Research Journal of Fisheries and Hydrobiology, 3(2): 41-47, 2008

Induction of Triploidy in *Gymnocorymbus Ternetzi* (Boulenger)

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Abstract: The black tetra, *Gymnocorymbus ternetzi* (Boulenger) is being widely used in the ornamental fisheries industry. Therefore, studies have been conducted to obtain a greater knowledge of its biology and production improvement, since growth and food conversion are compromised by the precocious sexual maturation, interfering with somatic growth. Thus, the possible use of sterile triploids is an interesting option for its culture. In the present study triploids are produced for *G.ternetzi* using thermal shock. The best triploid rate (63.6%) and yield (58.2%) were obtained with the heat shock regime of 38.°C for 4 minutes at 2.75 minutes of zygote age; the actual and relative survival rates were 47.2 and 91.5% respectively. With cold shock, the maximum percentage of triploid rate (60.0%) and yield (11.9%) had been obtained with the shock regime of 9°C for 10 minutes at 2.75 minutes of zygote age; the actual and relative survivals were 12.6 and 19.8% respectively. The nucleoli staining with silver nitrate ($AgNO_3$) and karyology are proved to be practical and efficient tools to investigate the ploidy of the fish from the treatments.

Key words: Triploidy - Thermal shock - Karyology - Nucleoli count - G.ternetzi.

INTRODUCTION

The objective of the present study is to produce triploids for *Gymnocorymbus ternetzi*, an ornamental fish, by standardising the thermal shock protocols, since triploidy can overcome the effects of sexual maturation on the growth of the fish [1] ; thereby reducing the maintenance cost involved in fish culture . In many fish species, sexual development and somatic growth are thought to be antagonistic processes that compete for available nutrients and energy [2,3]. Genetic and physiological manipulations can be carried out easily in teleost fishes due to their external fertilization and embryogenesis, which ultimately result in altered phenotype[4]. Perusal of literature reveals lacuna in the triploidization of G.ternetzi; hence the present attempt.

MATERIAL AND METHODS

Collection and maintenance of fish: *G.ternetzi*, obtained in their immature stage (30-45 days old), from local private ornamental fish dealers, were stocked in outdoor concrete tanks till they attained maturity. Later, they were transferred to indoor glass aquaria and maintained at 28 ± 1°C and 14L: 10D photothermal cycle. One week prior to breeding, sexes were maintained separately as it may considerably enhance the willingness to breed, besides avoiding breeding on their own without our eye on it.

Breeding: *G.ternetzi* grows to a length of 4-5cm. The male is smaller and black and the female silvery with a smoky tinge. Temperature range of 27-29°C ensures breeding in captivity. Glass as well as plastic aquaria of 2-50 l can serve as breeding tanks and according to the size of it, the fish were set up to spawn in a pair to few pairs or as many as several dozens of pairs in case of using 50-200 l tanks. Floating plants (*Ceratophyllum* sp.*)* were put into the tank to give better environment to the spawners. Fish pairs in the ratio of 1-2 male(s) to 1 female were put in the breeding tank in the evening and spawning occurred usually in the early hours of next morning. After spawning, water column was lowered to a minimal level and few drops of 2% methylene blue was added to prevent fungal infection. The young ones hatched out after 18-22 hours of incubation at 28 ± 1 °C.

Artificial fertilization: Milt from 4 or 5 males was stripped into an embryo cup containing 1ml of Fish Physiological Saline (FPS)[5]. at 24°C and the motility of the sperm was checked under a phase contrast microscope (Nikon, Japan). Artificial fertilization was carried out using milt(10-20 microlitres) and eggs(number used is given in tables 1 &2) stripped from the females. Fertility was assessed by counting the number of eggs at two-cell stage.

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Heat shock: To ensure effective and uniform heat shock, glass bowls having the fertilized eggs were immersed into a thermoregulated hot water bath (Julabo SW 210) and, after a definite time, were put back immediately into plastic aquaria with water having normal ambient temperature.

Cold Shock: For cold shocking, a very simple and modified method[6]. was used. Glass bowls having fertilized eggs were immersed into moderately cold water filled in small plastic troughs kept ready in the refrigerator. Desired temperature was set using the regulator in the refrigerator and the temperature of the water was checked before starting the experiments.

Based on a report that a depression of about 19-21°C from ambient temperature successfully induces a cold shock in tropical Cyprinids and Cichlids[7], a depression of 19°C ,from 28°C (ambient temperature) to 9°C (treatment temperature), was chosen as the cold shock in the present study. Further, based on the results of the heat shock, only optimised zygote age of 2.75 minutes after fertilization, has been tried for cold shock.

Karyotyping: Determination of ploidy[8]. (2n or 3n) was based on 15±5 metaphase spreads per specimen.

Nucleoli counting: Embryos or small pieces of tissues were fixed in methanol: acetic acid mixture (3:1) overnight or longer and slides were prepared[8]. The slides were stained using silver nitrate[9]. Number of nucleoli/cell was recorded for 100 cells per fry.

RESULTS AND DISCUSSION

Heat shock: The three variables of heat shock treatment - temperature, zygote age and duration of shock - were optimised. Of the 45 regimes (Table 1) triploidy was induced only in 12 combinations. Among the 12, the best result for the triploid rate (63.6%) and yield (58.2%) was obtained with the heat shock of 38°C for 4 minutes at 2.75 minutes of zygote age; the actual and relative survival rate were found to be 47.2 and 91.5% respectively. Securing 100% triploidy was found to be difficult in this fish; percentage of deforms increased with unfavorable heat shock regimes. The 'time window' period sensitive to heat shock was found to be very narrow for this species as triploids were obtained only at the zygote age of 2.75-3 minutes, with the shock duration of 3-4 minutes (beyond this time window no triplods were obtained with change in either of the zygote age or shock duration or temperature - data of preliminary observations not shown) at the ambient temperature of 28.5°C.

Cold shock: Table 2 present details of induction of triploidy using cold shock. The maximum percentage of triploid rate (60.0%) and yield (11.9%) was obtained with the cold shock regime of 9°C at 2.75 minutes of zygote age for 10 minutes; the actual and relative survival were found to be 12.6 and 19.8% respectively and a treatment duration of 20 minutes proved to be lethal.

A comparison of efficacy of heat and cold shock in triploidy induction, is given in Table 3. In terms of triploid yield as well as % relative survival of hatchlings, heat shock was found to be better (58.2%, 91.5%) than cold shock (11.9%, 19.8%). However, the percentage of triploid rate obtained was found to be almost the same for both heat shock (63.6%) and cold shock (60.0%) treatments.

Identification of triploidy:

Karyotyping: While the diploid metaphase (Figure 1) had the modal number of 50 the triploid metaphase consisted of 72-75 (Figure 2) chromosomes; the three marker chromosomes could be clearly seen.

Nucleoli counting: Figures 3 and 4 show the cells with nucleoli in diploid and triploid specimens respectively. Table 4. shows the percentage occurrence of cells, with varied number of nucleoli in diploid and triploid and the increased percentage of cells (56.3%) with three nucleoli seen only with triploid individuals.

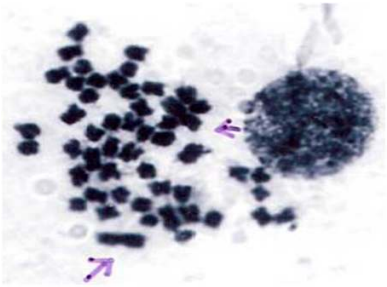

Fig. 1: Diploid metaphase 100X (arrows indicate marker chromosomes).

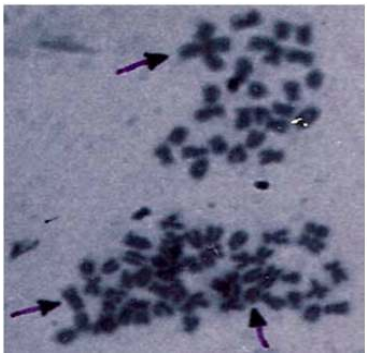

Fig. 2: Triploid metaphase 100X (arrows indicate marker chromosomes).

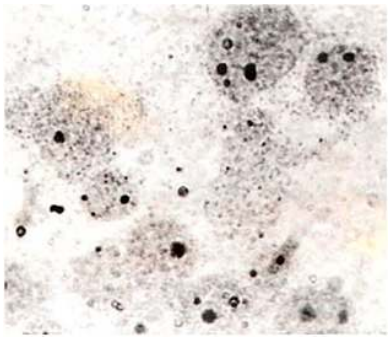

Fig. 3: Diploid cells with nucleoli 100X.

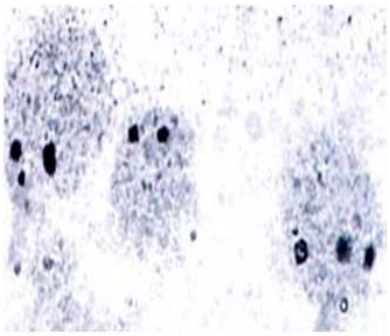

Fig. 4: Triploid cells with nucleoli 100X.

Table 1: Determination of optimum parameters of heat shock in the induction of triploidy in G.*temetzi*

	Heat shock				% survival at		Hatching							
Ambient Temp (N C)	Treatment Temp (N C)	Time after Inseminat ion (min.)	Duration (min)	No. of ova	Blastula	Organo genesis	Normal	Deformed	Hatching (expt)	control	% survival hatch relative to control	Sample (No. of hatchlings)	%Triploid rate	%Triploid Yield
29.0	36	2	3	89	55.1	50.6	31.5	19.1	50.6	28.1	180.1	10	0	0
29.0	36	2	4	60	16.7	10.0	3.3	6.1	10.0	28.1	35.6	2	0	0
29.0	36	2	5	162	45.7	29.0	25.3	3.1	29.0	29.6	98.0	10	0	0
29.0	36	2.5	2	59	74.6	64.4	39.0	25.4	64.4	28.1	229.2	10	0	0
29.0	36	2.5	3	170	38.2	30.6	27.1	3.5	30.6	29.6	103.3	10	0	0
29.0	36	2.5	4	126	38.1	26.2	22.2	4.0	26.2	29.6	88.5	10	0	0
29.0	36	2.5	5	100	48.0	46.0	46.0	-	46.0	60.0	76.7	10	0	0
29.0	36	3	3	95	67.4	63.2	35.8	27.4	63.2	28.1	224.9	10	0	0
29.0	36	3	4	97	16.5	16.5	13.4	3.1	16.5	65.6	25.2	9	0	0
29.0	36	3	5	172	52.3	50.6	45.9	4.1	50.6	65.6	77.1	10	0	0
29.5	37	2.75	4	118	38.1	13.6	11.0	2.5	13.6	28.8	46.9	10	0	0
29.5	37	3	5	12.4	81.5	70.2	68.5	1.6	70.2	32.4	216.4	10	0	0
29.0	38	2.5	3	117	94.0	76.1	74.3	1.8	76.1	24.0	317.1	10	0	0
29.0	38	2.5	4	165	673	65.5	60.0	5.5	655	36.2	180.9	10	0	0
29.0	38	2.75	3	84	85.7	56.0	54.8	1.2	56.0	60.0	93.3	10	40.0	37.3
28.5	38	2.75	4	127	48.8	47.2	46.5	0.7	47.2	51.6	91.5	11	63.6	58.2
29.0	38	3	3	75	68.0	45.0	38.7	6.6	45.3	65.5	69.2	11	27.0	17.9
29.0	38	3	4	163	25.8	22.7	19.6	3.1	22.7	65.5	34.7	9	44.4	15.4
28.5	39	2.5	3	128	24.2	3.9	3.9	-	3.9	33.3	11.7	5	0	0
28.5	39	2.5	4	124	44.4	12.9	11.3	1.6	12.9	33.3	38.7	10	0	0
28.5	39	2.75	3	148	493	45.3	43.9	1.4	45.3	51.6	87.8	15	53.3	46.8
30.0	39	2.75	4	218	20.6	12.4	12.4	-	12.4	29.4	42.1	15	45.7	19.7
28.5	39	3	3	119	31.9	13.4	9.2	4.2	13.4	33.3	40.2	7	429	17.2
30.0	39	3	4	219	53.0	19.2	15.1	4.1	19.2	29.4	65.3	15	26.7	17.4
28.5	40	2.5	3	92	55.4	6.5	4.3	2.2	6.5	42.4	15.3	4	0	0
28.5	40	2.5	4	105	57.1	13.3	11.4	1.9	13.3	42.4	31.4	7	0	0
28.5	40	2.75	2	66	59.0	30.3	24.2	6.0	30.2	42.4	71.1	7	0	0
28.5	40	2.75	3	60	36.7	25.0	20.0	5.0	25.0	42.4	66.7	126	8.3	3.5
28.5	40	2.75	4	118	22.0	8.5	5.9	2.5	8.4	42.4	19.8	6	0	0
28.5	40	3	2	134	44	17.9	9.7	8.2	17.9	39.6	45.2	10	10	4.5

Table 1: Continue

28.5	40	3	3	138	32.6	13.8	12.3	1.4	13.7	42.4	32.5	10	30	9.75
28.5	40	3	4	106	10.4	-	-	-	-	42.4	-	-	-	-
29.0	41	2.5	1	107	95.3	57.9	57.0	0.9	57.9	2S.1	206.0	15	0	0
29.0	41	2.5	2	47	72.3	31.9	14.9	17.0	31.9	2S.1	113.5	3	0	
0														
29.0	41	2.5	3	95	95.8	65.3	61.1	4.2	65.3	28.1	232.4	10	0	0
29.0	41	2.5	4	72	63.9	12.5	9.7	2.8	12.5	2S.1	44.5	3	0	0
29.5	41	2.75	2	136	25.0	7.4	5.1	2.2	7.3	2S.8	25.4	7	0	0
29.5	41	2.75	3	167	14.9	4.8	2.9	1.8	4.7	2S.8	16.3	5	0	0
29.0	41	3	2	135	5.1	3.7	2.2	1.5	3.7	28.8	12.8	3	33.3	4.3
29.S	41	3	3	95	3.2	3.2	1.1	2.1	3.2	28.8	11.1	1	0	0
30.5	42	2	2	125	56	36.8	25.6	11.2	36.8	4.3	855.8	10	0	0
30.5	42	2	3	144	45.8	23.6	7.6	16.0	23.6	4.3	548.8	7	0	0
30.5	42	2.5	2	70	44.3	25.8	22.9	2.9	25.8	4.3	87.2	1	0	0
30.5	42	2.5	3	102	32.3	2.9	1.9	1.0	2.9	4.3	69.8	1	0	0
30.5	42	2.5	4	80	11.3	3.8	1.3	2.5	3.8	4.3	87.2	1	0	0

Table 2: Details of Induction of triploidy in *G.ternetzi* using cold shock

Ambient Temp (NC)	cold shock			No. of ova	% survival at						% survival hatch relative to control	Sample (No.of hatchlings)	% Triploid rate	% Triploid yield
							Hatching							
	Treatment temp. (N C)	Time after insemination (min.)	Duration (min.)		Blastula	Organogenesis	Normal	Deformed	Hatching (expt.)	Control				
29	9	2.75	10	151	40.4	12.6	10.6	2.0	12.6	63.6	19.8	15	60.0	11.9
29	9	2.75	15	159	45.9	13.8	1.9	11.9	13.8	63.6	21.7	3	33.3	7.2
29	9	2.75	20	165	15.2	-	-	-	-	63.6	-	-	-	-

Table 3: Comparative efficacy of heat & cold shock in the induction of triploidy in *G.ternetzi*

Ambient Temp (N C)	Thermal shock parameters	No.of ova	% survival hatch relative to control	Sample (No.of hatchligns)	% Triploid rate %	Triploid yield
	Heat shock					
28. 5	38 (N C) 2. 75min A.F 4 min duration	127	91.5	11	63.6	8.2
	Cold shock					
29	9 (N C) 2.75min A.F 10 minduration	151	19.8	15	60.0	1.9

A.F - After fertilization

Table 4: Average fractions of cells (%) with one, two and three Ag-stained nucleoli

Ploidy level	Growth stage	No.of nucleolus per cell			No.of cellsper larvae	No.of individuals scored
		1	2	3		
Diploid	Larvae	22.8 ± 1.4	77.2 ±2.0	-	100	10
Triploid	Larvae	9.6 ± 1.0	36.1 ±2.3	56.3 ± 2.3	100	10

Discussion: *G.ternetzi* triploids are obtained in the present study using heat shock , with temperature range of 38-41°C, zygote age of 2.75 - 3 minutes, given for a duration of 2-4 minutes, at an ambient temperature of 28.5 - 29°C. Among these various combinations of regimes, the best result of 63.6% triploid (3n) rate and 58.2% triploid (3n) yield are obtained with 38°C for 4 minutes at 2.75 minutes of zygote age. Securing 100% triploidy has been difficult. Similar result of less than 100% 3n rate has been obtained using heat shock in some of the earlier studies in fishes like for e.g., *Oncorynchus mykiss*[10]. (76.5%), *Cyprinus carpio*[11]. (80%), and chinook salmon, *Oncorhynchus tshawytscha* [12] (< 80 %).

In the present study, a cold shock regime of 9°C for 10 minutes at 2.75 minutes of zygote age resulted in 60% 3n rate and 11.9% 3n yield. Similar lower triploid rates have been reported for cold shock in some studies involving *Clarias macrocephalus* [13] (80%), *Tinca tinca*[14]. (83.4%), *Pagrus major*[15]. (57.5%) and turbot, *Scophthalmus maximus*[16]. (70%).

In terms of triploid yield as well as % survival of hatchlings, heat shock has been found better (58.2%, 91.5%) than cold shock (11.9%, 19.8%) in the present

study though the triploid rate obtained is almost the same. The general consensus is that cold shock can successfully induce polyploidy in warm water fish and heat shock in cold water fish[17]. While the efficacy of cold shock has been equal to heat shock in triploid yield in *Oreochromis aureus*, cold shock excelled heat shock in *O.niloticus*[18]. These are indeed warm water species.

Low percentage of triploidy obtained in the present study may be attributed to lower sensitivity of the eggs to triploidising treatments[19]. and to the asynchronous development of eggs observed in this fish species. It has also been pointed out that differential maturity stages of eggs may be the reason for non-induction of polyploids in some specimens[20]. The ripeness of the eggs, the method of stripping, the sperm of the donor male or the water quality may have its impact on the production of triploids[21]. Further, diploids observed among lots treated to induce triploidy may result from sperm rejection as well as from failure of second polar body (2 PB) retention[22].

The temperature susceptibility of the cellular events related to the polar body (PB) extrusion has been located within the narrow range of 1°C in *Oreochromis aureus*[23]. At 1°C below this optimal temperature no triploids could be detected and 1°C above, the survival rate has been low, though still triploids are produced. A similar situation has also been observed in the present study and hence the above arguments hold good for the present case as well.

Further, triploids are obtained in the present study only when thermal shocks are given at the zygote age of 2.75 - 3 minutes and no triploids are obtained at other embryo ages higher or lower than this. A similar situation has been reported in *Oreochromis aureus*[23]. The window width for shock may be species – specific and in some cases brood stock – specific [24]. In addition, heat shocks have the effect of narrowing the window of opportunity for triploidization making time of application of shock more critical[25,26].

Survival has been found to be lower for the triploid treated lots than the control. Similar survival rates have been reported in species like *Salmo salar* [27]. *S.trutta* and its hybrids[28]. *Barbus barbus*[29]. and *Perca flavescens*[2]. One of the important factors that could be responsible for the reduced triploid survival may be inbreeding depression resulting from retention of 2PB.and further, the reason(s) underlying the low viability of triploids and the occurrence of malformations could possibly be due to ploidy other than triploidy[30]. in addition to the damages inflicted by thermal shock[31]. or triploidy *per se* or a combination of all these factors[32].

In triploid metaphase, in the present study, 77 chromosomes are found, but 3n are expected to have 75, as the diploid modal number (2n) is 50. A similar case has been reported in *Oncorhynchus mykiss*[33]. wherein the 3n has 90 or 91 or 93 chromosomes and the diploids are found to have two diploid modal numbers of 60 and 61. Robertsonian translocation has been suggested as the cause for the two modal numbers. These two extra chromosomes might have arisen through non-disjunction of only one or two chromosomes during meiosis II, as it is suspected to occur in fishes like *Salmo gairdneri*[34]. and *Pleuronectes platessa*[35]. Three marker chromosomes present in Figure 2 of the present study confirms the triploidy.

In somatic interphase cells there exists a close relationship between the number of visible nucleoli and the number of chromosome sets and the cells of any given species usually have a fixed number of nucleoli [36]. Based on this fact, nucleoli count has been made in the present study and no cells from diploid larvae are found to have 3 nucleoli but 56.3% of cells from triploid larvae have 3 nucleoli indicating the 3n ploidy status. While, more or less similar percentage of cells having 3 nucleoli has been reported for the triploids of *Cyprinus carpio* (51.6%) and *Tinca tinca* (40%), a higher percentage in the range of 70-80% has been reported for *Oncorhynchus mykiss* (70.6)[37].

Conclusion: For the first time triploidy has been induced using thermal shock in the black tetra, *Gymnocormbus ternetzi* successfully and identified using the conventional methods of karyology and nucleoli counting. This ornamental fish, is highly preferred by fish culturists for it very well contrasts with the other bright colored fishes and can co-exist with other fishes. As triploids, are expected to grow faster ,it can have a good impact on the trade.

ACKNOWLEDGEMENT

Author wish to thank Prof. M.R.Chandran and Prof.T.J.Pandian for their support and guidance and CSIR, New Delhi for the financial assistance.

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