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Reproductive performance and progeny testing of androgenetic clones of widow tetra, *Gymnocorymbus ternetzi* Boulenger (Characidae) generated using cryopreserved sperm

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Abstract
Production of androgenetic clones using preserved sperm is advantageous in resurrecting a lost population and germplasm conservation. Generation of androgenetic clones to produce 100% males in economically important species is a desirable practice in aquaculture. However, reasons for failure of the clones to generate all male population remains unknown. We investigated reproductive performances of androgenetic widow tetra, *Gymnocorymbus ternetzi* generated using cryopreserved albino widow tetra (WT) sperm and their ability to generate all male population were assessed. Survival of albino androgenotes at hatching and sexual maturity was 11% and 8%, respectively. Gonado-somatic-index (GSI) and fecundity of F_0 androgenetic females (X^2X^2) was significantly ($P<0.05$) lower and exhibited delayed sexual maturity compared to controls (X^1X^2) contrastingly, androgenetic males (Y^2Y^2) had higher sperm-count and performed superior to controls (X^1Y^2). Progeny testing crosses between F_0 androgenetic males (Y^2Y^2) and heterozygous control females (X^1X^2) generated 96-99% males and 2-4% unexpected females confirming the role of paternal autosomes upon sex determination. Reasons for delayed maturity, reduced reproductive performance and generation of unexpected females by progeny testing are discussed.

Keywords: androgenotes, paternal autosomes, sex-ratio, progeny testing, Gonado-somatic index, cryopreserved sperm

1. Introduction
Androgenesis is a genetic manipulation technique, wherein the clone receives genetically identical 'diploid' copies of exclusively paternal genome by thermal shocking of haploid embryos for arresting the first mitotic division [10]. Maternal genome-inactivation by UV-irradiation provides egg cytoplasm alone for development of homologous or heterologous fresh or preserved sperm [24, 10, 12], a desirable practice in aquaculture to generate economically important and commercially valuable strains. Several scientists across the world had already established multiple family lines of androgenetic clones in different fish species using fresh [8, 35, 19, 23, 13], cadaveric [27, 12], cryopreserved [33, 4, 10], dispermic [15] or post-mortem preserved sperm [23, 9, 12]. After Scheerer *et al.* (1991) [33] induced androgenesis in rainbow trout, *Oncorhynchus mykiss* using cryopreserved sperm, many others used cryopreserved and post-mortem preserved sperm [31, 34] in Carps [5], Trouts [3, 4], Loaches [2, 37] and Tetras [10]. Very few authors like Bercsenyi *et al.* (1998) [5], Arai et al (2001) [2] and David and Pandian (2006a, b) [12, 13] have studied reproductive performance and recorded reduced gonadal development and hatchability of the F_1 androgenetic clones generated using preserved sperm. Hence, studies on whether homozygous doubling of paternal genome affects gonadal development, sexual maturity and reproductive performance of androgenetic clones and unexpected female progenies (X^2Y^2) production were required [18, 24, 26, 12, 13]. In fishes, sex-chromosomes are undifferentiated and cytologically indistinguishable [36, 3] therefore progeny testing, a time-consuming procedure for confirming paternity is required [26, 12]. Progeny testing of adult males or females require ~3 years in *O. mykiss* [33], 3 to 8 months in *O. niloticus* [35] and ~4 months in barbs [24-27] and tetras [10-14]. Kirankumar and Pandian (2004b) [26, 27] crossed androgenetic rosy barb, *Puntius conchonicus* males (Y^2Y^2) with control females (X^1X^2) to confirm paternity and recorded 3-15% unexpected female progenies by progeny testing.

In Buenos Aires tetra (BT) *Hemigrammus caudovittatus*, (Ahl, 1923) [1], crosses between control males (X^1Y^2) and females (X^1X^2) produced equal (males 0.5:0.5 females) sex ratio [12, 13], but androgenetic clones (Y^2Y^2) when crossed with control females (X^1X^2) generated unexpected heterozygous females (X^1Y^2) by unknown mechanism [31-35]. Our understanding of the mechanism by which genetically heterozygous females are produced in low frequency during progeny testing remains obscure. In the present study, our objectives were to (i) estimate survival and sexual maturity of androgenotes generated using cryopreserved sperm, (ii) assess reproductive performance of androgenetic clones and (iii) conduct progeny testing to confirm paternity by crossing homozygous androgenetic males (Y^2Y^2) with heterozygous (X^1X^2) control females and record the sex ratio and unexpected females.

2. Material and Methods

2.1. Androgenesis

Intra-specific androgenotes of ornamental Widow Tetra (WT), *Gymnocorymbus ternetzi* (Boulenger 1895) [7] were generated. Briefly, genome-inactivated eggs of dominant black WT were activated by cryopreserved sperm of recessive albino WT followed by diploidization of embryos by thermal shocking [10]. Reproductive parameters such as gonado-somatic index, fecundity, sperm count and hatchability were estimated. Progeny testing of androgenetic clones was conducted to confirm paternity and generate 100% males by crossing with control females, following established protocols [24, 12, 15].

2.2. Reproductive performances

Androgenotes, control progenies and unexpected female progenies were raised in cement tanks at 28 ± 1 °C, until sexual maturity (8%). After maturity, fish were brought indoor, acclimatized at 14:10 hr Light: Dark conditions. Androgenetic males (Y^2Y^2) and females (X^2X^2) were reared separately after sexual maturity for assessing gonadal development and breeding performance [9, 15, 34]. Gonado-somatic index (GSI) was calculated by expressing gonad mass as percentage of body mass [12, 39].

2.3. Sperm Quality Parameters

Control or androgenetic males were stripped to collect milt in 2 ml of Ringer's solution for sperm analysis. Estimation of sperm count, motility duration and pattern for fresh and cryopreserved samples were carried out using a haemocytometer (New Bauer, Germany) [12]. Approximately, total number of sperms immediately active upon addition of water were counted and calculated as percentage of motile sperm while their motility duration was estimated using a stopwatch (Titan, India). Sperm quantity was estimated by counting sperm inside the large squares of the chamber and calculated based on the dilution factor to assess total number of sperms/ml [27, 12, 23]. Motility was estimated by diluting milt in 100 µl of Ringer's solution in a concave slide followed by addition of 300µl of tap water for activation and observed under a phase contrast microscope (Nikon, Japan) in a slide for motility pattern ranging from straight, zig-zag or circular, following standard protocol [12, 23].

2.4. Egg quality parameters

Tetra oocytes are translucent and sticky. Good quality oocytes were identified by shape, optical clarity, and presence of ovarian fluid. Briefly, for evaluating egg quality and quantity, the females were stripped in a petri dish containing Ringers buffer, washed with Ringers to remove ovarian fluid and counted under a microscope (Nikon, Japan) [23, 9, 12, 13].

Table 1: Reproductive performance of the normal male (X^1Y^2), androgenetic female (X^2X^2) and males (Y^2Y^2) generated using fresh* and cryopreserved ** sperm, respectively; the androgenetic males were crossed with either normal females (X^1X^2) or androgenetic females (X^2X^2) of widow tetra.

Parameters	Control males (X^1Y^2)	Androgenotes F_0 (Y^2Y^2) fresh*	Androgenotes F_0 (Y^2Y^2) cryopreserved**
Sexual maturity (day)	110 ± 3.2^a	125 ± 4.4^b	128 ± 2.3^b
GSI	0.41 ± 0.03^c	0.49 ± 0.06^d	0.47 ± 0.03^d
Sperm count (no/ml)	$7.4 \pm 0.3 \times 10^{5e}$	$7.1 \pm 0.4 \times 10^{6f}$	$6.8 \pm 0.8 \times 10^{6f}$
Fertilizability (%)	92 ± 1.2^g	90 ± 2.3^h	88 ± 3.2^h
	Control females (X^1X^2)	**F_0 (X^2X^2) fresh***	**F_0 (X^2X^2) cryopreserved****
Sexual maturity (day)	120 ± 3.6^l	152 ± 2.9^j	152 ± 1.2^j
Inter-spawning period (day)	18 ± 2.1^k	28 ± 1.9^l	29 ± 2.4^l
GSI	0.48 ± 0.06^m	0.32 ± 0.04^n	0.27 ± 0.09^n
Fecundity (egg no/spawn)	110 ± 3.7^o	90 ± 3.9^p	89 ± 2.1^p
Hatchability (%)	94 ± 1.6^q	78 ± 2.6^r	77 ± 3.4^r

Each value is the mean (±SD) of a minimum of 10 estimates using 10 sires/dams. Values in each row followed by different superscripts differ significantly from that of control (P<0.05). GSI—gonado-somatic index.

Table 2: Progeny testing of F_0 androgenetic albino WT males. A randomly selected androgenetic male from each of the 7 family lines (FL) was crossed with 3 randomly selected normal black dams

♂Parent / FL (Y^2Y^2)	♀ Parent (X^1X^2)	Fry (no)	Sex distribution (no)		Sex ratio
			♀	♂	♀ : ♂
Control (X^1Y^2)	3	102	52	50	0.5 : 0.5
	3	104	53	52	0.5 : 0.5
	7	106	54	52	0.5 : 0.5
	7	104	53	51	0.5 : 0.5
G1	6	98	0	98	0.0 : 1.0
	6	94	0	94	0.0 : 1.0
	2	102	4	98	0.04 : 0.96
	2	98	2	96	0.02 : 0.98
	5	105	0	105	0.0 : 1.0
	5	103	0	103	0.0 : 1.0
G2	1	102	0	102	0.0 : 1.0
	1	97	0	97	0.0 : 1.0
	3	102	0	102	0.0 : 1.0
	3	106	0	106	0.0 : 1.0
	9	96	0	96	0.0 : 1.0
	9	104	0	104	0.0 : 1.0
G3	4	106	0	106	0.0 : 1.0
	4	104	0	104	0.0 : 1.0
	7	98	0	98	0.0 : 1.0
	7	101	0	101	0.0 : 1.0
	6	94	3	91	0.03 : 0.97
	6	102	3	99	0.03 : 0.97
G4	5	98	0	98	0.0 : 1.0
	5	101	0	101	0.0 : 1.0
	6	102	0	102	0.0 : 1.0
	6	96	0	96	0.0 : 1.0
	5	103	2	101	0.02 : 0.98
	5	94	3	91	0.03 : 0.97
G5	3	106	0	106	0.0 : 1.0
	3	104	0	104	0.0 : 1.0
	1	92	0	92	0.0 : 1.0
	1	98	0	98	0.0 : 1.0
	9	103	2	101	0.02 : 0.98
	9	102	4	98	0.04 : 0.96
G6	2	101	0	101	0.0 : 1.0
	2	98	0	98	0.0 : 1.0
	7	102	0	102	0.0 : 1.0
	7	104	0	104	0.0 : 1.0
	6	103	0	103	0.0 : 1.0
	6	96	0	96	0.0 : 1.0
G7	4	98	2	96	0.02 : 0.98
	4	94	3	91	0.03 : 0.97
	1	96	0	96	0.0 : 1.0
	1	102	0	102	0.0 : 1.0
	5	104	0	104	0.0 : 1.0
	5	94	0	94	0.0 : 1.0

* Each cross was repeated twice to provide the male to fertilize 2 successive broods

2.5. Progeny testing

Progeny testing of androgenetic males were conducted to confirm genotype and generate 100% males by crossing homozygous male androgenotes (Y^2Y^2) with control (X^1X^2) [35, 26, 13, 39]. Androgenotes generated using cryopreserved sperm were used for the experiments. For progeny testing, 7 sexually mature F_0 androgenetic males (Y^2Y^2) were randomly selected from 17 androgenetic family lines, crossed with 3 randomly selected control dams (X^1X^2), with each male crossed twice with same female to make sure that occurrence of unexpected females is not because of selected females but of the androgenetic sires.

2.6. Statistical analysis

Values were expressed as Mean ± SD and for estimation of level of significance (P<0.05) among the corresponding data one-way ANOVA and Tukey's multiple range test was used following Zar (1984) [38]. Estimation of the level of significance of deviations from the theoretical 1:1 sex ratio was analyzed using Chi- square test with Yates correction.

3. Results

3.1. Survival, maturity and reproductive performance

Shocking of 22 min (after fertilization) old eggs fertilized with cryopreserved sperm at 41°C for 2 min resulted in diploidization (2n=48) as confirmed by karyotyping [20, 3]. Survival of androgenotes at hatching (11%) and sexual maturity (8%) was significantly (P<0.05) lower than the controls (78%) [10, 12]. Of the established 17 family lines of androgenotes, only 7 family lines were randomly selected for progeny testing. The F_0 androgenetic females generated using cryopreserved sperm attained sexual maturity after considerable delay. Besides delayed puberty (152 ± 2.9) and prolonged inter-spawning period (28 ± 1.9), total fecundity and hatchability were significantly (P<0.05) reduced compared to control females (Table 1). Reproductive performance of the F_0 androgenetic males (Y^2Y^2) was significantly (P<0.05) superior to controls (X^1Y^2), as evidenced by a higher GSI and sperm count while androgenetic females (X^2X^2) were inferior to control females (X^1X^2). Histological analyses of androgenetic males confirmed dense accumulation of spermatids supporting earlier evidences that F_0 androgenetic clones had increased fertilizability and hatching rate [24, 26, 35, 34, 12, 13] while females exhibited immature ovaries with lots of atretic oocytes still in developing phase.

3.2. Progeny testing

Preliminary experiments confirmed widow tetra (WT) males are heterogametic (X^1Y^2) and females homogametic ($X^1X^{2)}$ and *XX/XY* is the sex-determination system [13]. In control experiments, males comprised of ~ 49-51% of the total (100%) progenies (Table 2). In the present study, female progenies with male genotype (X^1Y^2/X^2Y^2)) were generated in low frequencies (4%) when F_0 androgenetic males (Y^2Y^2) and normal females (X^1X^2) were crossed and frequency of unexpected female progenies sired by *G. ternetzi* androgenetic males (X^1X^2♀x Y^2Y^2♂) increased in simultaneous crosses (Table 2). Of the 21 crosses made, only 6 (~29%) resulted in the production of ~2–4% females in F_1 progenies (Table 2) indicating the genetic role of paternal autosomes in homozygous conditions upon sex determination in tetras.

4. Discussion

Sexual maturity can serve as an index for determination of proper development of gonads in androgenotes. However, in androgenetic WT females, maturity was considerably delayed and fecundity was significantly lowered. Histological analysis of androgenetic WT male testes showed no difference in testicular structure and exhibited increased sperm count with dense accumulation of spermatocytes in the spermatic duct than controls [2, 13, 34, 39]. Our present study also confirms our earlier findings on increased fertilizability and hatching rate of androgenetic males and reduced reproductive performance by females i.e. reduced fecundity and hatchability [8, 29, 26, 13, 39]. (Table 1). Reasons for delayed maturity and prolonged inter-spawning period in female androgenotes may be attributed to asynchronous oocyte development and or abnormal

folliculogenesis during oogenesis in the androgenotes [25, 26, 12,].

Progeny testing provides a more comprehensive evidence for conforming paternity since fish chromosomes are cytologically indistinguishable. Yoshikawa *et al.* (2007) [37] have generated gynogenetic females using diploid sperm obtained from sex reversed clones in loaches while others [29, 30] conducted meiotic hybridogenesis in triploid loaches and obtained unexpected female progenies by progeny testing; frequency (~3-25%) of unexpected female progenies sired varied within each species [26, 12- 16]. The frequency of unexpected female progenies was limited to ~25/30 crosses involving androgenetic males (Y^2Y^2) and normal females (X^1X^2) in *Oreochromis niloticus* [21] and to ~16/20 crosses in the cyprinid loach, *Misgurnus anguillicaudatus* [22]. We limited progeny testing to ~21 crosses in *G. ternetzi* (involving androgenotes from 7 family lines), of which only 6 crosses generated unexpected progenies (~2-4% frequency); crossing androgenetic males with control females in progeny testing trials, expected to yield 100% male population, yielded 2-4% heterogametic females (X^2Y^2), confirming the role of paternal autosomes in homozygous conditions on skewing the expected sex ratio.

Production of unexpected females may also indicate molecular role of paternal autosomes in homozygous conditions or contamination of maternal genome [33, 35, 26, 12]. Upon inducing androgenesis, few authors like Scheerer et al (1991) [33], Corley-Smith and Lim (1996) [8] and Ezaz *et al.* (2004) [19] undertook progeny testing and limited their observations to F_1 progenies alone (reviewed by Devlin and Nagahama 2002) [18, 32] while, Kirankumar and Pandian (2004) [26] extended the observations up to F_3 progenies. Similarly, Goudie *et al.* (1995) [21] have produced mitotic gynogens from sex reversed (X^1Y^2) females of *Ictalurus punctatus*; its gynogenetic male (Y^2Y^2) with comparable genotype of androgenetic male (Y^2Y^2), when crossed with normal dams, also sired unexpected female progenies. Expectedly, frequency of unexpected females sired by *G. ternetzi* androgenetic males (Y^2Y^2) in the present study also increased with simultaneous crosses with control females. Therefore, the present study confirms earlier reports [10-14] on the effect of paternal genome homozygosity on sexual maturity and reproductive performance using *G. ternetzi* androgenetic clones, generated using cryopreserved sperm. Further, we also confirm that control WT female autosomes which carried equal number of paternal and maternal autosomes produced equal sex ratio in crosses with normal males (X^1Y^2) while, paternal autosomes in homozygous condition inherited by androgenetic males (X^2Y^2) generated female progenies with male genotype (X^2X^2 /X^1X^2)) in more crosses with heterozygous control females (X^1X^2) [26, 10]. But, the role of heterologous paternal genome in inducing unexpected females with X^1Y^2 genotype is still obscure.

From all the above studies we infer that it is ubiquitous feature of androgenetic males to sire unexpected female progenies. Using sex-specific markers, we have confirmed that genotype of inter-generic hybrids, triploids and inter-specific androgenotes and unexpected female progenies generated by androgenetic WT sires were truly genetic males and hence the probability for maternal contamination in F_0 androgenetic males is nil [25, 26, 13-15]. Interestingly, in all the studies, presence of homozygous paternal autosomes in androgenetic males (Y^2Y^2) overrides the modifying effect of autosomes on genetic sex determination, but maternal autosomes in unexpected female progenies override the single Y^2 present with either X^1 or X^2 and induced the production of unexpected females. However, further studies are required to establish the role of autosomes and allosomes on regulation of sex-determination in tetras.

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