The spawning method affects the reproductive efficiency of piracanjuba fish (*Brycon orbignyanus*) in restocking programs

El método de desove afecta la eficiencia reproductiva de piracanjuba (Brycon orbignyanus) en programas de repoblación

O método de desova afeta a eficiência reprodutiva de piracanjuba (Brycon orbignyanus) em programas de repovoamento

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Abstract

Background: Preserving the genetic diversity of wild fish is an important consideration for restocking programs, as inbreeding can compromise progeny survival as well as impact the resilience of natural populations. **Objective:** To evaluate the influence of spawning method: semi-natural (SN) or strip-spawning (ST), and the number of breeders ($1♀:3♂$ and $2♀:6♂$) on the reproductive efficiency and genetic diversity of *B. orbignyanus* progeny destined for restoration of wild stocks. **Methods:** Rates of fertilization, hatching and broodfish mortality were recorded. For genetic evaluations (allele frequency, observed and expected heterozygosity, Shannon index, inbreeding coefficient, molecular variance analysis, and genetic differentiation), breeders (n=24), and their progenies (90 larvae/treatment) were sampled and analyzed using eight microsatellite markers. **Results:** Higher fertilization and hatching rates, and lower broodfish mortality were observed for the SN method ($p<0.05$), whereas the number of breeders did not affect these parameters ($p>0.05$). Interaction between spawning method and number of breeders was not significant ($p>0.05$). The amplified microsatellite loci produced a total of 30 alleles, with sizes between 80 and 225 bp and their frequencies indicated an increase ($p<0.05$) of genetic diversity in the progenies, but low genetic differentiation

between treatments (p>0.05). **Conclusion:** The spawning methods and number of breeders tested increased equally the genetic diversity of the progeny, with low genetic differentiation between treatments. In contrast, rates of fertilization, hatching and brood fish mortality revealed that the SN method resulted in the best reproductive efficiency due to the handling stress and injuries caused by ST. Thus, SN proves to be the most suitable spawning-method for *B. orbignyanus* in restocking programs.

Keywords: *aquaculture; breeders; broodfish; broodstock; Brycon orbignyanus; fertilization; fish; genetic diversity; hatching; incubation; microsatellite markers; mortality; reproductive efficiency; restocking; spawning; strip-spawning.*

Resumen

Antecedentes: Mantener la diversidad genética de los peces salvajes es una consideración importante para los programas de repoblación, ya que la endogamia puede comprometer la supervivencia de la progenie y afectar la supervivencia de las poblaciones naturales. **Objetivo:** Evaluar la influencia del método de desove (seminatural - SN o en franjas - ST) y el número de reproductores (1♀:3♂ y 2♀:6♂) sobre la eficiencia reproductiva y la diversidad genética de progenies de *B. orbignyanus* destinados a la restauración de poblaciones silvestres. **Métodos:** Se monitorearon las tasas de fertilización, eclosión y mortalidad de reproductores. Para las evaluaciones genéticas (frecuencias alélicas, heterocigosidad observada y esperada, índice de Shannon, coeficiente de endogamia, análisis de varianza molecular y diferenciación genética) los reproductores (n=24) y su progenie (90 larvas/tratamiento) se muestrearon y analizaron utilizando ocho marcadores microsatélites. **Resultados:** La interacción entre el método de desove y el número de reproductores no fue significativa (p>0,05). Se obtuvieron mejores tasas de fecundación y eclosión (p<0,05), y una menor mortalidad de reproductores (p<0,05) con el método SN, mientras que el número de reproductores no tuvo efecto (p>0,05). Los loci de microsatélites amplificados produjeron un total de 30 alelos con tamaños entre 80 y 225 pb, y sus frecuencias indicaron un aumento (p<0,05) en la diversidad genética de las progenies, pero una baja diferenciación genética entre tratamientos (p>0,05). **Conclusión:** Los métodos de desove y el número de reproductores evaluados aumentaron de la misma manera la diversidad genética de las progenies, con baja diferenciación genética entre tratamientos. En contraste, las tasas de fecundación, eclosión y mortalidad de peces reproductores revelaron que el SN tuvo la mejor eficiencia reproductiva, un hecho relacionado con el estrés del manejo y las lesiones causadas por el ST. Por lo tanto, el SN demuestra ser el método de desove más adecuado para *B. orbignyanus* en los programas de repoblación.

Palabras clave: *acuicultura; Brycon orbignyanus; desove; desove en franjas; diversidad genética; eclosión; eficiencia reproductiva; fertilización; incubación; marcadores microsatélite; mortalidad; pez; pez de cría; repoblación; reproductores.*

Resumo

Antecedentes: A manutenção da diversidade genética dos peixes selvagens é uma importante consideração para programas de repovoamento, já que a endogamia pode comprometer a sobrevivência da progênie, além de impactar na resiliência das populações naturais. **Objetivo:** Avaliar a influência do método de desova (semi-natural – SN ou por extrusão – ST) e número de reprodutores (1♀:3♂ e 2♀:6♂) na eficiência reprodutiva e na diversidade genética de progênie de *B. orbignyanus* destinados à recuperação de estoques selvagens. **Métodos:** Taxas de fertilização, eclosão e mortalidade de reprodutores foram monitorados. Para avaliações genéticas (frequências alélicas, heterozigosidade observada e esperada, índice de Shannon, coeficiente de endogamia, análise de variância molecular e diferenciação genética), reprodutores (n=24) e sua progênie (90 larvas/tratamento) foram amostrados e analisados utilizando oito marcadores microssatélites. **Resultados:** Interação entre método de desova e número de reprodutores não foi significante (p>0,05). Melhores taxas de fertilização e eclosão (p<0,05), e menor (p<0,05) mortalidade de reprodutores foram observados para o método SN, enquanto que o número de reprodutores não afetou esses parâmetros (p>0,05). Os loci microssatélites amplificados produziram um total de 30 alelos, com tamanhos entre 80 e 225 pb e suas frequências indicaram aumento (p<0,05) da diversidade genética nas progênies, mas baixa diferenciação genética entre os tratamentos (p>0,05). **Conclusão:** Os métodos de desova e números de reprodutores avaliados aumentaram igualmente a diversidade genética das progênies, com baixa diferenciação genética entre tratamentos. Em contraste, as taxas de fecundação, eclosão e mortalidade de reprodutores revelaram que SN obteve a melhor eficiência reprodutiva, fato relacionado com o estresse de manipulação e injurias causadas por ST. Por isso, SN se mostrou como o método de desova mais adequado para *B. orbignyanus* em programas de repovoamento.

Palavras-chave: *aquicultura; Brycon orbignyanus; desova; desova por extrusão; diversidade genética; eclosão; eficiência reprodutiva; fertilização; incubação; marcadores microssatélites; mortalidade; peixe; repovoamento; reprodutores.*

Introduction

South American rivers have suffered significant anthropogenic impacts in recent decades. The construction of hydroelectric dams, pollution, and overfishing have strongly affected their natural fish populations (Reynalte-Tataje *et al.*, 2013). To mitigate these impacts, re-stocking programs aimed to release captive-produced progeny can be implemented (Lopera-Barrero *et al.*, 2016). However, these programs may pose genetic risks to wild populations. Mainly, ill-conceived reproduction strategies -such as mating genetically similar individuals- may result in inbreeding (Lopera-Barrero *et al.*, 2014).

Reproductive propagation of captive neotropical fish most often is performed by hormonal induction using two spawning methods: strip or semi-natural. The strip-spawning method consists of hormonal induction, gametes collection by abdominal pressure, incubation, and egg fertilization (Zaniboni-Filho and Nuñer, 2004; Reynalte-Tataje *et al.*, 2013). Semi-natural spawning is characterized by little intervention of the handlers (Lopera-Barrero *et al.*, 2014); after reproductive induction, males and females are placed together in a custom-designed tank with a re-circulation system simulating riverine flow that induces spontaneous spawning, and the eggs are collected by a pipe system that deposits them directly in the incubators (Zaniboni-Filho and Nuñer, 2004; Castro *et al.*, 2019).

Some studies have shown the advantages of semi-natural over strip-spawning on reproductive efficiency and genetic diversity. For example, Zanoni *et al.* (2016) obtained 87.2 and 8.17% viable eggs for *B. orbignyanus* in semi-natural and strip- spawning methods, respectively. Zanoni *et al.* (2019) noted that the percentages of *Salminus brasiliensis* females who survived after reproduction procedures were 100 and 62.5% for semi-natural and strip-spawning, respectively. Those researchers associated their results with the higher stress suffered by strip-spawned fish. Other studies have suggested that some individuals produce more larvae in

a mating than others in some South American species. Doubling male and female numbers in strip-spawning practices could decrease this reproductive dominance (Souza *et al.*, 2018). However, the effect of spawning methods and the number of breeders on the genetic diversity of *B. orbignyanus* progeny is not completely known.

Therefore, the objective of this study was to evaluate the influence of the reproductive method and number of breeders on the reproductive efficiency and genetic diversity of *B. orbignyanus* progeny intended for restoration of wild stocks.

Materials and Methods

Ethical considerations

The methodologies used complied with the National Council's Ethical Guidelines for the Control of Animal Experimentation and were approved by the Ethics Committee on the use of animals of the State University of Londrina, Parana, Brazil (CEUA/UEL 17156.2012.50).

Reproductive efficiency

The study was conducted between November and December 2014, the natural reproduction period of this species. A factorial design was used, consisting of two spawning methods (semi-natural or strip-spawning), two different number of breeders (1♀:3♂ and 2♀:6♂) at the same-sex ratio (1♀:3♂), and three replicates. Females (935 ± 0.19 g) and males (915 ± 0.08 g) of *B. orbignyanus* were selected from the broodstock maintained at AES-Tiete fish farm (Promissao, Sao Paulo, Brazil) based on reproductive traits (abdominal diameter, sperm production and color of the urogenital papilla). The animals were fed a 32% protein commercial extruded diet during the experiment (Guabi nutrition, Orlandia, Sao Paulo, Brazil). Water quality, temperature (26.92 ± 0.94 ºC) and oxygen (5.1 ± 0.12 mg/L) were monitored daily in the breeding tanks.

To induce spawning, females were injected with 5.5 mg kg^{-1} carp pituitary extract (CPE) in

two applications (10% in the first dose, and 90% 12 hours later). Males received 2.5 mg kg^{-1} in a single dose concurrently with the second dose administered to females (Lopera-Barrero *et al.*, 2014). In strip-spawning, after the second dose of hormonal induction, the breeders were held separately and approximately six hours later caught, their urogenital papilla dried to prevent contamination, and subjected to gentle cephalo-caudal abdominal massage to release oocytes on females and sperm on males. Then, gametes were mixed (50 g of oocytes + 1 mL of semen) in the presence of water (twice the volume of oocytes), and the eggs transferred to incubators (Lopera-Barrero *et al.*, 2014). In semi-natural spawning, after second hormone administration, the breeders were housed in a circular tank with a forced-circulation water system designed to induce semi-natural spawning conditions. Post spawning (about six hours after pituitary injection), the eggs were collected via an outlet pipe at the center of the spawning tank and transferred to incubators (Castro *et al.*, 2019).

Fertilization and hatching rates were calculated in duplicate by counting the number of viable embryos six hours and number of hatched larvae 12 hours after fertilization (n=200) (Godinho, 2007). The percentage of breeder mortality (males and females) at 24 h post-breeding was also recorded.

Genetic diversity

Caudal fin clips (about 1 cm²) of all (n=24) brood fish and 360 (n=90/treatment) 36 h post-hatch larvae were sampled for genetic analysis. Samples were preserved in 70% ethyl alcohol. The DNA of breeders and larvae was extracted using the sodium chloride and alcohol-based protocol described by Lopera-Barrero *et al.* (2008). After extraction, DNA was quantified in a PICODROP® spectrophotometer (Picodrop Limited, Hinxton, United Kingdom), and standardized to 10 ng/µL final concentration. DNA integrity was assessed in 1% agarose gel, stained with SYBR Safe™ DNA Gel Stain (Invitrogen, Carlsbad, CA, USA).

Eight heterologous microsatellite loci were evaluated: two (BoM5, BoM13) described for *Brycon opalinus* (Barroso *et al.*, 2003), five (Bh5, Bh6, Bh8, Bh13, Bh16) described for *Brycon hilarii* (Sanches and Galeti Jr., 2006), and one (Bc48-10) described for *Brycon insignis* (Matsumoto and Hilsdorf, 2009). Amplifications were performed in a thermocycler Veriti® (Applied Biosystems®) using Platinum Taq DNA Polymerase (Invitrogen, Carlsbad CA, USA) for a 15 µL final reaction volume, with 1X buffer Tris-KCl, 2.0 mM MgCl2, 0.8 µM of each primer (forward and reverse), 0.4 mM of each dNTP, one unit of Platinum Taq DNA Polymerase and 20 ng of DNA (Invitrogen, Carlsbad, CA, USA), according to Castro *et al.* (2017). The amplified products were subjected to 10% denaturing polyacrylamide gel electrophoresis (0.5X TBE buffer) for seven hours at 180 V. The gel was then stained with silver nitrate according to Bassam *et al.* (1991), and photographed on a Gel Doc EZ Imager (BioRad, USA). The number and size of alleles were recorded by Kodak EDAS 290-software using a 50 bp DNA ladder (Invitrogen, Carlsbad, CA, USA).

Statistical analysis

The reproductive efficiency assessed by fertilization, hatching and brood fish mortality rate was determined by analysis of variance using a randomized 2x2 factorial design. The variables studied were tested for normal distribution and the averages were compared by Tukey test using the ExpDes package (Ferreira *et al.*, 2018) of the R statistical program. The allele frequency (number and size) was calculated for each locus using the FSTAT 2.9.4 software (Goudet, 2005). The presence of null alleles was tested by the Micro-Checker software (Van Oosterhout *et al.*, 2004). Observed and expected heterozygosity, Hardy-Weinberg equilibrium and Shannon index, were calculated for each locus using the GENEPOP 1.2 software (Raymond and Rousset, 1995). Analysis of molecular variance and Genetic differentiation was conducted with the Arlequin 3.0 software (Excoffier *et al.*, 2005).

Results

Reproductive efficiency

The interaction between spawning method and number of breeders was not significant (p>0.05); thus, only main effects are presented and discussed. The best fertilization and hatching rates (p<0.05) were observed for semi-natural (71.12 and 61.23 %) compared to strip-spawned (53.93 and 40.29 %), whereas the number of breeders (1♀:3♂ or 2♀:6♂) did not affect (p>0.05) these parameters within spawning method (Table 1).

Despite the great difference in female mortality rate between semi-natural (33.33%) and strip-spawned (75.00%), this was not statistically relevant (p>0.05). In contrast, male mortality rates were higher (p<0.05) for strip-spawned. Once again, the number of breeders did not affect the male mortality rates (p>0.05) (Table 1).

Genetic diversity

The eight amplified microsatellite loci produced 30 alleles, ranging from three (Bh5 and Bh13) to four alleles per locus (BoM5, BoM13, Bh6, Bh8, Bh16 and Bc48-10), with size between 80 (Bc48-10) and 225 bp (Bh5). Exclusive alleles were not found. The analysis of allele frequencies showed the maintenance and/or increase of genetic diversity between breeders and progenies in all treatment groups, with both sharing more than 80% of the most frequent alleles. Null alleles were found for six loci (BoM5, BoM13, Bh5, Bh6, Bh8 and Bh16), mainly in BoM13, where the 140 bp allele did not appear in the breeders, but was present in all progenies (Table 2).

Higher values for expected heterozygosity (He) compared to observed heterozygosity (Ho) were found in all groups, except for 1♀:3♂ semi-natural breeders. The highest average of He and Ho values was obtained for 1♀:3♂ strip progeny (0.669) and 1♀:3♂ semi-natural breeders (0.594). Deviation from the Hardy-Weinberg equilibrium (p<0.05) was observed only in 1♀:3♂ strip breeders (Table 3). The Shannon index values ranged from 0.8378 for 1♀:3♂ semi-natural breeders to 1.1877 for 1♀:3♂ strip progenies, with the progenies, always having higher values than their parents. Similarly, the inbreeding coefficient (Fis) ranged from -0.027 for 1♀:3♂ semi-natural breeders to 0.269 for 1♀:3♂ semi-natural progenies (Table 3). Molecular variance analysis showed that most of the variation was within rather than between groups, with low genetic differentiation (Fst), 0.004 to 0.046, among groups (Wright, 1978) (Table 3).

Table 1. Fertilization, hatching, and mortality rates of brood *B. orbignyanus* subjected to two spawning methods with different number of breeders.

Spawning method	Spawn quality (%)		Brood fish mortality (%)	
	Fertilization	Hatching	Female	Males
Semi-natural spawning	71.12a	61.23a	33.33	8.33b
Strip-spawning	53.93b	40.29b	75.00	55.55a
Number of breeders				
1♀:3♂	62.20	49.25	50.00	33.33
2♀:6♂	62.85	52.28	58.33	30.55
Probability				
Fertilization method	**0.04489**	**0.00322**	0.15250	**0.00066**
Number of breeders	0.93014	0.56542	0.75992	0.75998
Interaction	0.36189	0.46911	0.75992	0.75970
Coefficient of variation (%)	20.05	17.23	84.27	47.62

Different letters within columns represent differences (p<0.05) between spawning methods.

Table 2. Number (N), size (S), and allele frequencies of *B. orbignyanus* subjected to two spawning methods with two different number of breeders.

Locus	N	S	Strip-spawning				Semi-natural spawning			
			1♀: 3♂		2♀: 6♂		1♀:3♂		2♀:6♂	
			Breeders	**Progeny**	**Breeders**	**Progeny**	**Breeders**	**Progeny**	**Breeders**	**Progeny**
BoM5	04	107	**0.000**	**0.183**	**0.000**	**0.213**	0.250	0.161	0.062	0.337
		112	0.250	0.500	0.250	0.410	0.250	0.458	0.375	0.331
		117	0.562	0.256	0.562	0.292	0.250	0.327	0.375	0.230
		122	0.188	0.061	0.188	0.084	0.250	0.054	0.188	0.101
BoM13	04	140	**0.000**	**0.072**	**0.000**	**0.142**	**0.000**	**0.017**	**0.000**	**0.046**
		145	0.562	0.333	0.562	0.318	0.875	0.461	0.750	0.420
		150	0.188	0.256	0.188	0.352	0.125	0.522	0.188	0.414
		160	0.250	0.339	0.250	0.188	0.000	0.000	0.062	0.121
Bh5	03	205	0.125	0.333	0.125	0.176	**0.000**	**0.256**	0.188	0.106
		215	0.750	0.450	0.750	0.659	0.875	0.506	0.625	0.800
		225	0.125	0.217	0.125	0.165	0.125	0.239	0.188	0.094
Bh6	04	180	0.312	0.500	0.312	0.589	0.500	0.656	0.625	0.478
		185	0.500	0.194	0.500	0.222	**0.000**	**0.100**	0.125	0.128
		190	0.125	0.172	0.125	0.189	0.125	0.139	0.188	0.250
		195	**0.000**	**0.133**	0.000	0.000	0.375	0.106	0.062	0.144
Bh8	04	185	0.688	0.183	0.688	0.572	0.625	0.386	0.750	0.652
		195	0.188	0.489	0.188	0.178	0.125	0.375	0.188	0.174
		205	0.062	0.233	0.062	0.161	0.250	0.238	**0.000**	**0.146**
		215	0.062	0.094	0.062	0.072	0.000	0.000	0.062	0.028
Bh13	03	150	0.125	0.292	0.125	0.227	0.333	0.456	0.250	0.178
		160	0.625	0.376	0.625	0.477	0.667	0.544	0.625	0.700
		165	0.250	0.331	0.250	0.295	0.000	0.000	0.125	0.122
Bh16	04	150	0.071	0.300	0.071	0.233	**0.000**	**0.300**	0.188	0.128
		160	0.714	0.472	0.714	0.572	0.250	0.244	0.438	0.461
		165	0.143	0.094	0.143	0.100	0.250	0.300	0.188	0.144
		170	0.071	0.133	0.071	0.094	0.500	0.156	0.188	0.267
Bc48-10	04	80	0.214	0.417	0.214	0.268	0.167	0.311	0.125	0.299
		85	0.500	0.306	0.500	0.451	0.500	0.456	0.500	0.384
		90	0.143	0.211	0.143	0.201	0.333	0.233	0.312	0.244
		95	0.143	0.067	0.143	0.079	0.000	0.000	0.062	0.073

Bold: Null alleles.

Table 3. Observed heterozygosity (Ho), expected heterozygosity (He), Shannon index (H'), inbreeding coefficient (Fis), analysis of molecular variance (AMOVA), and genetic differentiation (Fst) of *B. orbignyanus* subjected to two spawning methods with a different number of breeders.

			Ho	He	H'	Fis	AMOVA (Within/Between groups)	Fst
ST	1♀:3♂	Breeders	0.8570	0.5420*	0.6050	0.133	96.12/3.88	0.0040
		Progeny	1.1877	0.5730	0.6690	0.145		
	2♀:6♂	Breeders	0.9742	0.5670	0.5810	0.026	97.37/2.63	0.0260
		Progeny	1.1408	0.4580	0.6310	0.276		
SN	1♀:3♂	Breeders	0.8378	0.5940	0.5780	-0.027	95.38/4.62	0.0460
		Progeny	1.0564	0.4500	0.6150	0.269		
	2♀:6♂	Breeders	0.9922	0.5150	0.5920	0.136	98.22/1.68	0.0170
		Progeny	1.0754	0.4850	0.5940	0.185		

ST: Strip-spawning; SN: Semi-natural spawning. * Significantly different to Hardy-Weinberg equilibrium ($p < 0.05$).

Discussion

Reproductive efficiency

Qualitative spawning parameters obtained during reproductive management of fish can be very variable, mainly due to individual breeding conditions and spawning method. First, the greatest stress suffered by animals subjected to the strip-spawning results in considerable metabolic changes, such as cortisol and glucose levels, as well as hematological parameters, which directly affect gamete quality and breeder mortality (Zanoni *et al.*, 2016). Furthermore, in strip-spawning oocytes can be collected immature or in late to final maturation (Samarin *et al.*, 2015), besides being prone to contamination by feces, blood, or urine (Zaniboni-filho and Nuñer, 2004) that could impair fertilization and hatching rates.

The best fertilization and hatching rates observed for semi-natural spawning in our study is consistent with previous reports. For example, Reynalte-Tataje *et al.* (2013), working with native Brazilian species, showed that this method had better egg quality than strip-spawned, with 98.7 and 14.7% average fertilization rates, respectively. Zanoni *et al.* (2019) obtained 91.7% fertilization rate for semi-natural, and only 40.6% for strip-spawning in *Salminus brasiliensis*. For *B. orbignyanus*, Zanoni *et al.* (2016) obtained

87.2 and 8.17% viable eggs in semi-natural and strip-spawning methods, respectively.

The number of breeders did not affect fertilization or hatching rates within spawning method. Improvement was expected, due to the increase in the number of animals may compensate the reduction of the damage caused by the non-participation of animals in the reproductive process, because they are not yet able to reproduce or have poor gamete quality (Castro *et al.*, 2019).

Mortality of breeders during reproductive handling can be detrimental to stock recovery plans and should be avoided, especially when the species is at risk of extinction. Mortality is highly affected by handling stress -especially in sensitive species, such as *B. orbignyanus*- and injuries caused by stripping -such as loss of scales- which favors fungi and bacteria proliferation leading to animal death (Zanoni *et al.*, 2019; Castro *et al.*, 2019). Sato *et al.* (1997) attributed 83.3% death of stripped breeders to lesions and hyperemia accompanied with secondary bacterial and fungal infections. We observed high mortality of strip-spawned males. Regarding females, although there was no statistical difference, the strip-spawning method also showed greater mortality; the small number of animals in the 1♀:3♂ groups may have contributed to this lack of

significance. Reynalte-Tataje *et al.* (2013) also found mortality rates similar to those observed in this study, with up to 100% mortality of *B. orbignyanus* breeders used in strip-spawning. Zanoni *et al.* (2019) noted that the percentage of *Salminus brasiliensis* females that survived after reproduction procedures was 100 and 62.5% for semi-natural and strip-spawning, respectively; notably, all the females died 24 h after strip-spawning.

Genetic diversity

The size and number of alleles per locus produced by the eight amplified microsatellite loci were similar to previous studies with the same heterologous markers in *B. orbignyanus* (Castro *et al.*, 2019). The absence of alleles exclusive to any of the groups suggests there was uniformity of contribution by breeders to progeny across treatments regardless of spawning method (Lopera-Barrero *et al.*, 2014). The presence of null alleles can be related to mutations in the primers' binding region, preventing the annealing during PCR, resulting in unexpected size or no gene product (Chapuis and Estoup, 2007). Often, non-specific microsatellite, such as heterologous, can result in null alleles (Henriques *et al.*, 2017). However, null alleles did not affect heterozygosity in these groups.

The higher values for expected compared to observed heterozygosity in all groups were anticipated considering the dwindling number of the fish in the wild and limited number of breeders available for restocking programs of this fish (Romana-Eguia *et al.*, 2004). In general, the mean values of He and Ho were similar to recent studies with the genus *Brycon* (Bignardi *et al.*, 2016; Castro *et al.*, 2019).

The deviation from the Hardy-Weinberg equilibrium observed only in the 1♀:3♂ strip-spawned may be common in captive stocks, given the possible genetic drift of these animals, which are isolated and differentiate in these environments (Wahlund effect) or by non-random sampling of animals for reproduction (Romana-Eguia *et al.*, 2004). However, the heterozygous deficit observed indicates a likehood of a decline in genetic diversity in the next generations. Therefore, breeder replacement or acquisition is recommended before this occurs (Souza *et al.*, 2018).

The Shannon index and inbreeding coefficient indicate increase of genetic diversity in the progenies of all treatments. Lopera-Barrero *et al.* (2010) observed similar results, with increased Shannon index values and expected progeny heterozygosity, that involved individual mating of 1♀:1♂ of *B. orbignyanus* in semi-natural spawning. On the other hand, analysis of molecular variance and genetic differentiation (Fst) revealed low genetic differentiation between treatments since most of the variation is present within treatments and the Fst had close-to-zero values.

Implications for restocking programs

Genetic monitoring in conservation programs is necessary to optimize reproductive strategies to minimize mortality of broodfish and improve genetic diversity of wild populations. High mortality observed in strip-spawning can reduce future genetic diversity. This is more critical for highly endangered species, which will inevitably force mating between related animals, increasing the inbreeding rate and reducing progeny's survival and adaptability. Genetic analysis, even without differences between treatments -as observed in this study-, are very important for restocking programs since it provides essential information for reproductive management, such as incorporation or replacement of breeders that contribute with adequate genetic variability of progeny. Constant genetic monitoring of the next generations of progeny is strongly recommended. For all these reasons, the results of this study show that semi-natural spawning is the most suitable reproduction method for *B. orbignyanus* in restocking programs.

Declarations

Conflicts of interest

The authors declare they have no conflicts of interest with regard to the work presented in this report.

Author contribution

PLC, NMLB and RPR conceived and designed the experiment. PLC, ESRG and FPS conducted the experiment. PLC, ESRG and FPS did the laboratory analyses. PLC, NMLB and ALJF did the bioinformatic analysis. PLC, FPS, SCB and ALJF wrote the manuscript. PLC, SCB, ALJF and RPR did the final revision.

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