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Successful Induction of Intra-specific Androgenesis in Widow Tetra, *Gymnocorymbus ternetzi* (Boulenger 1895) Using UV Irradiation

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Abstract

A protocol for elimination of the maternal egg genome in widow tetra (WT), *Gymnocorymbus ternetzi* (Boulenger 1895) using contrasting black and albino strains was successfully optimised. Maternal genome elimination is a pre-requisite for inducing androgenesis. In the present study, UV-irradiation for 3 min inactivated the maternal nuclear genome in eggs of the black WT. A single layer of black WT eggs exposed to a UV-G bulb (254 nm, 40 W) at a height of 25 cm and at a final intensity of 4.2 $W \cdot m^{-2}$ successfully eliminated the WT egg genome. Androgenotes were generated by fusing genome-inactivated eggs with fresh sperm. Fertilisation was achieved by mixing fresh albino tetra sperm with irradiated eggs and adding water to initiate sperm motility. Following fertilisation by fresh albino WT sperm, 22-min-old embryos were shocked at 41 °C for 2 min to restore diploidy. Survival of androgenotes was 11% and 8% at hatching and maturity while that of the controls were 94% and 67%, respectively. Maternal genome inactivation was confirmed by (i) albino body colour in the diploid fry and adult, (ii) haploid karyotype and (iii) embryonic development. Hatched haploid androgenotes showed normal embryonic development but exhibited severe haploid syndrome while diploid androgenotes resembled albino WT and displayed a diploid karyotype.

Introduction

Of the approximate 20,000 extant teleost fish species available, the Indian subcontinent contains icthyofauna of 2,546 species (Khoshoo 1984) and this contributes ~11% of the total world fish germplasm (Ponniah et al. 1998). Conservation Assessment and Management Plans (CAMP) have identified 1 extinct, 45 critically endangered, 91 endangered and 81 vulnerable Indian fish species, that emphasises the need for fish genome conservation (Das 1994). Cloning and cryopreservation of gametes and embryos has yet to be developed for most fish taxa. Due to non-visibility of the egg nucleus, established protocols for nuclear transplantation to clone fish have also proven difficult. Due to the presence of high amounts of egg yolk, fish eggs and embryos are also not often amenable to cryopreservation. In contrast, fish sperm have a unique advantage over fish eggs and are amenable for preservation thus facilitating induction of

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androgenesis. Owing however, to the availability and amenability of gametes and external fertilisation, fish gametes can withstand techniques such as sperm preservation at -196 °C and androgenesis, by arresting mitotic division by applying thermal shock to embryos.

Androgenesis is a developmental process that facilitates the inheritance of an exclusively paternal genome. While diploid embryos are formed by genomic contributions from both parents (i.e., the maternal and paternal genomes), androgenotes contain genetic information that is exclusively paternal. Androgenesis can be used to restore a species or valuable brood stock using preserved sperm and genome-inactivated eggs from an alien strain/species. Over the years, many protocols for successful induction of androgenesis have been optimised for more than a dozen fish species (Pandian and Kirankumar 2003). Androgenesis can also be induced in post-mortem preserved (David and Pandian 2006a) or cryopreserved spermatozoa (Scheerer et al. 1991), which may be useful in short-term preservation of valuable breeders for aquarists. Scientists in some developing countries are developing simple protocols for successful elimination of the egg genome that could have wide applications for farmers and aquarists (Pandian and Kirankumar 2003). In the present study, UV irradiation, which is known to result in production of cyclo-butyl rings between adjacent pyrimidines (mostly thymine) in DNA strands was used to inactivate the egg genome. Dimers that form can often after UV irradiation be split *in-situ* after application of a specific enzyme repair system (photolyase). Hence, this process of enzymatic photo-reactivation can restore the mono pyrimidines (Voet and Voet 1990) to prevent photo-reactivation of the inactivated maternal genome of the egg. Hence, in the present study the entire process of UV-irradiation and subsequent activation and diploidisation were completed without light.

After fertilisation of genome-inactivated eggs with fresh sperm, haploids were generated without pigmentation that exhibited haploid syndrome. All hatched haploid embryos did not survive however, due to the presence of only a paternal set of haploid chromosomes. Clearly, there is a need for a detailed study of sperm: egg ratio, sperm motility and fertilisability of fresh vs preserved sperm (data not shown) and their use for induction of androgenesis (see also David and Pandian 2006b). Hence, the objectives of the present study were to optimise protocols for (i) inactivating black WT egg genome using UV-irradiation, (ii) optimise fertilisation with fresh spermatozoa and (ii) diploidisation by heat shock and (iii) to confirm paternity using (a) karyotyping, (b) RBC measurements and (c) embryonic development. In addition, the hatched androgenotes were raised to sexual maturity and their reproductive performance was compared with control WT males and females.

Materials and Methods

Fish

Widow tetra (WT), *Gymnocorymbus ternetzi* (Boulenger 1895) is a popular ornamental fish and was chosen as a model for the current study. A short generation time (4 months), oviparity and amenability to stripping of eggs and sperm, fortnightly breeding cycle and availability of strains with contrasting colours made the tetra an ideal candidate for the present

study. Of the three *G. ternetzi* strains available, only two, namely the dominant 'black' and the recessive 'albino' were chosen. The experimental design was to use genome-inactivated eggs from female black WT and to activate them with either fresh or stored (4 °C) sperm from male albino WT individuals (Fig. 1)

Genome inactivation by UV irradiation

After females showed mating behaviour in a plastic tank, they were removed and dried with tissue paper. Fish were cleaned of any excess moisture to prevent activation of eggs with water while stripping. For each exposure treatment, approximately 60 -120 eggs were stripped from a ripe female and placed into a thin plastic Petri dish (1 mm thickness) as a monolayer in Ringer's solution. Eggs were then irradiated using a UV-G lamp (254 nm; 40 W) for different treatment durations in a dark room. For the experiment, eggs were exposed to UV after first placing the Petri dish at a constant distance of 25 cm from a light source for 1.0, 1.5, 2.0, 2.5, 3.0 or 3.5 min. After UV exposure, embryos were removed and kept in the dark until they were fertilised with albino WT sperm.

Donor sperm collection

Healthy and mature albino WT males were anaesthetised in 1 L of water containing 2 mL of clove oil. When their opercular movement rate had slowed down and fish showed very little activity, they were presumed to have been anesthetised. Semen was collected from males by gently stripping the abdomen inside an embryo cup containing 2 mL of Ringer's solution. Semen collected in the embryo cup was used to activate the genome of inactivated eggs (Rosenthal et al. 1998). For the experiment, albino WT males were sacrificed after they had first been anesthetised in clove oil. Males were dissected and the two lobes of the testis, separated. One lobe was used to estimate the sperm count and motility duration, while the other was used to estimate fertilisability and hatchability (see also David 2004). Briefly, to obtain sperm, the lobe was gently macerated in 2 mL of Ringer's, and the sample generated was used to measure the sperm count and for activation of irradiated eggs after the required dilution.

Fertilisation

Fertilisation was achieved following wet and dry fertilisation procedures for fresh and stored (4 °C) sperm, respectively. For this, irradiated eggs were mixed with 1 mL of fresh milt and 2 mL of fresh tap water in a Petri dish. Subsequently, the embryos in Petri dishes were gently swirled for 45 sec to ensure activation of embryonic development that was confirmed under a microscope (Nikon, Japan).

Embryonic development

Activated eggs were maintained at 28 ^{o}C inside an incubator. After embryos had reached the 1, 000 cell stage, they were washed partially in fresh sterile water to remove dead and unfertilised eggs and then live eggs were transferred immediately to a plastic tray filled with sterilised tap water maintained at a constant temperature (28 ^{o}C). Embryonic development of

haploid embryos was followed for synchronised developmental duration with control embryos. Developing embryos were examined for presence or absence of melanocytes under a stereo-zoom microscope (Nikon, Japan).

RBC nuclear measurements

Blood smears were prepared from samples of young hatched larvae. Prior to fixing in acetic acid for karyotyping, larvae were gently pressed on to glass slides for obtaining RBC smear. Slides were stained with Giemsa and observed under the microscope. The length diameter of 20 erythrocytes and associated nuclei were then measured using an ocular micrometre calibrated with a stage micrometre.

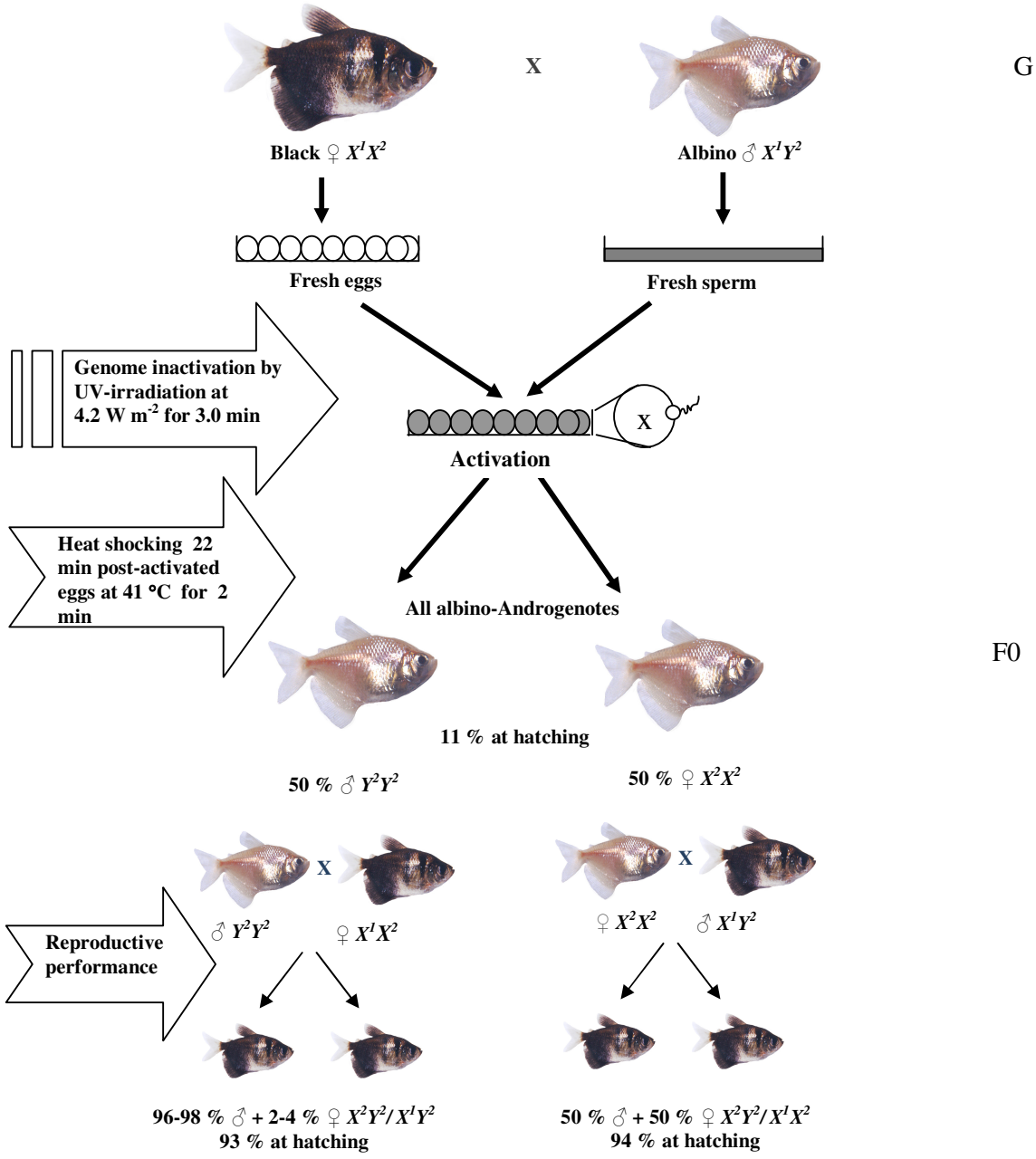

Fig. 1. Protocol for inducing intra-specific androgenesis in *Gymnocorymbus ternetzi*.

Karyotyping

For each respective treatment group, samples of freshly hatched fry were transferred into 0.01 % colchicine solution for 6 h. Karyotyping was conducted following the protocol of Kligerman and Bloom (1977). Ploidy level of each selected individual was determined from 25±5 metaphase spreads.

Pigmentation

Developing embryos were observed for synchronous development and the presence or absence of melanocytes under a stereozoom microscope (Nikon, Japan).

Statistical analysis

All data are expressed as Mean±SD. All statistical analyses were carried out using Sigma Stat software Ver 7.0. Estimation of the level of significance for corresponding data followed Zar (1984). Data were considered to be significant at $P < 0.05$.

Results

Maternal genome inactivation

After exposure to UV irradiation, black WT eggs were activated subsequently using fresh sperm from albino WT and 100% albino haploids were generated (Fig. 1). When eggs were irradiated as a monolayer, UV irradiation penetrates sufficiently well to induce thymine dimers thereby inactivating their genome. Hatchability of these haploids was very low (11%).

Effect of UV –irradiation on hatching success of genome inactivated embryos

Table 1 shows that UV irradiation for a sub-optimal durational period induced deformed aneuploids in all embryos exposed and only a slight pigmentation pattern was evident. However, all embryos that hatched following 2 and 2.5 min exposure duration to UV exhibited haploid syndrome. Embryos exposed to 3 min duration were androgenetic haploids that succumbed before or shortly after hatching. Haploidy of fry was checked via karyotyping; almost all haploid embryos and hatchlings suffered from 'haploid syndrome' and other deformities. Just prior to hatching or within 48 h following hatching, almost all haploids had died due to the cumulative effects of one or more of the deformities (Fig. 2). Heat shocking of 22 min old embryos at 41 °C for 2 min resulted in 100 % diploid albino WT (Table 2). Aneuploidy increased with increasing temperature and duration of heat shock. More aneuploids were generated when irradiated eggs were shocked at 42 °C and correspondingly, the number of diploid albino androgenotes decreased with increasing shock duration (Table 2). Hatched diploid androgenetic embryos resembled paternal Albino WT and were raised to sexual maturity for progeny testing to confirm paternity (data not shown). All embryos, as confirmed from karyotypic analyses were aneuploids and consequently showed severe deformities; no haploids or aneuploids survived until feeding stage.

Confirmation of Ploidy

Karyotype

The earliest stage at which karyotyping of WT could be conducted was in 2-day old fry. Black and albino WT individuals have identical chromosome numbers (2n=48; see also Klinkhardt 1998; Fishbase 2011). Ploidy status of haploids and aneuploids was confirmed by karyotyping randomly selected fry from the various irradiated batches of genome-inactivated eggs. Karyotyping of control fry confirmed the diploid (2n) number of chromosome to be 48 (Fig. 2) while that of haploids (n) was 24 (Fishbase 2011).

RBC nuclear measurements

Haploid syndrome produced smaller erythrocyte and nuclear diameters in recently hatched larvae. Erythrocyte measurements clearly showed that haploids had smaller mean nuclear diameters (2.4 µm) compared with their diploid (4.3 µm) counterparts. Mean erythrocyte diameter was 7.26 µm in diploids while that of haploids was 3.97 µm.

Table 1. Effect of UV–irradiation on the black widow tetra eggs that were subsequently fertilised with sperm of albino widow tetra. Each value is the Mean ± SD of six replicates. Values followed by different superscript are significantly different (P<0.05 % level) from control.

UV exposure	Hatched fry	Ploidy (%)		
		Haploid	Deformed	Diploid
(min)	(%)			
0.0	94±2.7^{f}	-	-	100
1.0	70±3.6^{e}	-	11±2.6^{a}	89±3.1^{f}
1.5	57±2.8^{d}	-	7±2.2^{a}	93±3.1^{f}
2.0	31±4.3^{c}	16±2.1^{b}	79±3.4^{f}	5±2.4^{a}
2.5	17±1.5^{b}	86±2.4^{f}	14±2.6^{a}	-
3.0	11±2.4^{a}	100	-	-
3.5	8±1.7^{a}	22±3.4^{b}	68±3.4^{e}	11±1.3^{a}

Pigmentation pattern

Pigment formation commenced earlier at 28 h post fertilisation (hpf) while body pigmentation became visible to the naked eye at 68 hpf. Body pigmentation was totally absent however, in all albino WT. Hence, it took about 28 hpf to identify haploids and aneuploids using body pigmentation as the critical index. UV-irradiation of black WT eggs for 'sub-optimal' durations (e.g. 1.5 min) led to incomplete inactivation of the maternal genome and embryos showed light pigmentation at 28 hpf (Fig. 2).

Embryonic development

We estimated the periods of time for black and albino strains of WT to reach each selected developmental stage. At 28 °C, diploid control black WT had reached the blastula stage at 3 hpf

while the 12 somite stage was reached at 11 hpf and 20 somite stage was reached at 13 hpf. In contrast, haploids required 4 hpf to reach the blastula stage but only 11 and 13 hpf to reach the 12 somite and 20 somite stages, respectively.

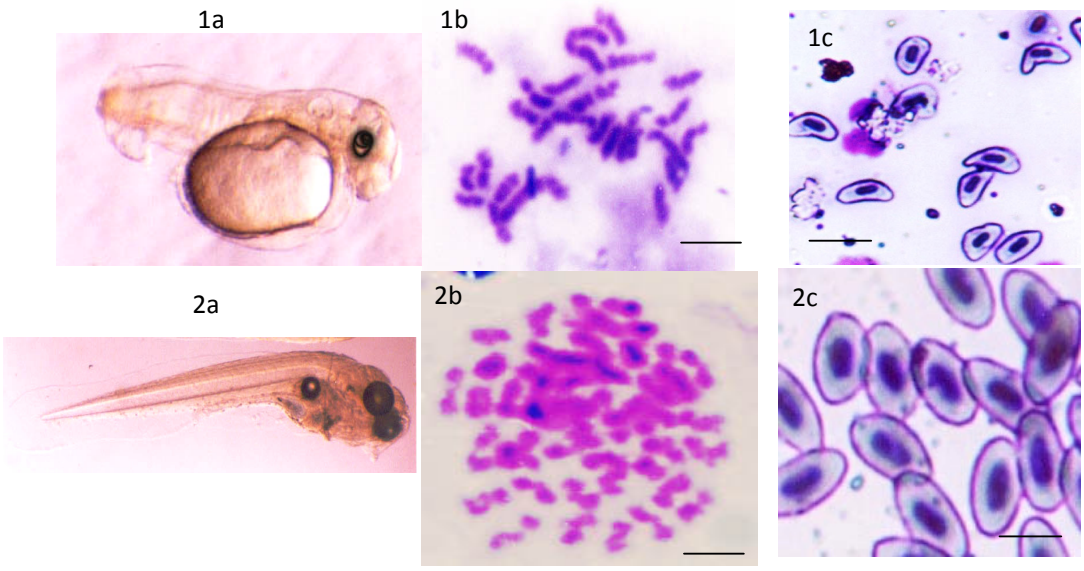

Fig. 2. Representative haploid (1a) embryo of genome- inactivated eggs of black widow tetra fertilised with albino widow tetra sperm and their respective karyotypes (1a and 2a) and erythrocytes (1c, 2c). Scales in the 2nd column and in 3rd column represent 5 and 10 µm, respectively.

Table 2. Estimation of optimum temperature, age of embryo and shocking duration required to restore diploidy in the albino androgenetic haploid zygote; the UV-irradiated eggs of black widow tetra were fertilised by fresh sperm of albino widow tetra. AF = after fertilisation. Each value represents mean ± SD of eight replicates. Values followed by different superscripts are significantly different (P<0.05 % level) from control.

Treatment protocol			Hatched fry	Ploidy (%)		
Temperature (°C)	Commencement time (min AF)	Duration (min)	(%)	Haploid	Aneuploid	Diploid
40	22	2.0	9±1.5^{c}	14±2.2^{c}	86±2.8^{h}	-
		2.5	6±1.4^{b}	-	96±2.6^{i}	4±1.3^{a}
	23	2.0	7±1.9^{a}	-	68±2.7^{g}	32±2.1^{e}
		2.5	4±1.7^{a}	-	39±2.3^{e}	61±2.1^{g}
41	22	2.0	11±2.5^{c}	-	-	100
		2.5	9±1.6^{b}	-	12±1.5^{c}	88±3.4^{h}
	23	2.0	10±1.5^{c}	-	18±2.1^{c}	82±3.6^{h}
		2.5	8±1.7^{b}	-	6±1.9^{a}	94±3.7^{i}
42	22	2.0	7±2.4^{b}	-	28±2.2^{d}	72±2.8^{g}
		2.5	6±1.4^{a}	-	47±2.4^{f}	53±2.6^{f}
	23	2.0	5±2.2^{b}	-	74±3.6^{g}	26±1.8^{d}
		2.5	4±1.2^{a}	-	81±2.9^{h}	19±2.1^{d}

Discussion

A protocol was developed for successful maternal genome inactivation of black WT, *G. ternetzi*. Previously, eggs from the Gray Buenos Aires Tetra, *Hemigrammus caudovitattus* Ahl 1923 had been used successfully to induce androgenesis after maternal genome inactivation using UV irradiation (David and Pandian 2006a). Earlier, Kirankumar and Pandian (2004a) had also demonstrated that UV irradiation can produce complete maternal genome inactivation. Here, we show that UV irradiation applied to the inactivated maternal genome of black WT eggs successfully eliminated the maternal genome. Results of the present study clearly indicate that fish eggs are susceptible to genome inactivation via UV irradiation which is an easier, safer and more economical cost effective approach to producing androgenesis. Maternal genome inactivation of black WT eggs via UV irradiation effectively inactivated maternal DNA thereby resulting in progenies that expressed the genotypic and phenotypic features of only the male albino WT parent when fertilised with this sperm.

Since mean egg size of WT was 1.0 mm which is smaller than that of *Puntius tetrazona* Bleeker 1855 (1.2 mm) (Kirankumar and Pandian 2003), it is understandable that the optimum durations required for inactivation of their maternal genomes by UV irradiation were different, 3.0 and 3.5 min for *G. ternetzi and P. tetrazona*, respectively. In all cases for embryos treated with durations of less than 3.0 min, individuals suffered a variety of deformities and died at some stage during their development. Completing embryonic development with only a haploid set of recessive paternal homozygous chromosomes is remarkable. Hence, hatching success of androgenetic haploids was lower than control diploids. Hatching rate of haploid fry was higher in the 2.0 min treatment group than that of the diploid androgenotes, indicating that enhanced homozygosity (Y^2Y^2) is perhaps more deleterious than haploidy (Y^2)

Paternal origin of haploid individuals that were generated from albino WT sperm was confirmed via (i) phenotypic markers (namely pigmentation), (ii) karyotyping and (iii) embryonic developmental duration. Colour and appearance of the corresponding pigments have been used in earlier studies to confirm paternity of androgenetic progeny (e.g. Corley-Smith et al. 1996; Bercsenyi et al. 1998; David and Pandian 2006a, b). In gray *H. caudovittatus,* pigments became visible by 28 hpf. Black *G. ternetzi* showed a similar pattern with black pigmentation on the body and eye becoming visible from the 28th hpf onwards. In the present study, haploids did not develop any pigmentation even after 30 hpf thus confirming that a haploid set of chromosomes had been inherited from the paternal genome alone. Of interest, eggs that were exposed to UV irradiation for less than 3 min were aneuploid and had pigmentation similar to controls. Diploid albino androgenotes were reared to sexual maturity to estimate sex-ratio and for progeny testing following Ezaz et al. (2004; Fig 1).

Karyotyping has been used to distinguish species at the earliest possible stage of development (Kirankumar and Pandian 2003; David and Pandian 2006a). Karyotyping of fry in the current study showed that haploid chromosome number (n=24) was the same as albino WT. Karyotyping was possible in fry that were only 24 to 48 hpf. However, when both the parents possessed identical chromosome numbers, karyotyping could not be used to differentiate them;

hence, we relied on other phenotypic characteristics to identify paternal genome. As both parents in the present study had the same chromosome number, other phenotypic characters including pigmentation and embryonic developmental duration were used as markers of paternal parent (Pandian and Koteeswaran 1998). Haploid embryonic development pattern and duration were similar to control embryos. Fry with haploid syndrome had completed their embryonic development within 24 hpf and even diploids after shocking completed their development by 24 hpf (David 2004). Hence, in the present study, haploid chromosome number did not alter the embryonic development rate due to haploidy but individuals completed their larval developmental stages within 24 hpf - a result identical to paternal albino WT individuals.

Conclusion

Development of protocols for inducing androgenesis can aid in resurrecting (and propagating) endangered or economically important fish species. Use of androgenetic restoration can be used to produce male breeders of economically important fish species in addition to healthy and economically important fish phenotypes and strains of importance.

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References

Bercsenyi M., I. Magyary, B. Urbanyi, L. Orban and L. Horvath. 1998. Hatching out goldfish from common carp eggs: interspecific androgenesis between two cyprinid species. Genome 41:573-579.

Corley-Smith G.E., C.J. Lim and B.P. Brandhorst. 1996. Production of androgenetic zebrafish (Danio rerio). Genetics 142:1265-1276.

Das, P. 1994. Strategies for conserving threatened fishes. In: Threatened Fishes of India (eds. P.V. Dehadrai, P. Das and S.R. Verma) pp. 307-310. Nature Conservation Publication. No. 4, Muzaffarnagar.

David C.J. 2004. Experimental sperm preservation and genetic studies in selected fish. Ph.D. thesis submitted to Madurai Kamaraj University, Madurai. 122 pp.

David C.J. and T.J. Pandian. 2006a. Cadaveric sperm induces intergeneric androgenesis in the fish *Hemigrammus caudovittatus*. Theriogenology 65:1048-1070.

David C.J. and T.J. Pandian. 2006b. GFP reporter gene confirms paternity of the androgenetic Buenos Aires tetra, *Hemigrammus caudovittatus*. Journal of Experimental Zoology 305(A): 83-95.

Ezaz M.T., J.M. Myers, S.F. Powell, B.J. McAndrew and D.J. Penman. 2004. Sex ratios in the progeny of androgenetic and gynogenetic YY male Nile tilapia, *Orecochromis niloticus* L. Aquaculture 232:205-214.

FishBase. (eds. R. Froese and D. Pauly.) 2011. World Wide Web Electronic Publication. http://www.fishbase.org/Summary/SpeciesSummary.php?ID=4682. Accessed 20 April 2014.

Khoshoo T.N. 1984. Environmental concern and strategies. Current Science 25:577-582.

Kirankumar S. and T.J. Pandian. 2003. Production of androgenetic tiger barb, *Puntius tetrazona*. Aquaculture 228:37-51.

Kirankumar S. and T.J. Pandian. 2004a. Interspecific androgenetic restoration of rosy barb using cadaveric sperm. Genome 47:66-73.

Kligerman A.D. and S.E. Bloom. 1977. Rapid chromosome preparations from solid tissues of fishes. Journal of Fisheries Research Board Canada 34:266-269.

Klinkhardt, M. 1998. Some aspects of karyoevolution in fishes. Animal Research and Development 47:7-36.

Pandian T.J. and R. Koteeswaran. 1998. Ploidy induction and sex control in fish. Hydrobiologia 384:167-243.

Pandian T.J. and S. Kirankumar. 2003. Androgenesis and conservation of fishes. Current Science 85:917-931.

Ponniah, A.G., P. Das and S.R. Verma. 1998. Fish Genetics and Biodiversity Conservation. Nature Conservators, Muzaffarnagar. 474 pp.

Rosenthal H., D. Klumpp and J. Willfuhr. 1998. Influence of sperm density and contact time on herring fertilization. Journal of Applied Ichthyology 4:79-86.

Scheerer P.D., G.H. Thorgaard and F.W. Allendorf. 1991. Genetic analysis of androgenetic rainbow trout. Journal of Experimental Zoology 260:382-390.

Voet, D. and J. Voet, 1990. DNA replication, repair and recombination. In: Biochemistry, (eds. D. Voet and J. Voet), pp. 967-969. John Wiley, New York.

Zar H. J. 1984. Biostatistical analysis. Prentice–Hall International, New Jersey. 718 pp.

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