Dispermic induction of interspecific androgenesis in the fish, Buenos Aires tetra using surrogate eggs of widow tetra

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Induction of interspecific androgenesis in fish involves introduction of one or more sperm of a species into genome-inactivated eggs of another compatible species that serve as surrogate. Incubation of milt of Buenos Aires tetra (BT), *Hemigrammus caudovittatus* (recessive golden strain) for a constant period of 7 min in selected concentrations of polyethylene glycol (PEG) facilitated entry of two fused haploid sperm into genome-inactivated egg of widow tetra (WT), *Gymnocorymbus ternetzi* (dominant black strain). Following incubation at 2.5% PEG, the milt contained 78% motile sperm, of which 37% remained single; of the 41% fused sperm, 14% alone were completely fused from head to tail subsequently activating 61% of UV-irradiated (3 min) WT eggs. Hatched F_0 androgenotes were morphologically identical to the golden BT but were distinguishable from hybrid diploids and triploids by colour and shape. Karyotype, erythrocyte measurements and sex ratio 1♀ (X^2X^2) : 2♂ (X^2Y^2 or Y^2Y^2) (the superscript '2' indicates paternal origin) of androgenotes confirmed their diploid status. Reproductive performance of androgenetic males was equal to normal males but that of the females was inferior. Of six crosses involving the androgenetic males (X^2Y^2 or Y^2Y^2) and normal females (X^1X^2) (the superscript '1' indicates maternal origin), four produced 100% male progenies by sperm bearing Y^2 genotype; two generated 50% (X^1X^2/X^2X^2) females and 50% (X^1Y^2/X^2Y^2) male progenies by sperm bearing either X^2 or Y^2 genotype. Only 1.8% dispermic androgenotes survived at hatching. Causes for low survival at hatching were traced to the death of 39% inactivated eggs, 49% haploids and about 10% embryos suffering developmental incompatibility. Hatching success increased to 4.3% with availability of 19% completely fused double sperm, when sperm concentration was quadruplicated.

Keywords: Dispermic induction, maternal genome inactivation, progeny testing, reproductive performance, sex ratio.

ABSENCE of acrosome in spermatozoa of teleostean fishes[1] and the presence of micropyle in egg[2] facilitate not only heterogamy, but also polyspermy[3]. Hence fishes are ideal vertebrate models to study genome manipulation, as many are known to tolerate multiple genomes, or maternal or paternal genome alone, or even genome of an alien but compatible species[4,5]. As maternal genome is either inactivated or eliminated before fertilization, androgenotes inherit the paternal genome alone[6]. Following monospermic activation, the genome-inactivated homologous or heterologous eggs obligately require diploidization by suppression of the first mitotic cleavage. The diploidization procedure introduces considerable homozygosity and stress, and causes high mortality during early embryonic development of the androgenotes (e.g. ref. 7). Such high mortality may be avoided with activation by a single diploid (e.g. ref. 8), or two haploid sperms (e.g. ref. 9), which may also enhance heterozygosity. When normal diploid male and female are paired, 95% of eggs are hatched out (e.g. ref. 5). The hatching success is reduced to 11% when fresh sperms were used to activate heterologous eggs (e.g. ref. 10). It was further reduced to 8 or 4% when (cadaveric) sperms from post-mortem preserved specimens were used to fertilize homologous eggs or activate heterologous eggs[11]. A common but decisive cause for the observed high embryonic mortality is the enhanced homozygosity suffered by the androgenotes due to suppression of the first mitotic division. To avoid this, activation of heterologous eggs by two haploid sperms, known to enhance heterozygosity was resorted[12]. Unexpectedly, dispermic induction of androgenesis also resulted in equally low hatching success (e.g. ref. 13), the causes for which are not yet known.

Being extremely hydrophilic, polyethylene glycol (PEG) has the property of fusing similarly charged membranes. Since the cell membrane of sperms is negatively charged, PEG facilitates fusion of adjoining biological membranes of cells like spermatozoa[14]. Hence PEG incubation facilitates dispermic induction of androgenesis. Indiscriminate fusion of two or more sperm cells, however, may alter the pattern of motility and/or reduce the effective facilitation of entry of double sperms. This article reports the effect of PEG incubation on the frequency of fused sperms of the ornamental fish, *Hemigrammus caudovittatus* and the

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Table 1. Phenotypic markers in *Hemigrammus caudovittatus* BT male and *Gymnocorymbus ternetzi* WT female chosen for induction of interspecific dispermic androgenesis. For comparison the markers for diploid and triploid hybrids are also included

Marker		*G. ternetzi* WT♀	*H. caudovittatus* BT ♂	Diploid hybrid	Interspecific androgenote	Triploid hybrid
	Strain	Black	Gold	–	Gold	–
Body	Shape	Fusiform	Slender	Fusiform	Slender	Fusiform
	Colour	Black	Gold	Black	Gold	Black
	Vertical stripes	Present	Absent	Absent	Absent	Absent
Colour of fins		Black	Golden yellow	Yellow-rimmed with black	Golden yellow	Golden yellow
Tail morphology		Deeply forked	Normal	Partially forked	Normal	Partially forked
Eye colour		Black	Red	Black	Red	Black

ability of fused sperms to generate dispermic androgenotes using heterologous eggs.

Materials and methods

Fish

To induce interspecific androgenesis, tetras with contrasting phenotypic markers were chosen (Table 1). The sex determination mechanism[10,11] operating in these fishes is XX/XY and generates male : female in the ratio of 1 : 1. Eggs of the golden strain of Buenos Aires tetra (BT), *H. caudovittatus*[15] activated by black widow tetra (WT), *Gymnocorymbus ternetzi*[16] failed to develop. Hence BT was chosen as the sperm donor and WT as the recipient. The design was to activate the genome-inactivated eggs of dominant black WT by previously PEG-incubated sperm of the recessive golden BT (Figure 1).

Preparation of gametes

Responding males and females of these tetras were allowed complete courtship, but just prior to spawning, the females were gently stripped and fresh eggs were collected. For each treatment, a ripe female was stripped and 100 randomly selected eggs were arranged in a thin plastic petri dish (1 mm thickness) as a monolayer in Ringer's solution and were irradiated using UV-G lamp (254 nm; 40 W) in a dark room[4]. UV-irradiation at the intensity of 4.2 W/m^2 and exposure duration of 3 min has been shown to inactivate the genome of WT eggs[11].

To facilitate dispermic entry, milt of BT was subjected to incubation in increasing concentrations of PEG for a constant duration of 7 min. By gently stripping the golden BT male, milt was collected in 2 ml of Ringer's solution. The solution was centrifuged at 594 *g* for 3 min and to the pellet, 0.5 ml PEG solution (4000 Sigma, USA) of the selected concentration was added and incubated at room temperature (26 ± 1°C). Then the milt was diluted to 1.5 ml with Ringer's solution. One ml of the diluted milt was used for activation and the remaining for microscopic observations.

In a final set of experiments, an attempt was made to increase the hatching success of the dispermic androgenotes by doubling or quadruplicating sperm concentration. For this, milt collected in 2 ml of Ringer's solution from a single male was considered as a unit. The unit was doubled and quadruplicated by collecting milt in 2 ml Ringer's solution by stripping two and four males respectively. However, the number of WT eggs to be activated was kept constant, i.e. 100 per experiment and the experiments were limited to the batch of eggs which were activated by sperm incubated at 2.5% PEG.

To estimate the frequency of live and motile sperms, a sample (20 µl) of fresh or PEG-incubated milt was released into a haemocytometer and to it 2 µl of 0.2% Tryphan blue was added to distinguish live sperms from dead ones. To estimate motility duration, a sample of 20 µl of fresh or PEG-incubated milt was kept on a clean glass slide, and a drop of water was added. Using a stop-clock (Shinco, Japan), both pattern and duration of motility were observed. Incidentally, the frequency of sperm completely fused from head to tail and incompletely fused was also estimated. The remaining clumped or immotile sperms were not taken into account as they could not fertilize the egg.

To facilitate dispermic activation, the irradiated eggs of black WT were mixed with 1 ml of PEG-incubated milt of golden BT in a petri dish. Subsequently, 3 ml of water was added into the petri dish. Gentle swirling of the dish for 45 s ensured activation of embryonic development. The developing embryos were observed for the mode of mitotic division and the presence or absence of melanocytes under stereozoom microscope (Nikon, Japan).

To collect gametes for each experiment, 10–15 individuals were used. Correspondingly, estimations reported in Tables 2 to 5 are based on (means ± SD) 10–15 replicates. For assessing reproductive performance, four pairs were used. Appropriate controls were kept for each experiment/estimation.

Confirmation of polyspermy

Karyotyping and erythrocyte measurements were used as an index of ploidy. For karyotyping, live hatchling/fry

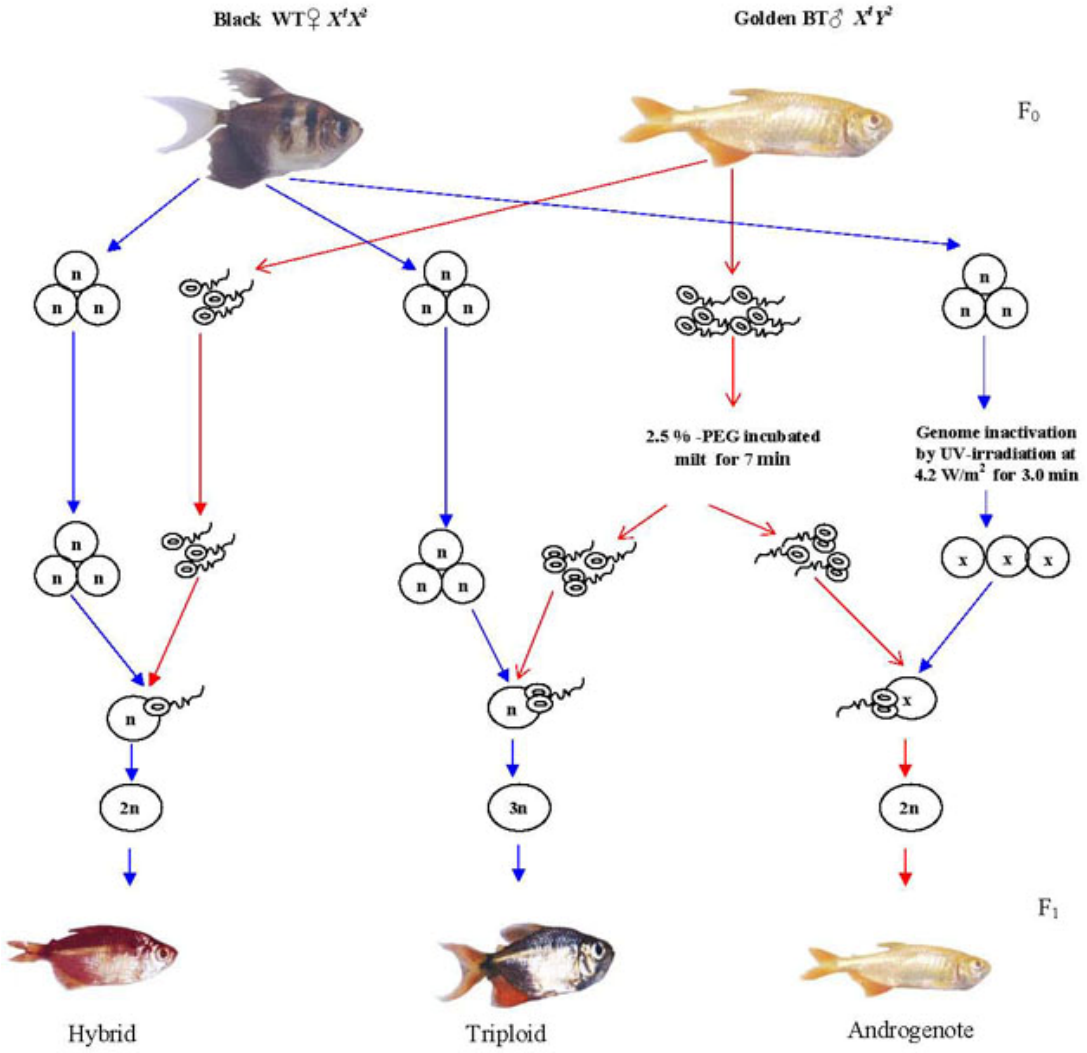

Figure 1. Induction of dispermic androgenesis using sperm of golden *Hemigrammus caudovittatus* (BT) incubated in 2.5% PEG and genome-inactivated eggs of black *Gymnocorymbus ternetzi* (WT). For comparison, procedures adopted for production of hybrid diploids and triploids are also shown. Note the phenotypic markers in the F_0 and F_1 progenies.

Table 2. Effect of 7 min of sperm incubation at different concentrations of polyethylene glycol (PEG) on sperm count, pattern of motility, motility duration and frequency of activation of black WT eggs by golden BT sperm

						PEG incubated BT sperm			
	Motile sperm count**						Fused double sperm		
PEG (%)	(%)	(no./ml)	Motility pattern	Motility duration (s)	Activation (%)	Single sperm (%)	Complete (%)	Incomplete (%)	Triple sperm (%)
0.0	100[a]	7,600,000	Zig-zag	95 ± 10[c]	100[e]	100[g]	–	–	–
1.0	96 ± 1.7[a]	7,296,000*	Zig-zag	90 ± 8[c]	96 ± 0.3[e]	95 ± 1.2[g]	–	1	–
1.5	96 ± 1.3[a]	7,296,000*	Zig-zag	86 ± 10[d]	94 ± 1.1[e]	93 ± 0.8[g]	–	2 ± 0.4	–
2.0	63 ± 0.7[b]	4,788,000*	Zig-zag	82 ± 7[d]	72 ± 3.2[f]	63 ± 1.4[h]	2 ± 0.4	16 ± 0.4	–
	18 ± 0.6[b]	1,368,000*	Circular						
2.5	47 ± 1.2[b]	3,572,000*	Zig-zag	72 ± 4[d]	61 ± 2.3[f]	37 ± 1.5[h]	14 ± 0.4	27 ± 1.3	0.7 ± 0.03
	31 ± 0.8[b]	2,356,000*	Circular						
3.0	64 ± 1.2[b]	4,864,000*	Irregular	56 ± 6[d]	49 ± 2.1[f]	6 ± 0.4[h]	3 ± 0.4	6 ± 0.4	49 ± 1.6

Values (mean ± SD) in each column followed by superscripts differ significantly from that of the control ($P < 0.05$).
*Calculated values; **Clumped immotile and dead sperm % not included.

Table 3. Dispermic activation of genome-inactivated eggs of WT, *G. ternetzi* by PEG-incubated sperm of BT, *H. caudovittatus*. The BT sperms were earlier incubated for 7 min in selected concentrations of PEG

PEG (%)	Irradiated eggs (no.)	Hatched** (%)	Activation*						Survival** at maturity	
			Karyotyped*		Monospermic		Dispermic			
			No.	%	No.	%	No.	%	No.	%
0.0	1016	10 ± 1.3	11	19	11	100	0	0	0	0
1.0	1026	17 ± 1.5	31	18	31	100	0	0	0	0
1.5	1028	14 ± 2.1	17	12	144	100	0	0	0	0
2.0	1034	12 ± 1.4	16	13	118	95	6[#]	5	0	0
2.5	1021	11 ± 1.3	22	19	91	81	21	19	13	1.3
3.0	1022	4 ± 1.1	9	22	41	100	0	0	0	0

*As % of hatched fry. **As % of irradiated eggs. [#]Hypodiploids.

Table 4. Reproductive performance of the dispermic androgenetic male (Y^2Y^2/X^2Y^2) and female (X^2X^2) BT*

Parameter	Normal male X^1Y^2	Androgenetic male Y^2Y^2/X^2Y^2
Sexual maturity (days)	120 ± 2.3^a	132 ± 3.4^b
Gonado somatic index	0.44 ± 0.02^c	0.52 ± 0.04^d
Sperm count (no./ml)	$7.6 \pm 0.3 \times 10^{6e}$	$6.6 \pm 0.8 \times 10^{6f}$
Fertilizability (%)	95 ± 1.4^g	94 ± 0.3^g
	Normal female X^1X^2	Androgenetic female X^2X^2
Sexual maturity	120 ± 3^h	145 ± 6^i
Inter-spawning period (days)	15 ± 2.4^j	28 ± 1.8^k
Gonado somatic index	0.56 ± 0.04^l	0.44 ± 0.02^m
Fecundity (no./spawn)	160 ± 3.6^n	135 ± 1.4^o
Hatchability (%)	98 ± 1.2^p	76 ± 2.3^q

*Values (mean ± SD) in each row followed by superscripts differ significantly from those of the control ($P < 0.05$).

were kept in 0.01% colchicine for 6 h and the typing was made following Kligerman and Bloom[17]. Ploidy level of each selected individual was determined from 25 ± 5 metaphase spreads. Just before immersing the selected fry in acetic acid for karyotyping, it was streaked on a glass slide for preparation of blood smear, which was then fixed and stained with Giemsa. The longer diameter of erythrocyte nucleus was measured.

Statistical analysis

All values are presented as means ± SD. Level of significance among the corresponding data was estimated by one-way ANOVA and further analysed by Dunnet's multiple comparison method. Statistical analysis was performed using Sigma STAT (ver. 2.0).

Results and discussion

Effect of PEG incubation

Sperm of golden BT was recognized with a head (1.86 ± 0.06 μm), a short cytoplasmic collar and a long thread-like motile tail (72 ± 0.03 μm) (Figure 2 *d*(1)). It became motile as soon as water was added. Incubation of milt with increasing PEG concentration significantly ($P < 0.05$) reduced the count of motile sperms and their motility duration (Table 2). PEG incubation also altered the mobility pattern of the sperms. Incubation up to 1.5% PEG sustained the usual 'zig-zag' motility pattern. Sperms displaying circular/irregular movement progressively increased from 18 to 64% with increasing PEG concentration from 2.0 to 3.0%. Consequently, 49% of WT eggs were alone activated by BT sperms incubated in 3% PEG.

Tendency for clumping of the PEG-incubated BT spermatozoa increased with increasing PEG concentration (Figure 2 *b*, *d*(5)). Due to attachment (Figure 2 *d*(2)), or possible fusion (Figure 2 *d*(3, 4)), the frequency of spermatozoa with almost two times larger head (3.43 ± 0.08 μm) was higher in samples that were PEG-incubated at concentrations > 1% (Figure 2 *a*). At 2.5% PEG, about 41% of the sperms were with larger heads, due to lateral attachment/fusion of the heads with two independent (Figure 2 *d*(2)) or fused (Figure 2 *d*(3)) tails; a few were seen with head-to-head attachment/fusion (Figure 2 *d*(4)). Frequency of sperm completely fused from head to tail displaying unidirectional motility decreased from 14 to

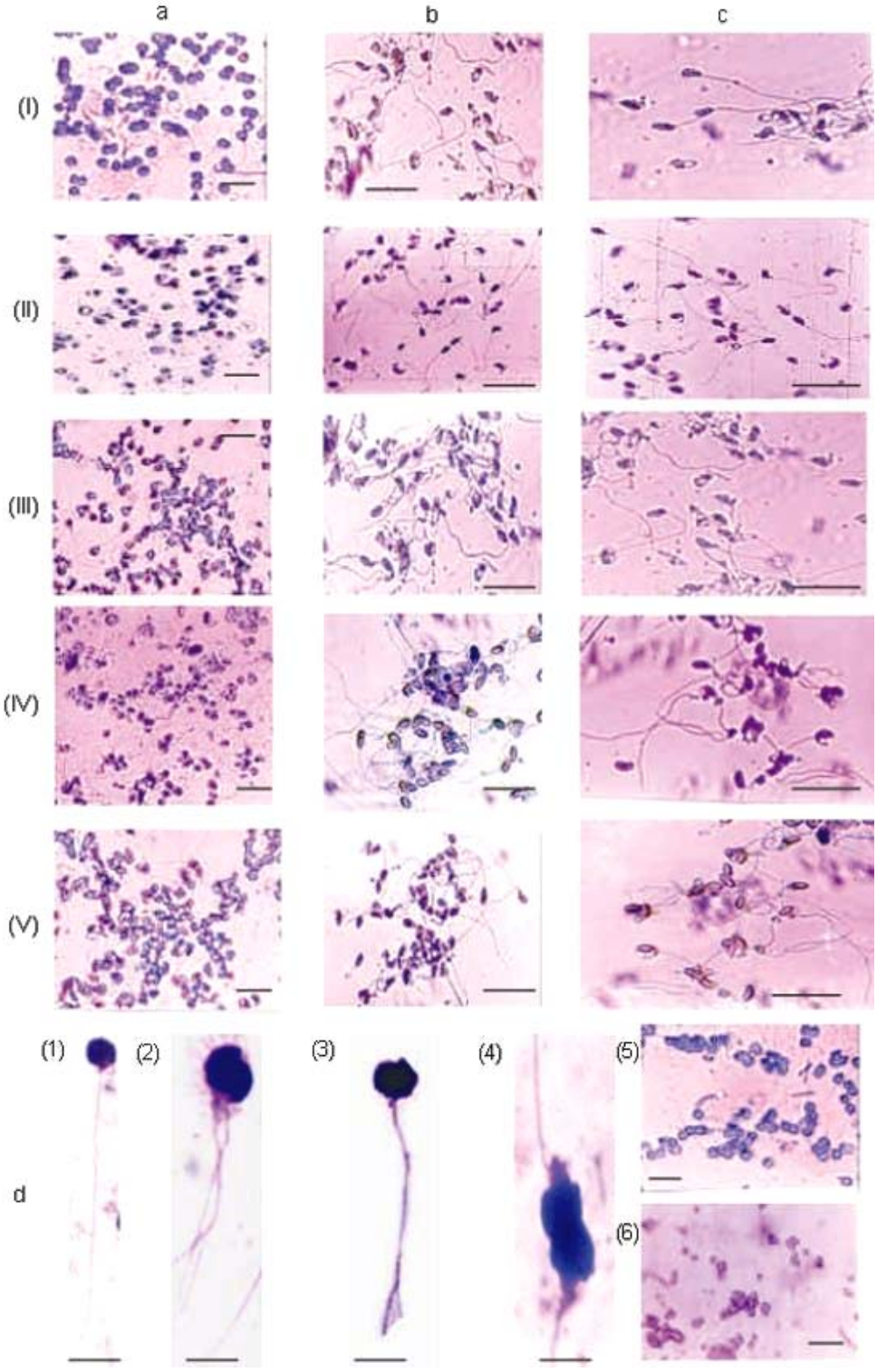

Figure 2. Effect of PEG incubation on clumping, attachment/fusion of BT sperm. Photomicrographs under columns a and b show the sperm stained with 0.02% – Tryphan Blue and Giemsa respectively. Column c represents magnification of photos shown in column b. Pictures in the rows indicated by letters I, II, III, IV and V show the sperm after 7-min incubation in PEG concentration of 0.0, 1.0, 1.5, 2.0 and 2.5 respectively. Row d shows representative magnifications of (1) a single sperm, (2) attached/fused sperm each with two tails, (3) head to tail fully attached/fused sperm, (4) head to head fused sperm, and (5) sperm incubated at 3.0% PEG, in which more than two sperm are clumped. (6) 2.5% PEG incubated milt with four times higher sperm concentration. Scale: 100 μm (a, d(5) and d(6)), 150 μm (b, c) and 20 μm (d(1) to d(4)).

Table 5. Progeny testing in dispermic androgenetic F_0 'golden' BT males (X^2Y^2 or Y^2Y^2) obtained from black WT eggs by dispermic-activation. Each of the six males was crossed with two randomly selected grey BT (X^1X^2) females. Each cross was repeated twice to enable the male to fertilize two successive broods

Identity number of ♂	Identity number of ♀ X^1X^2	Fry (no.)	Sex distribution (no.) ♀	Sex distribution (no.) ♂	Sex ratio ♀ : ♂	Inferred genotype of ♂
Control X^1Y^2	4	141	71	70	0.5 : 0.5	X^1Y^2
	4	147	74	73	0.5 : 0.5	
	9	138	68	70	0.5 : 0.5	
	9	144	71	73	0.5 : 0.5	
1	3	111	0	111	0.0 : 1.0	Y^2Y^2
	3	113	0	113	0.0 : 1.0	
	6	108	0	108	0.0 : 1.0	
	6	116	0	116	0.0 : 1.0	
2	1	102	0	102	0.0 : 1.0	Y^2Y^2
	1	112	0	112	0.0 : 1.0	
	5	112	2	110	0.02 : 0.98	
	5	102	3	99	0.03 : 0.97	
3	4	112	0	112	0.0 : 1.0	Y^2Y^2
	4	114	0	114	0.0 : 1.0	
	11	104	3	101	0.04 : 0.96	
	11	107	4	103	0.03 : 0.97	
4	1	116	59	57	0.5 : 0.5	X^2Y^2
	1	106	53	53	0.5 : 0.5	
	9	110	54	56	0.5 : 0.5	
	9	118	58	60	0.5 : 0.5	
5	4	114	0	114	0.0 : 1.0	Y^2Y^2
	4	112	0	112	0.0 : 1.0	
	9	108	0	108	0.0 : 1.0	
	9	114	0	114	0.0 : 1.0	
6	8	116	58	58	0.5 : 0.5	X^2Y^2
	8	112	54	58	0.5 : 0.5	
	2	96	48	48	0.5 : 0.5	
	2	104	51	53	0.5 : 0.5	

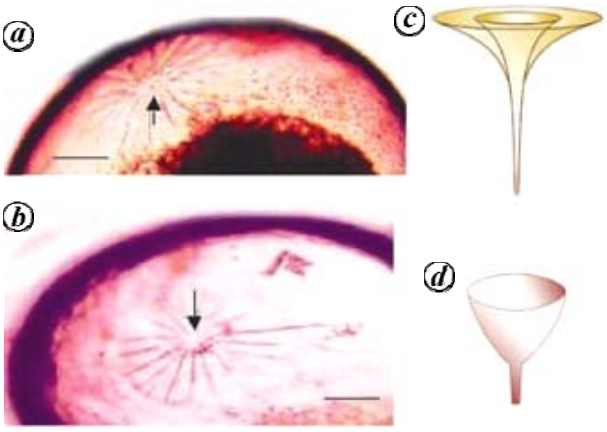

Figure 3. Photograph showing the all view of micropyle in the eggs of (***a***) normal gray BT and (***b***) normal black WT. Note the dot in the centre of the micropyle in eggs of BT. Schematic representation of the micropyle of BT (***c***) and WT (***d***). Scale represents 50 µm.

3%, when PEG concentration was increased from 2.5 to 3.0% (Table 2). The long tail, which was clearly visible in the normal sperm, became less apparent with increasing PEG concentration; at 3% PEG the tails of most sperms were detached either totally or partially (Figure 2 *d*(5)). The clumped or immotile sperms were not taken into account, as they could not fertilize the egg.

Dispermic activation

The micropyle, through which the sperm enters, is situated on the animal pole of the egg; in BT eggs, it is a saucer-like pit, into the centre of which the proximal end of the long and narrow micropylar tube projects (Figure 3). But in WT eggs, it is a broad and deep pit leading into a broad, short tube, which opens on the inner surface of the chorion[18]. This may be a reason for the inability of BT eggs to serve as recipients of WT sperms. The micropyle of WT eggs is large enough to accommodate successive/simultaneous entry of two or three fused PEG-incubated sperms of BT (Figure 3 *b*).

Maternal genome inactivation

Maternal nuclear genome of the eggs of black WT can be inactivated[10] by exposing them to UV-irradiation at the

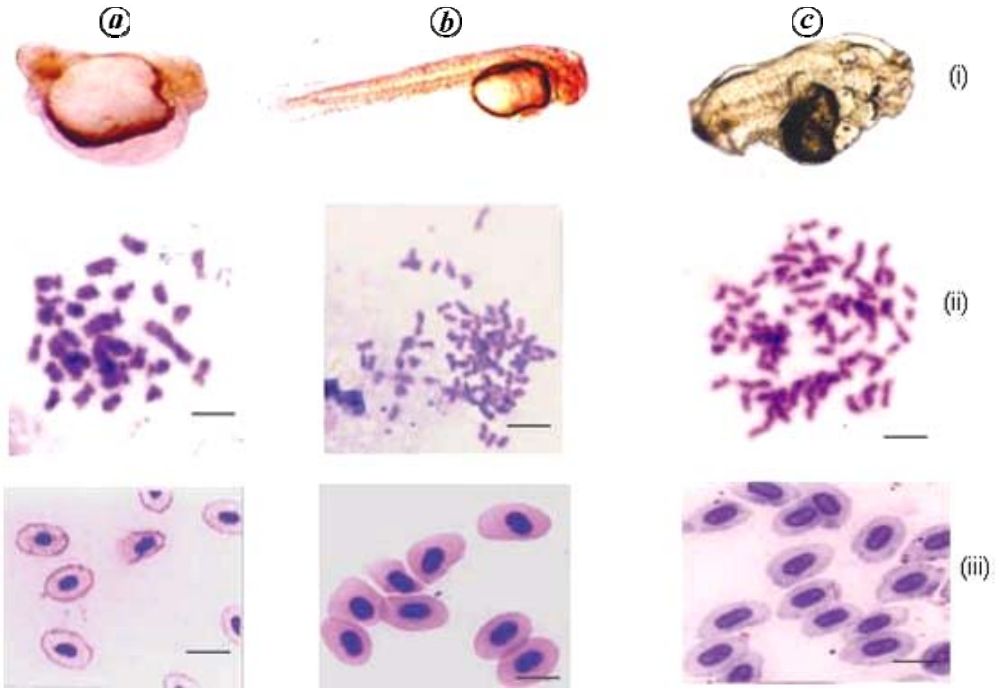

Figure 4. Photographs showing representative (***a***) hatched haploid BT embryo, its karyotype and erythrocytes, (***b***) normal diploid BT fry, its karyotype and erythrocytes and (***c***) abnormal hypo-triploid BT fry, its karyotype and erythrocytes. Note the increase in nuclear and cell size of erythrocytes with increasing ploidy level. Scales in second and third row represents 5 μm respectively.

intensity of 4.2 W/m^2 for a period of 3 min. Cortical reaction indicated successful activation of genome-inactivated eggs of black WT by the entry of BT sperms. Successful inactivation of maternal genome was confirmed by the generation of golden haploids and karyotyping.

Confirmation of dispermic androgenesis

Of the 112 fry produced using BT sperms that were incubated in 2.5% PEG, 91 and 21 progenies were generated by monospermic and dispermic activation respectively (Table 3). This is evidenced by (i) phenotypic markers (Figure 1), (ii) karyotyping and erythrocyte measurements (Figure 4) (iii) sex ratio (see below) and (iv) progeny testing (Table 5, Figure 5). All the diploid hatchlings showed the same phenotypic markers in terms of colour and shape, and could easily be distinguished from the diploid and triploid hybrids (Figure 1, Table 1). They were active and healthy even after absorption of yolk. None of the karyotypes of the hatchlings generated by 2.5% PEG-incubated sperms showed any sign of aneuploidy (Figure 4). Erythrocyte nucleus of the haploid, diploid and triploid androgenotes measured 2.4 ± 0.08 μm, 4.3 ± 0.06 μm and 6.2 ± 0.04 μm respectively (Figure 4). Thus, phenotypic markers as well as karyotyping and erythrocyte measurements confirmed dispermic activation and generation of diploid androgenetic golden BT.

Sex ratio

In both BT and WT, males are heterogametic (see also ref. 19). Hence the sex ratio of intraspecific and interspecific androgenetic golden BT[9,10] is also given as 0.5 X^2X^2♀ : 0.5 Y^2Y^2♂ ('2' indicates paternal origin). Of the 13 dispermic androgenotes which survived until maturity (Table 3), four were females and nine were males, i.e. the observed sex ratio in the present study was 0.307 X^2X^2♀ : 0.693 X^2Y^2/Y^2Y^2♂. Since the sperms bearing X^2 and Y^2 genotypes have equal opportunity of entering the black WT eggs, the expected combinations of genotype were $X^2X^2 : X^2Y^2 : Y^2Y^2$ and the sex ratio was 0.33 X^1X^2♀ : 0.67 X^2Y^2/Y^2Y^2 ♂ (the '1' indicates maternal origin; see also Figure 5).

Reproduction

Of the 19% dispermic androgenotes hatched, 1.3% alone underwent normal post-hatching development and attained sexual maturity (Table 3). Reproductive performance of the dispermic androgenetic males (X^2Y^2 and Y^2Y^2) was significantly ($P < 0.05$) inferior to that of normal males (X^2Y^2), in terms of sperm count, and puberty was also delayed by 12 days (Table 4). But the performance of dispermic androgenetic females (X^2X^2) was also not equal to that of normal females (X^1X^2); for instance, puberty was

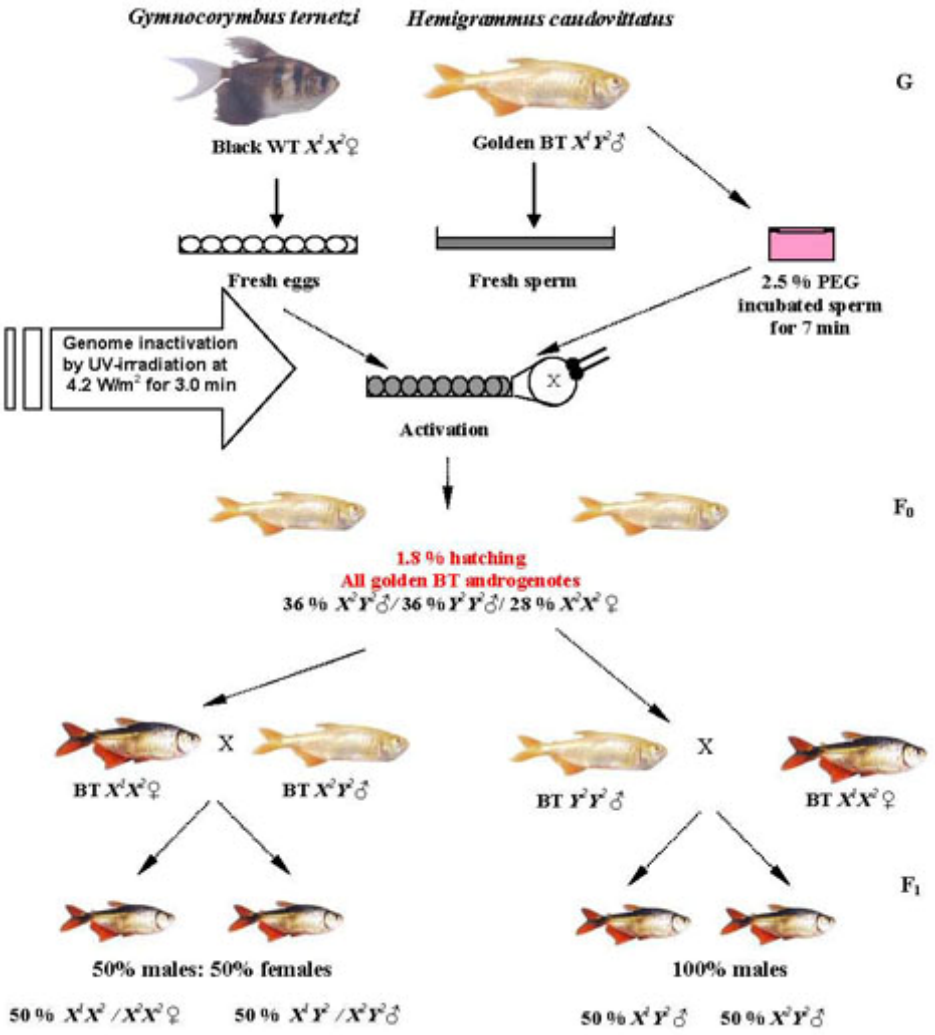

Figure 5. Progeny testing of inter-specific androgenotes by crossing Y^2Y^2 and X^2Y^2 golden BT sires with normal gray BT dams X^1X^2 through F_0 to F_1 and F_2 generations.

delayed by 25 days and inter-spawning period was prolonged to 28 from 15 days. Fecundity and hatchability of F_1 progenies of these female androgenotes were also significantly ($P < 0.05$) reduced.

Progeny testing

Though time-consuming, the testing provides a decisive evidence for the entry of two haploid BT sperms into the genome-inactivated WT eggs. In BT, X^1X^2/X^1Y^2 sex determination mechanism is known to operate[5,10,]. Hence a cross between X^2Y^2 male and normal female (X^1X^2) is expected to generate 50%♂ : 50%♀, but that between Y^2Y^2 male and normal female (X^1X^2) will produce 100% male progenies. Hence, progeny testing of F_0 dispermic androgenotes was complicated by the fact that while a third of them had the X^2X^2 genotype, the others were either X^2Y^2 or Y^2Y^2 (Table 5). From nine dispermic androgenetic males, six were randomly chosen for progeny testing. Of these, two crosses produced progenies with the sex ratio of 50%♂ : 50%♀ (Table 5). Apparently, these males were X^2Y^2 (Figure 5). Of the other four crosses, 96–100% males were generated indicating that the males used in these crosses were evidently Y^2Y^2. Hence progeny testing confirmed the production of truly dispermic androgenetic BT.

Multiple paternal genome

In the control WT and BT, over 93% of the fertilized eggs hatched successfully. About 78% diploid hybrid fry, which had equal genomic contributions from BT (♂) and

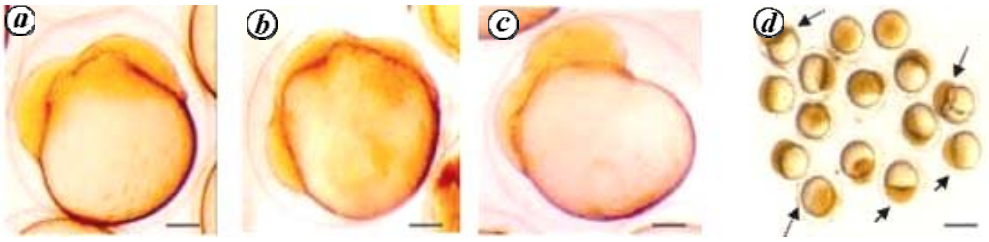

Figure 6. Photographs showing normal first mitotic division in the genome-inactivated oocytes of WT, which were evidently activated by successive/simultaneous entry of (***a***) two BT sperm; (***b***) three sperm, and (***c***) abnormal first mitotic division of an WT egg activated by two BT sperm. (***d***) 3 h old developing eggs of WT activated by 2.5% PEG incubated BT sperm. →, indicate normal development; ⇢, indicate oozing out of cytoplasmic content due to defective development. Scale represents 20 μm (***a***, ***b*** and ***c***) and 100 μm (***d***).

WT (♀), hatched out. However, the hatching success of either intra- or interspecific androgenotes[10,11] was reduced to 10–11% and that of dispermic androgenotes to 1.8%.

The causes for such low hatching success of dispermic androgenotes are as follows:

(1) Only 78% of the 2.5% PEG-incubated BT sperms was motile. Among them, 31% displayed not the usual zig-zag, but circular movement (Table 2). Hence, they were motile, but not as mobile as those displaying zig-zag motility. Consequently, the remaining 47% motile (zig-zag) BT sperms were able to activate 61% of irradiated WT eggs, i.e. 39% of the eggs remained inactivated and succumbed subsequently.

(2) Of the 61% WT eggs thus activated; (a) 37% suffered haploid syndrome due to activation by single sperm; most of these haploids succumbed prior to hatching and the others after hatching (Table 2); (b) 27% succumbed due to activation by incompletely fused sperm; (c) 0.7% suffered activation by triple sperm, as evidenced by the simultaneous appearance of two furrows dividing the cytoplasm into three blastomeres (Figure 6 *b*) owing to the entry of three BT sperms. It resulted in monster-like triploids (Figure 4 *c*(i)) with corresponding triploid karyotype (Figure 4 *c*(ii)) and nearly three times larger red blood cells (Figure 4 *c*(iii)). In many cases, however, it led to delayed and distorted mitotic divisions until the 16 blastomere stage, and subsequent arrest of development, and finally oozing out of cytoplasm due to bursting (Figure 6 *d*). Very few others successfully hatched as triploids, but died after hatching. Further, there was another 11% mortality of developing dispermic androgenotes during embryonic development, perhaps owing to genetic incompatability.

(3) On the whole, more than 88% of the WT eggs, which either remained inactivated or activated by single/incompletely fused double sperms/triple BT sperms, died before hatching. Hence, 1.8% of the irradiated WT eggs alone hatched out as diploid (dispermic) androgenotes (Table 6). Thus, low hatching success of dispermic BT androgenotes is due to problems associated with PEG incubation rather than enhanced heterozygosity.

An attempt was also made to check whether the hatching success of the dispermic androgenotes could be increased by doubling and quadruplicating BT sperm concentration. The frequency of motile sperm displaying zig-zag motility significantly ($P < 0.05$) increased from 47 to 63%, when the sperm concentration was quadruplicated (Table 6). Correspondingly, the frequency of completely fused double sperms also increased significantly ($P < 0.05$) from 14 to 19%. Consequently, hatching success also increased significantly ($P < 0.05$) to 4.3%.

The idea of artificially inducing polyspermy in fish was first tested by Ueda *et al.*[20], who induced paternal triploid, *Oncorhynchus mykiss* by facilitating fertilization by double sperms, which were previously PEG-incubated. PEG has the property of fusing similarly charged membranes due to its hydrophilic nature; since the sperm membrane is negatively charged[14], PEG incubation facilitates fusion of adjoining membranes of spermatozoa. To induce dispermic activation and generate intraspecific androgenetic *O. mykiss*, Araki *et al.*[9] tested 2-min incubation in 50% PEG and 5-min incubation at 85 mM $CaCl_2$ and found the latter more effective. Finding 50% PEG incubation too strong, Kirankumar and Pandian[13] produced interspecific androgenetic *Puntius conchonius* facilitating activation of genome-inactivated eggs of *P. tetrazona* by two sperms which were previously incubated for 10 min in 2.5% PEG. For BT, 7-min incubation in 2.5% PEG was found adequate. While our observations confirm those of Kirankumar and Pandian[13], the study has provided photographic evidence for attachment/fusion of the sperm heads and tails. Besides, the evidence for successful generation of dispermic BT androgenotes hails from phenotypic markers and karyotyping. Thirdly, progeny testing and the observed sex ratio of F_0 androgenotes is another evidence.

The first evidence showed that the fused/attached sperm successfully activated heterologous eggs. With the absence of acrosome in fish sperm[1], entry of the sperm is made possible through the micropyle[2] during activation/fertilization. Hence fertilization/activation may not be a species-specific event in fishes. Heterospecific in-

Table 6. Effect of increasing milt quantity on motile sperm count and frequency of activation of WT eggs by single, double or triple PEG-incubated BT sperm

Parameter		2.5% PEG-incubated sperm		
		1 unit*	2 units*	4 units*
Motile sperm count (%)	Zig-zag	47 ± 1.2^{a}	54 ± 1.2^{b}	63 ± 1.2^{c}
	Circular	31 ± 0.8^{d}	29 ± 1.2^{d}	26 ± 1.2^{e}
Single sperm (%)		37 ± 1.5^{f}	39 ± 1.8^{f}	44 ± 1.3^{g}
Double sperm (%)	Complete	14 ± 0.4^{h}	15 ± 0.8^{h}	19 ± 0.7^{i}
	Incomplete	27 ± 1.0^{j}	27 ± 1.7^{j}	24 ± 1.3^{k}
Triple sperm (%)		0.7 ± 0.03^{l}	0.9 ± 0.07^{l}	1.6 ± 0.04^{m}
Motility duration (s)		72 ± 10^{n}	72 ± 7^{n}	66 ± 3^{o}
Activation (%)		61 ± 2.3^{p}	64 ± 1.4^{p}	73 ± 1.7^{q}
Hatching (%)		1.8 ± 0.4^{r}	2.1 ± 0.6^{r}	4.3 ± 0.7^{s}

Values (mean ± SD) in each row followed by superscripts differ significantly ($P < 0.05$) from those of respective control (1 unit). *Clumped immotile and dead sperm % not shown.

semination[21] and the consequent interspecific and intergeneric hybridization[22] are known to generate fertile F_1 hybrids in about 300 fish species. A cross between WT female and BT male producing hybrids clearly indicated genomic compatibility between the two species. Hence compatibility between micropyle of egg and sperm head is one of the critical factors in the facilitation of heterospecific insemination and hybridization. Observations of sperm head and micropyle showed that BT sperm could easily enter the WT eggs. However, the diameter of the distal end of the micropylar tube in BT eggs was too narrow to accommodate the WT sperm; conversely, the distal end of the micropylar tube of WT eggs was broad enough to allow successive and/or simultaneous entry of one, two or rarely three individual or fused BT sperms[23]. Photographic evidence for such activation is obtained from the first mitotic division of WT eggs. Evidences for such dispermic and polyspermic activation are reported in several earlier studies; for instance, the eggs of fishes activated by three sperms contained three pronuclei and centromeres[24,25] or three fertilization cones[3]. The present study has also provided more evidences like karyotype and erythrocyte size for dispermic and trispermic activation by PEG-incubated BT sperm.

The causes for high frequency of embryonic mortality in the dispermic androgenotes of BT, despite the enhanced heterozygosity, have been traced to the presence of 47% motile/mobile sperms alone displaying normal zig-zag motility pattern and the consequent reduction of activated eggs to 61%, and to the production of 37% golden haploids. Araki *et al.*[9] too found that ~20% of the rainbow trout sperms, when incubated for 2 min at 50% PEG, were not motile. They recorded that the fused trout sperm fertilized about 47% eggs. Briefly, PEG incubation not only facilitated the desired fusion of sperm, but also significantly reduced ($P < 0.05$) the count of mobile sperms, which resulted in a large number of eggs (>39%) remaining inactivated/unfertilized. It was shown that the number of WT eggs remaining inactivated, significantly decreased ($P < 0.05$) on increasing BT sperm concentration.

A second major cause is the production of 37% haploids and <1% triploids. Hence, dispermic induction of androgenesis, despite introducing heterozygosity, will not improve hatching success of androgenotes. An alternative to induce androgenesis without involving diploidization is the introduction of diploid sperms. Thorgaard *et al.*[26] produced androgens using haploid or diploid sperms of *O. mykiss* and found that the hatching success of androgenotes was significantly higher for the dispermic androgenotes than that monospermic androgenotes.

A cause for the high frequency (11%) of embryonic mortality in the dispermic BT androgenotes is the absence of maternal genome. The presence of a single maternal genome in the paternal triploid led to 7% hatching[27], but its absence resulted in the poorest hatching success of 1.8% (present study). Whereas the two genomes drawn from the pronuclei of the second polar body and the egg proper in meiotic gynogenesis are compatible and result in more than 70% hatchlings[4], the causes for incompatibility between the two haploid BT sperms in the presence or absence of a single maternal WT genome reducing activation to 61% have to be traced. Many fishes are known not to tolerate Y^2Y^2 genome. For instance, George *et al.*[28] recorded the inviability of the Y^2Y^2 zygote of *Betta splendens.* Hence it is equally not clear why the multiple paternal genomes, especially the Y^2Y^2 genotype, result in low frequency of successful activation and hatching. Likewise, it is also not clear why reproductive performance of androgenetic male carrying Y^2Y^2 genotype is superior, while that of its female counterpart (X^2X^2) is inferior.

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