Journal of Aquatic Biology & Fisheries, Vol. 2 (1) 2014: 215-228

PRODUCTION OF STERILE FEMALETRIPLOID BLUE GOURAMI, *TRICHOGASTER TRICHOPTERUS* BY HEAT SHOCK AND THE EFFECT OF TRIPLOIDIZATION ON GROWTH, SURVIVAL AND APPEARENCE

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Received on: 12.08.2013; accepted on: 12.12.2013

Abstract: Triploidy has been induced in a number of species to improve the performance and quality of fish in commercial cultivation. Blue gourami, *T. trichopterus*is a popular ornamental fish which exhibits distinct sexual dimorphism. Triploid blue gourami, *Trichogaster trichopterus* (Pallas) were produced by heat shocking freshly fertilized eggs. The optimum heat shock treatment required based on highest percentage triploidy (90%) and survival (46%) was at 41°C for 3 minutes duration on 1 minute old fertilized eggs. Induction of triploidy was confirmed by measurements of erythrocyte's nuclear volume and chromosome count. The triploid blue gourami had 69 chromosomes against the diploid chromosome number of 46. The triploid larvae were more viable (46 %) than diploid larvae (27%) after first feeding. Mean length and weight of one year old triploid fish were not significantly higher than diploids. All the triploid fish were sterile females with reduced GSI (1.09%) compared to diploid (15.77%). The triploid females were as attractive as diploid males and less aggressive. The triploid blue gourami is an ideal species for ornamental fish culture.

Key words: Blue gourami, ornamental fish, chromosome, diploid, GSI

INTRODUCTION

Triploidy is a common chromosome manipulation technique used in fish culture. Reasons for inducing triploidy usually relate to either sterility, improved growth performance or some other improved triploid quality. The sterile triploids can be released to the wild without danger of contaminating local gene pools. But generalization cannot be made about performance characteristics of triploids because triploids of different species have given different results. The results of triploidy induction depend apparently on the biological features of the species as well as on experimental conditions, including the method of obtaining triploid progenies (Wolters *et al;* 1982; Benfey and Sutterlin, 1984a; Thorgaard, 1986; Sugama *et al;* 1992; Fast, 1998; Johnson *et al.,* 2004; Taylor *et al*., 2011). So it is necessary to determine the procedures for mass production of triploid fish in each species. The study of impact of triploidy on various biological aspects in each species is also necessary.

The technique of triploidisation by heat shock method has been applied successfully in many species of fish (Valenti, 1975; Chourrout, 1980; Thorgaard *et al*., 1981; Lincoln and Scott, 1983; Benfy and Sutterlin, 1984b; Johnstone, 1985; Hollebecq *et al*., 1988; Kavumpurath and Pandian, 1990; Hussain, *et al.,* 1991; Holmefjord *et al.,* 1997; Anitha *et al*., 2000; Basavaraju *et al.,* 2002; Haffray *et al*., 2007). Heat shocking method is an inexpensive and less sophisticated method for triploidy induction. The present investigation was undertaken to determine the optimum heat shock treatment required for producing triploid blue gourami, *Trichogastertrichopterus*and to evaluate the effect of triploidy on survival, morphology, growth and gonadal development.

Blue gourami is a popular fresh water ornamental

fish. It is a good specimen for the aquarium, especially for beginners. This fish is a bubble nest builder exhibiting parental care and is a prolific breeder. The subsequent spawning period is an average of 20 days in females. Each male participates in the spawning of several females,but only one at a time. The male and female fish become fully mature at about five months age. Some of the major problems associated with rearing blue gourami are - during breeding,if the female fish is not responding to a male or if smaller in size than the male, may be attacked and killed by the male. Moreover,blue gourami exhibits distinct sexual dimorphism, females are not so attractive as males. So aqua rium keepers prefer to males . But in a population majority are females. The larvae are exceptionally small in size and their survival is also very low. The present study was also aimed to check whether triploidy induction can solve these problems associated with blue gourami rearing.

MATERIALS AND METHODS

Spawning and triploidisation

Brood stocks of blue gourami reared in separate cement tanks were stocked together at 1:2 female to males ratio in aquaria. This fish usually breeds 18 hours after stocking. After each mating a batch of eggs was laid. First and last few batches contain very small numbers of eggs (10 -100 each). So the eggs of third to sixth or seventh batches each consists of 200 - 600 eggs were collected by using fine meshed hand nets and immersed in pre heated water in a water bath. Shock was applied from 1 minute to 3 minutes after fertilisation. The temperature and duration of shock were 39°C to 43°C and 1 to 4 minutes respectively. After treatment, the eggs were immediately returned to water at normal temperature (25ºC to 28ºC) for incubation. One batch of eggs without heat shock treatment was used as control for each fish.

Hatching and survival.

The eggs hatched in about 22 hours at 25ºC. Just hatched larvae were extremely small and measure about 2.5 to 3 mm in length. Yolk was completely absorbed by the fourth day and then the larvae started feeding. Hatchability of eggs, number of deformed fry and survival of feeding fry were monitored.

Ploidy assessment.

Efficiency of heat shock treatment was ascertained by chromosome counts on 4 - 5 month old fish from each treatment group. Chromosome slides were prepared according to the method described by Kligerman and Bloom (1977). Ploidy was also determined by cell and nuclear volume measurements of erythrocyte's (RBC). Morphometric measurements of both control and triploidy induced fish of same age group (4 month) were also calculated to ascertain the impact of triploidy on morphology. Student's ¿t¾ test was conducted to find any significant difference between diploids and triploids in volume measurements of erythrocytes and morphometry.

Growth analyses

Triploidy induced and diploid larvae of *T .trichopterus* were reared separately in similar troughs of 50 litre capacity for three weeks. Fifty percentage of water was exchanged daily. After three weeks nursing, the fry were transferred to cement tanks of 500 litre capacity, in equal numbers of 20 each to undertake a comparative study of growth. About 25% of water was replenished once in every week after siphoning out the accumulated detritus and faecal matter. Three replicate samples were maintained for each group. The larvae were fed initially with boiled hens' egg and then with cultured plankton, powdered dry pellets and at one month age they were fed with 35% protein feed pellets to satiation once daily. Total weight and total length measurements were recorded monthly for one year starting at one month. The data were analysed using ANOVA. The percentage composition of the different ingredients in the 35% protein feed are given in Table 1.

Gonadal development, Sex ratio and Breeding behaviour studies

On termination of the growth analysis all the fish were sacrificed and weighed. The sex of each fish was determined by dissection of the gonads. The gonads were weighed to calculate the GSI using

the following formula.

Gonado-somatic Index(GSI) = Weight of ovary (g) / Weight of Fish (g) x 100

Development ofgonads was assessed using histological procedure. The relationship between gonad weight and body weight was analysed by simple regression analysis.

A series of breeding experiments were conducted to study the breeding behaviour of triploid fish. Six month old triploid fish were paired with diploid males of same age and the reproductive behaviour was noted.

RESULTS

The effect of heat shock on triploidization and survival

The optimum heatshock treatment based on highest percentage triploidy (90%) and survival (46%) was at 41° C for 3 minutes duration on 1 minute old egg. Most of the deformed embryos died soon after hatching. Exposure of eggs to heat shock higher than 41° C and 4 minutes or higher resulted in sharp decline in survival of embryos. The percentage of hatching was high in control eggs (94%) than shock treated eggs (69%). Whereas the viability was high in triploids after first feeding (46 versus 27). The results are summarised in Table 2.

Ploidy status

Chromosome counting and measurement of nuclear volume of erythrocytes confirmed triploidy induction. Diploid cells had 46 chromosomes and triploid cells had 69 chromosomes. The typical metaphase spreads of diploid and triploid *T. trichopterus* are shown in Figs. 1a, 1b. Triploid erythrocytes and erythrocyte nuclei were larger than diploid erythrocytes and erythrocyte nuclei (Figs. 2a, 2b). The differences observed for the cellular as well as nuclear measurements of diploid and triploid *T. trichopterus* are shown in Table 3 and Figs. 3a, 3b, 3c & 3d. Volume of the triploid nucleus($p < 0.01$) and cell ($p < 0.01$) was significantly larger (1.56 and 1.46 times respectively) than that of diploids.

Morphological markers in triploids

Juvenile triploids were not strikingly morphologically different from diploids. However, at two months age the triploid fish could be identified from their diplod siblings by the size and shape of dorsal fin, which was intermediate between that of diploid males and females, ie; it was more elongated and pointed than that of normal diploid females, but shorter than that of normal diploid

males. In triploid fish there was a shallow depression between head and trunk region. The snout was shorter than that of diploids. Moreover, during sexual maturity of diploids, ie; at about 4 to 5 months age, the triploids could be very clearly distinguished from diploids. The normal diploid females had rounded bulged belly, but the triploids had flat and compressed belly like that of diploid males. Moreover the triploids were more brightly coloured than normal diploid females. At about one year old the triploids showed close resemblance to normal diploid males in size,shape and colour.

Comparative morphometric measurements of diploid females and triploids also showed that the dorsal fin length, pectoral fin length and caudal fin length were significantly higher ($P<0.01$) in triploids. Whereas the head length, head breadth and snout length were significantly lower ($P<0.01$). The diploid male, female and triploid *T. trichopterus* are shown in Figs. 4, 5 and 6. The difference in morphometry is presented in Table 4 and Fig. 7 .

Growth

Slow growth of triploids was apparent initially (up to 4 months). Later, during the sexual maturation and active breeding period of diploids (4 -8 months), the triploids showed significantly fast growth. Afterwards (8 -9 months) the growth of triploids decreased, but the growth rate was slightly higher than diploids. However the final mean length and weight of triploid fish were not sigficantly higher than diploids. The growth of diploid and triploid *T. trichopterus* for one year is presented in Tables 5 and 6. The growth pattern is presented in Figs.8 & 9. Regression equation

Table 1. Proportion of feed ingredients and proximate composition of feed

Ingradients	Proportion (gm)	Protein content (%)	Proximate composition of diet (Dry weight basis)	
			Parameter	Percentage
			Moisture	14.59
Rice bran	17.8	1.23	Protein	34.96
Tapioca flour	17.8	0.37	Fat	5.90
Fish meal	32.2	18.06	Carbohydrate	10.13
Groundnut oil cake	32.2	15.44	Fibre	4.20
			Ash	2.70

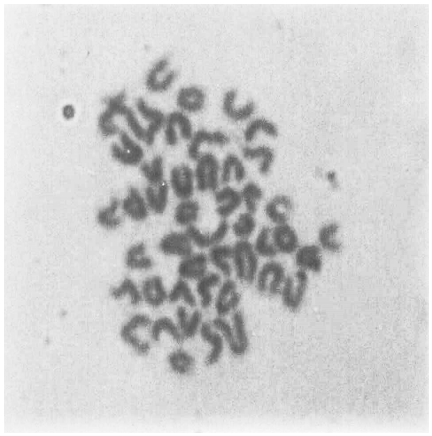
Table 2. Effect of heat shock on triploidy and survival in *T. trichopterus*

Heat shock (^{0}C)	Duration of shock (min)	Time after Fertilization (min)	Hatching (%)	Feeding fry survival (%)	Triploidy (%)	Deformed fry (%)
30 (Control)	-	-	94	27	0	2
40	2	1	80	23	0	2
	3	1	40	23	40	2
	4	1	35	25	67	5
41	2	1	54	25	0	2
	3	1	69	46	90	2
	4	1	31	23	67	4
	3	1.5	68	38	52	2
	3	2	69	37	36	1
	3	2.5	44	32	0	4

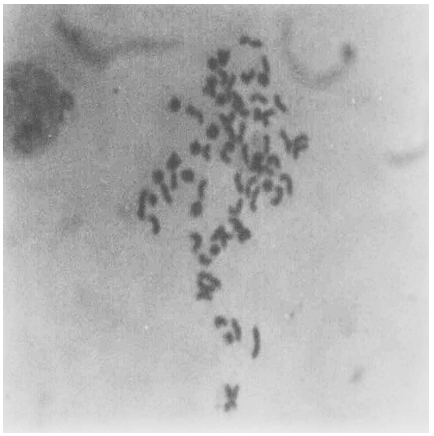
Table 3. Comparative cellular and nuclear measurements of diploid and triploid *T. trichopterus*

Sl. No.	Parameters	Diploid		Triploid		t value
		Mean	± SD	Mean	± SD	
1	Nuclear length (mm)	4.77	0.49	6.15	0	- 8.999**
2	Cellular length (mm)	10.46	0.65	12.61	0.65	- 7.411**
3	Nuclear width (mm)	3.15	0.24	3.38	0.4	-1.567
4	Cellular width (mm)	6.46	0.65	7.07	0.32	- 2.683**
5	Nuclear volume (mm^3)	24.9	4.49	39.05	9.04	- 3.959**
6	Cellular volume (mm^3)	227.81	30.52	332.57	50.6	- 3.638**
7	Nuclear area (mm^2)	11.79	1.39	16.34	1.92	- 6.079**
8	Cellular area (mm^2)	52.73	1.57	70.19	7.05	- 7.648**

** $P < 0.01$

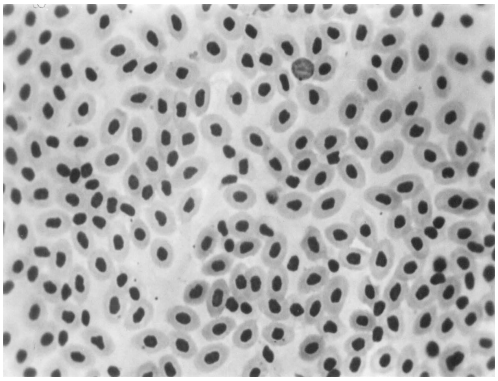


1a. Diploid *T. trichopterus*

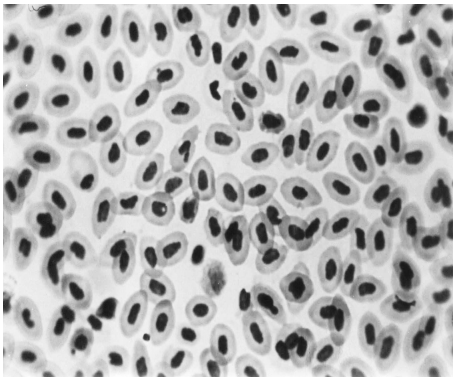


1b. Triploid *T.trichopterus*

Fig. 1. Chromosomes



2a. Diploid



2b. Triploid

Fig. 2. Erythrocytes

relating culture days and percentage length and weight increment of diploid and triploid *T. trichopterus* are given in Figs. 10, 11, 12 and 13.

Gonadal maturation, sex ratio and breeding

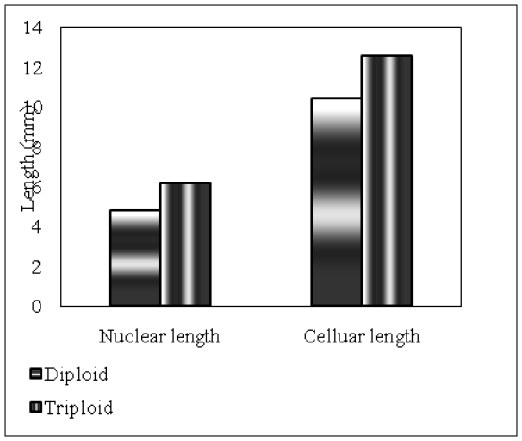
The ovaries of triploid fish were very small, paired and triangular in shape. They were reddish in colour and occupies only quarter of the abdominal cavity (Fig. 14). Much more fat was observed around digestive tracts .

The ovaries of diploid fish were very much broad, paired and triangular in shape. They were deep yellow in colour and completely filled the abdominal cavity. The stomach and other internal organs get pressed to one side of the abdominal wall (Fig. 15). The ovaries of diploid and triploid fishes are shown in Fig. 16.

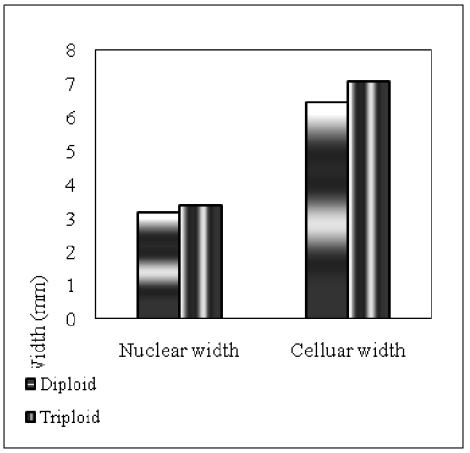
In the present study all the triploids were females. The weight of ovary was sinificantly low in triploid fish compared to diploid fish. The histological observations of ovaries showed that the triploid ovary had extensive connective tissue with a few atretic oocytes (Fig. 17). Whereas the diploid ovaries were ripe with mature, maturing and immature oocytes (Fig. 18).

The breeding experiments confirmed that the triploid fish were sterile and they could not breed. Immediately after paring with diploid males they showed mild courtship behaviour for a very short duration (5 -10 minutes), then they ignored the males.

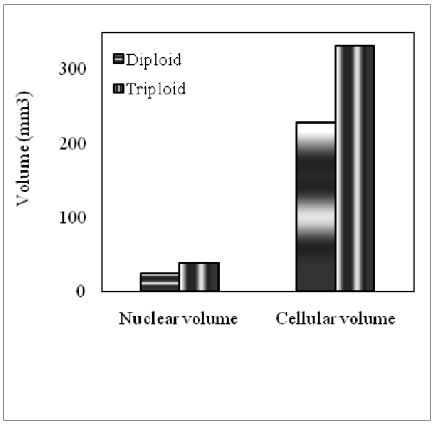
The weight of ovary of triploid fish was significantly lower than that of diploid fish. The



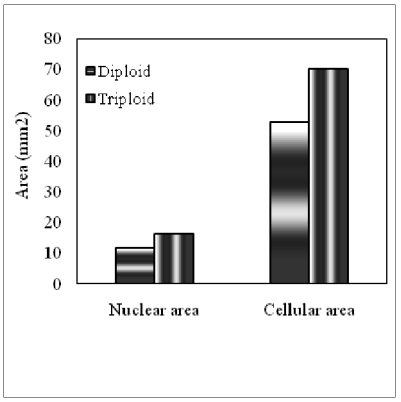
3a



3b



3c



3d

Fig. 3. Comparison of nuclear and cellular measurement of diploid and triploid *T. trichopterus*

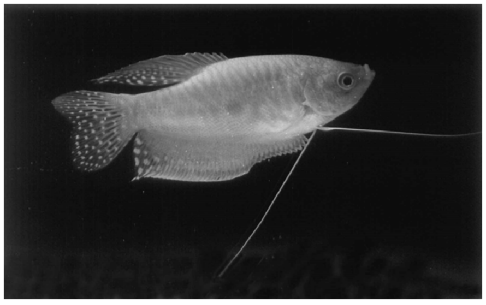


Fig. 4. *T. tricchopterus* male

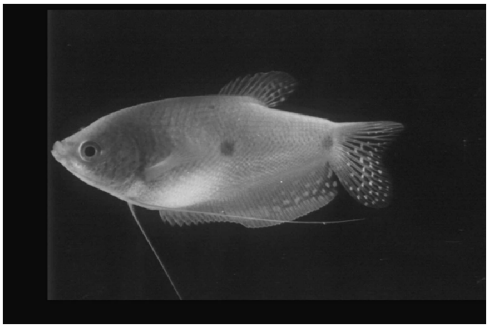


Fig. 5. *T. trichopterus* female

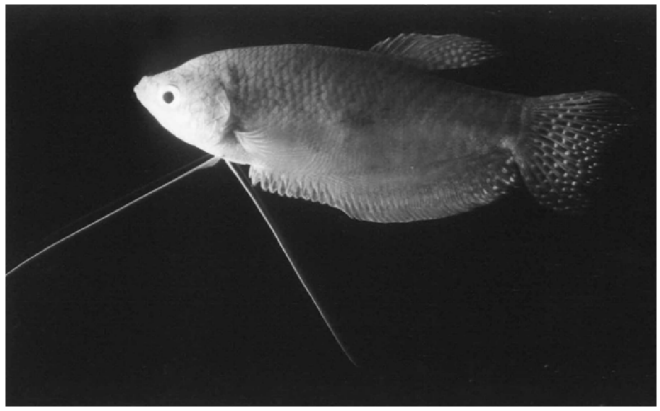


Fig. 6. *T. trichopterus* triploid

Table 4. Comparative morphometric measurements of diploid and triploid *T. trichopterus*

Sl. No.	Parameters (cm)	Diploid		Triploid		t value
		Mean	± SD	Mean	± SD	
1	Total length	11.5	0.35	11.48	0.31	0.142
2	Standard length	8.7	0.24	8.54	0.21	1.485
3	Head length	2.5	0.12	2.04	0.09	10.182**
4	Head breadth	2.8	0.11	2.56	0.21	3.969**
5	Snout length	1.2	0.05	0.86	0.05	12.991**
6	Predorsal length	5.0	0.19	5.04	0.18	-0.761
7	Body depth	3.6	0.16	3.70	0.16	-1.499
8	Dorsal fin length	2.5	0.19	3.16	0.15	-9.193**
9	Pectoral fin length	2.4	0.05	2.00	0.07	16.500**
10	Anal fin length	2.8	0.13	2.92	0.13	-1.819*
11	Caudal fin length	6.0	0.14	6.86	0.27	-9.458**

* $P < 0.05$

** $P < 0.01$

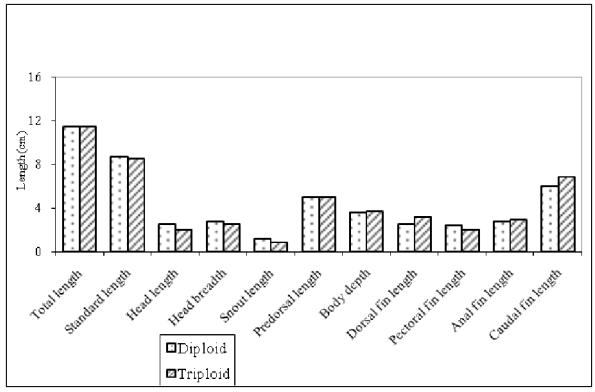


Fig. 7. Morphometric characters of diploid and triploid *T. trichopterus*

Table. 5. Growth of diploid and triploid *T.trichopterus*

Parameter	Fish Genotype		Culture period (Days)											
			0	30	60	90	120	150	180	210	240	270	300	330
Length (cm)	Diploid	Mean	0.20	1.68	3.19	4.77	6.00	6.85	7.48	7.94	8.29	8.55	8.79	9.00
		±SD	-	0.44	0.85	1.34	1.71	1.99	2.21	2.36	2.43	2.50	2.52	2.55
	Triploid	Mean	0.20	0.93	1.68	2.60	3.81	5.21	6.46	7.43	8.11	8.54	8.83	9.11
		±SD	-	0.18	0.41	0.76	1.24	1.75	2.19	2.52	2.78	2.91	3.00	3.06
Weight (g)	Diploid	Mean	0.04	0.75	1.72	3.05	4.23	5.15	5.89	6.43	6.91	7.28	8.04	7.86
		±SD	-	1.13	0.78	1.16	1.46	1.76	2.01	2.11	2.21	2.32	2.06	2.49
	Triploid	Mean	0.04	0.17	0.41	1.05	2.09	3.52	5.00	6.13	7.33	7.54	7.96	8.33
		±SD	-	0.12	0.35	0.71	1.25	1.81	2.21	2.56	2.69	2.96	3.06	3.12

Table 6. Percentage increment of *T.trichopterus*

Parameter	Fish Genotype	Culture period (Days)										
		30	60	90	120	150	180	210	240	270	300	330
Length (%)	Diploid	742	1496	2283	2900	3325	3642	3871	4046	4175	4296	4400
	Triploid	363	738	1200	1804	2504	3129	3613	3954	4171	4317	4454
F value		30.868**	30.82**	23.81**	12.88**	4.61*	1.302^{NS}	0.27^{NS}	0.03^{NS}	0^{NS}	0.001^{NS}	0.009^{NS}
Weight (%)	Diploid	1775	4192	7525	10463	12775	14629	15963	17171	18108	20004	19546
	Triploid	319	933	2515	5129	8692	12400	15213	18233	18754	19796	20733
F value		3.15^{NS}	27.79**	14.81**	5.04*	1.07^{NS}	1.07^{NS}	0.098^{NS}	0.179^{NS}	0.057^{NS}	0.006^{NS}	0.003^{NS}

* P<0.05 ** P<0.01 NS Not significant

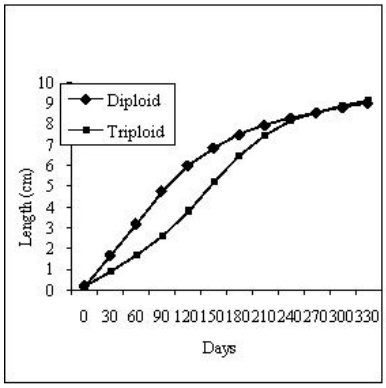


Fig. 8. Mean length of diploid and triploid T. trichopterus

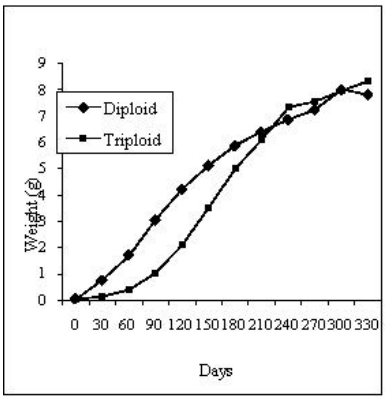


Fig. 9. Mean weight of diploid and triploid of T. trichopterus

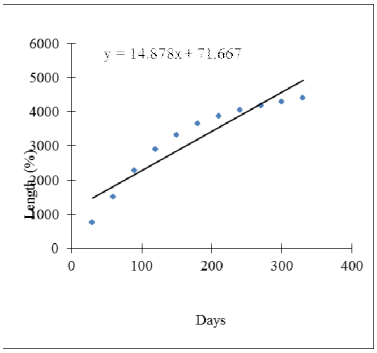


Fig. 10. Regression between days and percentage length increment of triploid *T. trichopterus*

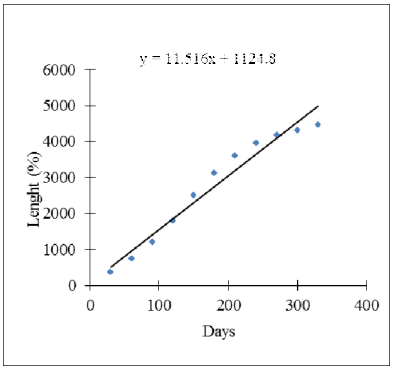


Fig. 11. Regression between days and percentage length increment of diploid *T. trichopterus*

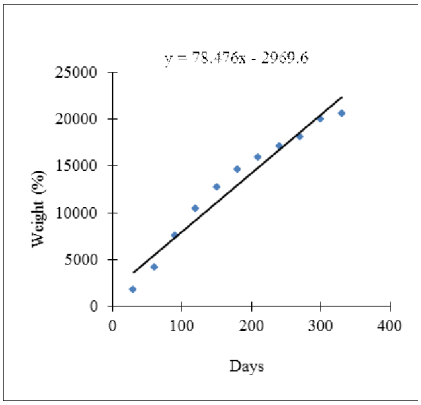


Fig. 12. Regression between days and percentage weight gain of triploid *T. trichopterus*

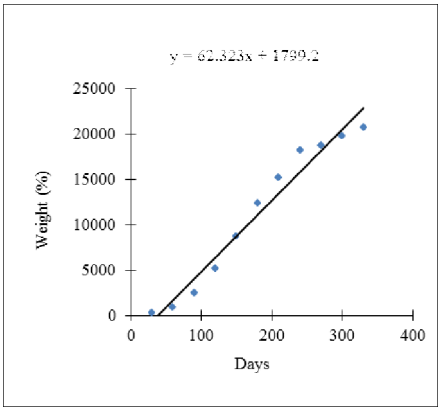


Fig. 13. Regression between days and percentage weight gain of diploid *T. trichopterus*

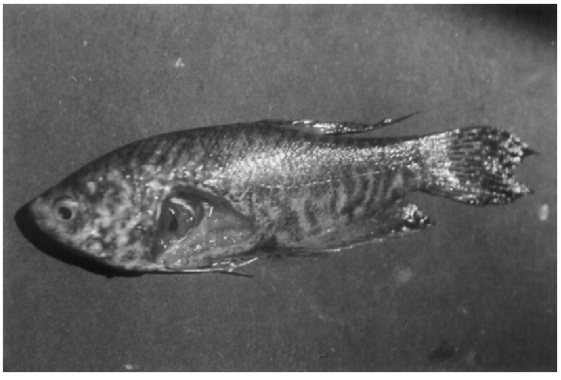


Fig. 14. Triploid *T.trichopterus* female with visceral cavity cut open to show the ovary

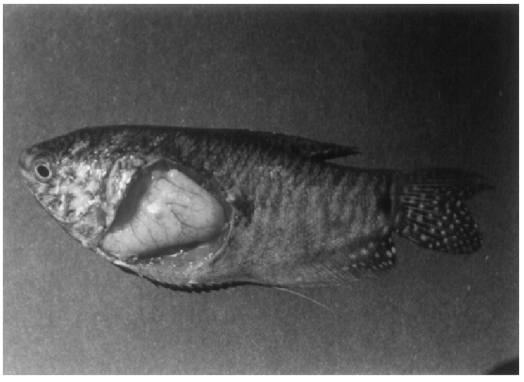


Fig. 15. Diploid *T.trichopterus* female with visceral cavity cut open to show the ovary

Fig. 16. Ovaries of diploid and triploid *T.trichopterus.*

Table 7. Gonodo Somatic Index (GSI)of *T. trichopterus*

	Diploid	Triploid
Body weight	4.74	9.78
±SD	1.47	3.06
Weight of Ovary	0.81	0.09
±SD	0.48	0.03
GSI	15.77	1.09
±SD	5.45	0.75

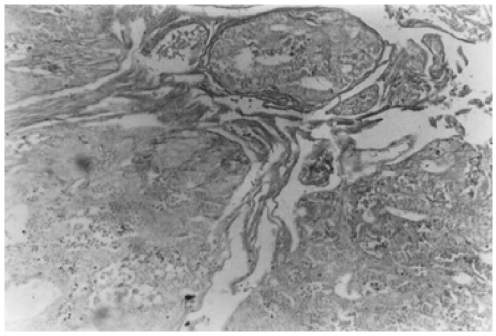


Fig. 17. Cross section of triploid ovary

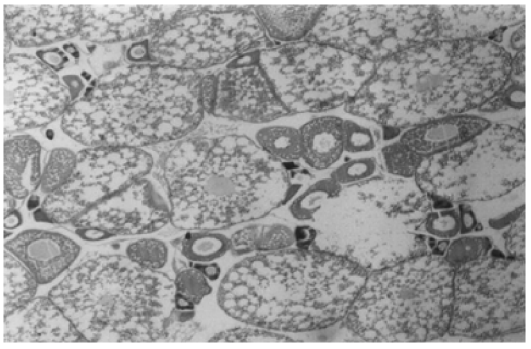


Fig. 18. Cross section of diploid ovary

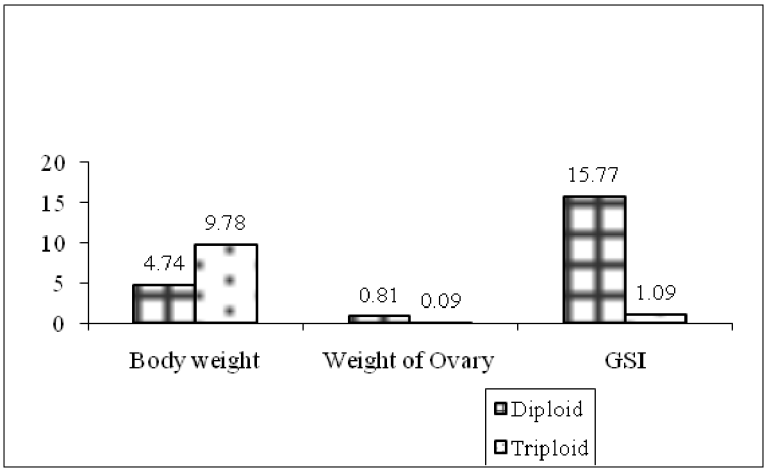


Fig. 19. Weight of body and ovary and GSI of *T. trichopterus*

mean ovary weight was 0.09g in triploids and 0.81g in diploids. The triploid fish had reduced gonado-somatic index (GSI), 1.09 compared to diploids (15.77). Table.7 shows the average gonad weight and gonado-somatic indices of triploids and diploids. The differences observed in GSI between 2n and 3n fish are presented in Fig.19. In triploid fish, the ovary weight decreased with increase in body weight. Where as in diploid fish, the ovary weight increased with increase in body weight.

DISCUSSION

The high levels of triploidy achieved in the experiments conducted here confirm the effectiveness of heat shock treatment (41° C for 3 minutes on 1 minute old eggs) in supressing meosis II in *T. trichopterus.* In certain species like *O. mossambicus*and *B. rerio*heat shock is 100% effective in inducing triploidy (Pandian and Varadaraj, 1988a; Kavumpurath and Pandian, 1990). But in the present study, heat shock induced 90% triploidy in *T. trichopterus*. As reported by Lou and Purdom,(1984b), the difference in the percentage of triploidy may be related to egg quality or to the susceptibility of eggs of different origin to triploidization treatment.

Mortality of heat shocked eggs was comparatively higher than control groups. This may be due to the deleterious effects of heat shock on early embryologlcal development. Eventhough the adult fish were hardy the young ones were very delicate. Major losses irrespective of treatment occurred between hatching and feeding stage, as in the case of rainbow trout (Chourrout, 1980; Chourrout and Quillet, 1982) *Bettasplendens* (Kavumpurath and Pandian, 1992) and Atlantic cod (Opstad *et al.,* 2013). However, the survival of triploids was higher than diploids after first feeding. Better survival due to triploidy is reported only in interspecific hybrids (Thorgaard, 1983; Chevassus *et al*., 1983; Dobosz and Goryczko, 1988).

Most triploidy induction techniques result in some diploids mixed with triploids. Eventhough a number of techniques like karyotyping, flowcytometry, silver staining etc. exist for separating diploid and triploid fish, they are generally impractical for screening large numbers of fish. In the case of *T.trichopterus* the triploids could be visually identified from their diploid siblings at about 2 months old. An experienced person can easily distinguish the triploids from diploids from this stage onwards. The triploids were as attractive as diploid males. The morphological abnormalities due to triploidy were also reported by many authors (Swarup, 1959b; Sutterlin *et al.,* 1987; Tiwary *et al.,* 1999; Tiwary *et al.,* 2001). According to Ihssen *et al.* (1990), the morhological difference of triploids would have to be due to a dosage or co -dominant effect, because the diploids and triploids share the same alleles.

Cytological karyotyping is the most accurate method of determining ploidy, because actual chrosome numbers can be determined. The chrosome study revealed that the diploid *T.trichopterus* cells had 46 chromosomes and triploids had 69 chromosomes. Presence of one additional set of chromosomes confirmed the successful induction of triploidy in *T. Trichopterus*.

Triploid fish had larger erythrocytes than that of diploid individuals. This observation was confirmed by several works on other fish species (Wolters*et al*; 1982a; Small and Benfy, 1987; Kavumpurath and Pandian, 1990; Arai *et al.,* 1993; Kim *et al*., 1994; Svobodova *et al.,* 1998; Tiwary *et al.,* 1999; Felip *et al*., 1999). The increased cellular and nuclear volume also confirmed triploidy in *T. trichopterus*.

The juvenile triploid blue gourami exhibited inferior growth relative to diploids. The inferior growth may be due to the deleterious effect of heat shock. From 4 to 8 months the triploids showed significantly high growth rate than that of their diploid counterparts. This period coincide with the active breeding period of their diploid sibs. After this period slow growth was observed. However no significant difference in weight and length of triploid fish was observed at the end of one year growth studies. Similar observations were reported in rainbow trout *Oncorhynchus mykiss (*Myers, 1991)., in mud loach, *Misgurnusmizolepis* (Kim *et al.,* 1994) and in *O. Niloticus* (Hussain *et al.,* 1996). Peruzzi *et al*., 2004 also noticed inferior growth of triploids prior to maturation in Sea bass, *Dicentrarchus liabrax*

Usually aquarists prefer to small sized fishes, as they are the most suitable for home aquaria. Eventhough triploidy brings out large size in many species of fish, the triploid fish of blue gourami had no significant size difference than their diploid counterparts. This is a great

advantage for aquarists.

The most important observation of the present study is that all the triploids were females and were sterile. They had very small ovary and reduced gonado somatic index (GSI). While diploid male *T. trichopterus* were highly aggressive during breeding season and causing great mortality to females, triploid females were less aggressive and not attacked by males. Thus mortality during breeding season could be minimised. The reason for total absence of males in the present study is unknown. This may be due to the variation of sex determining mechanism among the different ploidy levels (Streisinger *et al*., 1981). Generally all female triploids are produced by inducing triploidy in eggs that had been fertilized with sperm from musculanised females(Lincoln and Scott, 1983; Okada, 1985; Galbreath and Thorgaard, 1995),or by the direct feminization of triploid embryo by direct oestrogen treatment shortly after hatching (Varadaraj and Pandian, 1990; Piferrer *et al.,* 1994). Where as in the present study, the triploidy induction by heat shock treatment alone produced all female population. All males produced by triploidy induction alone are reported in many fish species like, *Brachidaniorerio* (Kavumpurath and Pandian, 1990), and in brook trout, *Salvelinusfontinalis* (Warrillow *et al*., 1977). But there is no reports about all female triploids produced by heat shock alone. However in majority of fish species the sex ratio among triploids equals controls (Pandian and Vradaraj, 1989; Cherfas *et al*., 1994; Felip *et al*., 1998).

Large fat deposits were found around the digestive tract of trilpoid *T. trichopterus*. This tendency has also been reported in Rainbow trout (Thorgaard and Gall, 1979; Chevassus *et al.,* 1983; Lincoln and Scott, 1984), Coho salmon (Johnson *et al.,* 1986) and Tilapia*, O. niloticus* (Hussain *et al.,* 1995). According to Johnson *et al.* (1986), large fat deposition in triploid female may be due to the failure of lipid withdrawal from body reserves for vitellogenesis,which normally takes place during breeding season in normal diploid fishes.In conclusion , the high survival combined with the attractive appearance, less aggressiveness and reproductive sterility of triploid blue gourami may make them ideally suited for ornamental fish culture.

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