Bangladesh J. Fish. Res., 11(2), 2007: 131-140

Comparison of effectiveness of heat and cold shocks applied in the induction of gynogenesis in *Clarias gariepinus* (Burchell)

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Abstract
An experiment was conducted to optimize the procedure of gynogenesis in African catfish, *Clarias gariepinus* by suppressing meiotic and mitotic cell divisions in fertilized eggs. Gynogensis was conducted by fertilizing normal eggs with UV-irradiated sperm followed by either heat or cold shocking Irradiation of spermatozoa was given for a duration of 1 min and the eggs were fertilized *in vitro*. Cold shock at a temperature of 3±1°C for a duration of 30 and 60 min and heat shock at a temperature of 39±1°C for a duration of 1 and 2 min was applied to induce diploidy. Higher percentage of hatching (68.66) was observed for meiotic gynogens at a shock temperature of 39±1°C for a duration of 1 min, 5 min after fertilization (af). Higher percentage of mitotic gynogenetic induction (15.33) was observed at a temperature shock of 39±1°C for a duration of 1 min, 30 min af.

Key words: Gynogenesis, Sperm irradiation, Heat shock, Cold shock

Introduction

Gynogenesis is a parthenogenetic form of reproduction in which activation of the eggs is achieved by fertilization with genetically inactivited spermatozoa. According to Thorgaard (1986), gynogenesis is an "all-maternal" type of inheritance, where the genetic material of the sperm cell does not contribute to that of embryo. Stanley and Sneed (1974) suggested that this may be useful for the production of all female or monosex offsprings in the case of exotic species for release into the natural environment without risk of reproduction. Many workers have earlier attempted to induce gynogenesis in African and stinging catfish applying various shock treatments (Volkaert *et al.* 1994, Varadi *et al.* 1999, Galbusera *et al.* 2000, Gheyas *et al.* 2001). The aim of the present work was to compare the effectiveness of heat and cold shocks used at different times after fertilization, to duplicate the haploid set of chromosomes in

gynogenetic embryos for the production of all female monosex population in the exotic species, *C. gariepinus* of India.

Materials and methods

C. gariepinus broodstock were reared in cement cisterns of 5 meter dia. Ovulation was achieved through injecting Ovaprim, a synthetic analogue of salmon gonadotropin releasing hormone (sGnRH) and a dopamine antagonist 12 h before stripping of matured females at 28°C. Males were not induced extragenously. Testes were removed surgically and sperm was collected in an eppendorf tube and diluted 1:500 in HBSS (Hanks Balance Salt Solution without sodium bicarbonate and phenol red). Sperm concentration was estimated using a Haemocytometer.

2 ml of the diluted milt was exposed to ultraviolet (UV) irradiation in a simple UV irradiation chamber with 15W germicidal lamps-4 Nos. (Philips, Holland). Two lamps were fixed on the top and two lamps on the bottom at a distance of 27 cm from the centre. The sperm suspension was kept in the middle of the chamber for irradiation. Motility of the sperm was tested before (control) and after irradiation by microscopic examination. Arbitrary scoring system modified after Kurokura (1979) was used to asses the sperm motility and motility score of above 70% was recorded. The exposure of ultraviolet rays was done for different durations at 10 stages such as 30, 60, 90, 120, 150, 180, 200, 240, 270 and 300 sec and the duration of the motility was recorded. The irradiation process was carried out in full darkness to avoid genetic photo-reactivation of sperm (Kaastrup and Horlyck 1987). Maximum irradiation (60 sec) dosage with high motility was taken and used for activating the eggs for further study.

The female was hand stripped and the collected eggs were divided into 10 samples. The eggs were fertilized under dry condition with diluted and irradiated sperm. One sample was fertilized with the non-irradiated sperm to use as control, the remaining nine samples was fertilized with irradiated sperm. Eight of them were subjected to thermal shocks of heat or cold. One sample was fertilized with irradiated sperm and no shock treatment was given to serve as haploid. The induction was attempted for both suppression of meiotic and mitotic cell divisions to induce meiotic and mitotic gynogenesis. Cold shocking was performed by dipping a small netting basket (tea filter) containing the activated eggs in a water bath containing ice cubes (3 ± 1°C) for a duration of 30 and 60 min for the induction of meiotic gynogenesis 5 min a.f. and 30 min a.f. for the induction of mitotic gynogenesis. The heat shock was applied by submerging the basket in a water bath (39 ± 1°C) for a duration of 1 and 2 min, 5 min a.f. for the induction of meiotic gynogenesis and 30 min a.f. for the induction of mitotic gynogenesis. Subsequent to treatment, the embryos of treated and control groups were raised in small plastic troughs till hatching. The hatchlings were also reared in plastic troughs and frequent water exchange was done. The ambient water temperature was maintained at 28 ± 1°C throughout the study period. Viable embryos were counted at regular intervals of 4 h a.f. up to 24 h and at every week for a period of 4 weeks.

Survival percentage of haploid, gynogens and control fish were assessed after rearing the fry at a density of 100 individuals/10 l in plastic tub. The larvae were fed with freshly hatched *Artemia* nauplii. Haploid and diploid (gynogen and conrol) progeny status of *C. gariepinus* were assessed by the chromosome preparation from the hatchlings of each treatment according to protocol of Kligerman and Bloom (1977).

Results

The mean sperm concentration in *C. gariepinus* was found to be $3.2X10^9$ cells/ml and the values ranged from $2.7X10^9$ to $3.8X10^{10}$ cells /ml. The effect of UV irradiation on the motility of spermatozoa in *C. gariepinus* was depicted in Fig.1. More than 70% sperms were actively motile for a period of 60 sec after irradiation. The summary of the data observed in *C. gariepinus* subjected to various shock treatments for the induction of meiotic and mitotic gynogenesis and the percentage of hatching are given in Table 1. Chromosomes were prepared from the hatchlings for the determination of ploidy status. Haploid larvae of *C. gariepinus* possessed only one set of chromosomes (n=27 - Fig. 2), gynogens and controls had two sets of chromosomes (2n=54 - Fig. 3). In addition to normal hatchlings (Fig. 4) haploid syndrome was also observed in the treated groups with deformed hatchlings (Fig. 5). The data on the survival of meiotic and mitotic gynogens along with that of control and haploid for 24 h are presented in Table 2 and Fig. 6-9. The experiment showed that the hatching rates were high in both mitotic and meiotic eggs when the shock duration was minimum in all treatments. After a rearing period of 28 days the survival rate of gynogens and control group were 9.0 % and 52 %, respectively (Fig. 10).

Table 1. Summary of observation with various treatment for induction of meiotic and mitotic gynogenesis in *Clarias gariepinus* eggs fertilized with UV irradiated (60 sec) spermatozoa

Type of shock	Type of gynogenesis	Time of shock after fertilization (min)	Duration of shock (min)	Shock Temperature (°C)	Hatching (%)
Heat shock	Meiotic	5	1	39 ± 1	68.66
Heat shock	Meiotic	5	2	39 ± 1	60.66
Cold shock	Meiotic	5	30	3 ± 1	36.33
Cold shock	Meiotic	5	60	3 ± 1	30.33
Heat shock	Mitotic	30	1	39 ± 1	15.33
Heat shock	Mitotic	30	2	39 ± 1	11.66
Cold shock	Mitotic	30	30	3 ± 1	4.66
Cold shock	Mitotic	30	60	3 ± 1	2.33
Control	---	---	---	---	72.30

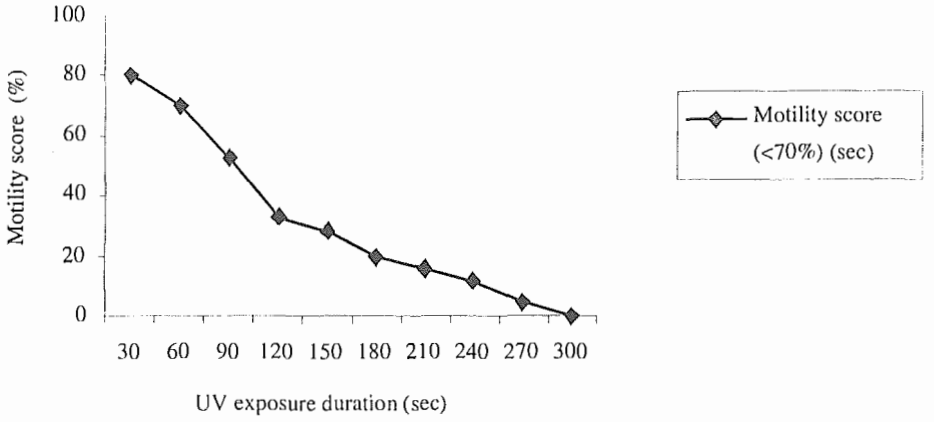

Fig. 1. Effect of UV irradiation on the motility of spermatozoa of *Clarias gariepinus*

Fig. 2. Haploid chromosomes of *Clarias gariepinus* (n=27)

Fig. 3. Diploid chromosomes of Gynogenetic *Clarias gariepinus* (2n=54)

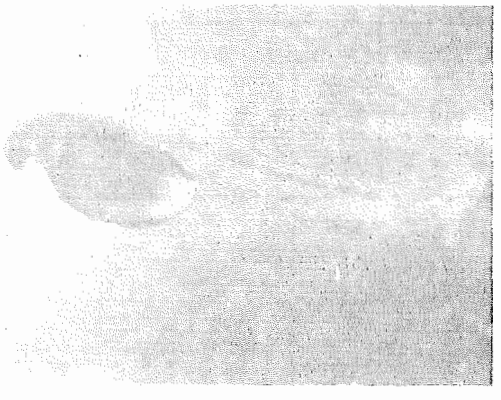

Fig. 4. Normal hatchling of *Clarias gariepinus*

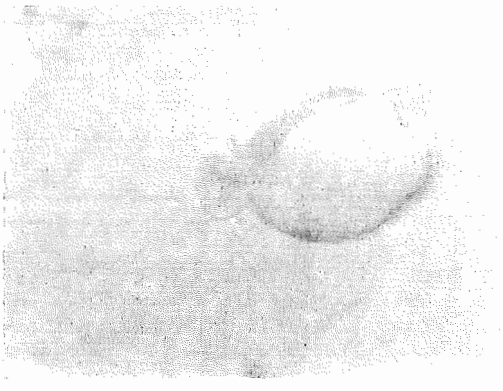

Fig. 5. Haploid syndrome of *Clarias gariepinus*

Table 2. Survival rate of meiotic, mitotic, haploid and control group embryos of *Clarias gariepinus* at different shock temperatures and duration.

Time after fertilization / treatment (hour)	Embryo survival (%)									
	Meiotic gynogenesis				Mitotic gynogenesis				Haploid (%)	Control (%)
	Heat shock (39±1°C)		Cold shock (3±1°C)		Heat shock (39±1°C)		Cold shock (3±1°C)			
	1 min	2 min	30 min	60 min	1 min	2 min	30 min	60 min		
4	98.00	96.00	98.66	90.66	98.66	69.66	82.00	80.33	96.33	98.66
8	95.66	94.33	95.33	88.33	50.33	46.33	42.00	60.66	85.66	96.00
12	92.33	90.66	89.33	80.33	49.66	32.33	29.33	27.33	76.66	90.66
16	87.66	82.66	49.33	40.66	33.33	24.00	18.66	16.66	40.00	83.33
20	71.00	76.33	35.33	33.33	28.06	16.33	5.33	8.33	16.66	75.66
24	68.66	60.66	36.33	30.33	15.33	11.66	4.66	2.33	4.33	72.30

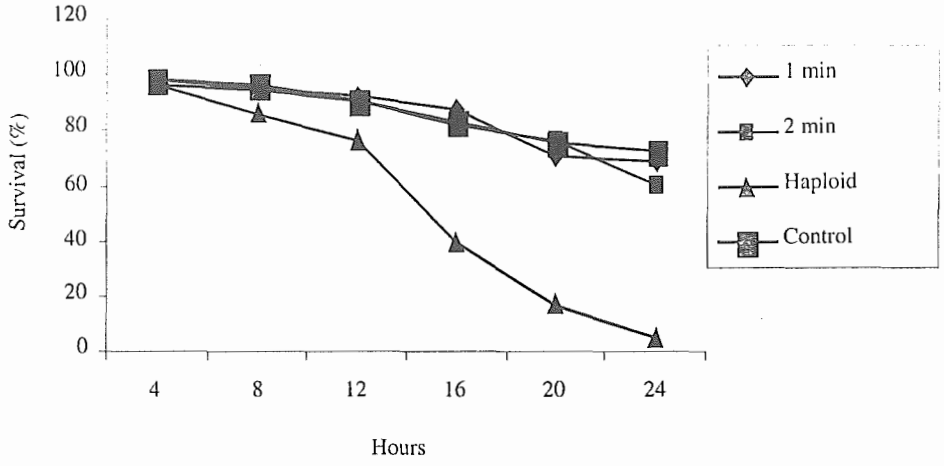

Fig. 6. Survival of meiotic gynogens by heat shock at 39±1°C for a duration of 1 and 2 min , haploid and control

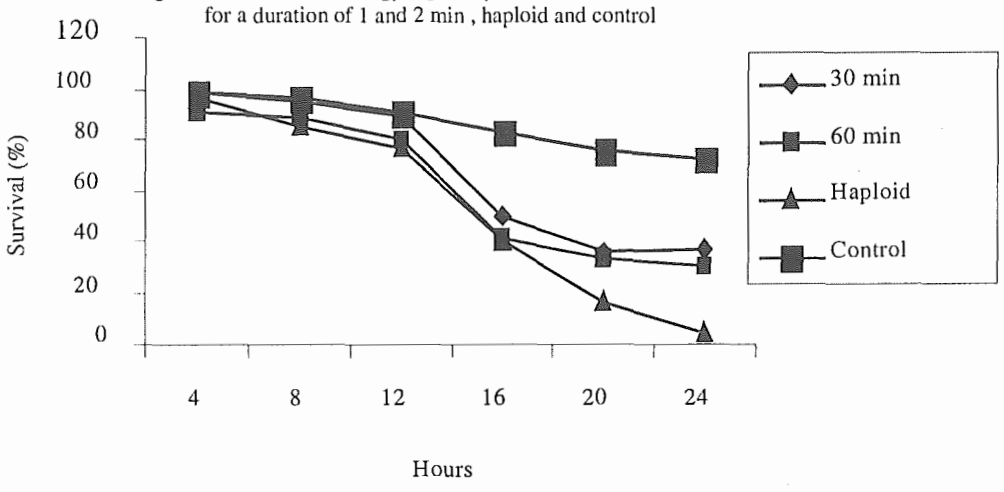

Fig. 7. Survival of meiotic gynogens by cold shock at 3±1°C for a duration of 30 and 60 min, haploid and control

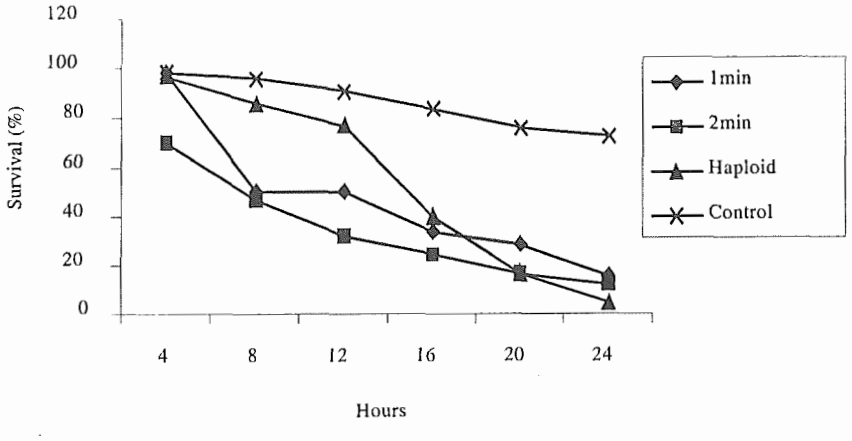

Fig. 8. Survival of mitotic gynogens by heat shock at 39±1°C for a duration of 1 and 2 min, haploid and control

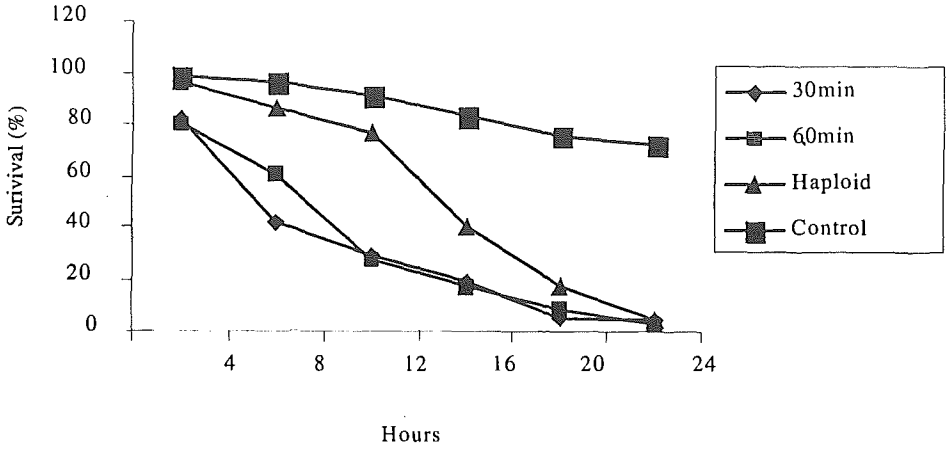

Fig. 9. Survival of mitotic gynogens by cold shock at 3±1°C for a duration of 30 and 60 min, haploid and control

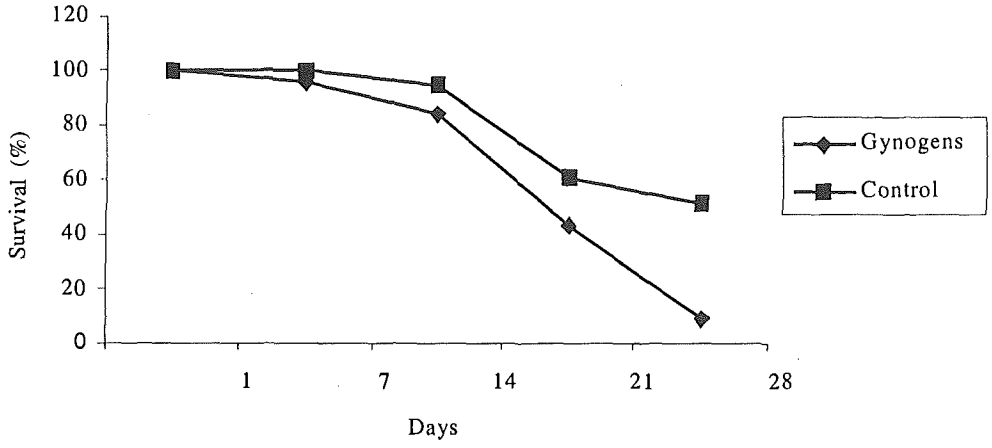

Fig. 10. Percentage survival of gynogens and control of *Clarias gariepinus* reared for 28 days in aquaria

Discussion

In the present study, the UV irradiated sperms were actively motile (70-100%-motility) for a period of 60 sec and motility was lost completely after 300 sec. For effective fertilization, mixing of gametes for a duration of 1.0 to 1.5 min is enough for *C. gariepinus*. In the case of *H. fossilis*, UV irradiation for 210 sec resulted in complete elimination of male genetic material (Godwin Christopher 2001). The viability of haploid fish embryo at various stages of development seems to be species specific. In this study, haploids in *C. gariepinus* were found to survive to hatching stage and died within 3-5 days. Similar results were also observed in *O. aureus* by Don and Avtalion (1988) and in *H. fossilis* by Godwin Christober (2001).

Karyological study revealed that haploid larvae of *C. gariepinus* possessed only one set of chromosomes (n=27), gynogens and controls had two sets of chromosomes (2n=54). Karyological studies performed by Ozouf-Costaz *et al.* (1990) revealed

karyotype, 2n=56. Similar results were also reported by Teugels *et al.* (1992) and Varadi *et al.* (1999). In the present study, meiotic and mitotic gynogenesis were induced by different treatment techniques and heat shock (39±1°C) at a short duration of 1 min was better than 2 min for diploidization. Similarly, cold shock (3±1°C) for a duration of 30 min gave better results than 60 min shock treatment. Higher percentage of hatching (68.66%) was observed for meiotic gynogens at a temperature shock of 39±1°C for a duration of 1 min, 5 min a.f. Higher percentage of mitotic gynogenesis induction (15.33%) was observed at a temperature shock of 39±1°C for a duration of 1 min, 30 min a.f. Bongers *et al.* (1995) reported that the mitotic division occurred at about 35 min a.f. an incubation temperature of 28°C in common carp, *Cyprinus carpio*.

Varadi *et al.* (1999) obtained 6.34 ± 2.35% survivors at swimming stage when they induced diploid gynogenesis using the inactivated sperm of rosy barb (*Barbus conchonius*) at a temperature of 40.5°C for 2 min. Heat shock at 42°C and 41.5°C did not produce any viable larvae, which might be due to their genetic incompatibility. However, fertilization with irradiated African catfish sperm resulted in haploid syndrome. In the present study also, when the irradiated sperm was used to fertilize the egg typical haploids showing the haploid syndrome were observed. The haploid embryos were grossly abnormal, showing in particular thick bodies with poorly-developed tails and with small underdeveloped eyes (Fig. 5). These abnormalities were already reported in fishes by Purdom (1969) and Godwin Christopher (2001).

In this study a very high mortality rate and a high incidence of distortions were observed in the gynogenetic fishes. This has been also reported by Purdom (1969), Nagy *et al.* (1978), Chourrout and Quillet (1982), Refstie *et al.* (1982), Onozato (1984), Suzuki *et al.*(1985), Don and Avtalion (1988). Meiogynogens in *C. gariepinus* was produced by Volckaert *et al.* (1994a, 1997) with a survival rate of 46% with a shock temperature at 41°C for a duration of 2 min and 3 min a.f. When compared to heat shock, cold and pressure (55MPa) shocks for 1.5 min applied 4 min a.f. generated higher survival rates (80% and 68% respectively).

Galbusera *et al.* (2000) obtained lower yield of meiogynogens (12%) at 40°C for 1 min when the shock applied after 22 and 38 min of fertilization. A cold shock applied 35-45 min after fertilization resulted in 5% mitogynogenetic offspring in the European catfish, *Silurus glanis* (Krasznai and Marian 1986). The first mitotic division in channel catfish occurred 90 min a.f. (Bidwell *et al.* 1985) whereas in *C. gariepinus,* first mitotic nuclear division was initiated at about 30 min a.f., while the first mitotic cleavage was initiated 45 min a.f. at 28°C (Volckaert *et al.* 1994b).

Survival rates varied considerably among the different treated group of eggs. The sharp boundary between success and failure in gynogenetic (especially in timing of the shock) creates steep survival curves. Timing of application of the shock is so critical that a difference of 1 min may strongly influence the outcome as reported by Volckaert *et al.* (1994a). Thus, reports on viability of gynogenitic fish are conflicting. Purdom (1983) cited poor survival rates in gynogenetic common carp, other cyprinids and loaches. Mia *et al.* (2001) reported survival rate of gynogenetic in Indian major carp within 5 to 7 days after hatching, ranged from 0-50%, whereas it was 30-60% in the controls. Another

reason for the poor survival attributed by Na-Nakorn *et al.* (1993) is the stage at which eggs were stripped might affect the tendency to retain the second polar body. Differences in the effectiveness of heat and cold shocks probably result from the different mechanisms of their effect on the dividing cells (Bieniarz and Epler 1984, Komen *et al.* 1988). Further standardization is required for the production of gynogenetic *C. gariepinus*. Thus, the production and farming of monosex (all female) population of exotic *C. gariepinus* either using genetic manipulation may solve the threat that its accidental escape into natural waters will affect the natural fauna of the aquatic ecosystem in future.

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(Manuscript received 14 March 2007)