

## Individual contributions to pooled-milt fertilizations of silver catfish *Rhamdia quelen*

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Supplementary stocking of fish in natural environments is a way to mitigate or compensate for the changes imposed on wild populations by river damming. Since little is known about the genetic composition of the supplementary stocks obtained by pooled-milt fertilization, the aim of this study was to determine the individual contributions of male jundiá *Rhamdia quelen* to offspring. Sperm from four males were mixed using equal volume of sperm from each of the males to fertilize eggs from only one female, kept in three blend with six males and three females. The proportions of larvae sired by the different males were quantified using five polymorphic DNA microsatellite *loci*. Analysis of these *loci* allowed paternal determination of 84% of the progeny, at a 0.972 combined exclusion probability. Broodstock milt had good fertilizing capacity when used alone, but when pooled the fertilizing capacities, its fertilizing possibility varied from 4 to 65%. Results show that milt pools favor gametes of some males over others, thus reducing the progeny's genetic variability.

A estocagem de suplementação de peixes em ambientes naturais é uma das maneiras de mitigar ou compensar as alterações impostas pelo represamento dos rios às populações selvagens. Pouco se conhece sobre a composição genética dos estoques repovoadores obtidos através do manejo reprodutivo com fertilizações com o uso de pool de sêmen, de maneira que este trabalho teve como objetivo verificar a contribuição individual de machos de jundiá (*Rhamdia quelen*) na progênie. O mesmo volume de sêmen de quatro machos foi misturado para fertilizar os ovócitos de uma única fêmea, sendo mantidos em três combinações com seis machos e três fêmeas. A proporção de larvas geradas de diferentes machos foi quantificada através de cinco marcadores microssatélites. A análise dos cinco *locus* permitiu a determinação da paternidade de 84% da progênie, com 0,972 de probabilidade de exclusão combinada. O sêmen dos reprodutores apresentou boa capacidade de fertilização quando utilizados separadamente, porém, quando utilizados em pool apresentaram capacidade de fertilização entre 4 a 65%. Os resultados revelam que o pool de sêmen favorece alguns machos sobre outros, reduzindo a variabilidade genética da progênie.

**Key words:** Jundiá, Milt pool, Paternity, Genetic diversity.

### Introduction

Environmental changes and overfishing have been broadly responsible for the reduction of genetic diversity in fish populations, which eventually leads to population decline and species extinction. These environmental changes are mainly caused by river pollution, eutrophication, construction of dams, fishing, and introduction of species (Agostinho *et al.*, 2005).

Another important factor is the inadequate reproduction management techniques that may reduce the genetic variability of the hatchery stock and directly induce deleterious genetic effects on the wild fish populations (Allendorf & Ryman, 1987; Hindar *et al.*, 1991). Effects of fish stocking for supplementation or restoration of native populations has been discussed by

several authors (Incerpi, 1996; Grimes, 1998; Waples, 1999). Miller & Kapuscinski (2003) established the genetic guidelines for hatchery supplementation programs to reduce the impact of these managements, suggesting pool of milt from overlapping pairs of males when it is suspected that many males are infertile. Pooled milt is frequently used in hatcheries to reduce the impact of males with low sperm quality on the overall fertilization rate; it not only increases the probability of fertilization but also can improve genetic variability (Gharrett & Shirley, 1985; Kaspar *et al.*, 2008).

However, due to the lack of information, limited space in hatcheries and difficulty in using wild broodstock fish, a small number of males are repeatedly used to fertilize the oocytes of a large number of females; *i.e.*, the semen of multiple males is added in sequence or as a pool just before water is added

(Withler, 1988). Additionally, optimized methods of artificial fertilization with knowledge about optimal sperm/egg ratio for fertilization are used. Paternal effects on early life history are often obscured and unexplained when sperm from multiple males are mixed during fertilization (Rideout *et al.*, 2004). Previous research has demonstrated that spermatozoa competition in pooled-milt results in different paternity contributions from potential male parents, which reduces the effective size of the broodstock (Campton & Utter, 1985; Allendorf & Ryman, 1987). Reported variations in the fertilizing capacity of pooled-milt were confirmed by the verification of family relationships for Chinook salmon *Oncorhynchus tshawytscha* (Walbaum) (Gharret & Shirley, 1985; Withler, 1988; Withler & Beacham, 1994), rainbow trout *Oncorhynchus mykiss* (Walbaum) (Gile & Ferguson, 1995; Herbinger, 1995), Atlantic halibut *Hippoglossus hippoglossus* (L.) (Ottesen *et al.*, 2009) and common carp *Cyprinus carpio* (L.) (Kaspar *et al.*, 2008).

Microsatellite markers are habitually used with success in paternity and identification tests because of the high frequency of polymorphisms and their wide distribution in the genomes of many eukaryotes (Tautz, 1989). Many authors have demonstrated the capacity of such markers to determine family relationships in cultured and wild fish populations (Herbinger *et al.*, 1995; Fessehaye *et al.*, 2006; Ottesen *et al.*, 2009).

The present study investigated the fertilization and survival rates of *R. quelen* in different artificial crossings and also determined the individual paternal contributions in fertilization with pool semen from overlapping males using microsatellite markers.

### Material and Methods

The study was conducted in the Laboratory of Freshwater Fish Biology and Fish Culture (LAPAD) and in the Laboratory of Plant Genetics and Developmental Physiology (LFDGV), at the Universidade Federal de Santa Catarina (UFSC). *Rhamdia quelen* (Quoy & Gaimard) broodstock were caught from wild populations in the upper Uruguay River, in January of 2006. The individuals were subsequently brought to LAPAD where hormonal induction and extrusion were performed according to Woyrnarovich & Horváth (1980). Gametes were collected from six males (M1, M2, M3, M4, M5 and M6) and three females (FA, FB and FC), in separate containers to quantify milt volume and number of oocytes.

To activate spermatozoa and stimulate fertilization, 100 ml of water was added to the mixture of gametes. The eggs were then washed, hydrated and transferred to individual incubators for each treatment. The milt from each male was evaluated separately. To estimate the sperm concentration, 10 µl of milt was diluted in 2 ml of 5% formalin (in buffered saline), and the number of spermatozoa was counted in a Neubauer chamber under a microscope (400x), according to Mylonas *et al.* (1997). Spermatozocrit values were determined for sperm collected in capillary tubes and centrifuged at 3000 rpm during 5 min. The result was expressed as the percentage of packed cell volume in relation to the total volume (Ciereszko &

Dabrowski, 1993). The duration of sperm motility after activation with distilled water was measured considering the time of cessation of 80% of active movement (Carolsfeld *et al.*, 2003).

One gram of oocytes from each female was fertilized with 40 µL of a pool of semen (equal volume of semen from each of the four males) to determine the individual contribution of the males. Oocytes from female FA were fertilized with a pool of semen from males M1, M2, M3 and M4 (pool A); oocytes from female FB with a pool of semen M1, M2, M5 and M6 (pool B); and the oocytes from female FC with a pool from M3, M4, M5 and M6 (pool C), as shown in Table 1.

**Table 1.** Directed crossings of female (F) *Rhamdia quelen* oocytes with semen of a single male (M) (control) and with pooled semen from four males (treatment). <sup>1</sup>pool A (M1+M2+M3+M4), <sup>2</sup>pool B (M1+M2+M5+M6) and <sup>3</sup>pool C (M3+M4+M5+M6).

	Control						Treatment		
	M1	M2	M3	M4	M5	M6	pool A <sup>1</sup>	pool B <sup>2</sup>	pool C <sup>3</sup>
FA	X	X	X	X			X		
FB	X	X			X	X		X	
FC			X	X	X	X			X

A control group in which 1 g of oocytes from one female was fertilized with 40 µl of sperm from one male was set to check egg quality, capacity of fertilization of each male and larval survival. For each crossing, sperm was mistured to oocytes in a beaker and 100 ml of freshwater was added, then the mixture was moisturized, washed, and transferred to individual incubators. Fertilization success (number of eggs in blastopore closure stage x 100 x number of eggs observed<sup>-1</sup>) was determined the next day (approximately 15 h after fertilization) and total larval survival was determined 72 h post hatching. All evaluations were done in triplicate, and results were submitted to analysis of variance. When significant differences were detected between treatments, means were compared with Tukey's test at the 5% significance level.

Samples of caudal fins of broodstock and eighty live larvae of each pool (sacrificed using overdose of eugenol) were fixed in 90% ethanol and stored in a freezer at -20°C. DNA was extracted following phenol/chloroform methodology (Monesi *et al.*, 1998) and quantified on 0.8% agarose gels. For all samples, DNA concentration was diluted to 10 ng/µl for further analysis. The following five microsatellite markers were used for DNA analysis: Pcor1 and Pcor2 (Revaldaves *et al.*, 2005), Pc17 and Pc97 (Moeser & Bermingham, 2005), and Rh1 (F: 5'-TTACTCGGGATACGATGC-3' and R: 5'-TTGTCAGAGTGCCAAAGG-3'). PCR amplifications involved 3 min of denaturation at 95°C, followed by 30 cycles of consecutively 1 min denaturation at 95°C, 1 min annealing at specific temperatures (Pcor1, 50°C; Pcor2, 51°C; Rh1, 56°C; Pc17, 53°C; Pc97, 54°C), 1 min elongation at 72°C and a final extension for 10 min at 72°C. Amplification products ran on 4% polyacrylamide denaturing gels and stained as described

by Creste *et al.* (2001).

Paternity inference was initially performed using an exclusion-based approach starting from the genotypes at five *loci*. We look for mismatches between parents and offspring using the matrix outputs from the software Cervus version 3.0 (Marshall *et al.*, 1998). The likelihood-based method implemented in the same program was applied to obtain the probabilities of the most likely parents or couples for comparison with the exclusion approach. Simulation parameters involved 10,000 bootstrap cycles from broodstock allele frequencies. A default genotyping error rate of 1% was used and it assumed that all parents were sampled. The analysis multilocus genotypes within family groups, diversity estimators (allele number, heterozygosity, polymorphic information content) and the two exclusion probabilities (Excl1, Excl2) for each *locus* and for all *loci*, were calculated using the allele frequency option of Cervus.

### Results

Spermatocrit values were not good indicators of fertilization capacity in the control crossings, as a positive correlation was found only in pool C ( $r = 0.44$ ) and not in any other treatment. M1 presented both a low spermatocrit value (7.5%) and a high fertilization rate in the crossing between female A and male 1 (FAM1) (93.4%) ( $p < 0.05$ ). In contrast, M6 presented a high spermatocrit value (23.9%) but produced the lowest fertilization rate in the FBM6 crossing (77.1%) ( $p < 0.05$ ) (Table 2). Sperm concentrations ranged from  $1.72$  to  $4.76 \times 10^{10}$  spermatozoa  $ml^{-1}$ , with higher concentrations for male M3 than M1, M4, M5 or M6 ( $p < 0.05$ ). Spermatocrit values ranged from 4.4 to 24.6%, with the highest values observed for males M3, M4 and M6 (24.6, 23.5, and 23.9%, respectively).

Sperm motility durations (20% of active sperm) ranged from 38.5 to 97.8 seconds (M1 and M5). The fertilization rate was above 77% in control and 83% in the pooled-milt crosses. There were no significant differences in the rates at which the sperm of different males fertilized the oocytes of female

FA ( $p > 0.05$ ), whereas the fertilization rates of crossings FBM1 and FBM5 (95.4 and 98.5%, respectively) were higher than that of crossing FBM6 (77.1%). Crossings with female FC presented a significant difference only between the fertilization rates with M4 and M6 (Table 2).

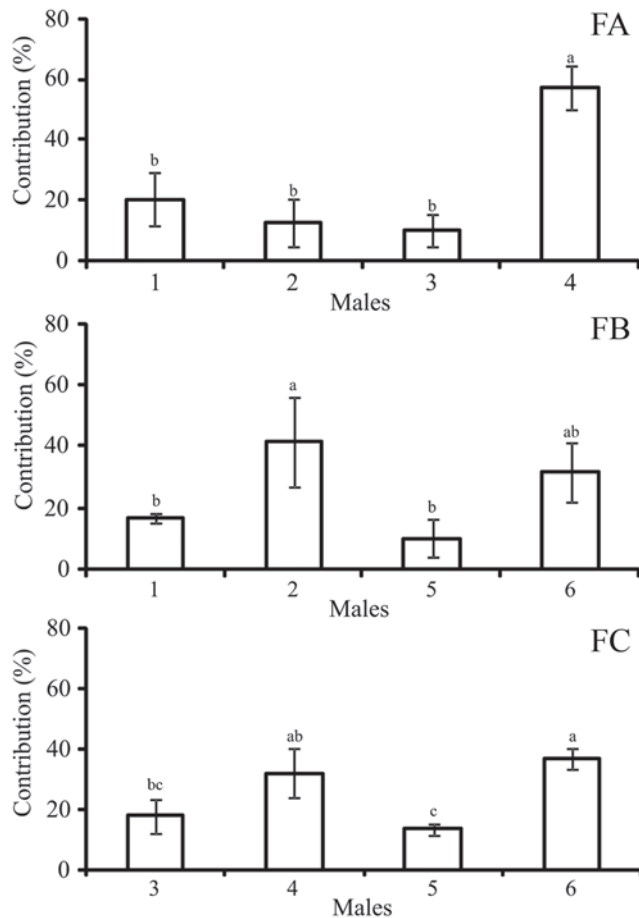
Larval survival 20 days post-hatching varied between 22 and 99%. The survival rate in pool B was higher than in pool A ( $p < 0.05$ ). No significant difference in survival was observed when the oocytes of a given female were fertilized by the milt of different males; however, in crossings FAM1, FAM3 and FAM4, survival was significantly lower than in FBM5, FBM6, FCM3, FCM4 and FCM5 ( $p < 0.05$ ) (Fig. 1).

Some males usually present higher fertilization capacity than others, but this ability can change when crossed with different females or the semen is pooled with distinct males. In pool A, male M4 had higher fertilizing capacity than the others, fathering 63% of the progeny ( $p < 0.05$ ). Males M1, M2, M3 contributed 20.4, 12.5 and 10.2%, respectively, but the control crosses did not show significant differences in the fertilization rates or larval survival among the male fish that fertilized oocytes from female FA. In pool B, male M2 had higher fertilizing capacity (41.7%) than males M1 and M5 (16.6 and 10.3%, respectively), but the fertilization rates in crosses FBM1 and FBM5 were higher than in cross FBM2 ( $p < 0.05$ ). The number of M6 descendants (36.7%) in pool C was higher than that of M3 (17.8%) or M5 (13.5%). In summary, males M4 and M6 contributed more in the pooled-milt fertilizations, male M2 had higher fertilizing capacity only in pool B, and M1, M3 and M5 behaved similarly in the two pooled crosses with consistently low contributions to the progeny (Fig. 1).

The total number of alleles per *locus* observed ranged from five (Pc17) to eight (Pcor2 and Rh1). Probabilities of exclusion per *locus* ranged from 0.129 to 0.474, when the information for the female parent was known (Excl). The mean heterozygosity was 0.770 and the mean polymorphic information content was 0.706. Combined exclusion probability was 0.972 when the genotype of the other parent was known (Table 3), determined 201 offspring (84%) from a

**Table 2.** Mean values and standard deviation of sperm concentration, spermatocrit, sperm motility duration, fertilization rate, and larval survival for each cross. <sup>abc</sup>In the same column and for each female, means followed by different letters are significantly different ( $p < 0.05$ ).

Female	Male	Concentration (cells/ml)	Spermatocrit (%)	Motility (S)	Fertilization (%)	Survival (%)
A	1	$1.89 \pm 0.31 \times 10^{10b}$	$7.5 \pm 0.57^c$	$38.5 \pm 8.3^{bc}$	$93.4 \pm 4.8^a$	$22.4 \pm 13.1^b$
	2	$3.10 \pm 0.89 \times 10^{10ab}$	$7.0 \pm 0.01^c$	$40.3 \pm 2.1^c$	$89.8 \pm 4.2^{abc}$	$62.5 \pm 20.6^{ab}$
	3	$4.80 \pm 0.85 \times 10^{10a}$	$24.6 \pm 0.48^a$	$62.8 \pm 8.8^{ab}$	$89.9 \pm 6.6^{abc}$	$26.1 \pm 11.3^b$
	4	$2.53 \pm 0.71 \times 10^{10b}$	$23.5 \pm 0.58^b$	$63.5 \pm 7.3^{ab}$	$86.1 \pm 7.3^{abc}$	$22.9 \pm 3.9^b$
pool A		-	-	-	$83.4 \pm 8.5^{abc}$	$38.1 \pm 19.1^b$
B	1	$1.89 \pm 0.31 \times 10^{10b}$	$7.5 \pm 0.57^c$	$38.5 \pm 8.3^{bc}$	$95.4 \pm 1.7^{ab}$	$54.7 \pm 34.7^{ab}$
	2	$3.10 \pm 0.89 \times 10^{10ab}$	$7.0 \pm 0.01^c$	$40.3 \pm 2.1^c$	$91.7 \pm 2.5^{abc}$	$68.4 \pm 4.8^{ab}$
	5	$2.78 \pm 0.36 \times 10^{10b}$	$4.4 \pm 0.48^d$	$97.8 \pm 8.1^a$	$98.5 \pm 1.0^a$	$89.8 \pm 10.8^a$
	6	$1.72 \pm 0.06 \times 10^{10b}$	$23.9 \pm 0.48^{ab}$	$74.5 \pm 5.2^{bc}$	$77.1 \pm 2.7^c$	$81.5 \pm 25.0^a$
pool B		-	-	-	$98.0 \pm 0.4^a$	$99.5 \pm 0.2^a$
C	3	$4.80 \pm 0.85 \times 10^{10a}$	$24.6 \pm 0.48^a$	$62.8 \pm 8.8^{ab}$	$93.6 \pm 2.3^{ab}$	$98.0 \pm 0.6^a$
	4	$2.53 \pm 0.71 \times 10^{10b}$	$23.5 \pm 0.58^b$	$63.5 \pm 7.3^{ab}$	$98.2 \pm 1.0^a$	$86.7 \pm 21.7^a$
	5	$2.78 \pm 0.36 \times 10^{10b}$	$4.4 \pm 0.48^d$	$97.8 \pm 8.1^a$	$91.7 \pm 1.7^{abc}$	$76.2 \pm 5.8^a$
	6	$1.70 \pm 0.06 \times 10^{10b}$	$23.9 \pm 0.48^{ab}$	$74.5 \pm 5.2^{bc}$	$81.2 \pm 6.4^{bc}$	$66.2 \pm 12.3^{ab}$
pool C		-	-	-	$91.7 \pm 1.8^{abc}$	$61.4 \pm 4.0^{ab}$



**Fig. 1.** Mean values and standard deviation of the relative contribution of each male to the progeny when the oocytes of each female (FA, FB, FC) were fertilized with pooled semen from four males (pool A: M1+M2+M3+M4; pool B: M1+M2+M5+M6; pool C: M3+M4+M5+M6).

total of 239 offspring analyzed. The remaining incompatibilities were considered as false exclusions due to null alleles and mutations, because all breeders had been genotyped and the number of incompatible *loci*.

Frequencies of null alleles in the broodstock as estimated by Cervus 3.0, ranged between 1 and 6% for all *loci* (Table 3).

**Table 3.** Locus SSR, number of alleles (*k*), number of individuals successfully genotyped (*n*), range of observed alleles in pb (size), observed heterozygosity (Ho), expected heterozygosity (He), polymorphic information content (PIC), probabilities of exclusion (EP) and null alleles estimated by Cervus version 3.0 at a frequency > 0.05.

Locus	k	n	Size (pb)	Ho	He	PIC	EP	Null alleles
Pcor1	6	229	82-102	0.878	0.823	0.797	0.466	-0.0359
Pcor2	8	219	180-280	0.941	0.826	0.800	0.474	-0.0672
Rh1	8	219	220-290	0.872	0.816	0.791	0.464	-0.0396
Pc17	5	227	240-262	0.714	0.734	0.686	0.300	+0.0164
P97	6	216	250-290	0.449	0.475	0.457	0.129	+0.0635

## Discussion

In crosses with pooled-milt, differential contributions from each male result in a reduction of the effective size of the founding stock. According to Falconer & Mackay (1996), this reduction depends on the total number of individuals that contribute with genes to the next generation. One of the main practical problems caused by pooled sperm is unequal representation of males used for fertilization, causing reduction of the effective size of the population (Kaspar *et al.*, 2008). In this study, we verified that pooling milt before insemination of oocytes does not confirm that the number of descendants of each male parent will be proportional to the volume of donated milt. All male parental fish demonstrated fertilization capacity in individual crosses, however, when pooled, this rate varied between 4 and 65%. Considering that all tested males exhibited high fertilization rates in the control crosses, the obtained results suggest the dominance of some males over others during the pooled-milt fertilization. Spermatozoa competition in the fertilization of an egg pool with pooled milt of Atlantic salmon was observed by Campton & Utter (1985). Withler (1988) found dominance of some males of *Oncorhynchus tshawytscha* in crosses with pooled milt, with variations of 1.4 to 75.6%. According to this author, variability among males is not simply the result of individual differences, but also of individual variation throughout the reproductive season, so that some males can fertilize the oocytes individually but cannot make a significant contribution when applied in a pool. Some studies have been presenting differences in the success of fertilizations with a pool of semen (Rideout *et al.*, 2004; Ottesen *et al.*, 2009).

Hormonal induction of the silvercatfish broodstock resulted in high fertilization rates in the control crosses, but differences in fertilizing capacity of the same male were observed when milt was pooled with different males and used to fertilize eggs from distinct female (*e.g.* M2 contributed with 12.5% in pool A and 31.9% in pool B). This is consistent with results of Withler (1988) for *O. tshawytscha*, who related the variation in fertilizing capacity observed in some species to the possible interaction among male gametes or to the different females involved in the fertilization. In fertilizations of pooled-milt, if all males had the same reproductive success, any offspring has an equal chance to be originated from each of the males, following a Poisson distribution:

$$N_{em} = N_m - 1 / K_m$$

where  $N_{em}$  is the number effective of males,  $N_m$  is the number of males used and  $K_m$  is the mean number of progenies per male. The theoretical  $N_{em}$  in the absence of competition was 3.98 on average for all pooled-milt of jundiá. Kimura & Crow (1963) exhibit a parameter that is modified by unequal paternal contributions and may be used to estimate the genetically effective size of a population created from progeny groups of pooled-milt fertilizations:

$$N_{em} = (N_m \cdot K_m - 1) / (K_m - 1 + V_m / K_m)$$

where  $V_m$  is the variance of the number of progeny per male. The true  $N_{em}$  was 1.93 on average (0.85-3.66) in Pool A; 2.92

(1.67-3.77) in Pool B and 3.40 (2.77-3.77) in Pool C. The fertilization procedure used in this research produced a mean reduction in the  $N_{em}$  of 30.91%. This results show that using a mixture of sperm leads to an important reduction of the effective number of males. Similar results were found by Withler (1988) and Withler & Beacham (1994) with average reduction in  $N_{em}$  as 31.4% for Chinook salmon and Kaspar *et al.* (2007) showing a mean reduction of 42.4% for common carp.

Among many factors that interfere in the success of fertilization, sperm motility is considered the main characteristic (Birkhead & Møller, 1998; Rakitin, 1999). In Atlantic halibut, sperm velocity and the number of motile sperm are major components in the competition for fertilization (Ottssen *et al.*, 2009), and in Atlantic salmon, sperm speed is considered the main factor determining the success in sperm competition (Gage *et al.*, 2004). The duration of sperm motility of silver catfish was not correlated with the fertilization rate in these crosses. Male M1 showed the lowest sperm motility time but not the lower ability of fertilization; nevertheless, male M5 had the higher motility time and the lowest fertilization rate in both pooled-milt fertilizations that it was used. Some studies collaborate with our results (Rakitin, 1999; Casselman *et al.*, 2006); however sperm motility is still considered the most important parameter for fertilization success in other fish species (Lahnsteiner *et al.*, 1998; Rurangwa *et al.*, 2001; Ottesen *et al.*, 2009). Sperm concentration and spermatocrit value were also not correlated with the fertilization rate for silvercatfish. According to Gharrett & Shirley (1985) and Withler (1988), the variations in male fertilization potential cannot be described by spermatocrit values and sperm cell size, which change between males.

The uneven participation of parental gametes cannot be attributed only to the quality of semen parameters, because it does not necessarily indicate the best one for competitiveness fertilizations in pooled-milt. The methodology used during fertilization can favor the gametes of a certain individual over others (Hill, 1979). The reproductive success of individual male of *R. quelen* in the pooled milt treatments was independent of the fertilized females and also the interactions with other silvercatfish male. These differences reflect the complexity of pooling sperm for fertilization. The maximum effective size of the foundation stock can be guaranteed by using controlled crosses with fertilizations in small individual recipient before being grouped into incubators. In this condition, all males have the same opportunity to leave their genetic information and, thus, this management can be recommended for maintain genetic diversity in the progeny.

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