North American Journal of Aquaculture 78:218–223, 2016

ISSN: 1522-2055 print / 1548-8454 online
DOI: 10.1080/15222055.2016.1150923

COMMUNICATION

Ploidy Variation and Viability of Aneuploid Ornamental Koi Carp Obtained by Crossing Triploid Females with Diploid Males

Boris Gomelsky,* Thomas A. Delomas,[1] and Jeffrey L. Warner
Aquaculture Research Center, Kentucky State University, 103 Athletic Drive, Frankfort, Kentucky 40601, USA

Abstract

Results of crossing triploid females of ornamental koi, a variant of Common Carp *Cyprinus carpio*, with diploid koi males in two consecutive spawning seasons (2014 and 2015) are presented. A total of seven progenies from six triploid females (one female was spawned twice) were produced and analyzed. The same as normal diploid females, triploid females were highly fertile and produced hundreds of thousands of eggs (up to 400,000 per female). Processes of embryo incubation and hatching of larvae in progenies obtained from triploid females proceeded normally; however, mass mortality of hatched larvae occurred at the swim-up stage. Nevertheless about 200,000 swim-up larvae were obtained and stocked for further rearing. A total of about 1,000 juveniles (or 0.5% of the number of stocked larvae) was collected from outdoor tanks. As expected, all analyzed larvae and juveniles in control progenies obtained by crosses of diploid females with diploid males were diploid. About 95% of analyzed fish (larvae and juveniles) obtained by crossing triploid females with diploid males were aneuploids with ploidy ranging from 2.14n to 3.0n; mean values of fish ploidy in progenies obtained from different triploid females varied from 2.47n to 2.63n. Since aneuploid fish have in their genomes one haploid set from parental males, the data obtained indicate that triploid koi females produced aneuploid eggs with ploidy range from haploid to diploid level and a modal ploidy level around 1.5n, similar to the production of aneuploid spermatozoa observed earlier for triploid males in fish. About 5% of juveniles obtained from triploid females had ploidy range from 3.21n to 4.0n. Apparently these fish resulted from spontaneous suppression of the second meiotic division in aneuploid eggs.

Usually, artificially obtained triploid females in fish have small ovaries that do not contain advanced vitellogenic oocytes (Benfey 1999; Piferrer et al. 2009). In contrast, Gomelsky et al. (2015) recently described that triploid females of ornamental koi, a variant of Common Carp *Cyprinus carpio*, from a heat-shocked progeny developed large ovaries that were filled with fully grown oocytes. Crosses of triploid koi females with normal diploid koi males yielded mass aneuploid progenies having low viability (Gomelsky et al. 2015). The present article presents more detailed data on ploidy variation and viability of aneuploid koi produced from triploid koi females. Gomelsky et al. (2015) described the production and analysis of aneuploid koi obtained by crosses of triploid females in 2013. The present article describes the data on ploidy variation and viability of aneuploids obtained by crosses of triploid koi females in the next two consecutive spawning seasons (2014 and 2015).

METHODS

Scheme of crosses for production of experimental progenies.—Characteristics of fish parents used in crosses in 2014 and 2015 as well as the list of obtained progenies are given in Table 1. As was indicated by Gomelsky et al. (2015), all fish from control (no shock) progeny to heat-shocked progeny, where fertile triploid females were identified, have previously been dissected. Therefore, for obtaining comparative data on viability and ploidy variation of fish obtained from triploid females, normal diploid koi females were also used in crosses in 2014. According to the general scheme of crosses in 2014, each of three diploid koi males (2n-1, 2n-2, and 2n-3; see Table 1) were crossed with one diploid and one triploid female; a total of six progenies (progenies 1–6) were obtained in 2014. In 2015, four triploid

*Corresponding author: boris.gomelsky@kysu.edu
[1]Present address: School of Environment and Natural Resources, The Ohio State University, Kottman Hall, 2021 Coffey Road, Columbus, Ohio 43210, USA.
Received November 16, 2015; accepted January 22, 2016

TABLE 1. Results of induced spawning of diploid and triploid koi females and their crosses with diploid koi males.

Progeny number	Parents		Female weight (kg)	Weight of stripped eggs		Number of eggs per g	Total number of stripped eggs	Weight and number of eggs used for cross		% of live embryos	Number of swim-up larvae		Number of juveniles from outdoor tanks	
	Female	Male		g	%[a]			g	*n*		*n*	%[b]	*n*	%[c]
Progenies of 2014														
1	2n-1	2n-1	2.2	350	15.9	734	256,900	287	210,658	38.0	47,796	59.7	1,620	34.6
2	3n-1	2n-1	4.1	627	15.3	642	402,534	304	195,168	87.0	27,700	16.3	7	0.03
3	2n-2	2n-2	1.7	268	15.8	LS[d]		210		43.3	37,987		1,845	36.3
4	3n-2	2n-2	4.6	484	10.5	650	314,600	302	196,300	51.0	19,040	19.0	21	0.1
5	2n-3	2n-3	2.5	355	14.2	701	248,855	300	210,300	50.7	47,670	44.7	998	17.0
6	3n-3	2n-3	3.6	352	9.8	636	223,872	300	190,800	43.7	8,840	10.6	8	0.1
Progenies of 2015														
7	3n-4	2n-4	3.7	602	16.3			534			46,500		278	0.6
8	3n-5	2n-5	4.4	360	8.2			360			32,800		230	0.7
9	3n-6	2n-6	4.2	840	20.0			600			45,000		222	0.5
10	3n-1	2n-7	5.0	960	19.2			600			19,300		236	1.2

[a]Determined from female weight.

[b]Determined from number of live embryos the next day after fertilization.

[c]Determined from the number of stocked larvae. All swim-up larvae obtained from triploid females (progenies 2, 4, and 6) were stocked for raising; the numbers of stocked larvae obtained from diploid females were as follows: progeny 1, 4,676; progeny 3, 5,087; and progeny 5, 5,857.

[d]Lost sample.

females were crossed with normal diploid koi males for production four progenies (progenies 7–10; see Table 1).

All triploid females used in crosses were marked with passive integrated transponder tags (Biomark, Inc., Boise, Idaho). Tag identification showed that the same triploid female (designated 3n-1; see Table 1) was used in crosses in 2014 and 2015 and produced progenies 2 and 10, respectively. Also, female 3n-2, which was used for production progeny 4 in 2014, was identified as having been used in crosses of 2013 as "female number 4" (see Gomelsky et al. 2015).

Artificial spawning, production, and raising of progenies.—The technique of artificial spawning was the same as described by Gomelsky et al. (2015). Ovarian follicle maturation in females and spermiation in males were induced by injecting fish parents with carp pituitary extract (Argent Chemical Laboratories, Redmond, Washington) at 3 mg/kg. Total weight of the eggs obtained from each female was recorded. In 2014, a sample of eggs taken from each female was weighed, and the number of eggs was enumerated to determine the number of eggs per gram. Eggs were artificially inseminated in plastic bowls and were treated with a water : cow milk mixture (8:1 by volume) to remove egg adhesiveness. Embryos were incubated in McDonald jars (Pentair Aquatic Eco-Systems, Apopka, Florida). In 2014, the percentage of live embryos was determined approximately 20 h after fertilization at an early organogenesis stage; at this stage, live embryos are easily distinguished from unfertilized white eggs. Hatched larvae were collected in mesh hapas (small, fine-mesh cages) placed in flow-through raceway tanks. All swim-up larvae obtained from triploid females in 2014 and 2015 and samples of swim-up larvae (approximately from 4,500 to 6,000 larvae) obtained from diploid females in 2014 were stocked in separate 20-m^3 outdoor tanks for rearing; the numbers of larvae were determined by volumetric method. After 3.5 months of rearing, tanks were drained and all juveniles were collected.

Ploidy determination.—Ploidy of all fish parents used in crosses as well as samples of the obtained progenies was determined by flow-cytometric analysis of nuclear DNA content by an Accuri C6 flow cytometer (Becton, Dickinson and Co.) according to a technique similar to the one described by Gomelsky et al. (2015). In progenies obtained in 2014, both larvae and juveniles were analyzed for ploidy, while in progenies obtained in 2015 only the ploidy of juveniles was determined. For analysis of fish breeders and juveniles, DNA content in erythrocyte nuclei was determined. From fish breeders and some of the juveniles, blood samples were collected from the caudal vein into 3.0-mL Vacutainer Tubes (Becton, Dickinson and Co.) containing lithium heparin. For some juveniles obtained in 2015, blood samples were obtained by ablating the tail and holding the caudal peduncle in 1.5 mL of 0.85% NaCl solution. For each sample, 0.5 μL of heparinized

blood (or 4 μL suspension of blood in salt solution) was placed into 12 × 75 mm polystyrene test tubes (Becton, Dickinson and Co.). Also added to each test tube was 0.5 μL of heparinized blood taken from Largemouth Bass *Micropterus salmoides* to act as an internal staining control. One hundred microliters of propidium iodide staining solution (Biosure) was then added to the tubes. After a 10-min incubation period in darkness at room temperature, 40,000 events were recorded for each sample. The relative DNA content was determined as the ratio of sample fluorescence peak intensity to that of the internal standard (Largemouth Bass). For flow-cytometric analysis of larvae, tissue was dissociated with a technique similar to the one used by Kerby and Harrell (1990) for determination of ploidy of striped bass larvae. A whole larva was placed in 500 μL of propidium iodide staining solution, and the larva was repeatedly drawn into a 1-mL syringe fitted with a 21G needle and then expelled to break up the tissue. The mixture of larval tissue and staining solution was filtered through a 200-μm screen, mixed with 0.5 μL of heparinized largemouth bass blood added to the sample as an internal control, and then incubated in the dark for 10 min. Immediately after incubation, the samples were analyzed with the flow cytometer as described above for blood samples.

RESULTS

Results of induced spawning of diploid and triploid koi females and their crosses with diploid koi males are presented in Table 1. The body weight of diploid females used in crosses in 2014 ranged from 1.7 to 2.5 kg, while the mean weight of triploid females used in crosses (in both 2014 and 2015) varied from 3.6 to 5.0 kg. The weight of stripped eggs from diploid and triploid females varied from 268 to 355 g and from 352 to 960 g, respectively. The relative weight of eggs to female body weight varied in diploid and triploid females from 14.2% to 15.9% and from 8.2% to 20.0%, respectively. Diploid females had 701–736 eggs per gram in samples (the sample from female 2n-2 was lost); from 636 to 650 eggs per gram were recorded in samples of eggs from triploid females. The total numbers of eggs obtained from diploid and triploid females (recorded in 2014) varied from about 249,000 to 257,000 and from 224,000 to 402,500, respectively. Percentages of live embryos on the next day after fertilization (recorded in 2014) in most diploid and triploid females were close and varied from 38% to 51%; only in one triploid female (3n-1) was this parameter much higher (87%). Processes of embryo incubation and hatching of larvae in progenies obtained from diploid and triploid females proceeded normally. However, mass mortality of larvae in progenies from triploid females was observed in hapas for several days. Yields of swim-up larvae from the number of live embryos the next day after insemination recorded in 2014 were 59.7% and 44.7% in two progenies from diploid females and varied from 10.6% to 19.0% in progenies from triploid females (Table 1). Yields of juveniles from outdoor tanks in progenies from diploid females in 2014 varied from 17.0% to 36.3% (mean value for three progenies, 29.3%) from the number of stocked larvae. In 2014, survival of fish in progenies obtained from triploid females in outdoor tanks was extremely low and varied from 0.03% to 0.1% (mean, 0.08%); a total of only 36 juveniles was obtained from triploid females. In 2015, the yield of juveniles from outdoor tanks in progenies from triploid females varied from 0.5% to 1.2% (mean, 0.85%). A total of 966 juveniles was obtained from triploid females in 2015 (Table 1).

Data on ploidy of fish from obtained progenies are presented in Table 2. As expected, all analyzed larvae and juveniles obtained by crossing diploid females with diploid males (progenies 1, 3, and 5) were diploid.

All analyzed fish (larvae and juveniles) from progenies 2 and 6 obtained from triploid females in 2014 were aneuploids; the ploidy range covered three ploidy classes (2.21–2.40n, 2.41–2.60n, and 2.61–2.80n), class 2.41–2.60n being the most numerous (from 60% to 72% of all analyzed fish; Table 2). Mean ploidy of larvae and juveniles in progenies 2 and 6 were close and varied from 2.47n to 2.61n. In progeny 4, also obtained from a triploid female in 2014, a considerable difference between the ploidy distributions of larvae and juveniles was observed. Ploidy distribution of larvae in this progeny was similar to ones observed in progenies 2 and 6; one larva (4.3% of all analyzed larvae in this progeny) had ploidy 4.0n. Juveniles from progeny 4 demonstrated a large range of variability. Sixty percent of juveniles had ploidy variability from 2.41n to 2.80n, while 35% of fish in this progeny had ploidy from 3.61n to 4.0n. Also, one analyzed juvenile (5%) from this progeny was diploid (2.0n). Mean values of ploidy in larvae and juveniles from progeny 4 (calculated with exclusion of ploidy values higher than 3.61n) were close (2.54 and 2.51n, respectively; Table 2).

The ploidy distributions of juveniles from progenies 7–10 obtained from triploid koi females in 2015 have been similar (Table 2). Classes 2.41–2.60n and 2.61–2.80n were the most numerous in fish ploidy distributions in all four progenies. Lower frequencies of fish (from 2.5% to 15%) were observed in three adjacent classes (2.14–2.20n, 2.21–2.40n, and 2.81–3.00n). In progeny 9, one fish was recorded in class 3.21–3.40n and another fish was found in class 3.81–4.00n. Mean values of fish ploidy in progenies 7–10 were close and varied from 2.54n to 2.63n (mean value of ploidy in progeny 9 was calculated after excluding ploidy values higher than 3.21n).

DISCUSSION

In 2014, both normal diploid and triploid koi females were artificially spawned and crossed with normal diploid males. Although the weight of diploid females was smaller than that of triploid females, a comparison of female fertility can still be

TABLE 2. Data on fish ploidy in progenies obtained from diploid and triploid koi females.

Progeny number	Female ploidy	Stage of analysis	Number of fish analyzed	Mean ± SD ploidy, n	Fish ploidy distribution (%)									
					2.0n	2.14–2.20n	2.21–2.40n	2.41–2.60n	2.61–2.80n	2.81–3.00n	3.01–3.20n	3.21–3.40n	3.41–3.60n	3.61–3.80n	3.81–4.00n
1	2n	larvae	8	2.0	100										
		juvenile	21	2.0	100										
2	3n	larvae	25	2.47 ± 0.08			20.0	72.0	8.0						
		juvenile	7	2.53 ± 0.09				71.4	28.6						
3	2n	larvae	4	2.0	100										
		juvenile	23	2.0	100										
4	3n	larvae	23	2.54 ± 0.11[a]			13.0	52.2	30.4						4.3
		juvenile	20	2.51 ± 0.18[a]	5.0			45.0	15.0					5.0	30.0
5	2n	larvae	4	2.0	100										
		juvenile	25	2.0	100										
6	3n	larvae	24	2.52 ± 0.10			8.3	62.5	29.2						
		juvenile	5	2.61 ± 0.09				60.0	40.0						
7	3n	juvenile	40	2.63 ± 0.13			2.5	50.0	42.5	5.0					
8	3n	juvenile	40	2.59 ± 0.17		2.5	15.0	22.5	60.0						
9	3n	juvenile	40	2.63 ± 0.15[a]			2.5	42.5	42.5	7.5		2.5			2.5
10	3n	juvenile	40	2.54 ± 0.14		2.5	15.0	45.0	37.5						

[a]Mean ploidy was calculated only for values that are less than or equal to 3.0n.

made. The same as diploid females, triploid females were highly fertile and produced hundreds of thousands of eggs (from about 224,000 to 402,500). In 2015, the number of stripped eggs from triploid females was not recorded but obviously these numbers were even larger since some females produced 850 and 960 g of eggs. The relative weight of stripped eggs to weight of females in diploid females varied from 14.2% to 15.9%. In triploid females this parameter was more variable and ranged from 8.2% to 20.0%. This variability was similar to the range of relative weight of stripped eggs for four triploid koi females spawned in 2013; Gomelsky et al. (2015) reported that this parameter varied from 12.0% to 23.6%. As mentioned above, some triploid females were spawned in two consecutive years. As shown in Table 1, female 3n-1 produced 627 g of eggs in 2014 and 960 g of eggs in 2015; also, female 3n-2 produced 450 g of eggs in 2013 (as female number 4; see Gomelsky et al. 2015) and 484 g of eggs in 2014 (see Table 1). This shows that triploid koi females have a cycle of ovary development that is typical for normal diploid females in the local climate conditions and are able to be spawned every year.

In both 2014 and 2015, the processes of embryo incubation and hatching of larvae produced by triploid females proceeded normally. The percentages of live embryos recorded the next day after fertilization in 2014 was similar in most diploid and triploid females and varied from 40% to 50%. However, in both years mass mortality of hatched larvae in progenies obtained from triploid females was observed; the same observation was made in the spawning of 2013 (Gomelsky et al. 2015). Apparently, the negative effect of aneuploidy starts to affect fish survival at this developmental stage. As a result, the mean yield of swim-up larvae in progenies obtained from triploid females in 2014 (15.3%) was less than one-third of that from diploid females (52.2%). During further rearing of fish in outdoor tanks, the difference in survival between progenies obtained from triploid and diploid females became even more drastic. Mean yield of juveniles from the number of stocked swim-up larvae in progenies from triploid females in 2014 was only 0.08% versus 29.3% of mean yield of juveniles in progenies from diploid females. Mean yield of juveniles in progenies from triploid females obtained in 2015 was 0.8% (range, 0.5% to 1.2%). The values of survival of fish from triploid females in outdoor tanks observed in 2015 are similar to those recorded in progenies from four triploid koi females in 2013; Gomelsky et al. (2015) reported that the yield of juveniles varied from 0.2% to 1.2% (mean, 0.6%). In general, the results of three annual, consecutive spawning seasons showed that survival of aneuploid fish obtained from triploid koi females is consistently low, the overall yield of juveniles from the larval stage being less than 1%. Nevertheless, because of production of large amounts of swim-up larvae from triploid females (up to about 47,000 from one female), a large number of juveniles remained in spite of high mortalities. As reported above, almost 1,000 juveniles were produced from triploid koi females in 2015.

All analyzed fish obtained by crossing diploid koi females with diploid koi males were diploid. This shows that diploid fish breeders used in crosses, as expected, produced haploid gametes.

Approximately 96% (253 from 264) of analyzed fish (larvae and juveniles) obtained from crosses of triploid females with diploid males in 2014 and 2015 were aneuploids with ploidy ranging from 2.14n to 3.0n; mean values of fish ploidy in progenies obtained from different triploid females varied from 2.47n to 2.63n. Gomelsky et al. (2015) observed similar ploidy variability in fish obtained from triploid koi females in 2013; in that study, most of the aneuploid fish had ploidy from 2.3n to 2.9n (mean, 2.6n). Given that one haploid chromosome set in the genomes of aneuploid fish originated from parental males, the data on ploidy of fish obtained from triploid females show that these females produced aneuploid eggs with ploidy range from haploid to diploid level with a modal ploidy level around 1.5n. Earlier similar ploidy variability was described for spermatozoa produced by triploid males in fish (Piferrer et al. 2009).

In progeny 4 obtained from a triploid female in 2014, one larvae and seven juveniles had ploidy in the range from 3.61 to 4.0n. Also, in progeny 9 obtained from a triploid female in 2015, one juvenile had ploidy in the range of 3.21–3.40n and one juvenile had ploidy in the range of 3.81–4.0n. Gomelsky et al. (2015) described that one juvenile obtained from one triploid koi female in 2013 had a ploidy level 3.9n and suggested that this fish resulted from the spontaneous suppression of the second meiotic division in aneuploid (1.45n) egg. The appearance of fish with ploidy larger than 3.21n in the present study could be caused by the same mechanism. If triploid females produce eggs with ploidy range from haploid (n) to diploid (2n) level, then theoretically, suppression of the second meiotic division in eggs (fertilized with sperm from diploid males) can result in the appearance of fish with any ploidy from 3n to 5n. Earlier, Ueda et al. (1991) suggested suppression of the second meiotic division in aneuploid eggs for the explanation of the appearance of aneuploid fish with 3.5n ploidy in progeny obtained by crossing allotriploid Rainbow Trout *Oncorhynchus mykiss* × Brook Trout *Salvelinus fontinalis* hybrid males with diploid Rainbow Trout females.

One diploid (2n) juvenile was recorded in progeny 4 obtained from a triploid female; also two diploid juveniles were reported by Gomelsky et al. (2015) in progenies produced by triploid koi females in 2013. These diploid fish can result from haploid eggs, which can arise with low frequency from atypical triploid meiosis. Earlier, Van Eenennaam et al. (1990) showed that rare surviving juveniles obtained by crossing triploid Grass Carp *Ctenopharyngodon idella* males with diploid females were diploid.

In three progenies from triploid females obtained in 2014 both larvae and juveniles were analyzed for ploidy. In two progenies (numbers 2 and 6) ploidy distributions in larvae and juveniles did not differ substantially. In one progeny (number

4) the difference in ploidy distributions in larvae and juveniles was considerable. This difference in ploidy distributions was apparently caused by selection based on better survival of fish with certain levels of ploidy; survival of juveniles in this progeny was only 0.1% from the number of stocked larvae. Only 1 from 23 analyzed larvae had ploidy 4.0n (4.3% from all analyzed larvae), while among juveniles 7 fish (35%) had ploidy in the range 3.61–4.0n. Apparently fish having ploidy range 3.6–4.0n increased their frequency due to their higher viability than that of most fish in the progeny with ploidy range 2.21–2.80n. Similarly, the initial frequency of diploid larvae could be very low; none was recorded in the sample of larvae (n = 23) analyzed for ploidy. However, analysis of surviving juveniles identified one diploid fish, which apparently had higher viability.

The important result of this study is a demonstration that the investigated triploid koi females consistently produced mass aneuploid progenies when crossed with normal diploid males. As far as we know, the results of the present study and of those presented earlier by Gomelsky et al. (2015) is the first case in which artificially produced triploid females in fish were able to develop large ovaries filled with fully grown oocytes and to produce mass aneuploid progenies. Further studies will be aimed at the investigation of additional reproductive features of triploid females and the influence of aneuploidy on the reproductive ability of fish.

ACKNOWLEDGMENTS

Support for this study was provided by U.S. Department of Agriculture/National Institute of Food and Agriculture grant KYX-80-12-24A to Kentucky State University (KSU) and Kentucky's Regional University Trust Fund to the Aquaculture Program as KSU's Program of Distinction.

REFERENCES

Benfey, T. J. 1999. The physiology and behavior of triploid fishes. Reviews in Fisheries Science 7:39–67.

Gomelsky, B., K. J. Schneider, A. Anil, and T. A. Delomas. 2015. Gonad development in triploid ornamental koi carp and results of crossing triploid females with diploid males. North American Journal of Aquaculture 77:96–101.

Kerby, J. H., and R. M. Harrell. 1990. Hybridization, genetic manipulation and gene pool conservation of Striped Bass. Pages 159–190 *in* R. M. Harrell, J. H. Kerby, and R. V. Minton, editors. Culture and propagation of Striped Bass and its hybrids, American Fisheries Society, Bethesda, Maryland.

Piferrer, F., A. Beaumont, J.-C. Falguière, M. Flajšhans, P. Haffray, and L. Colombo. 2009. Polyploid fish and shellfish: production, biology and applications to aquaculture for performance improvement and genetic containment. Aquaculture 293:125–156.

Ueda, T., R. Sato, M. Iwata, A. Komaru, and J. Kobayashi. 1991. The viable 3.5n trouts produced between diploid females and allotriploid males. Japanese Journal of Genetics 66:71–75.

Van Eenennaam, J. P., R. K. Stocker, R. G. Thiery, N. T. Hagstrom, and S. I. Doroshov. 1990. Egg fertility, early development and survival from crosses of diploid female × triploid male Grass Carp (*Ctenopharyngodon idella*). Aquaculture 86:111–125.