Advances in Bioresearch
Adv. Biores., Vol 5 (2) June 2014: 183-192
©2014 Society of Education, India
Print ISSN 0976-4585; Online ISSN 2277-1573
Journal's URL:http://www.soeagra.com/abr.html
CODEN: ABRDC3
ICV 7.20 [Poland]

Advances in Bioresearch

ORIGINAL ARTICLE

Effect of UV Irradiation on the Fertility of Eggs of *Danio sps.* and Ploidy condition of Embryos produced using intra- and inter-specific Sperms

V. Sridevi[1] and V. Kalarani[1*]
[1]Department of Biotechnology, Sri Padmavati Mahila Visvavidyalayam (Women's University), Tirupati - 517 502, A.P.
*Corresponding author: kala.dandala@gmail.com

ABSTRACT
Of 21,730 fish species described, the Indian ichthyofauna includes 2546 species, i.e. only 12% of the global fish germplasm. Due to habitat destruction, over exploitation, chemical and biological pollution many fish species are endangered or have become extinct. Androgenesis is one of the Ex Situ strategies that facilitate the conservation of endangered species through inheritance of exclusively paternal genome. But the technique followed to inactivate the egg genome during the process of androgenesis, plays an important role in determining the viability of androgenetic progeny. Hence the present work is carried out to observe the influence of UV irradiation on the viability of eggs of zebrafish, Danio rerio (grey) and Danio frankei (dotted). Distance (16 to 30 cms) Vs time of exposure (1.2 to 3.0 minutes) of eggs to UV radiation (254 nm) was tested for complete inactivation of the egg genome. Eggs of D. rerio (grey) and D. frankei (dotted) kept at a distance of 26cms from UV source and irradiated for 2.0min contributing to an intensity of 84,369 ergs.cm² showed 100% haploidy upon fertilization with sperms of D. rerio (albino) exhibiting maximum survival of 68% and hence was found to be suitable for inducing complete genome inactivation still retaining the viability. Insignificant differences in the fertility, survival and ploidy condition of eggs of D. rerio (grey) and D. frankei (dotted) upon UV irradiation and fertilization with intra/interspecific sperm, clearly suggest that both groups of eggs exhibited similar response to radiation. Prospects of using UV irradiation instead of X-rays or γ- rays for genome inactivation in the development of conservation strategies of androgenesis have been discussed.
Key words: *Danio rerio (grey / albino), Danio frankei (dotted), egg genome inactivation, U.V. irradiation, intra/interspecific sperm*

Received 09/12/2013 Accepted 21/04/2014

How to cite this article:
V. Sridevi and V. Kalarani. Effect of UV Irradiation on the Fertility of Eggs of *Danio sps.* and Ploidy condition of Embryos produced using intra- and inter-specific Sperms. Adv. Biores., Vol 5 [2] June 2014: 172-182. DOI: 10.15515/abr.0976-4585.5.2.172182

INTRODUCTION

Ex Situ strategies like nuclear manipulation (cloning) and chromosomal manipulation are known for their use in the production of clones and the conservation of germplasm. The scheme of nuclear manipulation remained as a difficult task in some groups such as fishes, due to dense yolk and non-visibility of egg nucleus. But androgenesis [1] which facilitates the inheritance of exclusively paternal genome and gynogenesis [2] that facilitate inheritance of exclusively maternal genome are the two well proposed chromosomal manipulation techniques useful for establishing fish conservation strategies. Androgenesis, in particular, proved useful for the production of viable supermales, inbred isogenic lines and intraspecific/interspecific androgenetic clones for conservation of germplasm. But since the fish eggs render them recalcitrant for enucleation and hence elimination of maternal genome needs efficient method to prevent the transmission of nuclear DNA from the eggs [3] in case of androgenesis.

To date, chemical methods tried, were not very successful [4]. Consequently, the induction of androgenesis in fishes obligately involved irradiation of eggs to eliminate the maternal genome and insemination with normal sperm. Physical methods, including UV, X-ray and gamma ray irradiations have

been proven to be relatively more successful [5]. Ionizing radiation such as gamma (γ) and X-rays had been widely used to induce androgenesis in fishes [1]. Higher energy irradiation (X-rays and gamma rays) penetrates aqueous media with less attenuation and fragments DNA by breaking covalent bonds [5, 6]. Moreover the application of γ- or X -radiation is technically difficult, because of safety issues [6]. γ- or X-rays because of their high penetrance,were also shown to destroy 'the maternal products' like proteins (e.g. enzymes), RNA (mainly mRNA) and mtDNA [7], obligately required for initial development [8]. For instance, Stroband *et al*. [9] demonstrated that the maternal products control development in common carp zygotes until the stage of epiboly, which occurs 5–6 h after fertilization. Further the presence of stable maternal DNA residues were detected in interspecific androgenetic Atlantic salmon (*Salmo salar*) developed using gamma rays [10]. Hence better alternatives for egg-genome inactivation are under exploration.

UV irradiation is proposed for its simplicity and safety and also because it dimerizes the DNA rather than fragmenting it [11]. It has been used earlier for the genetic inactivation of cyprinid oocytes [12]. Studies on hybrid tilapia, Nile tilapia, Mud loach, Tiger barb, Rosy barb, Buenos Aires tetra and Loach proved the importance of confirmation of complete inactivation of maternal genome during androgenetic cloning [13].

Radiation energy absorbed by the body depends on its distance from the source as well as their relative exposure time, as has been noticed in bacteria during their UV sensitivity assessment [14]. Hence determination of optimal UV dose required to generate 100% genome-inactivated eggs becomes important in the successful production of androgenic progeny. The present work is carried out to understand the impact of irradiation on the fertility of eggs of zebrafish, *D. rerio* (*grey*) and *D. frankei* (*dotted*) kept at variable distances (16 to 30cm) from UV source for different time periods (1.2 to 3.0min) and ploidy condition of embryos produced upon fertilization with sperms of *D. rerio* (*albino*).

MATERIALS AND METHODS

D. rerio (*grey*) and *D. frankei* (*dotted*) were obtained and maintained in the laboratory as explained earlier by Kalarani *et al*., [15].

a) UV irradiation of eggs for genome- inactivation

Egg genome inactivation was carried out using custom-built UV illumination chamber, fabricated in association with Labnet Scientific Services, Chennai. UV lamp of 254nm was used as a source of irradiation in the chamber. UV lamp was switched on for at least 30min prior to subjecting eggs for irradiation. Eggs collected from each female *D. rerio* (*grey*) / *D. frankei* (*dotted)* (160±30 / 320±45 nos) were suspended separately in petriplates containing 3 ml of synthetic ovarian fluid [12]. 5 ml of sterile water was added to each plate containing approximately 150 eggs to provide slight buoyancy. Dishes were placed separately in the chamber on a rotator - shaker at 25 rpm, permitting eggs to roll in the fluid and thus ensuring the uniform exposure of eggs to illumination (1mW at the surface of eggs). The distance of 16/ 18/ 20/ 22/ 24/ 26/ 28/ 30 cm between the light source and eggs was maintained by adjusting the height of the shaker. Irradiated eggs kept at each distance for a time period of 1.2/ 1.4/ 1.6/ 1.8/ 2.0/ 2.2/ 2.4/ 2.6/ 2.8/ 3.0 min were fertilized and later considered for the assessment of ploidy (Haploid / Aneuploid) condition as explained below.

b) Assessment of fertility and survival of UV irradiated eggs

Fertility of genome-inactivated eggs of *D. rerio* (*grey)* / *D. frankei* (*dotted)* was assessed through activating (inseminating) the irradiated eggs with freshly collected sperms of *D. rerio (albino)* and maintaining them at 28 ± 1°C on a rotator-shaker for 30min. Appearance of blastodisc, a thin region of yolk-free cytoplasm at the animal pole of the egg where cleavage occurred within 2 hours 30 minutes following activation has been considered as a sign of successful fertilization. Count of cleaving blastulae was done using the stereomicroscope (Olympus) and expressed as percent survival. Later the samples were transferred to plastic trays containing filtered tap water which were kept floating in the water bath for further development.

c) Assessment of ploidy condition of UV irradiated eggs upon fertilization

24 hr after fertilization, subsamples of embryos were collected for assessment of ploidy condition. The embryos were suspended in 0.01% freshly prepared colchicine solution. Incubated at 28 ± 0.5º C for 90 min in the dark and transferred to a container with 1.1% sodium chloride. Yolk sac of the embryos was punctured and after 8min, embryos were transferred onto ice and incubated for further 8min. Later the embryos were transferred to a container having Carnoy's fixative and incubated for 20min. The spent solution was replaced with fresh fixative and the embryos were incubated at 4º C overnight. Then the embryos were blotted partially dry and suspended in 45% acetic acid for 1min, aspirated using Pasteur pipette for cell dispersion. Cells were dropped onto a pre-warmed (45º C) glass slide for breakage

following the method of Kligerman and Bloom [16]. Slides were stained with 4% Giemsa for 30min. Metaphase chromosomal spreads were prepared and screened for assessing the ploidy condition.

RESULTS AND DISCUSSION

a & b) Fertility and Survival of UV irradiated eggs

Eggs of both *D. rerio* (*grey*) and *D. frankei* (*dotted*) upon UV irradiation from variable distances (16 to 30cm) for different time periods (1.2 to 3.0min), upon fertilization with fresh monosperms of *D. rerio* (*albino*) exhibited variations in survival rates (Fig. 1, 2 & 3).

Eggs of *D. rerio* (*grey*) UV irradiated for time periods viz., 1.2, 1.4, 1.6, 1.8, 2.0, 2.2, 2.4, 2.6, 2.8 and 3.0min showed significant increase in survival with increase in distance from UV source viz., 16, 18, 20, 22, 24, 26, 28 and 30cm; however at each distance with increase in the duration of irradiation / exposure time, the eggs showed significant decrease in survival (Table 1; Fig. 2) (Duncan's Multiple Range Test, $P<0.01$). Similar trend was noticed in case of eggs of *D. frankei (dotted)* (Table 2 Fig. 3).

Eggs of *D. rerio* (*grey*) exposed to UV irradiation for 1.2min keeping at a distance of 16cm showed survival of 56%, which significantly decreased to 50, 42, 36, 29 24, 18, 10, 8 and 6% upon irradiation for 1.4, 1.6, 1.8, 2.0, 2.2, 2.4, 2.6, 2.8 and 3.0min respectively (Table 1; Fig. 2) while *D. frankei* (*dotted*) eggs exposed to UV irradiation for 1.2min keeping at a distance of 16cm showed 58% survival, which significantly decreased to 52, 44, 38, 31, 26, 19, 12, 9 and 6% upon irradiation for 1.4, 1.6, 1.8, 2.0, 2.2, 2.4, 2.6, 2.8 and 3.0min respectively (Table 2; Fig. 3) with no significant variation between the species.

Radiation damage depends on how sensitive the cells are to radiation. Confirming the fact that all cells are not equally sensitive to radiation damage, cells which divide rapidly and/or relatively non-specialized were observed to show effects even at lower doses of radiation than those which are less rapidly dividing and more specialized. Eggs are the most potent dividing cells and, hence, are more prone for damage from radiation. Li Qi *et al*. [17] using optimum UV dose of 6480 erg/mm^2 observed 64% survival in pacific oyster eggs and noticed that fertilization rate decreases with increase in the duration of exposure. Xu *et al*. [18] also reported that a dose of 75 Jcm^{-2} is required for complete elimination of sperm genome of yellow croaker, *Pseudosciaena crocea.* The eggs of *D. rerio (grey)* and *D. frankei (dotted),* exposed to UV irradiation from a distance of 30cms for 1.2min showed 90% survival upon fertilization while those exposed for 3.0 min from the same distance showed 51 and 52% survival respectively. Though not similarly severe, such an effect (56% egg survival) was found on the eggs exposed to UV irradiation even for only 1.2min from a distance of 16cms. Further the eggs of both species exposed for 3.0min from a distance of 16cm showed only 6% survival, indicating that damage depends on the total amount of radiation received by the eggs beyond genome inactivation which might cause deleterious effects [19] leading to egg mortality. Some ionizing events were also known to produce substances not normally found in the cell [20] which might also result in the breakdown of the cell structure and its components.

Fig. 1. a) Fertilized stage and **b)** Blastula stage of eggs of *D. rerio grey* /*D. frankei dotted* fertilized with sperms of *D.rerio albino*

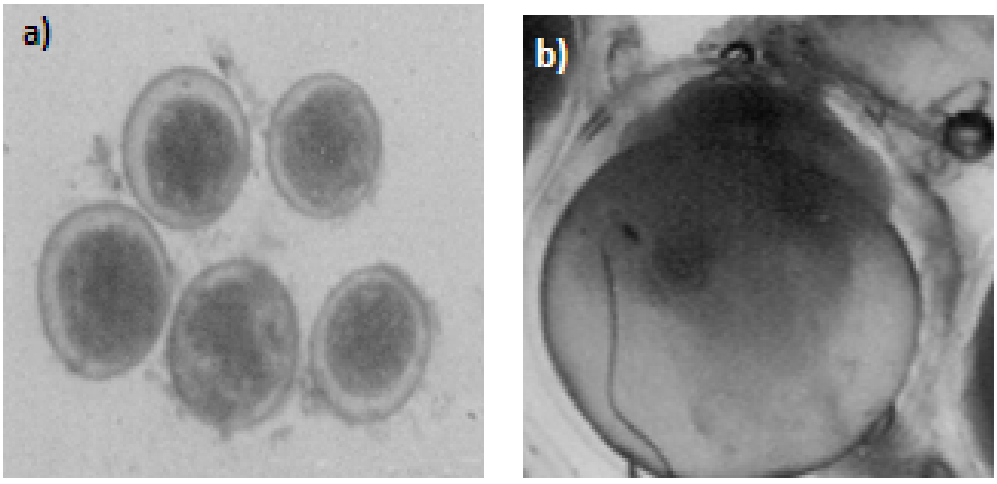


Table 1. Number of eggs of *D. rerio* (*grey*) upon UV irradiation for 1.2, 1.4, 1.6, 1.8, 2.0, 2.2, 2.4, 2.6, 2.8 and 3.0 min from a distance of 16, 18, 20, 22, 24, 26, 28 and 30 cm following fertilization.

Distance (cms) / Time (min)	16	18	20	22	24	26	28	30
1.2	89±4.2	102±4.0	121±4.2	130±4.1	139±4.2*	141±4.1*	142±4.3*	144±4.4*
1.4	80±4.1	93±3.5	113±3.8	119±3.9	125±3.6	131±4.0	138±4.1*	138±4.3*
1.6	67±3.6	85±3.0	102±4.1	109±4.0	116±3.7	124±3.6	134±4.0*	136±4.2*
1.8	57±3.2	71±3.3	92±4.0	98±3.8	107±3.7	116±3.8	125±4.0	135±4.1*
2.0	47±2.5	62±3.1	83±3.5	88±3.6	99±3.6	108±3.6	116±3.8	125±4.3
2.2	38±2.0	51±2.8	63±3.0	74±3.6	89±3.4	97±3.5	107±3.6	116±4.2
2.4	28±1.6	41±2.7	56±2.8	67±2.8	83±3.5	88±3.6	99±3.5	106±3.6
2.6	16±1.1	27±2.0	45±2.7	54±3.0	73±3.4	78±3.7	91±3.2	98±3.8
2.8	12±0.7	21±1.5	36±2.1	43±3.0	63±3.3	71±3.8	82±3.7	90±3.6
3.0	10±0.5	16±1.2	28±2.1	37±2.3	55±3.2	64±3.3	72±3.6	81±3.7

Values are Mean±SD of 6 individual observations.
Similarly marked values in a row / column are not significantly different from each other (P<0.05)

Table 2. Number of eggs of *D. frankei* (*dotted*) upon UV irradiation for 1.2,1.4, 1.6, 1.8, 2.0, 2.2, 2.4, 2.6, 2.8 and 3.0 min keeping at a distance of 16, 18, 20, 22, 24, 26, 28 and 30 cm following fertilization.

Distance (cm) / Time (min)	16	18	20	22	24	26	28	30
1.2	184±4.5	212±4.8	241±4.9	254±5.1	268±5.3	284±5.5*	286±5.4*	289±5.6*
1.4	166±4.1	192±4.7	222±4.6	238±4.9	256±5.0	276±5.1*	278±5.2*	282±5.4*
1.6	140±3.8	178±4.4	201±4.6	224±4.7	238±4.8	256±4.9	275±5.2	274±5.2*
1.8	120±3.5	148±4.1	184±4.5	202±4.6	220±4.6	236±4.8	242±4.7	267±5.2
2.0	100±3.4	130±3.8	145±4.0	182±4.4	198±4.6	222±4.5	230±4.6	250±5.0
2.2	82±3.2	108±3.5	132±3.7	151±4.1	176±4.3	191±4.4	208±4.5	228±4.6
2.4	62±2.6	88±3.3	118±3.5	140±3.8	166±4.2	182±4.3	192±4.4	216±4.5
2.6	38±2.3	60±2.6	96±3.0	130±3.5	152±4.1	162±4.1	182±4.3	198±4.2
2.8	30±2.0	48±2.4	78±2.9	114±3.2	130±3.4	148±4.0	166±4.1	184±4.1
3.0	18±1.3	38±2.0	62±2.6	80±2.9	110±3.1	138±3.8	146±4.1	166±4.0

Values are Mean±SD of 6 individual observations.
Similarly marked values in a row / column are not significantly different from each other (P<0.05).

Fig. 2. Per Cent fertilization of UV irradiated eggs of *D. rerio* (*grey*) using fresh sperms of *D. rerio* (*albino)* (Survival of the control group – 98.8%).

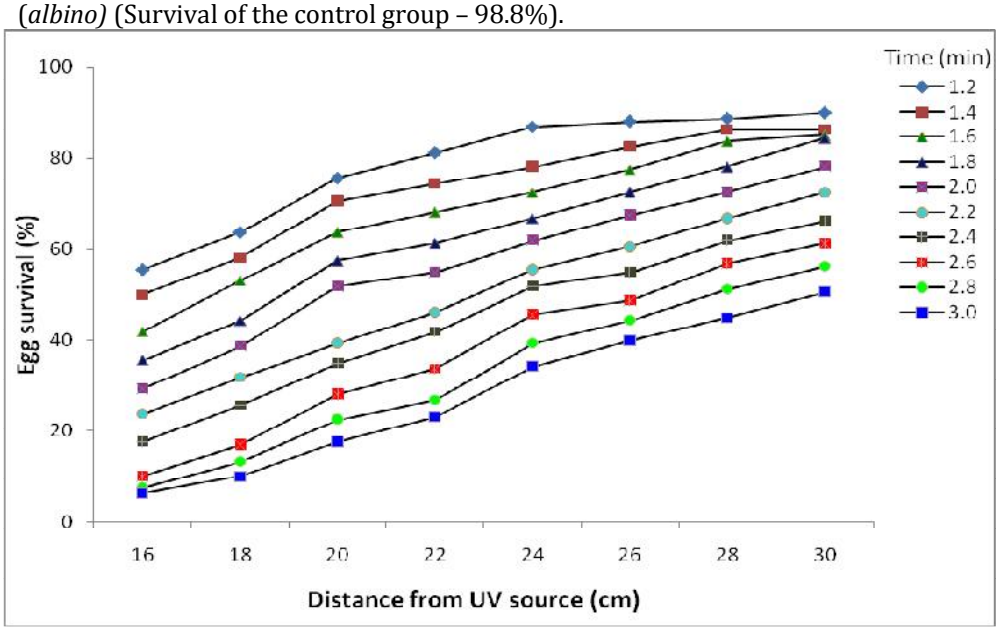


Fig. 3. Per Cent fertilization of UV irradiated eggs of *D. frankei* (*dotted*) using fresh sperms of *D. rerio* (*albino*) (Survival of the control group – 99.0%).

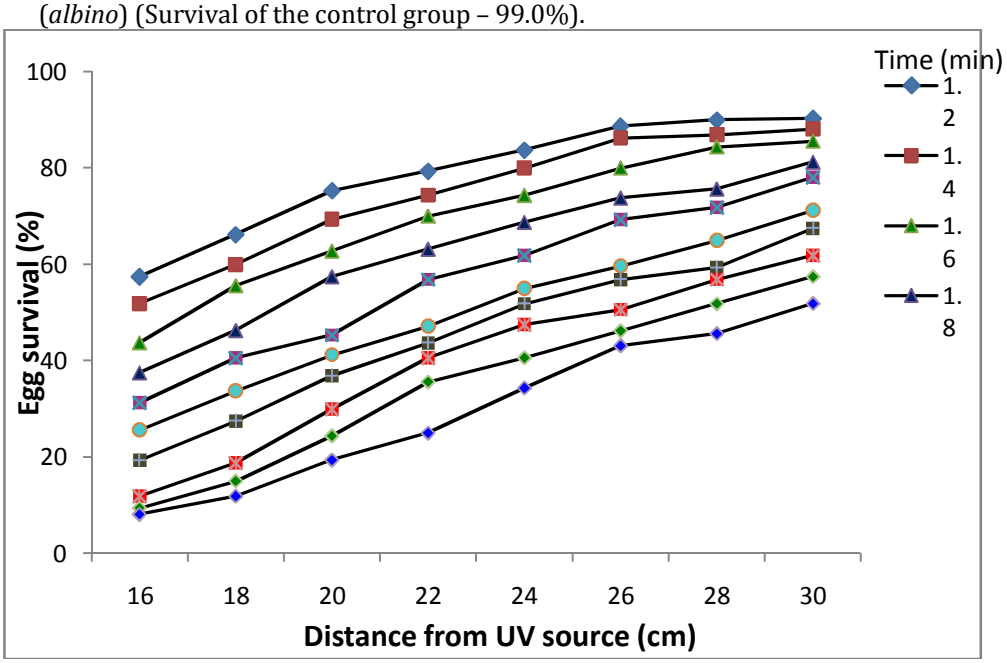


Table 3. Ploidy condition (%) of eggs of *D. rerio* (*grey*), UV irradiated for different time periods from variable distances, following fertilization. Values are Mean ± SD of 10 individual observations.

Time (min)	Ploidy condition	Distance (cm)							
		16	18	20	22	24	26	28	30
1.2	D	0	0	10	60	70	100	100	100
	A	100	100	90	40	30	0	0	0
	H	0	0	0	0	0	0	0	0
1.4	D	0	0	0	0	50	60	100	100
	A	100	100	100	100	50	40	0	0
	H	0	0	0	0	0	0	0	0
1.6	D	0	0	0	0	0	0	60	100
	A	100	100	90	80	70	60	40	0

	H	0	0	10	20	30	40	0	0
1.8	D	0	0	0	0	0	0	0	60
	A	100	100	80	70	60	70	100	40
	H	0	0	20	30	40	30	0	0
2.0	D	0	0	0	0	0	0	0	0
	A	0	0	0	0	0	0	60	100
	H	100	100	100	100	100	100	40	0
2.2	D	0	0	0	0	0	0	0	0
	A	0	0	0	0	0	0	40	100
	H	100	100	100	100	100	100	60	0
2.4	D	0	0	0	0	0	0	0	0
	A	0	0	0	0	0	0	30	100
	H	100	100	100	100	100	100	70	0
2.6	D	0	0	0	0	0	0	0	0
	A	0	0	0	0	0	0	20	100
	H	100	100	100	100	100	100	80	0
2.8	D	0	0	0	0	0	0	0	0
	A	0	0	0	0	0	0	0	100
	H	100	100	100	100	100	100	100	0
3.0	D	0	0	0	0	0	0	0	0
	A	0	0	0	0	0	0	0	100
	H	100	100	100	100	100	100	100	0

D- Diploid; A- Aneuploid; H- Haploid

Table 4. Ploidy condition (%) of UV irradiated eggs of *D. frankei* (*dotted*) UV irradiated for different time periods and from variable distances, following fertilization. Values are Mean±SD of 10 individual observations.

Time (min)	Ploidy condition	Distance (cm)							
		16	**18**	**20**	**22**	**24**	**26**	**28**	**30**
1.2	D	0	0	20	60	50	100	100	100
	A	100	100	80	40	50	0	0	0
	H	0	0	0	0	0	0	0	0
1.4	D	0	0	0	0	60	60	100	100
	A	100	100	100	100	40	40	0	0
	H	0	0	0	0	0	0	0	0
1.6	D	0	0	0	0	0	0	60	100
	A	100	100	80	70	80	70	40	0
	H	0	0	20	30	20	30	0	0
1.8	D	0	0	0	0	0	0	0	50
	A	100	100	80	60	60	70	100	50
	H	0	0	20	40	40	30	0	0
2.0	D	0	0	0	0	0	0	0	0
	A	0	0	0	0	0	0	70	100
	H	100	100	100	100	100	100	30	0
2.2	D	0	0	0	0	0	0	0	0
	A	0	0	0	0	0	0	30	100
	H	100	100	100	100	100	100	70	0
2.4	D	0	0	0	0	0	0	0	0
	A	0	0	0	0	0	0	20	100
	H	100	100	100	100	100	100	80	0
2.6	D	0	0	0	0	0	0	0	0
	A	0	0	0	0	0	0	20	100
	H	100	100	100	100	100	100	80	0
2.8	D	0	0	0	0	0	0	0	0
	A	0	0	0	0	0	0	0	100
	H	100	100	100	100	100	100	100	0
3.0	D	0	0	0	0	0	0	0	0
	A	0	0	0	0	0	0	0	100
	H	100	100	100	100	100	100	100	0

D- Diploid; A- Aneuploid; H- Haploid

Fig. 4. Per Cent survival and different ploidy conditions of eggs of *D. rerio* (*grey*), UV irradiated for different time periods keeping at variable distances, following fertilization.

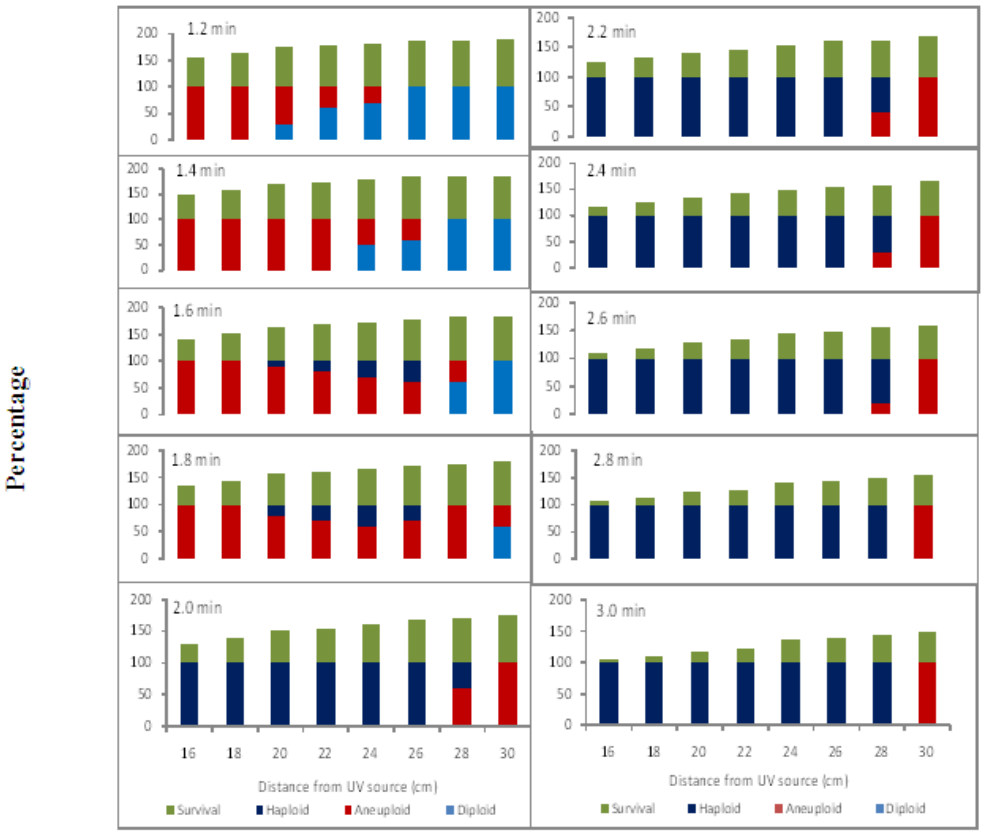
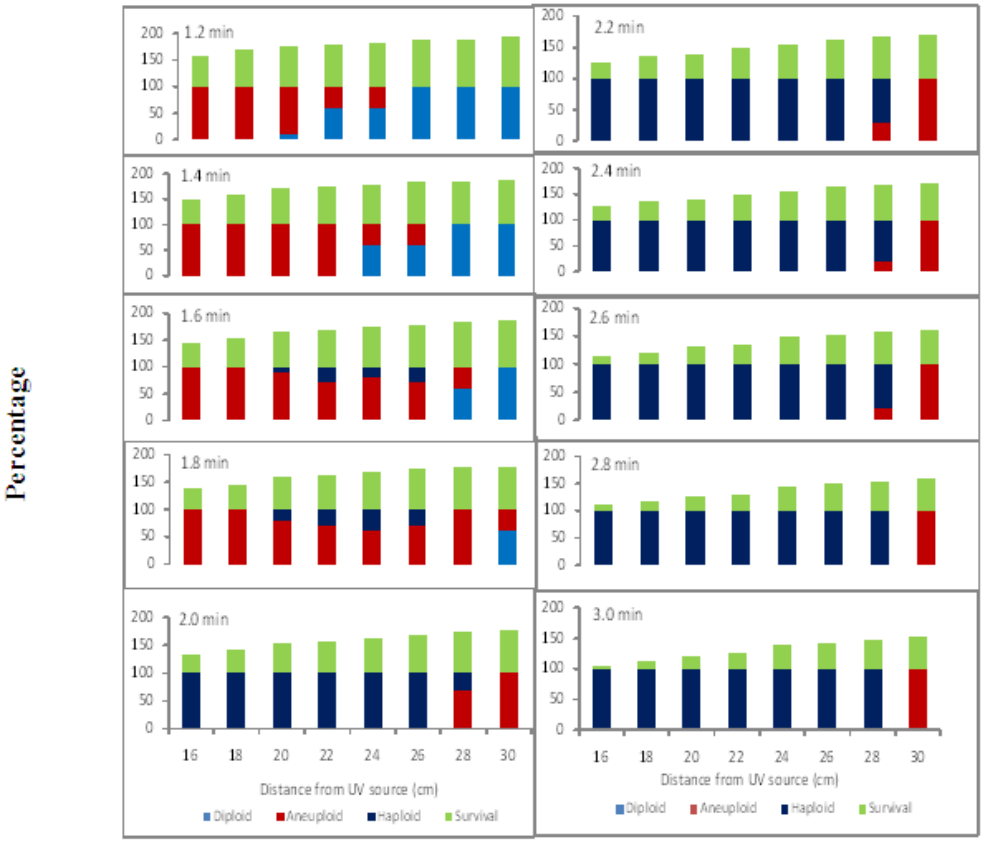


Fig. 5. Per Cent survival and different ploidy conditions of eggs of *D. frankei* (*dotted*), UV irradiated for different time periods keeping at variable distances, following fertilization.



c) Ploidy condition of UV irradiated eggs upon fertilization

Eggs of both *D. rerio (grey)* and *D. frankei (dotted)* upon UV irradiation from variable distances (16 to 30cms) for variable time periods (1.2 to 3.0min), following fertilization / activation with monosperms exhibited variations in ploidy condition. Haploids, diploids and aneuploids were obtained (Table 3, 4) though they were all expected to be haploid in nature.

i) Aneuploids

All (100%) the eggs of *D. rerio* (*grey*) / *D. frankei* (*dotted*) UV irradiated from a distance of 16 and 18cms for 1.2/1.4/1.6/1.8min, upon fertilization with monosperms of *D. rerio* (*albino*) showed aneuploidy (Table 3, 4). Closer distance from UV source was actually expected to have more effect on the genomic material causing complete fragmentation of chromosomes. This should lead to complete genomic destruction in the egg, which upon fertilization with sperm should possess only haploid condition containing genetic material contributed by the sperm alone. But aneuploid condition of n+2 to n+4 observed in the above groups (Table 3,4) clearly indicate that few chromosomes of these eggs remained unaffected under the above irradiation treatments and upon receiving chromosomal set from the sperm(n=25) might have resulted in aneuploid condition. Paternal transmission in gynogenetic fish, due to incomplete sperm genome inactivation was earlier by Carter *et al*. [21]. Li Qi *et al*. [17] also reported insufficient UV irradiation to generate aneuploids; and explained the occurrence of aneuploidy as partial involvement of UV irradiated egg chromosomes in cell division.

ii) Haploids

All the eggs (100%) of *D. rerio* (*grey*) / *D. frankei* (*dotted*), kept at a distance of 16, 18, 20, 22, 24 and 26cms and UV irradiated for 2.0 / 2.2 / 2.4 / 2.6 / 2.8 / 3.0min and kept at a distance of 28cm and UV irradiated for 2.8 / 3.0min attained ploid condition (Table 3, 4) upon fertilization. These results clearly indicate that the above exposures caused complete inactivation of egg genome resulting in haploid condition upon fertilization with monosperms of *D. rerio (albino).* Kucharczyk *et al*. [22] reported to use UV irradiation of 2700-3500Jm^{-2} for the production of pure androgenotes in common bream. Previously rosy barb eggs, UV irradiated for 3.5min were reported to produce 100% haploid androgenetic progeny without contamination of maternal chromosome fragments [23]. Results of the present study clearly show that zebrafish eggs kept at a distance of 26cms from UV source and irradiated for 2.0min contributing to an intensity of 84,369 $ergs.cm^2$ showed 100% haploidy upon fertilization exhibiting maximum survival of 68% (Fig. 4, 5) and hence was found to be suitable for inducing complete genome inactivation for genetic manipulations.

15000 $ergs/mm^{-2}$ was earlier found to be the optimum UV dose for irradiating sperms of *H. fossilis* for the production of gynogenotes [24]. On the other hand, 720mW exposure of sperms of Scallops for 60 seconds was found to result in 100% haploid gynogenesis [25]. The differences relative to the present observations further clearly indicate that in addition to species-specific variations, impact of UV irradiation differs based on the gamete size and shape.

iii) Diploids

All the eggs (100%) of *D. rerio* (*grey*) / *D. frankei* (*dotted*), kept at a distance of 26, 28 and 30cms and UV irradiated for 1.2min; kept at a distance of 28 / 30cm and UV irradiated for 1.4min and kept at a distance of 30cm and UV irradiated for 1.6min showed diploid condition (Table 3, 4) upon activation with monosperms of *D. rerio* (*albino*). This indicates that, the egg genome (haploid) did not get inactivated because of these treatments and remained undisturbed and contributed to the formation of diploid eggs upon fertilization with monosperms. Though Bongers *et al*. [12] used a dose of 250 Jm^{-2} for total elimination of maternal genome in common carp, it was not clearly reported whether the genetic contribution of the female was fully eliminated or not. 50% of androgenetic progeny of pacific oyster, produced upon UV irradiation of the eggs for 30 seconds were found to be diploid in nature indicating retention of complete maternal genome [17]. Kirankumar and Pandian [23] also reported the production of 95% diploids in interspecific androgenesis of rosy barb upon monospermic fertilization of eggs, which were UV irradiated for 1.5min and explained it as an insufficient inactivation of oocytes. Similarly the oocytes of *H. caudovittatus* upon exposure to UV irradiation for 1min followed by monospermic fertilization resulted in the production of 60% of diploid androgenotes [26].

Persistence of diploidy in the fertilized eggs of *D. rerio* even after UV treatment can be explained by the fact that when ionizing radiation interacts with eggs, it may or may not strike a critical part of the egg. Mild damage to the chromosomes is may get repaired [27] since there are noted number of very effective repair mechanisms that constantly repair cellular damage - including chromosome damage [20]. Further the alterations caused due to low intensity ionizing radiation might be the same as those that occur naturally in any cell [28] and might have not induced any negative effect

Sensitivity to radiation may differ from species to species due to differences in chorion structure, egg size, shape and relative position of the female pronucleus [29]. Both the distance from the source of radiation as well as total radiation intensity were found to affect both viability and fertility of fish eggs. No significant differences in fertility, survival and ploidy condition between the eggs of *D. rerio (grey)* and *D. frankei (dotted)* upon UV irradiation and fertilization with the sperms of *D. rerio (albino)* further clearly provide the scope for the use of the species/strain based on their availability. This proposes the exploration of sperms of a single fish species against the eggs of related species for the generation of intra- and inter-specific androgenotes, the practice which will have greater potential in the recovery of many endangered fish species.

ACKNOWLEDGEMENTS

The authors acknowledge The Department of Science and Technology for the financial support (Ref: DST.No. SR/SO/AS-36/2003 dated 17.05.2005). The authors are grateful to Prof. T.J. Pandian, National Professor, Madurai Kamaraj University, Madurai and Prof. D.C. Reddy, Former Head, Department of Fishery Science & Aquaculture, Sri Venkateswara University, Tirupati for their encouragement.

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