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A comparative study on the embryonic development of gynogen, triploid, haploid and normal diploid embryos of stinging catfish, *Heteropneustes fossilis*

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Abstract
UV irradiation and cold shock were applied on the eggs of stinging catfish, *Heteropneustes fossilis*, to produce haploid, gynogen and triploid embryos. A comparative account of the various features of embryonic development in chromosomally manipulated groups *viz.* haploid, gynogen and triploid and non-manipulated normal diploid group of *H. fossilis* has been discussed. A slow development and delayed hatching were observed in gynogen and triploid embryos compared to those in normal diploid (control) groups. Mass mortality was observed in all chromosomally manipulated groups particularly during the gastrulation stage. The hatchlings of the gynogen, triploid and normal diploid were similar in overall appearance.
Key words: Embryonic development, Gynogen, Triploid, Diploid, *H. fossilis*

Introduction

In fish breeding programs, chromosome manipulation techniques *viz.* gynogenesis, androgenesis and triploidy are now considered efficient tools for genetic improvement of fish stock. These techniques have been successfully applied to many commercially important fish species e.g. *Cyrinus carpio* (Nagy *et al.* 1978, Komen *et al.* 1988), *Labeo rohita* (John *et al.* 1984, Hussain *et al.* 1997), *Oreochromis niloticus* (Hussain 1995), *Ctenophryngondon idella* (Cassani and Caton 1986). However, frequent observations about the consequence of genetic manipulation are considerably lower hatchability of eggs and survivability of fry (Chourrout and Quillet 1982, Suzuki *et al.* 1985, Bieniarz *et al.* 1997). Mortality during the embryonic development of chromosomally manipulated groups of *Cyprinus carpio* was stage-specific and a rapid and mass mortality of gynogen embryos during the gastrulation stage of embryonic development was observed (Nagy *et al.* 1978). Moreover, a number of authors have reported a slow embryonic development in chromosomally manipulated groups (Ezaz *et al.* 1995, Goudie *et al.* 1995)

Recently, Gheyas *et al.* (2001a and 2001b) have established techniques for gynogenesis and triploidy induction in stinging catfish, *Heteropneustes fossilis* in Bangladesh. This catfish is a highly valued food fish in the Indian subcontinent. The establishment of the chromosome manipulation techniques for this species has paved the

way for production of fast growing superior female stocks for culture. But the problem of lower hatching and survival rates of ploidy-manipulated groups compared to normal diploids, like many other species, was observed for this species also. The present experiment was designed to compare the embryonic development in gynogen, triploid, haploid and normal diploid groups of *H. fossilis* to investigate the differences in the developmental features of embryos of chromosomally manipulated and non-manipulated control groups and also to identify the stage of mass mortality of embryos.

Materials and methods

The study was conducted in the Freshwater Station of Bangladesh Fisheries Research Institute, Mymensingh, Bangladesh. To induce ovulation the female fish received one PG injection of a dose of 70mg/kg body weight. The milt from the male was collected by dissecting out testes from body cavity and then macerating them in 0.85% saline solution. After collection of eggs and sperm chromosome manipulation techniques were applied to produce haploid, gynogen and triploid embryos by using UV irradiation and cold shock techniques used by Gheyas *et al.* (2001a and 2001b). The standard conditions for application of UV and cold shock treatment for haploidy, gynogenesis and triploidy induction applied were as follows:

UV irradiation

Intensity: 250 $\mu W\text{-}cm^{-2}$, duration: 2.5 minutes, sperm concentration: $1x10^8$/ml.

Cold shock

Temperature: 2^0C, duration: 10 minutes, time interval between fertilization and shock application: 3 minutes.

Eggs, after been collected from female fish, were divided into four groups, each containing 100 eggs approximately. Milt, on the other hand, was divided into two batches and each diluted to a level of $1x10^8$ sperms/ml by 0.85% saline solution. One of the sperm suspensions was UV irradiated to inactivate the sperm DNA and was subsequently used to fertilize two batches of eggs. One of these batches of fertilized eggs was incubated without further treatment and hence, they developed as haploid embryos. The other batch was subjected to cold shock to produce diploid gynogen embryos. The remaining two batches of the four groups of eggs were fertilized with non-irradiated normal sperm. One of these was subsequently cold shocked to induce triploidy and the other batch was spared from shocking to produce normal diploid embryos. The procedure for production of gynogen, triploid, haploid and normal diploid embryos is presented in Fig. 1.

In order to carry out a comparative study of the embryonic development in chromosomally manipulated and non-manipulated groups, eggs in all cases were fertilized at the same time. The time of onset of different embryonic stages in each group was carefully recorded. Rate of mortality in each developmental stage was also carefully

recorded. The embryonic development was observed under an Olympus Compound Binocular microscope.

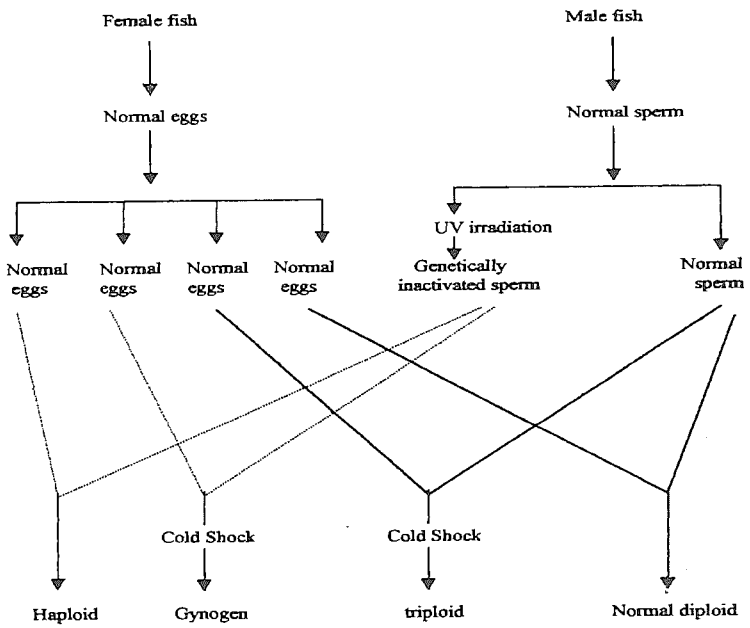


Fig. 1. Diagrammatic representation of production of haploids, gynogens, triploid and normal diploids.

Results

The study revealed differences in the embryonic development of the four groups *viz.* haploid, gynogen, triploid and normal diploid (control) in the following three dimensions:

- In the timing of onset and completion of the embryonic developmental stages
- In the mortality of embryos
- In morphological appearance of the embryos

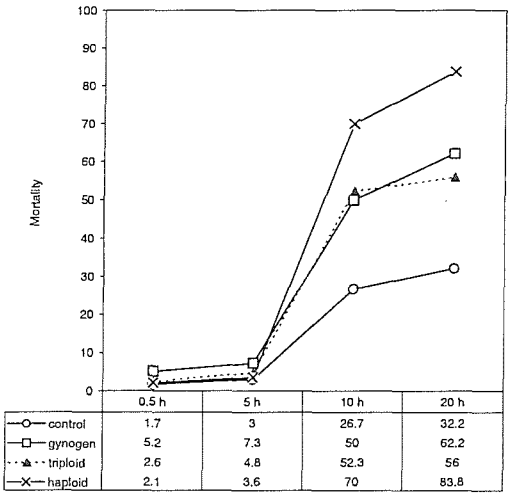
Table 1 presents a complete description of the sequential stages of the embryonic development in *H. fossilis* as well as a comparative picture of the development in chromosomally manipulated and control groups in respect to timing and morphology. The table shows that the first discernible embryonic developmental stage is the blastodisc formation stage, which normally comes about 25-30 minutes after fertilization (af). In haploid group the timing of the onset of this stage was similar to that in normal diploid or control group but in gynogen and triploid groups this developmental stage set in 10-15 minutes later than in control. Later stages in the gynogen and triploid groups

took more time to set in while those in control and haploid groups had more or less similar timing. As a result of delayed embryonic development the hatching in gynogen and triploid groups was delayed by about 2 hours. In control and haploid groups hatching started by about 20th hour af and was completed within 22nd hour. In gynogen and trioploid groups the event of hatching was observed in general between 22nd and 24th hours.

Table 1. Comparative description of the embryonic development in chromosomally manipulated groups vis-a-vis the non-manipulated group

Stage of development	Group			
	Normal diploid	Haploid	Gynogen	Triploid
Blastodisc formation	Brick red blastodisc formed within 25-30 minutes after fertilization at the animal pole	Appearance and timing of onset same as control	Same in appearance but takes 10-15 minutes more time for onset than that in control.	Same as gynogen
Two cell stage	Appears by first vertical cleavage and occurs in about 40 minutes after fertilization	Same as control in appearance and timing	Onset delayed by 15-20 minutes than that in control; appearance same	Same as gynogen
Four cell stage	Occurs in about 50-55 minutes after fertilization by second vertical cleavage	Same as control	Delayed by 15-20 minutes	Same as gynogen
Eight and sixteen cell stages	Eight cell stage comes nearly 60-65 minutes after fertilization and sixteen cell stage 70-75 min. after fertilization	Same as control	Both stages delayed by 15-20 minutes than in control	Same as gynogen
Blastula stage	Reached by repeated cleavage in the blastodisc in about 120-130 minutes after fertilization. Caplike blastoderm appears	Same as control	Takes 30-40 minutes more time for completion	Same as gynogen
Gastrula stage				
a. Spreading of blasto-derm	Gastrula stage begins by spreading of blastoderm over the yolk sac	Timing similar to control	Onset of gastrulation delayed by 30-40 minutes	Same as gynogen
b. Appearance of somites	From about 5 hours after fertilization somites started to appear	Same as control	From about 6 hours of fertilization somites started appearing	Same as gynogen
c. 12-16 somite stage	After about 10 hour fertilization embryos covered two-third of the yolk sac and had 16 somites	After 10 hour, embryos did not cover even half of the yolk sac; counting of somites was difficult	After about 10 hours embryos covered nearly two-third of the yolk sac but had 12 somites instead of 16	Same as gynogen
Hatching	Hatching started at around 20 h after fertilization and took about 2 h for completion	Hatching time similar but embryos were completely different in appearance showing deformed body	Hatching starts after 22 h of fertilization and takes about 2 h time for completion; hatchlings were same in appearance as the normal diploid hatchlings	Same as gynogen

Study on the mortality of embryo showed that death rates were very high in all the chromosomally manipulated groups. Fig. 2 depicts a comparative picture of the mortality in different groups at various stages of embryonic development. It appears from the figure that up to the 5 hours of development of which first 2-2.5 hours belong to blastula stage the mortality in all the four groups was very low. However, up to this 5 hours the highest mortality was observed in the gynogen group and least mortality was observed in the control and haploid groups. Regarding the mortality, the gastrula stage appeared to be very crucial as in this stage mass mortality occurred in all groups. Although, however, the gastrula stage starts from 3-3.5 hours af the figure 2 shows that major mortality in all groups occurred approximately between 5-10 hours. During gastrulation a very high rate of mortality was observed specially in haploid embryos. After 10 hours of development the percent of dead embryos in haploid, gynogen and triploid groups were approximately 70, 50 and 52 respectively whereas in control the mortality was only about 27%. After about 10 hours of development the mortality rates reduced considerably in triploid and control groups but were still quite high in haploid and gynogen groups. Hatching percentage in different groups were 16, 38 44, and 68 in haploid, gynogen control, and triploid groups respectively.



	0.5 h	5 h	10 h	20 h
control	1.7	3	26.7	32.2
gynogen	5.2	7.3	50	62.2
triploid	2.6	4.8	52.3	56
haploid	2.1	3.6	70	83.8

Figure 2. Mortality of normal diploid, gynogen, triploid and haploid embryos with the progression of development

Fig. 2. Mortality of normal diploid, gynogen, triploid and haploid embryos with the progression of development.

The third dimension of differentiation was in the morphology of the embryos. Deviation from normal morphology was observed only in the haploid embryos while the embryos of control, gynogen, and triploid were same in size and appearance. Since in most stages the embryos of different groups are similar in appearance, photographs of only control embryos showing different stages of development are presented in Plates 1 to 8. The difference in the embryonic development between the haploid and the other

three groups were visible after 10-12 hours of development. The germ ring of haploid did not cover even half of the yolk sac whereas in control and the other two groups the germ ring surrounded two-third of the yolk sac (Plates 9 and 10). The hatchlings of the gynogen, triploid and normal diploid were similarly normal in appearance (Plates 11, 12 and 13). However, the haploid hatchlings showed gross deformity of the body, a phenomenon called "haploid syndrome" characterized by short, bent tail and often watery yolk sac (Plate 14). Such deformity was visible in the later part of the Gastrula stage. The haploid hatchlings died within 24 hours after hatching.

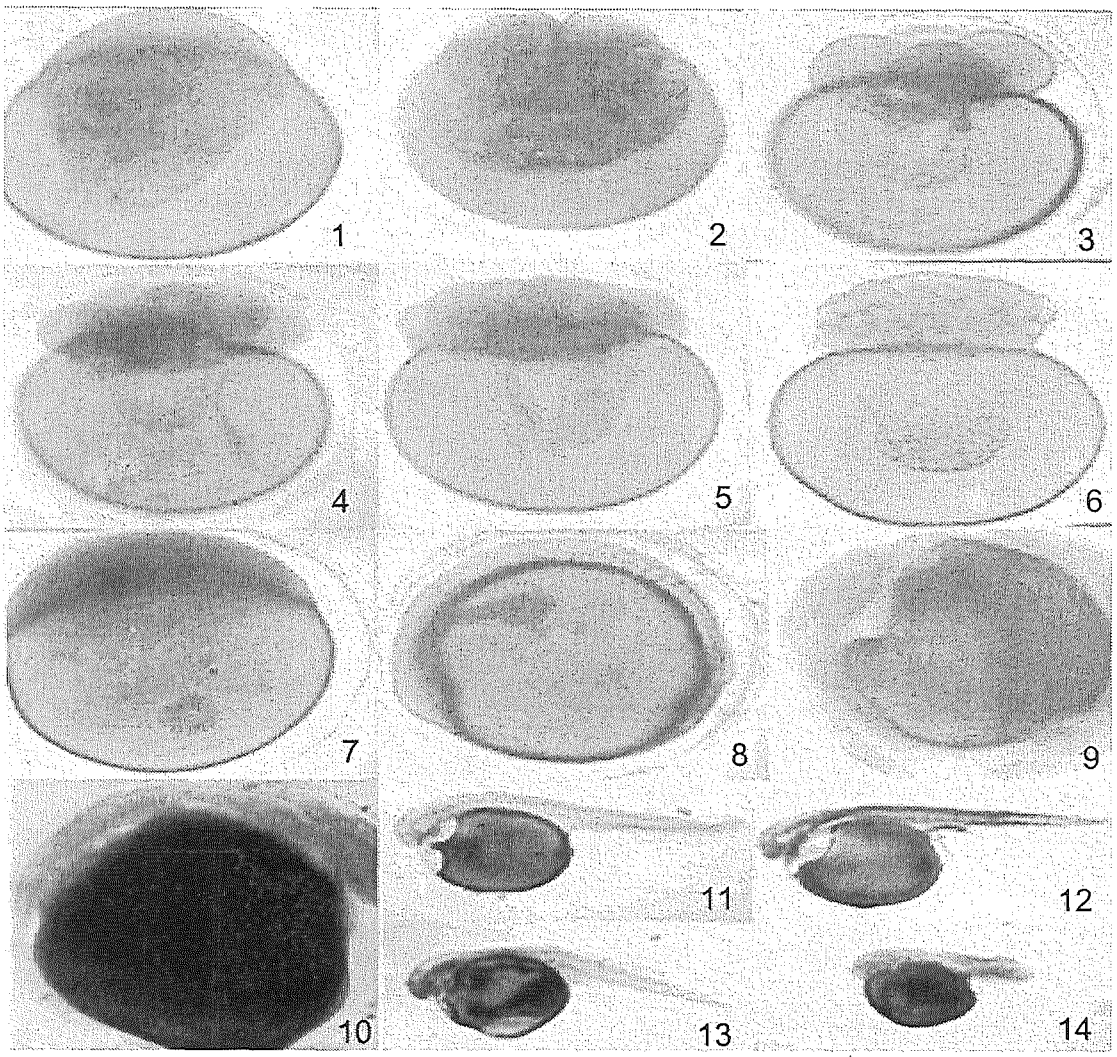


Plate 1. Blastodisc formation stage, Plate 2. Two cell stage, Plate 3. Four cell stage, Plate 4. Eight cell stagePlate 5. Sixteen cell stage, Plate 6. Blastula stage, Plate 7. Spreading of blastoderm, Plate 8. Sixteen somite stage Plate 9. Germ ring of control embryo, Plate 10. Germ ring of haploid embryo, Plate 11. Normal diploid hatchling, Plate 12. Gynogen hatchling, Plate 13. Triploid hatchling, Plate 14. Haploid hatchling

Plates 1-14. Photographs of different stages of embryonic development in normal diploid, gynogen, triploid and haploid *Heteropneustes fossilis.*

Discussion

Embryonic development in *H. fossilis* has been studied by Thakur *et al.* (1974) and Kohli and Vidyarthi (1990). The development pattern observed in the present experiment conforms to those reported by previous workers. The present study, however, was more interested in making a comparative investigation between the development of normal diploid embryos and chromosomally manipulated embryos. The following differentiation in the embryonic development in different types of embryos was observed. First of all in cold shock treated groups i.e. in gynogen and triploid the embryonic development was a slower process. In these two groups each stage came late compared to the normal situation and the hatching was delayed by about 2 hours. On the contrary, the embryonic development in haploid group was similar in timing to that in control. Such delay at the initial stage at least, can be attributed to the cold shock, as it is a known fact that at low temperature the cell processes slow down. Similar result of delayed development and hatching was observed by Ezaz (1995) in the embryonic development of triploid hybrid of *Clarias batrachus* and *Clarias gariepinus.* Kobayashi (1997) also observed delayed onset of cleavage in the development of gynogentic embryo of amago salmon, *Oncorhynchus rhodurus.* Goudie *et al.* (1995), observed a slow development in the haploids embryos of channel catfish *Ictalurus punctatus.* In the present experiment, however, no such delay in the development of haploid embryo was observed; rather the timing was quite similar to that in control group.

A very high mortality rate of embryos in all chromosomally manipulated groups particularly in haploid has been observed in the present study. Since haploids have only a single set of chromosomes, every detrimental allele is expressed as none is hidden by a dominant allele (Tave 1993). This probably is the reason for a very high mortality of haploid embryos compared to the mortality of embryos in other groups. On the other hand the high mortality in cold shock treated groups can be attributed to the stringent conditions of shock treatments (Gheyas *et al.* 2001).

Many workers have reported similar results of high mortality in chromosomally manipulated groups. For instance Suzuki *et al.* (1985) observed a lower hatching performance in the gynogenetic loach and carp embryos, Hussain *et al.* (1993) in the gynogenetic *Oreochromis niloticus,* John *et al.* (1984) in the gynogen embryos of *Labeo rohita and Catla catla,* Cherfas *et al.* (1993) in the gynogenetic and triploidy development of Israeli common carp line DOR-70. In the present experiment, the gastrula stage appeared to be especially crucial regarding the survival question as in this stage mass mortality of embryos occurred. Nagy *et al.* (1978) reported a high mortality of gynogenetic and normal diploid embryos of *Cyprinus carpio* between 10-16 hours of development, which is the gastrulation period in this species. After the 16th hour, mortality rates remained low until the time of hatching.

Gynogenetic and triploid embryos and hatchlings of *H. fossilis,* in the present study were found to be very similar in morphological appearance to that of control group larvae. However, the haploid showed gross morphological deformity and died within 24 hours of hatching. Such haploidy syndrome has also been reported by a number of

authors (John *et al.* 1984, Chao *et al.* 1986, Wu *et al.* 1986, Komen *et al.* 1988, Hussain *et al.* 1997) in different species of fishes.

The present experiment was a rather non-conventional study on the various features of differentiation in embryos of chromosomally manipulated and non-manipulated control groups. The authors believe that the results cited in the paper would give future workers some important baseline information and would facilitate to design for more sophisticated cytological studies to gain insight into the underlying causes of mass mortality during gastrulation and of slow development in cold shock treated groups.

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