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Triploidy induction using heat shock and its verification in *Schizothorax richardsonii*

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ABSTRACT

A field study was conducted to standardize triploidy induction and its verification in *Schizothorax richardsonii*, which is a potential indigenous fish of Himalayan region. Heat shock was applied on the fertilized eggs at three thermal levels i.e. 26 °C, 28 °C and 30 °C with four exposure timing i.e. 5, 8, 10 and 12 minutes for induction of triploidy and verification was done by chromosome number in karyotyping. The triploid rate (64-70%) was obtained with the heat shock of 28 °C for 10 minutes at 10 minutes of zygote age; hatching rate and survival rate up to swim-up fry stage were found as 62-66% and 50-58%, respectively in triploid, while in control (diploid) 80-88% hatching rate and 62-72% survival rate up to swim-up fry stage was observed from similar brood stock. Direct method of triploidy verification was done by karyotyping and chromosomes number, $3n = 144$ was observed in triploid while diploid contain $2n = 96$ chromosomes in the cells. The findings of the study would be useful for production of sterile fish for better growth in aquaculture practice of this fish species.

Keywords: Snow trout, Triploidy, Heat shock, Karyotyping, Sterile fish

Introduction

Snow trout (*Schizothorax richardsonii*) is one of indigenous fish species of the Schizothoracinae group, endemic to the Himalayas and true to its name, it is found in streams and lakes which receive snow melt water from the hills. In central Himalayas, this fish known as "Asela" and has commercial importance as food fish and sports fish. Though, this species contributes a major share in the wild catch-composition but does not consider as candidate species for cold-water aquaculture due to its slow growth in captive condition (Pandey *et al.*, 2010) [1]. Triploidy induction in fish has been found to be useful for the control of over population, for increasing growth in juveniles, and for extending survival and improving growth in mature fish. Triploidy induction refers to the production of individuals with three sets of chromosomes and can be induced in fish by inhibiting the extrusion of the second polar body by shocking eggs shortly after fertilization (Peruzzi *et al.*, 2000) [2]. Induction of triploidy may prevent the reduction in post-pubertal somatic growth and carcass quality due to gonadal maturation and secondary sex differentiation (Poontawee *et al.*, 2007; Fraser *et al.*, 2012; Cleveland *et al.*, 2014) [4, 5, 3]. The characteristics of triploid fish are thus known to have a beneficial impact on fish production economics (Berrill *et al.*, 2012) [6]. Inducing triploidy by using physical shocks such as abrupt changes in pressure and temperature is a safe and reliable method which does not involve potentially harmful chemicals or radiation (Benfey, 1999; Fraser *et al.*, 2012) [7, 5]. The underlying principle is that the application of thermal (heat or cold) and/or mechanical (pressure) shock to the zygote at appropriate time of post fertilization will prevent the extrusion of the second polar body during second meiosis, which results in three chromosome sets in every embryonic cell (Johnstone, 1992; Maxime *et al.*, 2008; Benfey, 2016) [9, 8]. It has also been proved that mass production of triploid salmonids is feasible through physical shock without major reduction of survival (Quillet *et al.*, 1991; Fraser *et al.*, 2012) [10, 5]. It was reported that triploidy rates closer to 100% could be achieved in brown trout through heat shock trials (Arai *et al.*, 1987; Quillet *et al.*, 1991) [11, 10]. Karyological studies have provided basic information on the number, size and morphology of chromosomes (Tan *et al.*, 2004) [12] that is important to undertake chromosome manipulations in fish (Khan *et al.*, 2000) [13]. Triploidy can therefore be determined by karyotyping (visualization and counting of chromosomes) and silver staining of nucleolar organizing regions (Garcia-Abiado *et al.*, 1999; Benfey, 1984 and Piferrer *et al.*, 2009) [15, 14]. Pandey *et al.* (1996) [16] reported the occurrence of natural tetraploidy in Asian catfish, *Clarius batrachus*. More importantly, the welfare, fitness and performance traits of triploid and diploid are not similar (Fraser *et al.*, 2012) [5]. There are no reports from India concerning induction of polyploidy or chromosomal manipulations in snow trout.

Materials and methods

The experimentation and field study was conducted at Central Institute for Coldwater Fisheries Research, Bhimtal (29° 21'N and 79° 34'E, Altitude 1370 msl) Uttarakhand, India during the year 2019 to 2025. Mature brooders of the age of 2-3 years having females of the length 204-325 mm and weight 118-340 g and males of 160-280 mm length and 50-110 g weight were collected from nearby streams and reared in the FRP tanks (year 2019) to obtain the maturity. Mature and healthy brooder of 3 years age group were selected for each breeding operation, and three operations were conducted at 18.5 to 21.3 °C water temperature in different years. After mixing of milt in freshly harvested eggs of snow trout, fertilized eggs were observed under microscope with 10X for the further development of embryo and observing the timing of extrusion of second polar body at 18°C water temperature. An inverted microscope was used to observe the 10 live eggs of the same lot for this purpose. Centigrade Temperature Minutes (CTMs) was calculated by multiplication of minutes of extrusion of second polar body and water temperature in °C.

Triploidy induction

In each operation, approximately 14,000 eggs were collected from mature brooders by dry stripping and were divided into thirteen (I to XIII) groups. Group I having 2000 eggs were kept as control without heat shock while 12000 eggs were divided into groups II to XIII with 1000 eggs in each group and exposed for heat shock after 10 minutes post-fertilization. Solar *et al.* (1984) [17] reported that heat shock

treatment at 26 and 28 °C for 10 minutes resulted 83-100% triploidy in rainbow trout. Due to the similar breeding behavior and breeding environment of snow trout with rainbow trout, three thermal levels i.e 26, 28 and 30 °C were decided for heat shock treatment. Thus, in the present experiment, three operations were carried out in 36 combinations (3 breeding x 3 thermal levels x 4 exposure timings) at 18.5 to 21.3 °C water temperature. All the treated eggs were kept for incubation in different trays fitted in troughs having continuous flow of water (0.5 L/min). The incubation period was calculated as degree days (water temperature °C x number of days). Trays were properly labeled and were carefully observed for survival and embryonic development. During the experiment, egg size, relative fecundity, fertilization rate, hatching rate and survival rate were also recorded and analyzed as per procedure followed by Joshi (2004) [18].

Triploidy verification

Direct method of karyotyping or metaphase spread preparation (visualization and counting of chromosomes) was carried out as triploidy verification in treated and control group by using fry of *S. richardsonii*. The method was standardized by Felip *et al.* (2009) [19] based on Kligerman *et al.* (1977) [20] for obtaining chromosome spreads followed for conformity of ploidy. The similar method was also followed by Pandey *et al.* (1996) [16] for Asian catfish.

Percent of triploidy induction (TR)

Metaphase chromosome slide preparation was done with 50 best slides observed and counted number of chromosomes with the given formula.

$$\text{Triploidy rate (\%)} = \frac{\text{Number of slides containing triploid plate}}{50} \times 100$$

Result and discussion

Initial success in artificial breeding of wild stocks of *Schizothorax richardsonii* was achieved by Shrestha (1978) [21]; Sharma (1989) [22], Pandey *et al.* (2010) [1] and Joshi *et al.* (2026) [23]. Feasibility of the artificial fecundation favours this species for the suitability of triploidy induction. In control group, observations on breeding performance in three operations reflect that fully mature females release orange and sticky eggs when gently pressed on the abdomen, but it requires a very specific time for spawning to get good quality of eggs. Degeneration of ripe eggs was started within 1-2 days, if not extruded in time. Growing brooders were also observed the presence of some seasonal pathogenic bacteria in captive condition, which was also recorded by Bisht *et al.* (2014) [24]. During the breeding operation (Table 1), the egg size was observed in the range of 3.5-3.8 mm which is in conformity, reported by Pandey *et al.* (2012) [25] and Joshi, (2006) [26]. The relative fecundity of the fish was observed as 17503 eggs/kg body weight. Pandey *et al.* (2010) [1] also observed fecundity of this fish as 10,560-22,120 eggs/kg at 14-18 °C water temperature. The fertilization rate of 82-90% was achieved at water temperature 18.5-21.3°C (Table 1). Joshi (2004) [18] also reported 80-94.2% fertilization at water temperature range of 22.5-24 °C. Developing embryo started to hatch on 8th day and complete hatching was observed within the 6 hrs in all three operations. Generally, 8-10 days have been taken for the egg incubation at the thermal regime of 18.5-21.3°C. Data showed an inverse relationship of incubation period with water temperature. Hence, degree days was calculated on the basis of days taken for the egg incubation and its consequent temperature. The degree days were observed in the range of 180-198. Joshi (2006) [26] reported 120-168 hrs or 5-7 days incubation period at 24 °C water temperature for the same species. Hatching rate was observed in the range of 80-88%. The length of the sac fry (alevins) varied from 7.3-8.4 mm; weight 0.014-0.022 g. Comparatively lower thermal regime supports the better cumulative survival rate from hatchlings to swim up fry (72% at 18.5 °C) rather than higher thermal regime (62% at 21.3 °C) (Table 1). Joshi (2004) [18] also reported the

58.0-81.0% overall survival during incubation at water temperature 20.0-24.5 °C.

For triploidy induction, after fertilization of eggs, the timing of extrusion of second polar body at 18°C water temperature was observed as 10-11 minutes of post fertilization. Hence, the centigrade temperature minutes (CTMs) in present study for the snow trout was calculated as 180-198 at 18°C. According to Padmaja *et al.* (2012) [27], triploidy induction rate (89.7%) in red tilapia was achieved at 164 CTMs. For rainbow trout, Loopstra *et al.* (2008) [28] standardized 300 CTMs after fertilization.

Triploidy is induced directly by blocking of second polar body extrusion during second meiotic division shortly after fertilization of fish eggs using various physical shocks and chemical treatments. The heat shock is one of the common techniques for triploidy in fish, but the application of heat shock to induce triploidy is not always 100% effective and can cause a detrimental side-effect and decreased viability. Induction of triploidy in rainbow trout by heat shock has been reported by various authors (Chourrout, 1980; Solar *et al.*, 1984 [17]; Don *et al.*, 1986 [29]; Dillon, 1988 [30]; Guner *et al.*, 2016 [31] and Pandey *et al.*, 2024) [32]. Dillon (1988) [30] reported that rate of triploidy induction ranged from 0-100%, and all variables affected triploid yield. In present study, among the 36 combinations (Table 2), the best result for the triploid rate (64-70%) was obtained with the heat shock at 28 °C for 10 minutes at 10 minutes of zygote age after post fertilization (Table 3). Heat shock at 30 °C completely resulted as immediate damage of eggs becoming white in color. Hatching rate (62-66%) and survival rate up to swim-up fry stage (50-58%) were found in different operations. The 'time window' (a frame of zygote age, heat shock regime and shock duration) period, sensitive to heat shock was found to be very narrow for this species as triploid of this fish were obtained only at the zygote age of 10-11minutes, with the shock duration of 10 minutes. Beyond this 'time window', success for triploidy induction was not found due to change in either of the zygote age or shock duration. Though, success of the triploidy induction was also found at low temperature shock i.e. 26 °C with exposure time of 12 min. But hatching rate was low (18%) with comparatively

low triploid rate (54%) and low survival (40%). The similar impact of temperature level and exposure time was observed by Solar *et al.* (1984) [17] for rainbow trout. In control group, hatching rate and survival rate up to swim- up fry stage were found to be 80-88% and 62-72%, respectively. In present study, the temperature susceptibility of the cellular vents related to the polar body extrusion has been located within the narrow range of 2 °C in snow trout. At 2 °C above the optimal temperature (28 °C) no survival could be detected and 2 °C below the optimal temperature, still triploids were achieved with low hatching rate and survival. The window width for shock may be species-specific and in some cases brood stock-specific (Seeb *et al.*, 1988) [33]. In triploid snow trout, survival has been found to be lower than the control group of diploids. Similarly, lower survival rates have also been reported in triploids of *Salmo salar* (Galbreath *et al.*, 1994) [34] and *S. trutta* and its hybrids (Mckay *et al.*, 1992) [35] in comparison to diploids of these species. One of the important factors that could be responsible for the reduced triploid survival may be inbreeding depression resulting from retention of second polar body (Ueda *et al.*, 1988) [36]. Retention of the second polar body by shock treatments for triploidy induction in a variety of species has shown different timings for initiation of the shock treatments, suggesting precise timings are required for each species (Padmaja *et al.*, 2012) [27]. The present study reports induction of triploidy in *Schizothorax richardsonii* and demonstrates how different shock conditions had different effects on triploidy rate and survival rate. Previous reports also revealed that securing 100% triploidy in fish by heat shock is difficult. Similar result having less than 100% triploidy rate has been reported using heat shock in some of the earlier studies in fishes *viz*; 76.5% in *Oncorhynchus mykiss* (Diaz *et al.* 1993) [37], 80% in *Cyprinus carpio* (Basavaraju *et al.*, 2002) [38]. Effects of rearing temperature on egg incubation was also observed in the present study, which is in the con-

formity of Das *et al.* (2021) [39] in another cold-water fish, Chocolate mahseer.

In present study, triploidy verification was done by direct method of karyotyping with colchicine treatment on fry stage of snow trout. The verification of triploidy in our experiment showed that the diploid has $2n = 96$ chromosomes (Fig 1) and triploid (treated) has $3n = 144$ chromosomes (Fig 2). Vasave *et al.* (2016) [40] also reported chromosome number $2n = 96$, while Lakra *et al.* (1997) reported the chromosome number $2n = 98$ for this species.

Conclusion

The manipulation of chromosome and chromosome sets are major components of aquatic animal genetics improvement. By manipulating chromosomes, we intend to create new genetic constructs that exhibit commercially useful traits. For the first time triploidy has been induced using thermal shock in the commercially important cold-water fish, snow trout, *Schizothorax richardsonii* successfully and identified using the conventional methods of Karyology. Thus, the study is concluded that the heat shock is an effective method for triploidy induction in snow trout, but the application of heat shock to induce triploidy is not always 100% effective and can cause a detrimental side-effect and decreased viability.

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Table 1. Details of the breeding operation of control group

Average weight of females (g)	Average weight of males (g)	Average number of eggs	Ova diameter (mm)	Water Temp. (°C)	Fertilization. Rate (%)	Incubation Period (degree days)	Hatching Rate (%)	Survival Rate (%)
340	109	5700	3.38	18.5	82	185.0	88	72
245	94	4600	3.16	21.3	90	170.4	84	62
312	110	5400	3.26	19.5	88	175.5	80	68

Table 2. Performance of triploid induction in different treatments

Temperature (°C)	Exposure Time (min.) of heat shock	Incubation period (degree days)	Hatching rate (%)	Survival rate up to swim- up fry stage (%)
First operation				
26	5	234	82	68
	8	235	72	64
	10	235	66	57
	12	234	18	40
28	5	232	80	62
	8	234	70	60
	10	234	62	58
	12	234	12	26
30	5 damage of all eggs immediately			
Second operation				
26	5	226	84	62
	8	226	70	60
	10	225	64	54
	12	All damaged	All damaged	-

(continued on next page)

Temperature (°C)	Exposure Time (min.) of heat shock	Incubation period (degree days)	Hatching rate (%)	Survival rate up to swim- up fry stage (%)
28	5	226	62	65
	8	225	72	62
	10	225	64	57
	12	All damaged	All damaged	-
30	5 damage of all eggs immediately			
Third operation				
26	5	228	82	64
	8	227	72	62
	10	226	64	62
	12	All damaged	All damaged	-
28	5	226	60	64
	8	226	68	60
	10	225	66	50
	12	All damaged	All damaged	-
30	5 damage of all eggs immediately			

Table 3. Conformity of triploid induction by Karyotyping

Temperature (°C)	Exposure Time (min.) of heat shock	Nos. of plates observed	Nos. of triploid plate	% of triploidy induction (TR)
Pool slides in all three operations				
26	5	100	0	0
	8	100	0	0
	10	100	0	0
	12	50	27	54
28	5	100	0	0
	8	100	12	12
	10	100	70	70

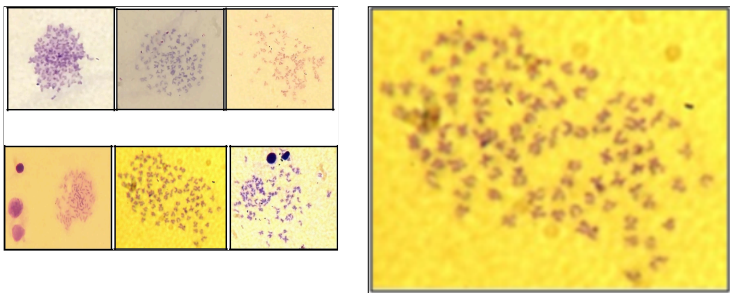


Figure 1. Diploid (2n = 96) Metaphase chromosome plates of *S. richardsonii* observed under 100x magnification

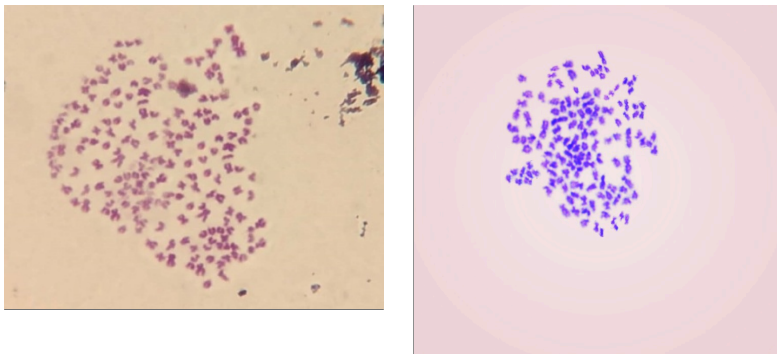


Figure 2. Triploid (3n = 144) Metaphase chromosome plates of *S. richardsonii* observed under 100x magnification

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