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Optimization of UV treatment to induce haploid androgenesis in the stinging catfish, *Heteropneustes fossilis*

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Abstract

The optimum UV irradiation duration for the complete inactivation of *Heteropneustes fossilis* egg's nucleus was developed. The unfertilized eggs were suspended in Hanks' balanced salt solution and were irradiated with UV light from both the dorsal and the ventral side for different exposure times ranging from 20 to 200 s (2,500 to 25,000 ergs/mm^2). Egg viability was assessed for the different exposure durations at fertilization, hatching, and haploidy. Majority of the larvae derived from irradiated eggs had an abnormal appearance. Complete inactivation of maternal genome was evaluated by haploid chromosome number and morphology which also exhibited haploid syndrome. Successful genome inactivation occurred from 100 s onwards. Larvae resulting from eggs irradiated above 12,500 ergs/mm^2 were 100% haploid, with better hatching (81.59%). The genetic inactivation of maternal chromosomes was confirmed by the chromosome count of the resulting embryo ($n = 29$). Completely anuclear embryos were obtained when the eggs were exposed with UV irradiation between 12,500 and 25,000 ergs/mm^2. We conclude that the optimum UV dosage for the complete genetic inactivation of the egg nucleus requires more than 12,500 ergs/mm^2.

Keywords: *Heteropneustes fossilis*, Androgenetic haploid, Haploid syndrome, UV irradiation

Background

Fish, by virtue of their high fecundity, external fertilization, and ability to tolerate manipulations of their chromosome sets, are suitable models to study different aspects of genetics. In addition, the production of polyploid fish and fish with uniparental inheritance has a significant impact in the aquaculture of various species (Thorgaard 1983). Androgenesis is a form of uniparental chromosome inheritance from males. Androgenetic development in fish can be triggered by fertilizing gamma-, X-ray-, or UV-irradiated eggs with normal spermatozoa, but the resultant embryos show inviable abnormality due to haploidy. Viable diploid androgens can be produced by doubling the paternal chromosome set through the suppression of the first cleavage using physical shocks such as temperature or pressure treatment or by the fertilization of inactivated eggs with diploid spermatozoa from tetraploid males.

Although complete genetic inactivation of egg nucleus is a prerequisite for a successful induction of androgenesis, the majority of experimental studies have concentrated on the

production of diploid androgenotes that are viable. Relatively few studies have focused on identifying the optimal conditions for the genetic inactivation of the egg nucleus.

UV light has been successfully used in irradiating eggs of white sturgeon, *Acipense transmontanus* (Kowtal 1987); common carp, *Cyprinus carpio* (Bongers et al. 1993, 1994); Nile tilapia, *Oreochromis niloticus* (Myers et al. 1995); loach, *Misgurnus anguillicaudatus* (Arai et al. 1992); African catfish, *Clarias gariepinus* (Bongers et al. 1995); and so on. Furthermore, UV irradiation results in no residual fragments, in contrast to gamma irradiation, and it is easy to use anywhere, inexpensive, and safer to apply (Ihssen et al. 1990; Thorgaard 1983; Myers 1995).

Haploid androgenesis has been induced in the loach, *M. anguillicaudatus* (Ihssen et al. 1990); flounder, *Pleuronectes flesus* (Purdom 1969); masu salmon, *Oncorhynchus masou* (Arai et al. 1979); rainbow trout, *Oncorhynchus mykiss* (Parsons and Thorgaard 1985); and brook trout, *Salvelinus fontinalis* (May et al. 1988).

The applicable potential of androgenesis involves the rapid establishment of inbred lines for breeding programs and research purposes (Purdom 1969; Ihssen et al. 1990), sex control using anticipated supermale (YY) in the male heterogametic species (Thorgaard 1983; Myers 1995), production of a nucleocytoplasmic hybrid between different species, and recovery of genotypes from those which are contaminated by hybridization (Ihssen et al. 1990; McAndrew et al. 1993).

Androgenesis can also be used to aid recovery of endangered species form its cryopreserved spermatozoa. This is done by fertilizing the endangered species' sperm with genetically inactived eggs followed by shock treatment to induce diploidy.

To induce viable androgenetic diploid individuals, it is essential to achieve both successful genetic inactivation of the egg nucleus and restoration of diploidy. Therefore, optimization of intensity and duration of irradiation is the first step for the successful production of androgens.

Heteropneustes fossilis (stinging catfish) is a commercially important freshwater catfish distributed in the tropical waters of the Indian subcontinent. *H. fossilis* is highly prized for its low-fat, high-protein, and high-iron content. It adapts well to water bodies with low dissolved oxygen and higher stocking densities, and its air breathing apparatus enables it to be sold as 'live fish' in the market. In *H. fossilis*, there are considerable studies on the reproductive biology and artificial breeding but only a very few studies on genetic manipulation. The family Heteropneustidae is represented by a single genus confined to the freshwaters of India, Sri Lanka, Bangladesh, and Burma. It has two species, *viz H. fossilis* and *H. microps*. Between the two, *H. microps* is rare and its distribution range is not well known, and *H. fossilis* is the only viable population of the family.

In the present study, the optimum UV irradiation required for the denaturation of egg genome to develop haploid condition was optimized.

Methods

Gamete collection and artificial fertilization

Specimens of *H. fossilis* which range from 40 to 80 g for male and from 80 to 120 g for female were collected during the month of June (early spawning phase) from the ponds around Alwarkurichi, Tamil Nadu (8°48′00″N; 77°27′30″E). On arrival to the laboratory, the fish were disinfected with formalin (50 ppm) for 2 h to remove external parasites. Mature female brood fish were primarily selected on the basis of a soft and bulging belly, which

yielded continuous ova of uniform size on gentle stripping. Then, these eggs were examined under a stereozoom microscope to check its maturity. These selected fish were stocked in a polyvinyl chloride tank (3 × 2 × 1 m) with continuous aeration, normal photoperiod (13.5-h L:10.5-h D), and an ambient water temperature (26 ± 2°C) to induce final ovulation. The induction of final maturation and ovulation was stimulated using a single intramuscular injection of Ovaprim marketed by GlaxoSmithKline Pharmaceuticals Limited (Mumbai, India), each milliliter contains salmon gonadotropin-releasing hormone of 20 mcg and Domperidone of 10 mcg at the rates of 0.3 and 0.5 mg kg^{-1} body weights for males and females, respectively. The gametes were collected separately 10 h after Ovaprim injection at an ambient water temperature of 26 ± 1°C (Godwin et al. 2009); gently pressing the abdomen towards the anus yields a copious stream of transparent greenish brown eggs. These eggs were collected in a glass bowl previously rinsed with Hanks' balanced salt solution (HBSS).

The males were anesthetized with 75 mg/l tricane methanesulfonate [MS-222] for 5 min and sacrificed for the collection of milt. The testes were surgically removed and were cut into small bits, then gently squeezed in a glass tissue homogenizer with a few drops of HBSS. The left out tissue particles were removed, and the milt was diluted to 1:10 with HBSS. The sperm motility was checked for each sample, by taking an aliquot of 10 μl in a microslide and activated by 3 μl of tap water, then viewed under the microscope at × 400. Samples with high motility (97%) alone were used for the experiment. The sperm suspension was prepared 1 h before the stripping of eggs and kept in the refrigerator at 4°C. The sperm remains viable for more than 6 h at 4°C (Rexlin 1996).

Genetic inactivation of egg genome

A simple glass UV irradiation chamber was designed with four UV-C, 15-W germicidal lamps (Philips, Amsterdam, Holland). Two lamps on the dorsal side and two on the ventral side were fixed at a distance of 27 cm from the center, fine threads passed across the center where the egg trays were placed for irradiation. The threads were connected to a vortex mixture in order to create vibration for the egg rotation. The lamps were switched on for half an hour before the experiment to stabilize the energy of the UV rays (Onozato and Yamaha 1983). Egg tray surface was measured with a UV meter (Ultra-Violet Products, Inc., San Gabriel, CA, USA) for dosage calculations.

The eggs were soaked in the HBSS to keep them wet always and were placed on a thin (25 μm), transparent film dish to a single layer without any overlap and placed at the center (between the dorsal and ventral lamps) for UV irradiation. During UV irradiation, the eggs were always rolling in the fluid due to a vibrational force created by the vortex mixture (stirrer). For each batch, approximately 300 to 400 eggs were used. The whole process was carried out in the dark to avoid genetic photoreactivation (at 24°C). After the irradiation of eggs for different durations from 20 to 200 s, i.e., 2,500 to 25,000 ergs/mm^2, the eggs were immediately mixed with a 200 μl of sperm suspension and allowed to fertilize by activating the sperm (adding water). One batch of eggs was not irradiated and served as the control. Then, they were transferred to egg baskets (diameter, 10 cm) with a mesh bottom (mesh size, 0.5 mm) and incubated in water temperature at 27°C in a dark room.

Ploidy determination

Ploidy was determined in 1-day-old post-hatch larvae. Haploid individuals were confirmed by chromosome counting followed by drop method (Kligerman and Bloom

1977). At least 20 karyotypes were counted per individual, and 10 individuals were sampled from each group. Also, the haploids had the characteristic haploid syndrome.

Results

UV irradiation and haploid androgenesis

The percentage of fertilization, hatching, and normal and deformed larvae head formation in the control and irradiated groups are shown in Table 1. This table also shows the percentage occurrence of haploids in each treatment group. In all the groups with UV irradiation above 5,000 erg/mm^2 of exposure, the fertilization rate decreased considerably compared with the control group. The rate of hatching also decreased with increasing UV dose, but a sharp decrease compared with the control group was noted at 12,500 erg/mm^2. In the control group, 95% of the larvae that hatched were normal. In the UV irradiation groups, few normal larvae hatched; the highest rate of normal larvae in the treated groups was seen in the 2,500 erg/mm^2 group in which 60.11% were normal embryo.

UV treatment of eggs with various doses caused various abnormalities in the external appearance of larvae 3 days after fertilization. In the control group, all hatched larvae exhibited almost normal appearance (Figure 1). In the larvae from UV-irradiated eggs, a range of abnormalities were observed: 'haploid syndrome,' showing dwarfing, microcephaly, and microphthalmus (microcephaly - a disorder where the head circumference is less than normal embryo; microphthalmus - abnormality in eye size); deformed head formation showing a range of defects in the head region. Abnormal larvae in the 5,000 erg/mm^2 group showed a wide range of appearances of body shapes (body axes). Most abnormal larvae in the 10,000 erg/mm^2 group showed the haploid syndrome; they were smaller than normal (Figure 2). Abnormal larvae classified as having 'deformed head formation' were observed at a higher percentage in the low-UV dose groups. The proportion of affected larvae in the 5,000 erg/mm^2 group (29.71%) was higher than that in the other irradiated groups. The percentage of deformed head decreased with the increase in the UV dose.

The groups that received a UV dosage of more than 12,500 erg/mm^2 showed 100% haploid syndrome. Deformed larvae were more frequent in groups which received a UV

Table 1 Stages and percentage survival of androgenetic embryos induced using UV irradiation

Serial number	UV dose	Dosage (ergs/mm^2)	Number of eggs[a]	Fertilization (%)	Hatching (%)	Normal (%)	Deformed (%)	Haploid syndrome (%)
1	Control	0	338 ± 12	93.7	98.12	99.05	0.82	0
2	20	2,500	413 ± 10	82.43	92.34	60.11	19.11	21
3	40	5,000	297 ± 21	73.44	91.65	39.06	29.71	31
4	60	7,500	311 ± 24	67.22	90.11	1.87	15.26	59
5	80	10,000	441 ± 21	63.15	86.78	0.5	4.17	87
6	100	12,500	287 ± 17	60.45	81.59	0.5	1.07	100
7	120	15,000	306 ± 22	58.23	80.11	0.6	0.19	100
8	140	17,500	293 ± 09	51.39	73.26	0	0.81	100
9	160	20,000	363 ± 16	50.61	61.39	0	0.44	100
10	180	12,500	426 ± 18	46.42	50.44	0	0.73	100
11	200	25,000	319 ± 18	39.68	32.69	0	0.54	100

[a]mean ± SD.

Figure 1 Normal diploid control *H. fossilis*.

irradiation of 2,500 to 7,500 erg/mm^2 than those with a higher dosage of UV irradiation (<10,000 erg/mm^2; Table 1).

Following irradiation of eggs, we observed a depression in the yolk of some embryos (Figure 3) in the UV treatment groups of 20,000 and 25,000 erg/mm^2. This depression was absent in the eggs of the control and low-UV irradiation groups.

Chromosome analysis

Chromosome analysis of normal larvae from the control group indicated a modal chromosome number of 58. In contrast, abnormal larvae from the 2,500 to 7,500 erg/mm^2 group did not have a clear modal number but showed a range of 29 to 58 chromosomes. Furthermore, chromosome fragments were also observed in the metaphase spreads. A clear modal chromosome number of 29 was obtained above 12,500 erg/mm^2. The frequencies of chromosome fragments decreased with increased UV dose. In the groups that receive more than 12,500 erg/mm^2, the chromosome number was clearly haploid without any chromosome fragment.

Figure 2 Haploid syndrome in *H. fossilis*.

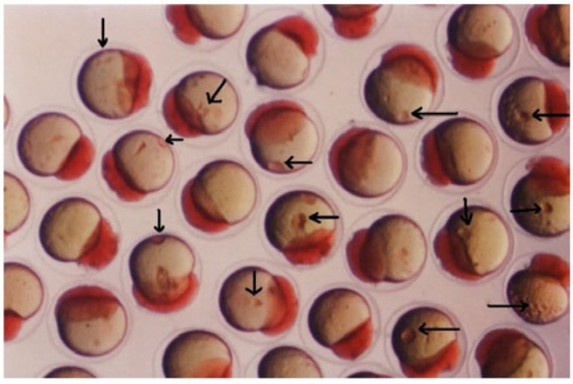

Figure 3 Arrows showing depressions in *H. fossilis* yolk.

Discussion

The present study indicates that UV irradiation successfully inactivated the nuclear DNA in *H. fossilis* eggs. The determination of optimal dose and duration of UV irradiation for the complete egg genome inactivation is the prerequisite. Many authors have successfully inactivated egg genome at different UV dosages. In African catfish, *C. gariepinus*, Bongers et al. 1995 worked on an optimum UV dose of 1,250 J/m^2 (corresponding to 12,500 ergs/mm^2). Lin and Dabrowski (1998) reported haploid androgenetic muskellunge, *Esox masquinongy* with optimal UV irradiation doses of 620 to 1,320 J/m^2 (corresponding to 6,200 to 13,200 ergs/mm^2). Marengoni and Onoue (1998) obtained androgenetic haploid *O. aureus* and *O. niloticus*, at total UV doses of 594 and 693 J/m^2 (corresponding to 5,940 to 6,930 ergs/mm^2); similarly, Myers et al. (1995) used 450 to 900 J/m^2 (corresponding to 4,500 to 9,000 ergs/mm^2) in the Nile tilapia; Kirankumar and Pandian (2003) used 3.5 min at 4.2 W/m^2 (corresponding to 8,820 ergs/mm^2) in the tiger barb, *Puntius tetrazona*; David and Pandian (2006) used 2.75 min at 4.2 W/m^2 (corresponding to 6,930 ergs/mm^2) in the Buenos Aires tetra. Fujimoto et al. (2007) reported 150 mJ/cm^2 (corresponding to 15,000 ergs/mm^2) in the loach, *M. anguillicaudatus.* In the present study, a UV dose greater than 12,500 ergs/mm^2 was required for the complete genetic inactivation of the maternal genome. The variations could be due to sensitivity to UV irradiation from species to species and could be explained by differences in the thickness, composition, and optical qualities of egg chorion, egg size and shape, and the relative position of the female pronucleus (Myers et al. 1995).

Rotation of the eggs to expose the animal pole to radiation from the UV lamp yielded better results of complete genome inactivation. Bongers et al. (1995) stirred the eggs of *C. gariepinus* in a synthetic ovarian fluid while exposing under UV irradiation and reported high yields of androgenetic haploid. The stirring did not negatively interfere with hatching but increased the efficiency of the UV treatment. Similar results were obtained in common carp, *C. carpio* using the same method of egg rotation (Bongers et al. 1994). Similarly, Arai et al. (1995) exposed *M. anguillicaudatus* eggs to a UV source from the upper and lower sides to get maximum exposure. The same methodology was adapted for *H. fossilis* by placing the eggs in HBSS which was more successful and also reduces the time of eggs in the UV chamber. This chamber has made it

possible to achieve more effective inactivation by ensuring that all of the eggs received equal irradiation. Moreover, the experiment was carried out in a complete dark chamber, which in turn prevents the photoreactivation of the inactivated chromosomes.

In general, survival of androgenetic haploid fishes after hatching is rare. Thus, it is analyzed till hatching. Arai et al. (1995) reported 22% hatched androgenetic haploids in loach, *M. anguillicaudatus.* In muskellunge, *E. masquinongy*, the yield of haploid androgenetic was 22.5% (Lin and Dabrowski 1998). Marengoni and Onoue (1998) obtained survival rates of 57% and 55% (relative to controls) in androgenetic haploid *O. aureus* and *O.niloticus*. Bongers et al. (1995) were able to produce higher numbers of androgenetic haploids (81% to hatching, relative to control) in African catfish, *C. gariepinus*. Fujimoto et al. (2007) reported 82% hatching from a fertilization success of 40%, which corresponds to 32.8%. Similarly, in the present study, 85% hatching from a fertilization success of 60%, which corresponds to 51% of the total number of eggs used at 12,500 ergs/mm^2, is reported.

The 'Hertwig effect,' a phenomenon in which there is a decreased survival rate at low doses of radiation and a paradoxical recovery in survival rate at higher doses, has been observed in experimental studies that have used X-rays and -rays to induce gynogenesis or androgenesis. In studies of androgenesis that used UV to inactivate the maternal genome, the Hertwig effect has only been observed in the tiger barb (Kirankumar and Pandian 2003). Survival rates decreased with increased doses of UV in other species also (Arai et al. 1992; Bongers et al. 1994, 1995; Myers et al. 1995; Lin and Dabrowski 1998; David and Pandian 2006; Kirankumar and Pandian 2003). However, it is unclear whether the decrease in survival rate was due to a UV irradiation effect on fertilization or on embryogenesis. In this study, a decrease in fertilization rate with increased UV dosage at 25,000 ergs/mm^2 (39%) was more apparent than a decrease in hatching rates (32.69%). Thus, this suggests that higher UV dosage impaired fertilization of irradiated eggs.

The use of higher UV doses to induce androgenesis in the zebrafish and muskellunge produced embryos that developed as a cell mass without a body axis (Lin and Dabrowski 1998; Ungar et al. 1998). In this study, larvae with deformed head formation frequently appeared at the low UV doses. The presence of depression in the yolk may be indicative of UV-induced cytological damage to the egg only at higher UV doses. This type of cytological damage is reported by Ungar et al. (1998) in zebrafish eggs exposed to 2- or 2.5-fold increased dosage.

When comparing the UV dosage required in sperm genome irradiation for haploid gynogenesis (Godwin et al. 2010) and egg genome inactivation for haploid androgenesis in *H. fossilis*, a minimum of 90 s corresponding to 11,250 ergs/mm^2 and 100 s (corresponding to 12,500 ergs/mm^2 for egg genome) were required, respectively. At the same time, sperms have the capacity to withstand a very high UV dosage of 270 s corresponding to 33,750 ergs/mm^2 (Godwin et al. 2010), but the eggs started developing cytological damages even at 22,500 ergs/mm^2.

Conclusions

We therefore concluded that a dose of 12,500 to 17,500 ergs/mm^2 of UV radiation is required to ensure genetic inactivation of maternal genome in *H. fossilis.*

Competing interests
The authors declare that they have no competing interests.

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Authors' contributions
All authors contributed equally. All authors read and approved the final manuscript.

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