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COMMUNICATION

Gonad Development in Triploid Ornamental Koi Carp and Results of Crossing Triploid Females with Diploid Males

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Abstract

Gonad development in 4-year-old triploid and diploid ornamental koi, a variant of Common Carp *Cyprinus carpio*, from corresponding heat-shocked and control progenies was investigated. Diploid males were normally mature. Triploid males from heat-shocked progeny demonstrated development of testes typical for triploid fish; triploid males did not release sperm and their testes had a pinkish color and were significantly reduced in size. Diploid females were normally mature and their gonadosomatic indices (GSIs) varied from 7.5% to 30.7% and the mean value was 21.3%. Triploid females had unexpectedly well-developed ovaries, which were filled with fully grown oocytes; their GSIs varied from 4.2% to 30.1% and the the mean value was 17.0%. Four triploid koi females released large quantities (from 260,000 to 394,500 eggs per female) of ovulated eggs after hormonal injection. Eggs from triploid females were fertilized with sperm from normal diploid koi males. Mass mortality of hatched larvae occurred at the swim-up stage, but about 32,000 swim-up larvae were obtained and stocked for further rearing. A total of 248 juveniles (or less than 1% from the number of stocked larvae) were collected from outdoor tanks. Ploidy analysis of juveniles ($n = 110$) showed that most of them were aneuploid with ploidy ranging from 2.3n to 2.9n with a mean value of 2.6n; two juveniles were diploid (2n). This shows that triploid koi females produced aneuploid eggs with a ploidy range from haploid to diploid level with the modal ploidy level around 1.5n, similar to the production of aneuploid spermatozoa observed earlier for triploid males in fish.

The primary purpose of induced polyploidy in aquaculture and fisheries is to obtain triploid fish, i.e., fish which have three haploid chromosome sets in karyotypes. Artificially obtained triploid fish are considered to be genetically sterile, i.e., they are not capable of producing viable progeny. Triploid fish are also characterized by complete or partial reduction of the gonads (Benfey 1999; Piferrer et al. 2009).

The present article describes gonad development in 4-year-old triploid and diploid ornamental koi, a variant of Common Carp *Cyprinus carpio*, from corresponding heat-shocked and control progenies and presents the results of crossing triploid females with normal diploid koi males. Initially, analyzed progenies were obtained in a study on the effect of ploidy on scale-cover pattern in linear koi (Gomelsky et al. 2012). In that study triploid linear fish in heat-shocked progeny exhibited a non-typical scale cover pattern characterized by the appearance of additional scales on the body; this type of scale cover was termed "multi-scaled linear." All analyzed multi-scaled linear fish ($n = 43$) were triploid. The control (not shocked) progeny obtained from the same koi parents consisted of typical linear and scaled fish (Gomelsky et al. 2012). In 2012, when the fish reached the age of 4 years, it was proposed to complete the experiment and collect data on gonad development. All fish from the control progeny were sacrificed and dissected; however, because dissecting multi-scaled linear females from heat-shocked progeny demonstrated unexpectedly well-developed ovaries, the remaining fish were saved in order to investigate the reproductive ability of triploid females in crosses.

METHODS

Gonad development in 110 scaled and typical linear fish from the control progeny and 57 multi-scaled linear fish from the heat-shocked progeny was investigated in 2012. Fish weight, TL, and gonad weight were recorded; the gonadosomatic index (GSI) was determined as the percentage of gonad weight to fish weight. Digital photographs of the two lateral sides of all multi-scaled linear fish from the heat-shocked progeny and of some fish from the control progeny were taken using a Nikon D7000 digital camera. Each multi-scaled linear fish has a unique scale distribution pattern that can be used to identify individuals much like a fingerprint, as was recently described for mirror carp, another variant of Common Carp, by Huntingford et al. (2013).

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In the previous study (Gomelsky et al. 2012), digital photographs of all multi-scaled linear fish, whose triploidy was determined by two methods (comparison of erythrocyte nuclear size using a Coulter counter and flow cytometric determination of DNA content), were taken for identification. Based on comparison of images, individual fish with proven triploidy were identified among the multi-scaled linear fish dissected in the present study.

In 2013, four, 5-year-old, multi-scaled linear females from heat-shocked progeny were artificially spawned and crossed with normal diploid koi males. Eggs from each female were fertilized with sperm taken from different koi males (one male per cross). The technique of artificial spawning was the same as described by Gomelsky et al. (2012). To induce ovarian follicle maturation in females and spermiation in males, fish parents were injected with carp pituitary extract (Argent Chemical Laboratories, Redmond, Washington) at 3 mg/kg. Total weight of the eggs obtained from each female was recorded; a sample of eggs taken from each female was weighed, and the number of eggs 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 (volumetric ratio 8:1) to remove egg adhesiveness. Embryos were incubated in McDonald jars. The percentage of live embryos was determined approximately 20 h after fertilization at the 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. Most swim-up larvae obtained in crosses were stocked in separate 20-m^3 outdoor tanks for rearing; some larvae were stocked in aquaria in a recirculation system. After 4 months of rearing, tanks were drained and all juveniles were collected, weighed, and measured.

Ploidy of fish breeders as well as samples of the obtained progenies was determined by flow cytometric analysis of erythrocyte nuclear DNA content using an Accuri C6 flow cytometer (Becton, Dickinson and Company). Blood samples were collected from the caudal vein in 3.0-mL Vacutainer tubes (Becton, Dickinson and Company) containing lithium heparin and placed on ice. For each blood sample, 1.0 μL of heparinized blood was placed into 12 × 75-mm polystyrene test tubes (Becton, Dickinson and Company); 0.5 μL of heparinized blood taken from Largemouth Bass *Micropterus salmoides* was also added to each test tube to act as an internal staining control. Then, 500 μL of propidium iodide staining solution (Biosure) was added to the tubes. After the 10-min incubation period in darkness, a sample was placed on the sample injection port and 40,000 events were recorded for each sample. The relative DNA content was determined as the ratio of sample fluorescence peak intensity to the internal standard (Largemouth Bass) fluorescence peak intensity.

RESULTS

Data on mean weight and GSI of 4-year-old fish are presented in Table 1. Diploid males from control progeny were normally mature and released sperm from the genital pore; GSI was 6.4 ± 1.8% (mean ± SD). Multi-scaled linear males from heat-shocked progeny demonstrated the development of testes typical for triploid fish; these males did not release sperm from the genital pore and their testes had a pinkish color and were reduced in size. Mean GSI of multi-scaled males from heat-shocked progeny (1.1%) was about six times smaller than that of males from control progeny (6.4%) (see Table 1). Among the 29 multi-scaled linear males analyzed from heat-shocked progeny, six fish whose triploidy was confirmed in the previous study (Gomelsky et al. 2012) were identified based on scale cover patterns in photographs. The GSIs of these males varied from 0.6% to 1.7% and had a mean value of 1.1%, which is exactly the same as the mean value of GSI for all dissected multi-scaled linear males from the heat-shock progeny (1.1%, see Table 1).

Diploid females from control progeny were mature and had ovaries filled with fully grown oocytes; GSI varied from 7.5% to 30.7% and had a mean value of 21.3% (SD, ±4.4%) (see Table 1). The appearance of one female from control progeny and its ovaries are shown in Figure 1. Investigation of multi-scaled linear females from heat-shocked progeny showed that many of them had well-developed ovaries, which were filled with fully grown oocytes; GSI varied from 4.2% to 30.1% and the mean value was 17.0 (SD, ±7.5%) (see Table 1). Among 28 multi-scaled linear females dissected in the present study, six fish were identified as those whose triploidy was determined earlier. The GSIs of these fish varied from 6.0% to 26.1% and had a mean value of 16.2%, which is close to the mean value of the GSI for all dissected multi-scaled linear females from heat-shocked progeny (17.0%, see Table 1). The appearance of one identified triploid multi-scaled linear female and its ovaries are shown in Figure 2.

In the spring of 2013, 5-year-old multi-scaled linear females from heat-shocked progeny were artificially spawned and crossed with normal diploid koi males. Six females with profoundly swollen abdomens were selected and hormonally injected. Four females ovulated after the injections and the stripped eggs looked typical for koi (Common Carp). Data on weights and reproductive characteristics of multi-scaled linear females as well as results of crosses are presented in Table 2. The weight of stripped eggs obtained from females varied from 450 to 778 g or from 12.0% to 23.6% of female weight. Numbers of eggs per gram, total numbers of eggs obtained from females, and percentages of live embryos are presented in Table 2. In general, the production of eggs from females, embryo incubation, and hatching of larvae proceeded normally. However, mass mortality of larvae occurred in the hapas for several days after hatching; large quantities of hatched larvae did not swim up and died. Nevertheless, from 3,000 to 14,950 swim-up larvae (or 2.9–5.5% from number of live embryos the next day after fertilization) were obtained in progenies (see Table 2); a total of about 32,000 swim-up larvae were obtained.

Most swim-up larvae obtained in crosses were stocked in separate 20-m^3 outdoor tanks for rearing. Survival of fish during

TABLE 1. Mean weight and GSI of 4-year-old koi from control diploid and heat-shocked triploid progenies.

Type of progeny	Scale cover types	Fish ploidy	Fish sex	Number of analyzed fish (*n*)	Mean ± SD weight (kg)	GSI (%)	
						Mean ± SD	Range
Control	Scaled and typical linear	2n	Males	53	1.57 ± 0.31	6.4 ± 1.8	1.4–10.0
			Females	57	2.03 ± 0.45	21.3 ± 4.4	7.5–30.7
Heat-shocked	Multi-scaled linear	3n	Males	29	2.14 ± 0.46	1.1 ± 0.6	0.3–2.9
			Females	28	2.61 ± 0.76	17.0 ± 7.5	4.2–30.1

4 months of rearing in outdoor tanks was low and varied from 0.2% to 1.2% in different progenies; a total of 248 juveniles were collected from the tanks. The numbers of collected juveniles per progeny and their mean TLs are presented in Table 2. About 40% of juveniles had different morphological abnormalities, which mostly were manifested as head deformities and a reduction of dorsal fins.

Some larvae (600 larvae from females 1–3 and 900 larvae from female 4) were stocked for rearing in aquaria of recirculation system. No survivors were observed in aquaria of the recirculating system at 5 months after larvae were stocked.

Triploidy of multi-scaled linear females, which were used in crosses, was confirmed by flow cytometric analysis. The relative DNA content of the four females varied from 2.54 to 2.58 and had a mean value 2.57 (SD, ±0.02). The relative DNA content determined for the five diploid koi males (four males used in crosses plus one male, which was hormonally injected but not used in crosses) varied from 1.70 to 1.75 and had a mean value 1.72 (SD, ±0.02). The ratio of mean DNA content in females to mean DNA content in diploid fish (2.57:1.72) was 1.49, which is very close to a theoretical value of 1.50, thus confirming triploidy of females.

Ploidy of 110 juveniles (from 2 to 61 fish per progeny, see Table 2) was determined by flow cytometry. Distribution of juveniles with regard to ploidy is shown in Figure 3. Most of the juveniles were aneuploid and ploidy ranged from 2.3n to 2.9n and had a mean value of 2.6n; two juveniles were diploid (2n) and one fish had ploidy of 3.9n.

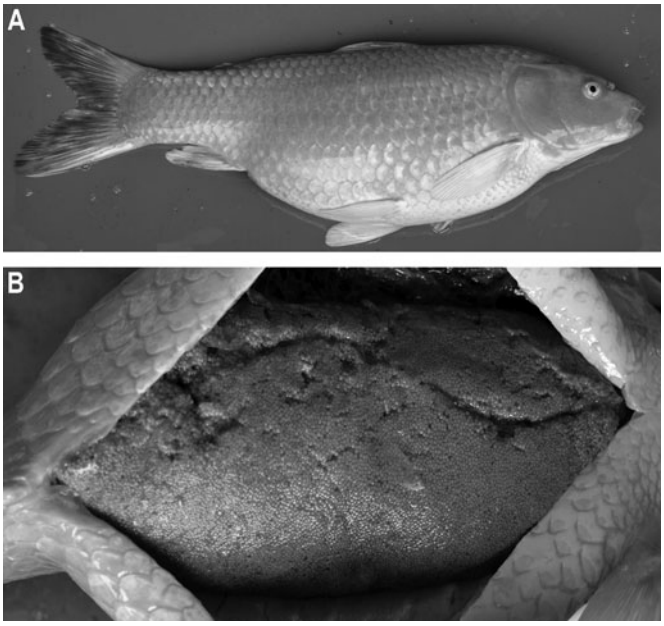

FIGURE 1. (**A**) Diploid scaled koi female from control progeny (TL = 51.1 cm, weight = 2.36 kg). (**B**) Appearance of ovary in female's body cavity (gonad weight = 644 g, GSI = 27.3%).

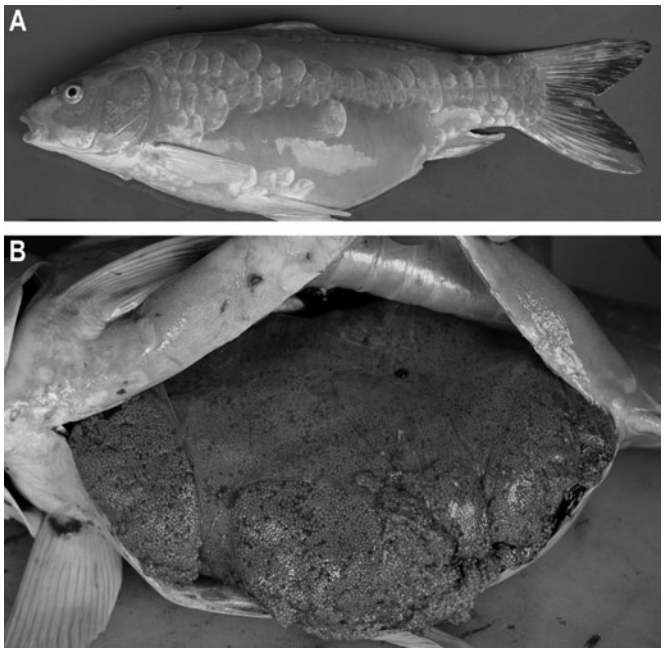

FIGURE 2. (**A**) Triploid multi-scaled linear koi female from heat-shocked progeny (TL = 52.7 cm, weight = 2.74 kg). (**B**) Appearance of ovary in female's body cavity (gonad weight = 714 g, GSI = 26.1%).

TABLE 2. Results of induced spawning of triploid koi females and their crosses with diploid koi males.

Female number	Female weight (kg)	Weight of stripped eggs		Number of eggs per gram	Total number of stripped eggs	% of live embryos	Number of swim-up larvae		Number of juveniles from outdoor tanks		Mean ± SD juveniles total length (cm)	Number of juveniles analyzed for ploidy
		g	%[a]				*n*	%[b]	*n*	%[c]		
1	3.30	778	23.6	507	394,446	69.0	14,950[d]	5.5	176	1.2	10.0 ± 3.2	61
2	3.60	492	13.7	640	314,880	64.0	5,850[d]	2.9	36	0.6	13.5 ± 4.0	23
3	3.72	714	19.2	509	363,426	50.0	8,580[d]	4.7	32	0.4	15.9 ± 2.9	24
4	3.76	450	12.0	578	260,100	26.0	3,000[d]	4.4	4	0.2	18.2	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.
[d]From these numbers 600 (from females 1–3) or 900 larvae (from female 4) were stocked in aquaria; the rest of the larvae were stocked in outdoor tanks.

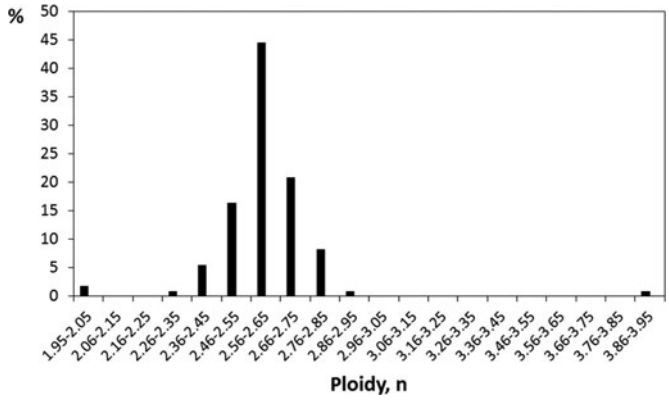

FIGURE 3. Percent distribution of juveniles ($n = 110$) obtained from triploid koi females with regard to ploidy.

DISCUSSION

As mentioned above, in the previous study (Gomelsky et al. 2012) all 43 analyzed multi-scaled linear fish from heat-shocked progeny were triploid. On this basis, it can be expected with a high probability that all multi-scaled linear fish dissected in the present study were also triploid. The 12 dissected multi-scaled linear fish (six males and six females), whose triploidy was confirmed in the previous study, had gonad development similar to other fish from this group. The present study used flow cytomery to confirm triploidy in four additional multi-scaled linear females, which were crossed with normal diploid koi males.

Multi-scaled linear males from heat-shocked progeny demonstrated a development of testes typical for triploids (Cherfas et al. 1994a; Benfey 1999; Piferrer et al. 2009). Testes of these males had pinkish (not white) color because of the absence of sperm and were reduced in size compared with the testes of diploid males. Mean values of GSI for triploid and diploid males recorded in the present study (1.1% and 6.4%, respectively) were close to the corresponding mean GSI values reported earlier by Cherfas et al. (1994a) for Common Carp (0.9% for triploid males versus 4.7% for diploid males). Cherfas et al. (1994a) observed that a few Common Carp triploid males released small amounts of sperm (with abnormal consistency) when stripped. In the present study, none of the 29 analyzed triploid males released sperm from the genital pore.

It is generally accepted that induced triploidy in fish affects the reproductive system of females more strongly than it does in males. Usually triploid females develop very small ovaries that do not contain advanced vitellogenic oocytes (Benfey 1999; Piferrer et al. 2009). In contrast, triploid koi females analyzed in this study developed large ovaries; in some triploid females GSI reached 25–30%. Earlier, Cherfas et al. (1994a) had also reported that some triploid Common Carp females had unexpectedly well-developed mature ovaries; in some triploid females GSI reached 10%. Wu (1990) also noticed that a few Common Carp triploid females had developed gonads containing oocytes at maturation stage. No information on any crosses of triploid Common Carp females has been previously reported.

In the present study, triploid koi females reacted normally to hormonal injection and released large quantities of ovulated eggs. Variability of ovulated eggs produced by triploid females was not studied especially, but visually no asynchrony in egg size was noticed as was described before for triploid females (Benfey 1996, 1999). Cases of artificially obtained triploid females in fish being able to produce some progenies in crosses are very rare. Goudie (1988) reported the production of triploid offspring in crosses of triploid females with triploid males in Grass Carp *Ctenopharyngodon idella* presuming that triploid females, the same as triploid males, produced aneuploid 1.5n gametes. Benfey (1996) described that no offspring survived through the prehatch period after eggs produced by one triploid Brook Trout *Salvelinus fontinalis* female were fertilized by normal haploid sperm. In contrast to females, reproductive ability of triploid males has been studied in many fish species. As a rule, triploid males produced aneuploid spermatozoa with ploidy range from haploid (n) to diploid (2n) level and the modal ploidy level around 1.5n. This was shown by flow cytometric analysis of sperm produced by triploid males in Rainbow Trout *Oncorhynchus mykiss* (Benfey et al. 1986), Barfin Flounder *Verasper moseri* (Mori et al. 2006), Tench *Tinca tinca* (Linhart et al. 2006), Atlantic Cod *Gadus morhua* (Peruzzi et al. 2009), and some other fish species. Aneuploidy of sperm produced by triploid males can result in the appearance of aneuploid progenies in their crosses with normal diploid females. For example, Peruzzi et al. (2009) showed that in Atlantic Cod the ploidy of larvae, generated by crossing triploid males with normal diploid females, ranged from nearly diploid (2.1n) to nearly triploid (2.75n) values and the mean ploidy level was around 2.4n. Most juveniles obtained from triploid females in the present study had ploidy ranging from diploid to nearly triploid (2.9n) levels and the mean ploidy value was 2.6n. This showed that triploid koi females produced aneuploid eggs with a ploidy range from haploid to diploid level and the modal ploidy level around 1.5n, similar to the production of aneuploid spermatozoa observed earlier for triploid males in fish.

Aneuploid progeny obtained from triploid koi females had low viability. Mass mortality of larvae was observed after hatching; the yield of swim-up larvae was about 3–5% of the number of live embryos the next day after fertilization (usually for normal koi progenies this index is 60–80%). Survival of fish in outdoor tanks was also very low, overall less than 1% (juvenile survival of normal koi progenies in tanks is usually 50–75%). Nevertheless, observed survival of some aneuploid koi juveniles can be considered as an unexpected result. Usually, crossing triploid male fish with diploid females results in the appearance of completely nonviable aneuploid progeny (Lincoln 1981; Lincoln and Scott 1984; Cherfas et al. 1994a; Peruzzi et al. 2009). Cherfas et al. (1994a) reported complete mortality of all embryos obtained after the fertilization of Common Carp eggs with sperm released by triploid males. In contrast, in the present

study some viable aneuploid juveniles from triploid females were obtained in the same species. It should be noted that progenies obtained by using sperm from triploid males are usually not numerous because of the small amount of sperm produced by triploid males and the low concentration of spermatozoa. In the present study, triploid koi females had large fecundity, which made it possible to obtain more than 30,000 swim-up larvae in spite of high mortality at the swim-up stage. When mass quantities of progeny are raised, the probability that some aneuploid fish could survive increases.

Not many cases have been recorded of aneuploid fish reaching the juvenile stage. Ueda et al. (1991) demonstrated the viability of aneuploid fingerlings with 3.5n ploidy which were obtained by crossing allotriploid Rainbow Trout × Brook Trout hybrid males with normal diploid Rainbow Trout females. Cherfas et al. (1994b) reported that fingerlings obtained by crossing allotriploid hybrid females of Prussian Crucian Carp *Carassius gibelio* × Common Carp with diploid Common Carp males were aneuploid and had chromosome numbers from 110 to 190, which corresponded to a ploidy range from 2.2n to 3.8n. Zhang and Arai (1999) obtained aneuploid progeny with fish ploidy ranging between 3n to 4n in the loach *Misgurnus anguillicaudatus* (also known as Oriental Weatherfish) by crossing artificially produced triploid males with natural tetraploid females.

Survivors in progenies arising from crossing triploid males with diploid females are typically believed to result from fertilization of eggs by euploid spermatozoa, which can arise with low frequency from atypical triploid meiosis. For example, Van Eenennaam et al. (1990) showed that rare surviving juveniles obtained by crossing triploid Grass Carp males with diploid females were diploid. In the present study, two diploid juveniles were found in progeny obtained from triploid females. One juvenile obtained from one triploid koi female had a ploidy level 3.9n. Apparently, this fish resulted from the spontaneous suppression of the second meiotic division in an aneuploid (1.45n) egg. Earlier, Ueda et al. (1991) suggested a similar mechanism for the explanation of the appearance of aneuploid fish with 3.5n ploidy in progeny obtained by crossing allotriploid Rainbow Trout × Brook Trout hybrid males with diploid Rainbow Trout females.

Further studies will be aimed at a more detailed investigation of reproductive features of triploid koi females. The influence of aneuploidy on the development of the reproductive system in fish obtained from triploid females will also be investigated.

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