid and mosaic white sturgeon.

COULTER COUNTER	Haploid mosaic	Diploid	Triploid	Tetraploid	Diploid-Tetraploid mosaic
Average	20.50/73.8	73.14	130.72	182.67	87/185
S.D.	1.38	6.32	9.01	8.99	---
Number	6	1127	1263	3	1
%	.25	46.96	52.63	.13	.04
Minimum	18	54	103	170	---
Maximum	22	93	159	190	---
FLOW CYTOMETER					
Average	25.5/47.9	49.46	72.15	---	52.7/93.7
S.D.	---	2.70	4.18	---	---
Number	1	71	89	---	1
Minimum	---	44.0	64.0	---	---
Maximum	---	55.4	81.7	---	---

Table 2. Neurulation and normal hatching frequencies, and ploidy analysis at 4 months by experimental treatment group in white sturgeon *Acipenser transmontanus* Richardson. Modified from Van Eenennaam et al., submitted. Aquaculture (Table 1).

Expt. (No.)	Design	Egg Treatment	Egg (No.)	Neurulation (%)	Hatch (%)	Ploidy (%)		Other (No.)
						2N	3N	
1	Diploid	Untreated	940	93	72	100		
	Haploid	Untreated	703	1	0			
	Triploid	34°C, 1 min	836	93	45	55	44	1 2N/4N
	"	34°C, 2 min	770	93	30	100		
	"	34°C, 5 min	845	77	9	20	80	
	Gynogen	34°C, 1 min	758	8	<1			
	"	34°C, 2 min	792	6	3	100		
"	34°C, 5 min	977	8	<1				
2	Diploid	Untreated	1489	88	74	100		
	Haploid	Untreated	1138	<1	0			
	Triploid	3°C, 60 min	2244	43	17	41	57	4 N/2N
	Gynogen	3°C, 60 min	6405	<1	<1	100		
3	Diploid	Untreated	968	89	84	100		
	Haploid	Untreated	2588	<1	0			
	Triploid	32°C, 2 min	1162	89	63	10	90	
	"	32°C, 3.5 min	1127	93	79	100		
	"	32°C, 5 min	1095	92	69	3	97	
	"	3°C, 15 min	1146	90	82	98	1	1 4N
	"	3°C, 30 min	1103	87	70	64	35	2 4N
	"	3°C, 60 min	1108	76	52	10	90	
	"	3°C, 180 min	1063	22	4	50	42	1 N/2N
	Gynogen	32°C, 2 min	1410	2	<1			
	"	32°C, 3.5 min	1475	2	2	100		
	"	32°C, 5 min	1178	<1	<1			
	"	3°C, 15-30min	2749	5	<1			
	"	3°C, 60 min	1290	6	4	100		
"	3°C, 180 min	1274	<1	<1				
4	Diploid	Untreated	1574	87	74	100		
	Haploid	Untreated	1840	19	<1	100		
	Triploid	34°C, 3 min	2255	87	53	29	71	
	Gynogen	34°C, 3 min	10631	26	21	98		1 N/2N

Triploid juveniles were phenotypically identical to the diploid fish, and no difficulties were experienced in raising them. The juvenile weight at 4 months of age was 39.6 ± 19.6 g (mean \pm s.d, n = 1948), and no significant difference was found between diploid, triploid, and gynogenetic fish (data not shown). Gynogenetic larvae displayed a behavioral peculiarity in that they appeared to swim randomly throughout the water column and at the water surface while the diploid and triploid control larvae exhibited the "normal" behavior of aggregating on the bottom of the tank. This aberrant behavior persisted in the gynogenetic juveniles throughout the first 40 days but was not as pronounced thereafter. At 40 days post-hatch average survival was 87%, 84%, and 62%, in the experiment 4 diploid, triploid and gynogen treatments, respectively. The 68 surviving gynogens from the first three experiments, and 40 randomly-selected gynogens from experiment 4 were screened for the presence of the appropriate RAPD sire-specific markers. There was no evidence of paternal inheritance in 105 of these fish (20/20, 14/15, 31/33 and 40/40 from experiments 1, 2, 3, and 4 respectively).

Discussion

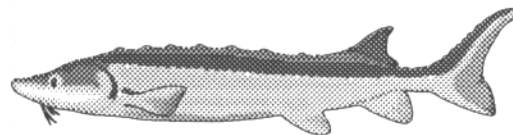
Measuring erythrocyte nuclear volume with a Coulter Counter and channelyzer is an indirect method to assess ploidy. We verified the accuracy of Coulter Counter ploidy determinations for this species in a subsample that was analyzed by flow cytometry, a direct method of ploidy determination. The ploidy determination data of the Coulter Counter and flow cytometer were in complete agreement and overall we found the Coulter Counter and channelyzer to be an inexpensive, rapid and accurate technique for ploidy determination in sturgeon.

When similar temperature shocks were applied to different batches of eggs we observed different rates of second polar body retention. The optimal timing of the temperature shock appeared to depend on the batch-specific stage of egg development. The stage of egg development at induced spawning may differ between individual females. A batch-specific treatment response was also seen with the male gametes. Identical UV-irradiation levels resulted in batches of sperm with differing abilities to initiate egg activation. The overall low number of gynogens produced in experiments 1-3 suggests that the sperm were unable to effect egg activation following UV-irradiation. This hypothesis is supported by the low rates of neurulation in the haploid control groups, and the observation that very few of the eggs receiving UV-irradiated sperm underwent even the first cleavage indicating that fertilization did not take place. The UV-irradiated sperm in Experiment 4 were more effective at egg activation, as evidenced by the 19% and 26% neurulation rates in the haploid control and gynogen treatment groups, respectively. Over 2000 putative gynogen hatched normally in the experiment 4 gynogen treatment group, and we will continue to rear several hundred through sexual differentiation. We are also rearing the 65 confirmed gynogens from experiments 1-3. Although our experiments were not designed to determine the optimal parameters for the induction of meiotic gynogenesis and polyploidy in white sturgeon, their results suggest that the optimal conditions may be dependent upon the unique developmental stage of the specific batches of gametes involved in each cross.

In the course of this experiment most of the embryos in the haploid control treatment groups died at an early stage of development before neurulation, or they displayed features of haploid syndrome (abnormal body shape, open blastopore) and failed to hatch normally. The six mosaics with predominantly-haploid erythrocytes that were detected in our ploidy analysis seemed to be fully viable, normal-appearing fish. Their weight at four months of age ranged from 15.33-26.86 g which was within the range of weights observed for the diploid and triploid fish in their respective treatment groups. RAPD analysis revealed that four of these predominantly-haploid mosaics had no apparent paternal inheritance (no analysis was done on the other two fish). The presence of a secondary population of diploid erythrocytes, and possibly the polyploid nature of white sturgeon may offer some explanation as to how these mosaic fish were able to tolerate the normally lethal gene dosage insufficiencies of the haploid condition.

Conclusion

We described the production of viable polyploid and gynogenetic white sturgeon. Although the effectiveness of our treatments to induce gynogenesis and second polar body retention was variable, the high percentage of verified gynogenetic and polyploid sturgeon in some progeny groups suggests that there is potential to use these techniques for further research and development in the domestic breeding of chondrosteans for aquaculture. We plan to continue rearing the gynogens produced in this study until gonadal sex differentiation has occurred. At this time we will analyze the sex ratios observed in the diploid, triploid and gynogenetic groups. These ratios will provide preliminary information regarding the sex determination mechanisms of white sturgeon, and could be of commercial interest to caviar producers.



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