BOLETIM DO INSTITUTO DE PESCA

ISSN 1678-2305 online version

Scientific Article

PARENTAL CONTRIBUTION OF CURIMBA OFFSPRING IN DIFFERENT REPRODUCTIVE SYSTEMS

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Received: July 27, 2017
Approved: November 29, 2017

ABSTRACT

The knowledge of appropriate reproductive managements is fundamental for the management and orientation of activities in restocking centers. The aim of the present study was to evaluate the influence of artificial and semi-natural reproductive systems and sex ratios on parental contribution progeny of *Prochilodus lineatus*. Samples of caudal fins from 25 breeders subjected to the artificial reproductive system and 10 breeders subjected to the semi-natural reproductive system were collected in different sex ratios (1♂:1♀ and 2♂:1♀, and 1♂:1♀, respectively). Two hundred and eight fingerlings from these matings were evaluated. Breeding mortality was recorded shortly after reproductive procedures. For paternity analysis, 10 microsatellite loci were amplified. Three breeders died in the artificial reproductive system (two in the 1:1 ratio and one in the 2:1 ratio). In the 1:1 ratio in the artificial reproductive system, one male (M1.4) had a higher contribution in the progeny formation (41.8%), whereas in the 2:1 ratio, four males contributed from 14.5 to 21.7%. Lower reproductive dominance was identified in the semi-natural reproductive system, with values ranging from 22.86 to 25.71% for four males. In the formation of families, both systems demonstrated a reproductive dominance of some couples. So, the semi-natural reproductive system allowed greater homogeneity in the contribution of progeny without losses due to mortality.

Key words: broodstock; conservation; paternity; *Prochilodus lineatus*; restocking programs.

CONTRIBUIÇÃO PARENTAL EM PROGÊNIES DE CURIMBA USANDO DIFERENTES SISTEMAS REPRODUTIVOS

RESUMO

O conhecimento de manejos apropriados para a reprodução é fundamental para a gestão e direcionamento das atividades em centrais de repovoamento. O objetivo deste estudo foi avaliar a influência dos sistemas reprodutivos por extrusão e seminatural e da proporção de sexo na contribuição parental de progênies de *P. lineatus*. Amostras de nadadeiras caudais de 25 reprodutores submetidos ao sistema reprodutivo por extrusão e 10 reprodutores submetidos ao sistema reprodutivo seminatural foram coletados em diferentes proporções de sexo (1♂:1♀ e 2♂:1♀, e 1♂:1♀, respectivamente). Duzentos e oito alevinos oriundos desses acasalamentos foram avaliados. A mortalidade dos reprodutores foi registrada logo após os procedimentos reprodutivos. Para a análise de paternidade, foram amplificados 10 *loci* microssatélites. Três reprodutores foram a óbito no sistema extrusão (dois na proporção 1:1 e um na proporção 2:1). No sistema extrusão 1:1 observou-se que um macho (M1.4) obteve maior contribuição na formação progênie (41,8%), enquanto que na proporção 2:1, quatro machos contribuíram entre 14,5 a 21,7%. A menor dominância reprodutiva foi identificada no sistema seminatural, com valores variando de 22,86% a 25,71% para quatro machos. Na formação das famílias, foi observada dominância reprodutiva de alguns casais em ambos os sistemas. Conclui-se que o sistema seminatural permitiu maior homogeneidade na contribuição na progênie sem perdas por mortalidade.

Palavras-chave: reprodutores; conservação; paternidade; *Prochilodus lineatus*; programas de repovoamento.

INTRODUCTION

Owing to the reduction of natural fish stocks in Brazilian basins, caused mainly from environmental pollution, overfishing, destruction of natural vegetation cover, and construction of hydropower plants, actions aimed at the replenishment of endangered fish populations are increasingly required for the conservation of these species in the natural environment (NOGUEIRA *et al*., 2010; FROTA *et al*., 2016). In this context, reproductive management adopted by restocking centers is a key element for fingerlings production, whereas different reproductive management systems can provide quantitative and qualitative variations (genetic diversity) in progeny formation, as well as infer information about the breeders' health (REYNALTE-TATAJE *et al*., 2013).

Curimba (*Prochilodus lineatus*), a rheophilic species, cannot reproduce naturally in captive conditions and requires spawning induction techniques (e.g., hypophysation) for gamete releases (PAULINO *et al*., 2011; SANTOS *et al*., 2013). In restocking programs, it is common to use two reproductive systems to commence spawning: artificial and semi-natural (POVH *et al*., 2010; REYNALTE-TATAJE *et al*., 2013). According to ZANIBONI-FILHO and WEINGARTNER (2007), the artificial reproductive system is one of the most applied methods in fish reproduction, due to the ease of gametes manipulation and the requirement of less infrastructures (i.e., no spawning tanks). However, this technique may bring about the mortality of breeders due to the stress caused by manipulation of the animals and can result in a decrease in genetic variability for some species (REYNALTE-TATAJE *et al*., 2013; LOPERA-BARRERO *et al*., 2014). The semi-natural reproductive system, on the other hand, consists of forced circulation of water inside a spawning tank, which simulates the natural environmental conditions, thus promoting gametes maturation. This methodology results in lower mortality of breeders and lower physical stress (POVH *et al*., 2010; ZANONI *et al*., 2015).

Research has shown that using different sex ratios (semen pool or individual spawning) for reproduction of some fish species can influence the parental contribution to progeny, thus exercising reproductive dominance of some males (RIBOLLI and ZANIBONI-FILHO, 2009). This fact is undesirable in conservation programs, since the homogeneity of genetic inheritance can minimize inbreeding that may compromise the progeny viability (LOPERA-BARRERO *et al*., 2014; GOES *et al*. 2017). In addition, this is fundamental for the control of mating aimed at genetic variability of the progeny, considering the importance of restocking programs in the replenishment of natural fish populations (LOPERA-BARRERO *et al*., 2014, 2016). However, no study has demonstrated of the influence of the sex ratio and reproductive system on parental contribution in *P. lineatus*.

The objective of the present study was to evaluate the broodstock mortality and influence of artificial and semi-natural reproductive systems and sex ratios in the parental contribution to progeny in *P. lineatus* obtained from the Tietê river restocking program.

METHODS

Reproductive systems

The reproduction of *P. lineatus* in the artificial and semi-natural reproductive systems was executed at the hydrobiology station (21°19′01.2″ S 49°47′22.8″ W) situated in Promissão city, São Paulo, Brazil. Molecular biology analyses were performed in the laboratories of the universities located in Londrina (State University of Londrina - UEL) and Maringá (State University of Maringá - UEM) city, Paraná, Brazil. The methodologies utilized during this experiment were approved by the Ethics Committee on the use of Animals of the State University of Londrina (CEUA_UEL nº17156.2012.50).

A total of thirty-five Curimba breeders were selected by physical condition of the animals, such as abdominal bulging and hyperemia of urogenital papilla from the hydrobiology station and these fish were divided according to the reproductive system and sex ratio: five males (M1.1, M1.2, M1.3, M1.4, and M1.5) and five females (F1.1, F1.2, F1.3, F1.4, and F1.5) (1:1 ratio) and ten males (M2.1, M2.2 …, M2.10) and five females (F2.1, F2.2 …, F2.5) (2:1 ratio) in the artificial system, and five males (M3.1, M3.2 …, M3.5) and five females (F3.1, F3.2 …, F3.5) (1:1 ratio) in the semi-natural system (Table 1).

A biometry of each breeder was performed to determine the hormonal dosage required to induce reproduction. Carp pituitary extract was administered to the dorsal fin base using a 1 mL syringe (intramuscular application) for induction of reproduction. For these proceedings, the animals were captured and held by the head and base of the tail. The females received 5.5 mg kg^{-1} divided into two applications: 0.5 mg kg^{-1} in the first application and 5 mg kg^{-1} administered 12 h later, while the males received 2.5 mg kg^{-1} in a single dose simultaneously with the second female application (adapted from PEREIRA *et al*., 2009).

Broodstocks from the artificial reproductive system were placed separately (males and females) in concrete tanks containing 2 m^3 of water and the temperature was measured every hour to obtain the accumulated thermal unit (ATU) necessary for spawning. Approximately 8 h after the second dose (220 ATU, corresponding to approximately 8 h at a water temperature of 27 °C), the artificial procedure was performed based on the methodology of LOPERA-BARRERO *et al*. (2014) by stripping. A semen pool (semen from all males) was used for fertilization of the immediate collected oocytes.

The matings in the semi-natural reproductive system occurred using the same procedure as the 1:1 artificial reproductive system.

Table 1. Characterization of breeders and fingerlings in artificial and semi-natural reproductive systems.

Reproductive System	N. of Breeders		Male: Female ratio	N. of fingerlings analyzed
	Males	Females		
Artificial	5	5	1:1	69
	10	5	2:1	69
Semi-natural	5	5	1:1	70

N.: Number.

Immediately following hypophysation, the broodstocks were placed in circular tanks (4 m diameter and 1.5 m depth) with a forced water circulation system (continuous flow) to simulate natural environmental conditions. Thus, semi-natural mating occurred between several couples enabling a random fertilization process. After mating and fertilization, which occurred after 220 ATU, the eggs had accumulated in the collector station via a tube placed in a central region of the tank. This facilitated egg collection and increased the suitability of the eggs to cylindrical-conical incubators (captation of 200 L with continuous water flow), where the formation and birth of the larvae occurred. The broodstock mortality in both reproductive systems were verified at 24 h after spawning.

Fin collection, DNA extraction, and quantification

DNA of caudal fins was extracted at UEL using an extraction protocol containing NaCl (LOPERA-BARRERO *et al*., 2008). A total of 35 caudal fin samples were collected from broodstocks in both systems, 69 fin samples were collected from fingerlings in each of the artificial reproductive systems (1:1 and 2:1), and 70 fin samples were obtained from fingerlings in the semi-natural reproductive system (Table 1). Caudal fin samples were collected from the fingerlings at 90 days after the eggs had hatched.

DNA quality was determined using agarose gel electrophoresis (0.01) with SYBR Safe™ DNA Gel Stain (Invitrogen, Carlsbad CA, USA) in 1 × TBE buffer for 2 h at 80 V. The gel was visualized in a transilluminator device with ultraviolet light and the image was photographed using Kodak EDAS software (Kodak 1D Image Analysis 3.5, Eastman Kodak Company, Rochester NY, USA).

The DNA quantification was performed using a PICODROP® spectrophotometer (Picodrop Limited, Hinxton, United Kingdom), with the samples standardized via dilution to a final concentration of 20 ng μL^{-1}.

DNA amplification and polyacrylamide gel electrophoresis

Amplification was performed in a final reaction volume of 15 µL containing 1 × Tris-KCl buffer, 2.0 mM of $MgCl_2$, 0.8 µM of each primer (forward and reverse), 0.2 mM of each dNTP, a half unit of Platinum Taq DNA Polymerase, and 20 ng of DNA, performed at UEM. Initially, the DNA was denatured at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 60 s, annealing for 60 s, and extension at 72 °C for 60 s, with a final extension at 72 °C for 20 min. A total of ten microsatellite loci were evaluated: Par12, Par14, Par15, Par21, Par43, Par80 (BARBOSA *et al*., 2006; BARBOSA *et al*., 2008), Pl01, Pli30, Pli43, and Pli60 (YAZBECK and KALAPOTHAKIS, 2007). The reactions were performed on a Veriti® thermal cycler (Applied Biosystems®, Austin, TX, USA).

The amplified samples were subjected to 10% polyacrylamide gel electrophoresis (acrylamide:bisacrylamide, 29:1) with denaturant (6 M urea) in 0.5 × TBE buffer at 180 V and 250 mA for 8 h. For allele observation, the gel was soaked in a fixation solution (10% ethanol and 0.5% acetic acid) for 20 min, followed by 6 mM of silver nitrate solution for 30 min, with revelation in a solution of 0.75 M NaOH and 0.22% formaldehyde 40% (BASSAM *et al*., 1991). The gel was photographed using a Nikon CoolPix 5200 camera. The allele size was calculated using a 100 base pair DNA ladder (Invitrogen). Paternity analysis was performed using PAPA software version 2.0 (DUCHESNE *et al*., 2002), enabling the elaboration of reproductive contribution analyses. PAPA is a software program that performs parental allocation. The calculation is based on the likelihood of a parental pair producing the multilocus genotype found in the progeny tested (DUCHESNE *et al*., 2002).

RESULTS AND DISCUSSION

Broodstocks mortality

Mortality was observed in two females from the 1:1 artificial reproductive system and one female from the 2:1 artificial reproductive system. There was no mortality in the semi-natural reproductive system. Comparing the two systems, a higher mortality of rheophilic fish species, such as *Leporinus macrocephalus*, *Piaractus mesopotamicus*, *Brycon orbignyanus*, and *P. lineatus* has previously been detected in animals subjected to the artificial system (REYNALTE-TATAJE *et al*., 2013). ZANONI *et al*. (2015) examined *B. orbignyanus* broodstocks subjected to reproduction in both systems and observed that the artificial system promoted metabolic changes related to stress, such as elevated cortisol levels, glucose, and hematological changes that was not evidenced in the semi-natural system. These studies and the results of the present study suggest that the semi-natural reproductive system provides a mortality decrease because of the lower stress placed on the fish, which becomes even more important when there is a limited number of breeders in the restocking program.

Parental contribution

In general, the semi-natural reproductive system facilitated better homogeneity of the male contribution to progeny formation, with M3.2 presenting the lowest contribution (Figure 1). Thus, this system did not provide dramatic reproductive dominance among males. In the 1:1 artificial reproductive system, M1.4 presented a greater reproductive contribution to progeny formation, whereas in the 2:1 artificial reproductive system this contribution was 71% dominated by four males (M2.4, M2.7, M2.5, and M2.9) (Figure 1).

In females, there was also a better balance in the contribution in the semi-natural reproductive system. Only F3.4 presented a low contribution value (Figure 1). In the 1:1 artificial reproductive system, a greater contribution of F1.4 was observed compared to other females. In the 2:1 artificial reproductive system, a greater contribution of F2.2 and F2.4 was observed compared to other females, demonstrating reproductive dominance of these females (Figure 1).

In both reproductive systems, each male was able to fertilize more than one female. These results characterize multiple paternity for the *P. lineatus* progeny, consistent with observations in other fish species by microsatellites markers, such as *B. orbignyanus* (LOPERA-BARRERO *et al*., 2014), *P. mesopotamicus*

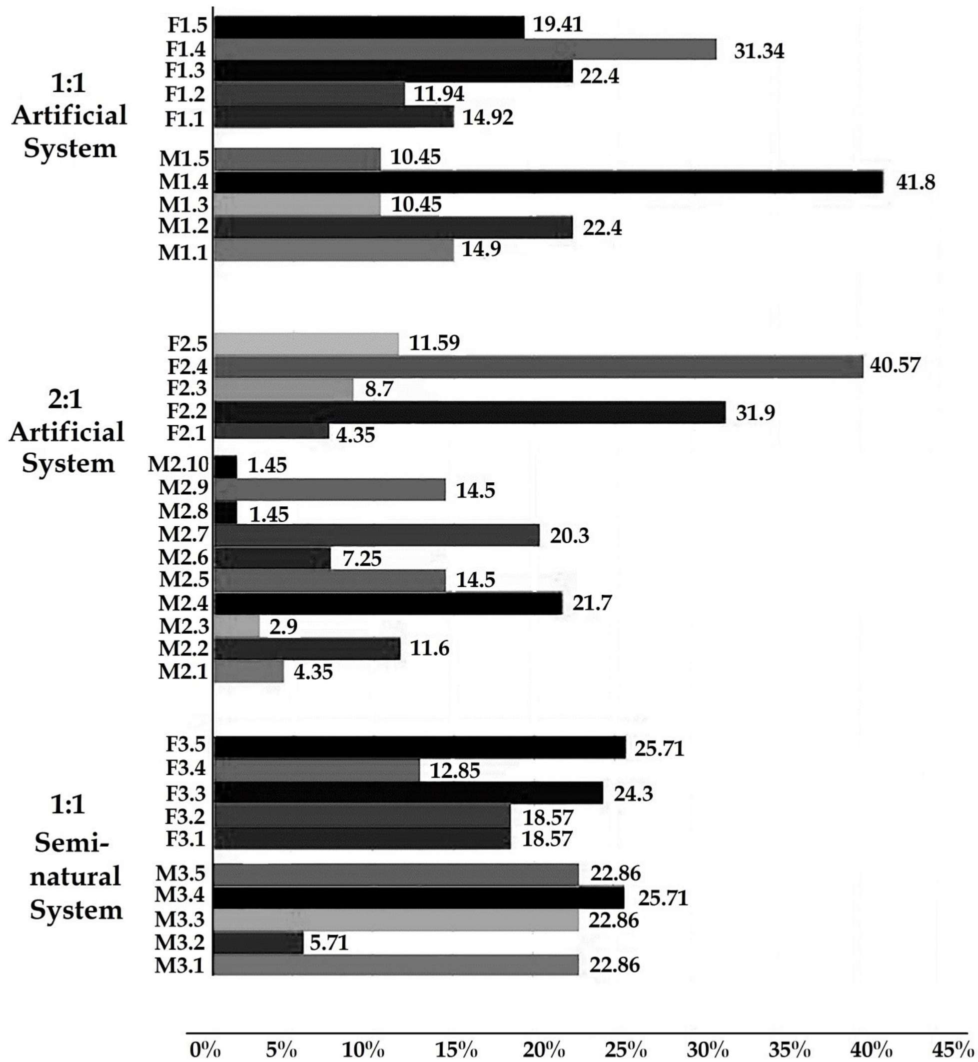

Figure 1. Reproductive contribution (%) of males (M) and females (F) in the *Prochilodus lineatus* progeny in 1:1 and 2:1 artificial reproductive systems and 1:1 semi-natural reproductive system. M: Males; F: Females.

(POVH *et al*., 2010), and *Rhamdia quelen* (RIBOLLI and ZANIBONI-FILHO, 2009; GOES *et al*., 2017).

The unequal participation of males in progeny generation has also been observed in other fish species. RIBOLLI and ZANIBONI-FILHO (2009) identified the unequal contribution of *R. quelen* males in the artificial system when the semen pool was used for oocyte fertilization, which did not occur when fertilization was performed individually, demonstrating that all males had high fertilization capacity. These authors attributed the dominance of some males to different seminal characteristics. LOPERA-BARRERO *et al*. (2014) identified a more homogeneous contribution of *B. orbignyanus* males in the semi-natural system (15 to 21.7%) than in the artificial system (5 to 25%). This finding agrees with the results of the present study, considering that in the semi-natural system both males and females similarly contributed to progeny formation.

According to REYNALTE-TATAJE *et al*. (2013), the artificial system increases the chance of errors when selecting breeders for spawning, reflecting the difficulty in determining the precise time interval of gamete maturation. The semi-natural system, however, reduces the unintentional selection of the gametes via natural synchronization promoted by this system, which favors a homogeneous contribution of broodstocks in the progeny constitution (POVH *et al*., 2010). In the present study, some breeders (males and females) more markedly contributed to progeny formation, particularly in the 1:1 artificial reproductive system, which supports the hypothesis of differentiated gamete maturation in some individuals and strengthens the reproductive dominance in this system in comparison to the 1:1 semi-natural reproductive system.

The composition of the families in both reproductive systems was influenced by the greater participation of some couples (Table 2). In the 1:1 artificial reproductive system, all males could fertilize the oocytes of all females, except for M1.3 with F1.3, and the

Table 2. Family composition (%) in the *P. lineatus* progeny using the artificial (1:1 and 2:1) and semi-natural (1:1) reproductive systems.

		M1.1	M1.2	M1.3	M1.4	M1.5	-	-	-	-	-
1:1 Artificial	F1.1	2.99	2.99	2.99	4.48	1.49	-	-	-	-	-
	F1.2	1.49	4.48	1.49	2.99	1.49	-	-	-	-	-
	F1.3	1.49	5.97	0	10.45	4.48	-	-	-	-	-
	F1.4	4.48	5.97	1.49	17.91	1.49	-	-	-	-	-
	F1.5	4.48	2.99	4.48	5.97	1.49	-	-	-	-	-
		M2.1	M2.2	M2.3	M2.4	M2.5	M2.6	M2.7	M2.8	M2.9	M2.10
2:1 Artificial	F2.1	0	0	0	2.90	2.90	1.45	0	0	0	0
	F2.2	1.45	1.45	1.45	10.14	2.90	1.45	7.25	0	5.80	0
	F2.3	0	0	0	0	5.80	4.35	4.35	0	0	0
	F2.4	0	8.70	0	7.25	1.45	4.35	5.80	1.45	4.35	1.45
	F2.5	1.45	1.45	1.45	1.45	2.90	0	2.90	0	0	0
		M3.1	M3.2	M3.3	M3.4	M3.5	-	-	-	-	-
1:1 Semi-natural	F3.1	10.00	1.43	2.86	1.43	2.86	-	-	-	-	-
	F3.2	1.43	0	7.14	5.71	4.29	-	-	-	-	-
	F3.3	2.86	1.43	4.29	7.14	8.57	-	-	-	-	-
	F3.4	4.29	1.43	1.43	2.86	2.86	-	-	-	-	-
	F3.5	4.29	1.43	7.14	8.57	4.29	-	-	-	-	-

F: Females; M: Males.

M1.4 × F1.4 mating showed a greater contribution to the progeny. However, in the 2:1 artificial reproductive system, some males were not able to generate offspring with certain females. In the semi-natural reproductive system, similarly to the 1:1 artificial reproductive system, only one female (F3.2) did not produce offspring with the M3.2 male, the other males were able to fertilize the oocytes of all females, and the M3.1 × F3.1 mating showed a greater contribution to progeny (Table 2).

The greater contribution of some couples demonstrates that both systems were subject to reproductive dominance, either by the intrinsic characteristics of the animals (SIVINSKI 1984; POVH *et al.*, 2010) or even dominance behavior (ALONSO *et al.*, 2012). In the 1:1 artificial reproductive system, the greatest dominance in the mating contribution (17.91% for M1.4 × F1.4) was observed in the 2:1 artificial reproductive system (10.14% for M2.4 × F2.2) and semi-natural reproductive system (10% for M3.1 × F3.1), which likely reflects the smaller number of males than in the 2:1 artificial reproductive system (Table 2). For this reason, the reproductive dominance in the 2:1 ratio was milder, to the point that the most dominant represented a contribution of approximately 20% compared to greater than 40% (M1.4) in the 1:1 artificial reproductive system.

In the semi-natural reproductive system, in general, the couples exhibited a homogeneous contribution; however, these results do not exclude reproductive dominance, since matings such as M3.1 × F3.1 and M3.5 × F3.3 contributed more effectively in relation to other couples. Behavioral factors, such as aggressiveness and social hierarchy (ALONSO *et al.*, 2012), and the seminal quality of the males may be involved in the greater contribution of these individuals.

These results demonstrate that spawning in forced-circulation tanks or using the doubling up of males in artificial reproduction allowed a greater number of breeders to contribute to offspring generation of *P. lineatus*. These findings are very important when considering the importance of the genetic diversity repass to the fingerlings, since the homogeneity in contribution of these individuals can minimize inbreeding. Furthermore, there was no mortality in the semi-natural reproductive system, which is very important in restocking programs, since usually there are few individuals and genetic losses by mortality could have incalculable effects, besides allowing a better fertility rate (REYNALTE-TATAJE *et al.*, 2013). However, the availability of restocking program resources should also be considered. Notably, the artificial reproductive system has a greater facility in the management of gametes and less investment in infrastructure, since it does not require spawning tanks with artificial current flow. The semi-natural reproductive system, in turn, demands greater investments in infrastructure such as tanks with a forced water circulation system and a collector station with access to the incubators.

Further studies are required to evaluate the number of breeders and different sex ratios in the semi-natural reproductive system in *P. lineatus* and in other fish species in other fish species that have economical and conservationist importance, such as *Salminus brasiliensis*, *Leporinus elongatus,* and *B. hilarii* among others. It is anticipated that these findings may assist in reproductive activities in conservation programs.

CONCLUSION

In conclusion, spawning in forced-circulation tanks allowed lower mortality and enabled broodstocks to contribute homogeneously to offspring generation of *P. lineatus* without presenting genetic losses by mortality.

ACKNOWLEDGEMENTS

The authors wish to thank the AES Tietê and CAPES for support during the research development.

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