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Author: Yang, Huiping

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PERFORMANCE AND FECUNDITY OF TRIPLOID EASTERN OYSTERS *CRASSOSTREA VIRGINICA* (GMELIN, 1791) AND CHALLENGES FOR TETRAPLOID PRODUCTION

HUIPING YANG*

Fisheries and Aquatic Sciences, School of Forest, Fisheries, and Geomatics Sciences, Institute of Food and Agricultural Sciences, University of Florida, Gainesville, FL 32653

ABSTRACT The goal of this study was to initiate a program for producing Florida tetraploid founders to meet the needs of the fast-growing oyster farming industry in the Gulf of Mexico. The objectives were to: (1) produce chemically induced triploids originated from Florida wild stocks and (2) screen and identify triploid females for induction of tetraploid founders. By use of Florida wild stocks from different locations, chemically induced triploids were produced in three spawn groups. Compared with diploid siblings, triploids at juvenile stages did not show significant fast growth (shell metrics and body weight) in every group, but at 1-y-old in spawning season did show significantly faster growth ($P < 0.0001$) than their diploid siblings. Among triploids, the occurrence of female averaged 1.66% (43 out of 2,597 triploids), ranging from 0.68%, 1.2%, and 4.26% in the three spawn groups. Oocytes from these 43 triploid females averaged 214,715 per female ranging from <100 to 1,800,000. Flow cytometry analysis of gonad from the nonfemale triploids ($n = 186$) showed five types of ploidy composition. A majority of these nonfemale triploids (66.1%) showed 3*n* only, indicating no gametogenesis occurred, and other different ploidy compositions showed initiation of gametogenesis with different chromosome segregations. Tetraploid induction was conducted by fertilizing the oocytes from triploid females with sperm from diploid males followed by polar body inhibition. The fertilization varied greatly ranging from 12% to 91%, and survival to D-stage swimming larvae ranged from 0% to 24%. Ploidy of pooled D-stage larvae indicated a tetraploid composition ranging from 38% to 80%. Heavy mortality of putative tetraploid larvae occurred at about 7–9 days and juveniles (1, 3, and approximately 200) were harvested in three groups. Unfortunately, these juveniles were lost during a hurricane in October 2018 at the growout site of one collaborating farm before individual ploidy examination. Although no confirmed tetraploid founders were harvested, the occurrence and fecundity of triploid females were documented in detail, which should be useful for future development of tetraploid stock for oyster aquaculture.

KEY WORDS: eastern oyster, *Crassostrea virginica,* fecundity, triploid, tetraploid, occurrence of female, ploidy

INTRODUCTION

Molluscan shellfish has been traditionally a major component of aquaculture with 17.5 million tonne annual production in 2018, accounting for 56.8% of the total marine aquaculture production (30.8 million tonnes) (FAO 2020). Cultured molluscan species include bivalves (clams, oysters, mussels, scallops, and pearl oysters), abalones, and other miscellaneous species. Sustainability of this large-scale aquaculture industry requires considerable efforts to increase product quality and production efficiency and improve aquaculture environments. Use of genetic breeding is one of the important strategies because selected broodstock can produce well-adapted offspring, and triploid-tetraploid technology has been widely recognized by the industry and used in many farming species, especially oysters (Guo 2004).

Triploid oysters can grow fast, have better meat quality during summer months, and probably withstand some diseases (Guo et al. 2009, Yang et al. 2019). So far, farming of triploids has become an important part of the global oyster industry. Triploids account for about 50% of the production on the west coast of the United States, 100% of the hatchery seed production in France (Degremont et al. 2016), and over 2.3 billion triploid seed in China (Yang et al. 2019). For eastern oysters *Crassostrea virginica*, triploids account for nearly 100% of the seed production in the Chesapeake Bay (Peachey & Allen 2016) and a majority of oyster seed in the Gulf of Mexico (Wadsworth et al. 2019). For Sydney rock oysters *Saccostrea glomerata*, triploids account for about 15% of the production in Australia (Peachey & Allen 2016). To meet the aquaculture industry needs of triploid seed, tetraploids are required for 100% triploid production through crossing with normal diploids (Guo et al. 1996).

In the Gulf of Mexico, the oyster industry has been traditionally reliant on the harvest of wild stocks, which has accounted for about 90% of annual U.S. landings. Since 2012, a dramatic decline of oyster landing has occurred in the Apalachicola Bay, FL, due to, at least in part, a prolonged drought (Pine et al. 2015). In December 2020, oyster harvesting in the iconic Apalachicola Bay, FL, was shut down and will stay closed for at least 5 y as a result of the depletion of wild oyster beds (Florida Fish and Wildlife Conservation Commission [FWRI] 2020). To cope with the fishery collapse, oyster aquaculture is expanding rapidly. Meanwhile, the demand for triploid seed is rapidly increasing.

Worldwide, oyster triploid seed in most hatcheries are produced through fertilizing oocytes (commonly called eggs in industry hatcheries) from normal diploids with sperm from tetraploids. Therefore, successful establishment of tetraploid breeding stocks is the key for application of triploid-tetraploid technology on shellfish aquaculture (Guo et al. 1996, Yang et al. 2000, Yang & Guo 2004, Yang & Guo 2006b, Yang & Guo 2006a, Yang et al. 2019). For oyster farming in the Gulf region, only tetraploid stock developed at Louisiana State University and 4C's technology Inc. (LSU/4Cs) can be used for triploid seed production, because oysters from the east coast, the United States, are prohibited by the Gulf states to prevent

*Corresponding author. E-mail: huipingyang@ufl.edu
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transferring of a deadly oyster parasite disease (MSX) caused by *Haplosporidium nelsoni*, including tetraploid stocks from Rutgers University and Virginia Institute of Marine Science. With the rapid increase of oyster farming, seed shortage has occurred in Florida. At this situation, shellfish farmers required tetraploid stocks originated from local populations for triploid production with enriched the genetic diversity of stocks beyond the existing LSU/4Cs stock. With the support of the Gulf States Marine Fisheries Commission, one project was initiated in 2017 aiming at producing Florida tetraploid stocks. Tetraploid induction in molluscan shellfish has been a challenge (Yang et al. 2019), and the viable tetraploid production was reported in the Pacific oyster through a unique approach in which oocytes from triploid females were fertilized with sperm from diploids, followed by the inhibition of polar body I (Guo & Allen 1994c).

The goal of this project was to initiate a program for producing Florida tetraploid founders to meet the needs of the fast-growing oyster farming industry in the Gulf region. The objectives were to: (1) produce chemically induced triploids originated from Florida wild stocks and (2) screen and identify triploid females for induction of tetraploid founders. Establishment of tetraploid founders requires at least two spawning seasons (triploid production in spawning season 1, and tetraploid induction in spawning season 2). Sometimes, several years are required due to few oocytes from triploid females, poor larval survival, or low tetraploid efficiency. In the current publication, results from this project performed during 2017–2018 were reported. Although no confirmed tetraploid founders were produced, the performance, occurrence, and fecundity of triploids were documented in detail. It is expected that the information in this report would be useful for academic or industry communities who wish to develop their own tetraploid stocks for oyster aquaculture.

MATERIALS AND METHODS

Production of Chemically Induced Triploids

Brood stock oysters were collected from three wild stocks located in Cedar Key and Oyster Bay, FL. Spawning was performed between April 5 and May 4, 2017, in collaborating hatcheries.

Gamete collection was performed by stripping of gonads directly in filtered seawater (T = 26°C). Oocyte suspension was filtered through a 250-μm screen to remove large pieces of tissues, and oocytes were collected on a 20-μm screen and washed into a 2-L beaker in a 500-mL volume for fertilization after 30-min hydration. Sperm were collected by suspending testis dissected from each male in filtered seawater in 100-mL beakers. After examining the quality of oocytes and sperm motility, fertilization was made by adding sperm into oocyte suspension at a 10:1 ratio of sperm to oocyte by viewing under microscopic at 100× magnification. After fertilization, a 20-mL of fertilized egg suspension was separated in a beaker as control.

Triploids were produced by inhibiting the release of polar body 2 of fertilized eggs with cytochalasin B (CB) (Guo et al. 2009). When the first polar body in fertilized eggs was observed using a microscope at 100× magnification (about 10–15 min after mixing of sperm and oocytes), CB (0.5 mg/L, stocking solution at 1 mg/mL in dimethyl sulfoxide) was added to the fertilized egg suspension (in 500 mL) and mixed well gently. When the second polar body in fertilized eggs were observed in the control group (without CB), CB treatment was ended by filtering through a 20-μm screen and fertilized eggs were washed into a 4-L pitcher in fresh seawater for embryo development. At two-cell embryo stage (about 1–2 h postfertilization) or beyond, embryo suspension (1 mL) was sampled and the fertilized eggs (which divided to 2-cell or beyond) and total eggs were counted by use of a 1-mL Sedgewick Rafter counting chamber. Fertilization rate was calculated as the percentage of fertilized eggs out of the total eggs. Fertilized eggs were cultured in the hatcheries after the routine practice for larval culture, seed harvest, nursery, and growout.

Ploidy Determination

Ploidy determination in this study was performed by use of a flow cytometer (Accuri C6, BD biosciences, San Jose, CA) equipped with 488-nm and 640-nm lasers. A preliminary experiment was conducted to generate an effective protocol for ploidy determination using fluorescent propidium iodide (PI, 50 mg/L) in 0.112% of sodium citrate with 0.1% Triton X-100 and 10 mg/L RNAase. Flow cytometer performance was validated each day using fluorescent validation beads (Spherotech 6-peak and 8-peak beads, BD Biosciences, Ann Arbor, MI) to ensure that coefficient of variation values for the fluorescence detectors were less than 3.0% (based on full peak height). Flow cytometry was performed using the CFlow plus® software (BD Biosciences, Ann Arbor, MI; version 1.0.202.1).

Ploidy determination was conducted by sampling a piece of gill (0.2 cm × 0.2 cm) for adult oysters, a pool of over 200 larvae for larval ploidy composition, or a smear of gonad tissue for gonad ploidy of nonfemale triploids. Briefly, tissue samples were suspended and minced in staining buffer (without PI), after syringing for 10 times with a 22-gauge needle and vortexing for 10 sec, the sample suspensions were filtered through a 20-um screen to get rid of large pieces of tissues, stained by mixing with an equal volume of staining solution (PI at 100 mg/L) for 20 min, and analyzed using flow cytometry. Ploidy was determined by viewing the histogram on FL3 (filter 670 LP) linear plot and comparing with the haploid/diploid controls of sperm and gill tissue from a wild diploid.

Comparison of Chemically Induced Triploids with Their Diploid Siblings

The oysters from CB treated group were sampled at juvenile stage (Fall 2017) and adult stage in the spring spawning season (March to May 2018, approximately 1 y old) to confirm the triploids and evaluate their growth, sex ratio, and fecundity. Oysters were brought back from nursery or grow-out sites in coolers and lined up in trays (20 oysters per tray) after being washed with freshwater and marked a permanent marker. Shell metrics (height, length, and width) were recorded using a digital caliper (0.01 mm accuracy, Mitutoyo, Aurora, IL), and the body weight was weighed individually using electronic scale (0.0001g accuracy, Mettler Toledo ME4002E).

After measurements, ploidy of each oyster was determined by flow cytometer. In March to May 2018, each oyster was opened, and the upper shell was removed carefully with a sterilized oyster knife to avoid gamete contamination.

Sex determination was conducted by sampling a piece of gonad with a sterilized toothpick and viewing the presence of gametes using a microscope at 100× magnification (Olympus, BX43, Tokyo, Japan). For triploids, "female" was defined when oocytes were observed (even only one oocyte was observed), and others without presence of oocytes were defined as "nonfemale." Within these nonfemales, "male" was not defined and separated because sperm motility was not examined. After sex determination, one piece of gill from each oyster was sampled for ploidy determination. The confirmed triploids were separated from the diploids in a new sample plate with females and nonfemales separated. Shell metrics and body weight of triploids and diploids were calculated and compared with each other.

The gonad from "nonfemale" triploids in one spawn group (out of the threes spawn groups) were sampled for examination of gonad ploidy composition using flow cytometer. The triploid females were used for oocyte collection for tetraploid induction.

Tetraploid Induction by Triploid Females × Diploid Males Following Polar Body 1 Inhibition

Tetraploid induction was conducted by crossing triploid females with diploid males and following inhibition of polar body 1 in fertilized eggs (Guo & Allen 1994c). Oocytes from triploid females were collected by strip spawn, and the total oocyte number was quantified and recorded. Fertilization was conducted as described for triploid induction with a sperm: oocyte ratio of 15–20:1. Oocytes from different triploid females were pooled for fertilization if the total oocyte number from individual oyster was lower than 100,000. Inhibition of polar body 1 in fertilized eggs was conducted using CB treatment (0.5 mL/L) at 5–10 min post fertilization min depending on temperature at until polar body 1 was observed in controls. Culture of fertilized eggs and swimming larvae was performed as the routine practice in the collaborating hatcheries. Ploidy of a pool of swimming larvae (approximately 200) was examined at Day 2–3 after water changes.

Data Analysis

Data collection, including shell metrics and body weight, were expressed as mean ± SD. Data analysis was performed by JMP pro software (version 15.0, SAS Institute, Cary, NC). Tests of homogeneity of variance were conducted for normalization before analysis. Analysis of variance ANOVA, (T-test), and independent Chi-square were used for data analyses. Significance level was set at $P < 0.050$.

RESULTS

Percentage of Chemically Induced Triploid and their Growth at Juvenile Stage

Juvenile putative triploids produced by chemical induction were harvested in three spawn groups and ploidy was determined individually at different months old (Table 1). The percentages of triploids varied among different spawn groups and changed over time, and comparison of shell metrics and body weight of triploids with their diploid siblings showed varied results. For Group I, no differences were found at 6-mo-old, but differences were found in shell width and body weight at 7-mo-old (see *P* values, Table 1). For Group II, no differences were found between triploids and diploids at 2-mo-old, after being cultured in three different growout farms (at 5–6-mo-old), significant differences in shell metrics and body weight were found between triploids and diploids in two growout locations (Cedar Key and Wakulla, FL), but not Terra Ceia, FL (Table 1). For Group III, no differences were found between triploids and diploids at 2-mo or 5-mo-old (Table 1).

Occurrence of Females and Growth of Triploids at About 1-y Old in Spawning Season

At about 1-y-old, triploids were significantly larger and heavier than that of diploid siblings regardless of growout locations and spawn groups ($P < 0.0001$) (Table 2). Triploid percentages were recorded as 58.3%, 61.3%, and 63.1% in spawn Groups I, II, and III.

Overall, the occurrence of triploid females was 1.66% (43 females out of 2,597 triploids). Among the confirmed triploids, the occurrences of females in Groups I, II, and III were 0.68%, 1.19%, and 4.26%, which were all significantly lower than that in their sibling diploids ($P < 0.0001$) and significantly correlated with (or different among) the spawn group ($P < 0.0001$). The occurrence of triploid females did not show correlation with the two different growout locations in spawn Group II ($P = 0.065$) or Group III ($P = 0.580$).

Among the diploids, the percentages of females in spawn Groups I, II, and III were 63.2%. 34.4%, and 23.1% ($P < 0.0001$), indicating that the sex ratio of diploids was correlated with the different parents. Additionally, the diploid oysters cultured in two locations showed significantly different female percentage in spawn Group II (38.3% versus 28.0%, $P = 0.004$) or group III (46.4% versus 20.9%, $P = 0.002$), indicating the sex ratio was also correlated with the culture locations.

Fecundity of Triploid Females and Production of Tetraploid

The oocytes collected from these 43 triploid females averaged 214,715 per oyster with a wide varying range from <100 to 1,800,000 (Table 3). Based on the microscopic observation of development of fertilized eggs at different temperatures, the starting time and duration of CB treatments were different for inhibition of polar body I (Table 3). The fertilization success varied greatly among groups ranging from 12% to 91%. D-stage swimming larvae (at day 2 unless specified in Table 3) were harvested in several groups at rates ranging from 0.024% to 24.00%, and complete mortality or no D-stage larvae were harvested in other groups (Table 3). Ploidy of pooled swimming larvae (mixture of trochophore and D-stage larvae) by flow cytometer was conducted during the first water change in groups with over 10,000 larvae, and a tetraploid composition ranging from 38% to 80% were recorded.

Putative tetraploid larvae suffered heavy mortality at about 7–9 days and a large amount of empty larval shells (some of them already reached umbo stage) were observed. Eventually, juveniles (after metamorphosis) were harvested in three groups with 1, 3, and approximately 200 (Table 3). Unfortunately, these juveniles were lost during a hurricane in October 2018 in a growout site of one collaborating farm before individual ploidy examination.

TABLE 1.

Production of chemically induced triploid eastern oysters *Crassostrea virginica* by inhibiting of polar body with cytochalasin B (0.5 mg/L) using Florida wild stocks from three different sites as parents. Comparison of triploids and diploids (siblings) indicated that significant differences in shell metrics and body weight did not show in all groups at juvenile ages.

Spawn info	Date	Ploidy	Number	Height (mm)	Length (mm)	Width (mm)	Weight (g)
Group 1		*2n*	69	43.83 ± 7.07	28.04 ± 4.37	NA	8.63 ± 4.31
04/05/2017	10/4/2017	*3n*	31	46.40 ± 5.85	29.44 ± 3.31	NA	9.28 ± 3.03
20 F × 6 M			*P values*	0.080	0.114	NA	0.444
75.5 million		*2n*	25	56.52 ± 5.37	36.19 ± 2.92	19.72 ± 5.27	22.22 ± 4.50
(Fert. 50%)	11/8/2017	*3n*	23	57.57 ± 6.25	40.18 ± 4.70	20.25 ± 3.47	26.98 ± 7.51
			P values	0.538	0.001	0.687	0.010
Group II		*2n*	10	NA	6.14 ± 0.74	NA	0.044 ± 0.015
05/04/2017	6/28/2017	*3n*	13	NA	6.43 ± 0.69	NA	0.050 ± 0.010
19F × 5 M			*P values*	NA	0.339	NA	0.284
31.1 million	10/04/2017 Cedar Key	*2n*	23	27.33 ± 3.33	39.55 ± 5.58	13.34 ± 2.78	10.37 ± 4.33
(Fert. 82%)		*3n*	27	30.49 ± 3.81	44.56 ± 6.89	15.22 ± 3.84	14.55 ± 6.12
			P values	0.003	0.008	0.050	0.008
	10/31/2017 Wakulla	*2n*	18	41.25 ± 7.40	26.63 ± 3.04	12.81 ± 3.79	11.73 ± 6.11
		3n	32	50.30 ± 9.95	32.89 ± 6.37	16.68 ± 3.38	22.94 ± 11.45
			P values	0.002	0.0003	0.0005	0.0004
	11/07/2017 Terra Ceia	*2n*	12	39.46 ± 3.15	74.92 ± 8.50	18.93 ± 3.50	41.94 ± 11.11
		3n	38	40.49 ± 2.93	78.27 ± 6.67	18.68 ± 2.85	45.47 ± 9.76
			P values	0.304	0.163	0.806	0.295
Group III		*2n*	22	5.60 ± 1.22	NA	NA	0.040 ± 0.023
05/30/2017	7/19/2017	*3n*	25	5.76 ± 1.48	NA	NA	0.035 ± 0.023
8F × 6 M			*P values*	0.142	NA	NA	0.548
93.0 million		*2n*	16	29.74 ± 6.42	21.68 ± 3.39	9.29 ± 1.49	4.13 ± 2.08
(Fert. 31%)	10/23/2017	*3n*	32	34.32 ± 8.51	21.12 ± 5.99	10.03 ± 1.84	5.31 ± 2.93
			P values	0.065	0.728	0.168	0.158

F × M, female × Male; Fert., fertilization rate.

TABLE 2.

Sex ratio, shell metrics (mm, height, length, and width), body weight (g) of triploid and diploid siblings of eastern oysters *Crassostrea virginica* at 1-y-old. Significant differences were found between triploids and diploids in shell metrics and body weight in all groups/growout sites. The occurrences of females in triploid oysters were associated with spawn groups but not the two growout sites within Group I or II.

				Triploid						Diploid					
				Nonfemale			Female			Male			Female		
Spawn group (spawn date)	Growout location	Total oyster#	Parameters	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD
Group 1 (04/05/2017)	Cedar Key 3/16–5/25/2018	1,536	Height	880	85.72	10.08	6	83.34	10.74	239	79.12	10.12	411	82.10	8.53
			Length		53.06	6.70		54.78	5.52		48.47	7.10		51.12	6.07
			Width		24.48	4.03		26.08	4.59		22.71	3.81		23.58	3.70
			Weight		57.61	16.00		53.65	8.49		44.28	15.49		46.82	11.84
Group II (05/04/2017)	Cedar Key 3/28–5/14/2018	821	Height	350	73.74	10.35	1	82.36		290	66.84	9.10	180	67.26	10.28
			Length		52.17	7.29		60.27			46.24	5.80		48.22	5.52
			Width		26.65	18.06		32.18			23.11	4.49		23.38	3.43
			Weight		51.62	16.66		65.90			36.39	10.55		41.57	9.85
	Wakulla 4/25–5/14/2018	1,122	Height	827	73.66	9.91	13	63.59	18.14	203	62.08	10.87	79	67.73	12.30
			Length		51.21	6.20		44.19	10.65		43.61	7.08		47.07	7.79
			Width		24.14	3.80		21.02	5.49		21.32	3.98		22.97	4.25
			Weight		43.44	17.10		32.09	24.28		28.06	13.68		36.04	17.17
Group III (05/30/2017)	Cedar Key 3/27–5/25/2018	69	Height	40	68.29	8.00	1	65.00		15	58.75	9.41	13	63.62	8.88
			Length		43.22	5.33		40.72			39.16	5.25		41.04	6.29
			Width		21.11	2.54		19.43			19.22	2.34		20.49	3.98
			Weight		29.42	10.05		24.50			18.86	6.72		23.16	10.21
	Alligator harbor 5/7–5/23/2018	823	Height	500	70.04	9.83	22	69.15	8.41	238	67.95	8.90	63	68.00	7.85
			Length		45.68	5.14		45.31	3.61		44.53	4.67		45.47	5.57
			Width		23.48	3.19		24.59	2.60		22.34	3.00		22.05	2.74
			Weight		33.63	11.79		35.20	11.16		29.55	10.05		28.93	8.58

TABLE 3.

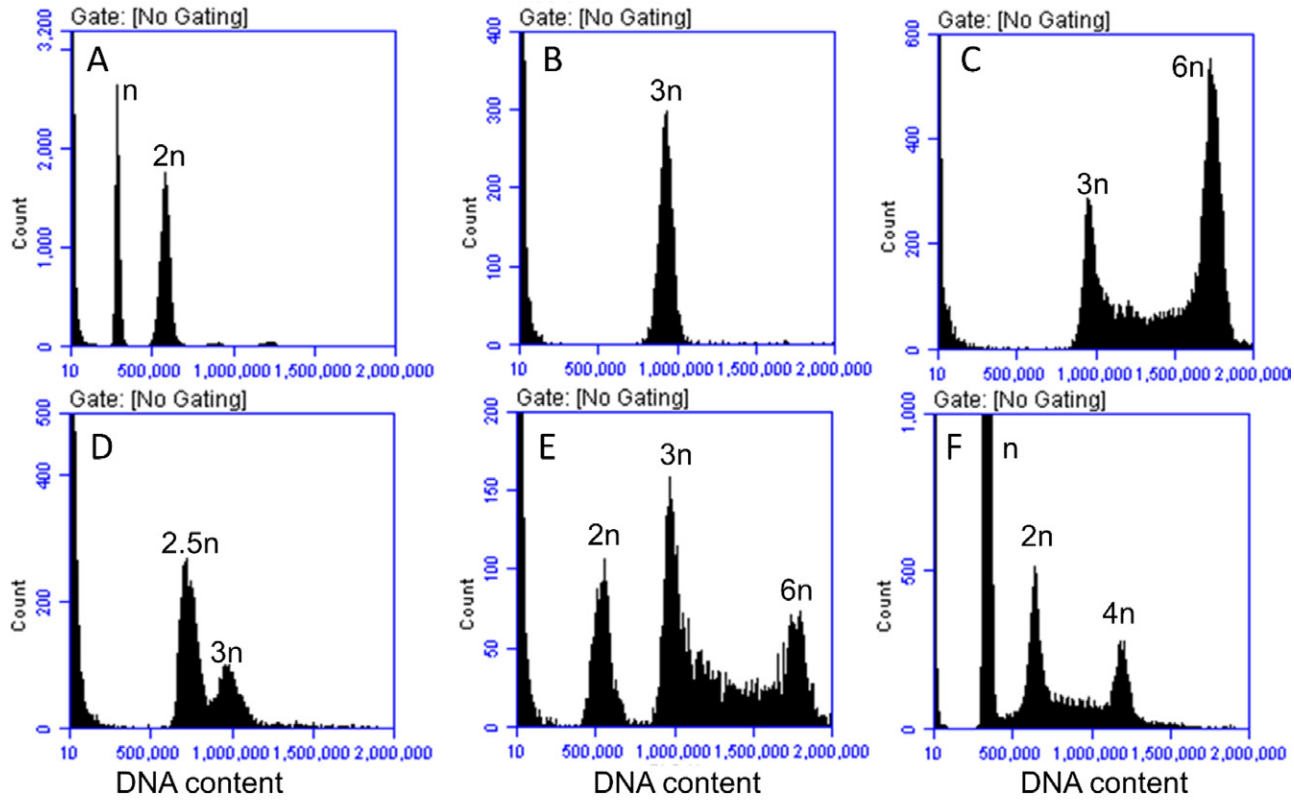
Oocyte numbers collected from each triploid female in three spawn groups of the eastern oyster *Crassostrea virginica*. Oocytes from triploid females were fertilized with sperm from diploid and the release of polar body of fertilized eggs was inhibited by cytochalasin B (CB, 0.5 mg/L, minutes after fertilization, mpf) for tetraploid induction. Fertilization temperature (temp), rate, and harvest of swimming larvae and spat (after metamorphosis) from each treatment group were recorded.

Spawn group	Date	Total oocytes	Treatment of CB (mpf)	Temp (°C)	Fertilization (%)	Larvae (D-stage)	Spat
Group I	2018_03_16	36,285 <100	7–25	23.2	36.2	533 (1.47%)	0
	2018_03_21	126,000	5–40	22.3	82.0	0	0
	2018_03_27	72,500 <100	6–31	26.2	37.9	0	0
	2018_04_19	18,600	13–30	26.0	41.9	0	0
Group II	2018_04_30	36,000	4–31	24.0	60.0	4200 (11.67%)	0
	2018_05_01	772,000 268,000	5.5–31 5.5–31	24.1 24.1	75.0 51.0	200 (Day 4; 0.024%) 100 (Day 4; 0.037%)	1
		4,200 2,200	NA NA	24.0 24.0	NA NA	NA NA	0 0
	2018_05_02	153,000	8–27	24.5	42.8	10,000 (6.54%)	0
	2018_05_10	732,000 88,000	6–27 6–27	25.0 25.0	77.0 64.0	300 (Day 4; 0.041%) 300 (Day 4; 0.034%)	1 spat
		320 600 100 400 100	NA	25	NA	NA	0
Group III	2018_05_08	16,600 1,000 1,000 1,000 1,000 1,000 1,000 400	NA	25.0	12.0	NA	0
	2018_05_17	1,600,000 1,800,000 1,340,000	8–33 8–33 8–34	23.8 23.8 23.8	88.0 78.0 88.0	220,000 (13.75%) 432,000 (24.00%) 224,000 (16.72%)	3 spat. Most died at Day 7–10
		214,000 340,000	8–34	23.8	91.0	38,400 (6.93%)	Approximately 200 Juveniles
		524,000 296,000	8–35	23.8	87.0	65,000 (7.93%)	
		22,400 9,600 51,000 5,000 35,000	8–35	23.8	73.0	0	0
	2018_05_23	100 18,000	NA 6–30	24 24	NA NA	NA 0	0 0
	2018_05_25	200	NA	24	NA	NA	0

NA, not available.

Ploidy Composition of Gonad from the "Nonfemale" Triploids

Flow cytometry analysis of gonads from the "nonfemale" triploids (n = 186, from spawn Group II) showed five types of ploidy composition (Fig. 1). Compared with the controls (n and 2n peaks) (Fig. 1A), the five types of ploidy composition were: (1) Type I: 3n only (Fig. 1B). This type of gonad ploidy indicated that no gametogenesis occurred. A majority of "nonfemale" triploids (66.1%) had this type of ploidy in their gonads. (2) Type II: 3n and 6n (Fig. 1C). This type of gonad ploidy composition indicated that gametogenesis initiated, and part of preliminary gametocytes were formed (6n). A 17.2% of "nonfemale" triploids had this type of gonad ploidy. (3) Type III: aneuploid (~2.5n) and 3n (Fig. 1D). A 6.5% of "nonfemale" triploids had this type of gonad ploidy. (4) Type IV: 2n, 3n, and 6n (Fig. 1E). A 7.5% of "nonfemale" triploids had this type of gonad ploidy. (5) Type V: ~n, ~2n, and ~4n (no 3n peak) (Fig. 1F). This type of ploidy indicated gametogenesis with an abnormal chromosome segregation. A 2.7% of "nonfemale" triploids had this type of gonad ploidy.



Ploidy		Oyster number	Gonad ploidy by FCM
Male diploid	A. Control (mix of sperm and gill)		n, 2n
Non-female triploids	B. Type I	123 (66.1%)	3n only
	C. Type II	32 (17.2%)	3n and 6n
	D. Type III	12 (6.5%)	2.5n and 3n
	E. Type IV	14 (7.5%)	2n, 3n, and 6n
	F. Type V	5 (2.7%)	n, 2n, and 4n

Figure 1. Flow cytometry analysis of gonad from the "nonfemale triploids (*n* = 186, from spawn Group II) showed a total five types of ploidy types (B–F) which were categorized based on their ploidy composition. (A) The ploidy of *n* and 2*n* peaks in control (mix of sperm and diploid gill); (B) 3*n* peak only, indicating no gametogenesis occurred. (C) 3*n* and 6*n* peaks, indicating gametogenesis initiated with preliminary gametocytes formed; (D) Aneuploid (~2.5*n*) and *3n* peaks; (E) 2*n*, 3*n*, and 6*n* peaks. (F) ~*n*, ~2*n*, and ~4*n* peaks without 3*n* peak.

DISCUSSION

Tetraploid induction in shellfish was initiated in the 1980s and has been a challenge due to the poor survival of tetraploid larvae to beyond metamorphosis (Guo et al. 2009, Yang et al. 2019) until a unique methodology was reported in Pacific oysters (Guo & Allen 1994c). This innovative method used oocytes from triploid females to fertilize with sperm from diploid males and followed by inhibition of the first polar body (Guo & Allen 1994c). So far, this method has been applied to several other shellfish species and produced viable tetraploids, such as pearl oysters *Pinctada margaritifera* (He et al. 2000), eastern oysters *Crassostrea virginica* (Guo et al. 2002), Sumino oysters *Crassostrea ariakensis* (Allen et al. 2005), bay scallops *Argopecten irradians* (Surier et al. 2012), and Catarina scallops *Argopecten ventricosus* (Maldonado et al. 2003). To date, tetraploid breeding stocks have been mostly established in oyster species by using this method and applied to commercial triploid seed production.

The procedure to produce tetraploids using this innovative method includes: (1) induction of triploids from normal diploids and culture to adult stage; (2) identification of fecund triploid females; (3) collection of oocytes from triploid females; (4) induction of tetraploids by inhibiting the release of polar body in the fertilized eggs of triploid females with diploids males; (5) culture of putative tetraploid larvae to juvenile and adult stage, and (6) identification of tetraploid individuals as tetraploid founders by nonlethal ploidy determination. The steps in this procedure are interconnected and problems at any step would result in failure of the tetraploid induction. The challenges included low occurrence of fecund triploid females, limited availability of oocytes from triploid females, and poor survival of putative tetraploid larvae to beyond metamorphosis (Yang et al. 2019), which were exactly reflected in the results in the current publication.

Obtaining of triploid females is the critical step and challenge for tetraploid induction, and large number of triploids

needs to be produced and screened as reported in this study. Theoretically, triploids are sterile because the three sets of chromosomes can yield abnormal chromosome synapsis and segregations during gametogenesis. But triploids are not 100% sterile and do exhibit gonad development to a certain extent. In the current report, the occurrence of female in 1-y-old triploid eastern oysters averaged as 1.66% (43 females out of 2,597 triploids) and were identified associating with the different spawning groups using different parents: 0.68% (six out of 840 in Group I), 1.2% (14 out of 1,177 in Group II), and 4.26% (23 out of 540 in Group III). For the same cohort of triploids, occurrences of fecund triploids varied in different culture locations but not significantly correlated. These results agreed with the results in a previous report in eastern oysters, in which only one female out of 1,600 triploids (i.e., 0.06%) was observed (Supan 2000).

Compared with eastern oysters, occurrences of females in triploids were reported much higher rate in other molluscan bivalve species. In Pacific oysters, a female percentage of 58% was observed in triploids (Guo & Allen 1994a). In *Crassostrea hongkongensis*, females were found in 30% (n = 40) and 37.5% (n = 40) of triploids cultured in two locations, and triploids exhibited polymorphic sterility with atrophic gonads and abnormal gametogenesis (Zhang et al. 2017). In *Saccostrea commercialis*, triploids were found to have highly retarded development, and females occurred differently in triploids sampled monthly ranging from 4% in September to 58% in February (Cox et al. 1996). In softshell clams *Mya arenaria*, females were found in 77% triploids (Allen et al. 1986), in Dwarf surfclams *Mulina lateralis*, 59% triploids were female (Guo & Allen 1994b), and in Noble scallops *Chlamys nobilis*, 25% triploid were females (Komaru & Wada 1989). The reason for the low occurrence of females in triploids in eastern oysters is not known, and this is the first big challenge for tetraploid induction.

The second challenge was the limited availability of oocytes from triploid females. Oocytes from triploid organisms usually undergo abnormal meiosis due to the three sets of chromosomes, thus, the number of oocytes from triploid females is usually low (Gong et al. 2004). In the current study, three triploid females (out of 43, at 7%) produced oocytes beyond 1 million; 12 triploid females (out of 43, at 28%) produced oocytes between 100,000 and 1 million, and 16 triploid females (out of 43, at 37%) produced 1,000 or fewer oocytes (Table 3). In Pacific oysters (Guo & Allen 1994a), triploid females produced oocytes ranging from 19,000 to 21.5 million (n = 19, mean = 2.3 million and median of 1 million), and nine out of 19 triploid females (47%) produced 1 million oocytes, which was significantly higher than results reported for eastern oysters in this study. At the same examination time, diploid siblings showed full gonad development and observation by the naked eye could easily distinguish triploids and diploids. Efforts have been made to improve gonad development in triploids, such as applying estradiol hormones to eastern oyster triploids (Quintana 2005, Young 2010), but no significant improvement was proved. One study on the mechanism of triploid female gametogenesis indicated that a specific gene (Nanos-like gene) was perhaps responsible for gametogenesis because expression of this gene in the diploid gonads matched with gonad seasonal development (Xu et al. 2018). Additionally, another two genes were identified in correlation with triploid gametogenesis (Jiang et al. 2017). In Pacific oysters, a high level (42%–50%) of gametogenesis was reported in triploids in all nine triploid batches from different culture locations (Houssin et al. 2019). Two types of gametogenesis were proposed in triploids: α-pattern for oysters displaying numerous proliferating gonia with abundant gametes and p-pattern for oysters displaying abnormal gonia with locked gametogenesis and few mature gametes at sexual maturity (Jouaux et al. 2010). Based on this classification, transcriptome profiling of gonad of triploid Pacific oysters were studied and disruption of sex differentiation and mitosis may be responsible for the impaired gametogenesis of triploids (Dheilly et al. 2014). In eastern oysters, development of triploid gametogenesis was classified based on the type of gonia, presence of spermatogenic cells or oocytes, and relative abundance of gametes (Matt & Allen 2021). But no relationship between the gonia and fecundity was identified in eastern oysters as that in Pacific oyster (Matt & Allen 2021).

In this report, the ploidy composition of gonads in the "nonfemale" triploids were analyzed to examine the gametogenesis situation. The five types of ploidy composition reflected the complicated gametogenesis in triploids. Most triploids (66.1%) were inactive of gametogenesis during the peak spawning season when diploid siblings were in full gonad development. Whereas other types of ploidy composition (Types II-V) reflected the ongoing gametogenesis process in these gonads. Possibly, a combination of histology observation, ploidy examination, and molecular analysis could be used to reveal the gametogenesis in triploids.

The third challenge was the poor survival of induced tetraploid larvae. In Pacific oysters, the survival of putative tetraploid to beyond metamorphosis was 0% in two replicates and 0.0739% in one replicate (Guo & Allen 1994c). Thus, it is essential to have sufficient oocytes from triploid females to start with the tetraploid induction for harvest of viable tetraploid juveniles (after metamorphosis). In this study, production of D-stage larvae was recorded as 0%–24%; putative tetraploid larvae were found to die off at Days 7–9 (some larvae were developed to umbo stage), and juveniles were harvested in only three groups with low numbers. In previous report on the eastern oyster, eight of the 13 treatment groups for tetraploid induction survived beyond metamorphosis and produced over 4,000 tetraploid spats (Guo et al. 2002). It reflected that larval culture could be an essential factor for survival of putative tetraploid larvae. In mussels *Mytilus galloprovincialis,* tetraploid larvae were found smaller than diploids and triploids in the same cohort (Scarpa et al. 1993) and the same situation was observed in Pacific oysters (Benabdelmouna & Ledu 2015) and Dwarf surfclams (Peruzzi & Guo 2002, Yang & Guo 2004). Therefore, screen sizes for water change during larval culture need attention to avoid accidental disposal of small sized larvae. But small-sized screens could also retain the dead larvae and subsequently affect the water quality for larval survival. Overall, extensive care is needed for culture of putative tetraploid larvae by saving slow growers and maintaining good culture conditions to ensure they can survive beyond metamorphosis.

Overall, this publication reported the results of a 2-y project on producing tetraploid founders to meet the need of the oyster industry in the Gulf of Mexico. The low occurrence and fecundity of triploid females and the low survival of putative tetraploid larvae were the major challenges for production of tetraploid founders in eastern oysters. Although this project did not yield live tetraploid founders, the data, generated by this study, are valuable for academic and industry communities for

the future production of tetraploid brood stocks. The workflow for tetraploid induction, methodologies (CB concentration, starting time, and duration) for triploid and tetraploid induction, and discussions about the challenges in tetraploid induction in eastern oysters can serve as guidance for future work. Additionally, the analysis of the gonad ploidy composition of nonfemale triploids proposed a new approach to understand the gametogenesis in triploids.

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