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Evidence of polygamy in the socially monogamous Amazonian fish *Arapaima gigas* (Schinz, 1822) (Osteoglossiformes, Arapaimidae)

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Arapaima gigas is one of the largest freshwater fishes of the world. It is socially monogamous, forming pairs, constructing a nest and providing parental care. We performed a paternity analysis under three scenarios in captive, semi-natural and natural areas using 10 microsatellite markers. As a positive control, we analyzed three pairs and their offspring isolated individually in artificial breeding ponds (*a priori* very high probability of monogamy). We then analyzed two samples of offspring from large artificial ponds with multiple adults but only one reproductive pair (*a priori* high probability of monogamy), two samples from semi-natural breeding station with multiple adults but only one reproductive pair (*a priori* high probability of monogamy), and a sample from a natural lake with multiple adults, some potentially breeding (*a priori* medium probability of monogamy). Analysis of patterns of Mendelian heredity suggested an extra-pair contribution for all broods except the positive controls. Similarly, results based on multilocus analysis estimated at least two sib-groups per nest. These results reject monogamy as a system of breeding in *Arapaima gigas*. From a management perspective, this behavior may be exploited to maintain genetic diversity in captive and as well in wild populations of *Arapaima gigas*.

O pirarucu *Arapaima gigas* é um dos maiores peixes de água doce do mundo. É socialmente monogâmico, forma casais, constrói ninhos e fornece cuidado parental. Com o objetivo de acessar o sistema de acasalamento do pirarucu, analisamos três cenários: em áreas de cativeiro, semi-naturais e naturais, utilizando 10 marcadores microssatélites. Como controle positivo, analisamos três casais e suas ninhadas isolados em açudes individuais (probabilidade *a priori* muito alta de monogamia). A seguir, analisamos duas amostras de ninhadas de um açude com vários adultos, mas somente um casal reprodutivo (probabilidade *a priori* alta de monogamia), duas amostras de estação de criação semi-natural com vários adultos mas somente um casal reprodutivo (probabilidade *a priori* alta de monogamia), e uma amostra de lago natural com vários adultos alguns potencialmente em fase de reprodução (probabilidade *a priori* média de monogamia). Análises de padrões mendelianos de hereditariedade sugerem contribuição extra-par para todas as ninhadas, exceto as do controle positivo. Similarmente, resultados baseados em análises multilocus realizadas no programa KINALYZER estimaram pelo menos dois grupos-irmãos por ninhada. Nossos resultados rejeitam a monogamia como sistema de acasalamento em *Arapaima gigas*. Da perspectiva de manejo, esse comportamento pode ser explorado para manter a diversidade genética em cativeiro assim como em populações naturais de *Arapaima gigas*.

Key words: Mate choice, Microsatellites, Multiple paternity, Pirarucu, Polygamy.

Introduction

Arapaima gigas (the 'pirarucu' or 'arapaima') is one of the largest freshwater fishes of the world reaching up to three meters, and weighing up to 200 kg. It is also one of the economically most important fish species in the Amazon basin, forming an integral part of the Amazon people's traditional diet, constituting an important food source, and thus making it subject to intense commercial and subsistence exploitation. Indeed, this species became scarce in the 70's and 80's, and in some cases even extinct near large Amazon cities (Goulding, 1980). In 1975, the 'arapaima' was listed in Appendix II of CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora). Currently on the red list of the IUCN (International Union for Conservation of Nature), *Arapaima gigas* is classified

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as "Data Deficient", a category that characterizes a species for which there is not enough knowledge for it to fit into one of the categories. Even though it has been exploited commercially for many years, much basic life history data are still lacking. However, several recent publications have made significant advances of our understanding of growth and reproductive behavior (Arantes *et al.*, 2010), population dynamics (Coutinho *et al.*, 2010; Castello *et al.*, 2011), spatial and temporal distribution (Arantes *et al.*, 2011) and trophic ecology (Watson *et al.*, 2013) as well as population structuring (Hrbek *et al.*, 2005; Hrbek *et al.*, 2007; Araripe *et al.*, 2013).

The first studies of reproductive biology of the *Arapaima gigas* in captivity were conducted by Oliveira (1944) and Fontenele (1948), who reported important biological data such as that it lays its eggs in lentic water, and belongs to the group of species with partitioned spawning and parental care. These data were confirmed by Imbiriba (1994), Imbiriba (2001), and Campos Baca (2001). Furthermore, these authors state that the spawning coincides with the rainy season, which, in the Amazon, extends from December to June. Imbiriba (2001) reports that the arapaima's reproduction is probably related with the water-level variation on the floodplains of the Amazon River. Sexual maturity occurs around the third to fifth year of life, when the fish reach an average length of 1.6 m and weigh 40 - 50 Kg (Lüling, 1964; Godinho *et al.*, 2005; Arantes *et al.*, 2010). Fontenele (1953) observed that during the spawning season secondary extra-genital sexual characteristics develop in the breeders, such as that males begin to present a dark coloring on the upper part of the head and flanks, and the abdomen and caudal peduncle turn reddish. However, as observed by Campos Baca (2001), these features may not always be observed in all specimens. In both sexes only the left testicle/ovary is functional, while the right testicle/ovary is atrophied (Imbiriba *et al.*, 1996; Campos Baca, 2001).

In accordance with Imbiriba (2001), the reproduction of the 'arapaima' occurs in a similar manner whether in nature or in captivity, including courtship, nest construction, mating and care of the offspring. In captivity, the sequence begins with a lack of interest in food on the part of the adults entering reproductive mode. In the following mating phase fights sometimes take place, although it not known if these are for the establishment of reproductive territories of they represent disputes for females. A four to five day period of tranquility follows, disturbed only at long intervals by the slow ascent of one or the other individual to breathe. Thereafter, the reproductive adults can be seen, one at a time, in a vertical position, head pointing down. During this period, the water becomes murky due to the excavation of the nest, which is circular, and commonly known as the "pan". The favorite place for preparation of the nest is the bottom of lakes, with clay soil and without vegetation, and where the water is shallow and still. After preparation of the nest, the female lays her eggs in it, and the male fertilizes them. During the nesting phase, the couple jealously guards the nest from up to a 10 m distance. After eclosion of the eggs, the larvae remain in the nest for five days, closely guarded by the male until absorption of the vitella/yolk. Brauner *et al.* (2004) also suggest that the males mouth brood or at least carry eggs in their mouths, a behavior observed by fishermen in distressed males. The larvae are black and swim over the head and dorsal region of the male protecting them, and after reaching one week of life, they become visible to the naked eye. In this period, they already surface to breathe (Imbiriba *et al.*, 1996). The juveniles are cared for exclusively the male, while the female circles in the vicinity of the group, defending against possible predators. The fry are cared for until their independence at between 35-40 cm in length and 4-5 months of age. During the free-swimming period, the couple swim slowly but continuously, guiding and protecting their offspring in what can be though of as a dynamic territory that can reach 1 ha/day. The size of this territory depends on the availability of food resources and refuge, including vegetation cover and shade. At the end of the parental care phase males are normally very thin, whereas females may have mature gonads and be able to spawn again in the very near future.

Based on what is known of the reproductive behavior of *Arapaima gigas*, it is assumed that the species is monogamous due to formation of couples and investment in parental care, however, monogamy has not been tested explicitly. There are many cases where apparently monogamous species in fact show high incidences of extra-pair mating, and thus levels of polygamy are high across a broad taxonomic range (Goossens *et al.*, 1998; Liebgold *et al.*, 2006; Ophir *et al.*, 2008; Sefc *et al.*, 2008).

The reproductive pattern and reproductive success of males is largely unknown in the majority of fish species. The physical nature of habitat normally hinders direct observation of reproductive behavior and kinship relations. Besides this, most fish species possess external fertilization, increasing the possibility of there occurring sperm competition (Stockley *et al.*, 1997). The study of reproductive behavior in fish species with manifestation of polyandry (a female copulating with various males), polygyny (a male copulating with various females) or the observation of a monogamous reproductive system, can be efficiently evaluated and tested through the use of molecular methods, such as the analysis of DNA microsatellite data. The use of molecular approaches has allowed addressing many questions that would otherwise have been difficult using only field observations. Additionally, molecular markers can be used to quantify the incidence of "hidden" reproductive behaviors, that otherwise is unlikely to be observed in the field.

Considering the importance for the management and conservation of the species, in the present study we assess the mating system of the 'arapaima' under three experimental conditions, which provide different levels of *a priori* confidence in assuming monogamous system of mating. In the first instance, breeding pairs were isolated

from all other individuals of their species (*a priori* very high probability of monogamy). In the second instance, breeding pairs were restricted to an area with other individuals of their own species, but no other breeding adults within the restricted area (*a priori* high probability of monogamy). In the third instance, offspring were collected from breeding pairs in nature (*a priori* medium probability of monogamy). To analyze mating system patterns under these three situations, we perform a parentage analysis of eight clutches of *Arapaima gigas* using 10 highly variable microsatellite markers developed for the 'arapaima' (Farias *et al.*, 2003).

Material and Methods

The samples utilized for this study come from an enclosed system, a breeding station, Fazenda Santo Antônio, in the municipality of Presidente Figueiredo near Manaus, Amazonas, a semi-natural system, a dammed lake in the municipality of Itacoatiara near Manaus, Amazonas, and a natural system, from the São Miguel Island, near the municipality of Santarém, Pará.

At the Fazenda Santo Antônio (FSA), 117 adult 'arapaima' individuals were maintained in breeding stations for approximately five years. Fourteen matched pairs were kept isolated from other adults and potential mates in individual 20 x 20 x 2 m tanks. From these fourteen pairs we sampled three groups of parents and offspring: Couple 1 (both parents plus 16 offspring), Couple 2 (both parents plus 19 offspring), and Couple 3 (both parents plus 23 offspring). With these samples we carried out a control analysis testing for the presence of any additional alleles not compatible with monogamy.

Two additional samples of offspring were obtained from Fazenda Santo Antônio Station, collected in different time periods, which we call brood FSA1 (25 offspring collected), and brood FSA2 (20 offspring collected). These samples were obtained from a communal 200 x 40 x 1.5 m tank containing 117 adults. In the collection period, only one brood under the care of one adult couple was observed at the breeding station.

In the Itacoatiara dammed lake, the breeders were kept for over seven years and there were 68 adults, but the proportion of males and females is not known. Two samples of offspring were obtained from the Itacoatiara dammed lake, collected in different periods, which we call brood Ita1 (20 offspring collected) and brood Ita2 (24 offspring collected). In the period of the two collections, only one brood under the care of one adult couple was observed in the breeding station.

In São Miguel Island (SMI), the offspring were collected from one couple and it was possible to collect only five individuals. Collection of additional individuals would have led to a major disturbance, resulting in the modification of behavior of the adults, and potential loss of all offspring. No other broods or spawning pairs were observed in the vicinity, however, given that this is a natural open system, other breeding adults may have been present in the area.

DNA extraction and genotyping. Total genomic DNA was extracted from scales and muscle tissues of the offspring preserved in 95% ethanol following the Qiagen Kit protocol. The microsatellite *loci* used were those developed by Farias *et al.* (2003): (CAm2, CAm13, CAm15, CAm16, CAm20, CTm3, CTm4, CTm5, CTm7 and Ctm8).

Polymerase Chain Reaction (PCR) was performed in 10 µl reaction volume containing 4.1 µl of MilliQ water, 0.8 µL of $MgCl_2$ (25mM), 0.8 µl of dNTP (2,5 mM for each dNTP), 1.0 µl of 10x Buffer (100 mM of Tris-HCl, 500 mM of KCl), 1.0 µl of each primer (2.0 mM), 0.2 µl of Taq Polymerase (5U/µl) and 1.0 µl of DNA (~10 ng). The PCR reactions were performed in a thermal cycler Thermo (PXE 0.2) with the following steps: initial denaturation at 92º C for 2 minutes, followed by 35 cycles at 92º C for 40 seconds, seconds at the locus specific annealing temperature (according to Table 1 of Farias *et al.*, 2003) and 72º C for 1 minute at 30 seconds. Last step was for 30 seconds a final extension at 72º C. PCR products generated with labeled primers were visualized on a MegaBACE 1000 Fragment Profiler v1.2 software (GE-Healthcare). Allele sizes were scored against the size standard ET-400 ROX (GE-Healthcare).

Allele size variation, observed and expected heterozygosity and Hardy-Weinberg equilibrium were implemented in the program Arlequin 3.5 (Excoffier & Lischer, 2010). These analyses allowed inferring the genetic variability in each family group. Additional genetic parameters like distribution and frequency of each microsatellite *loci* were also implemented in Genetix v.4 (Belkhir *et al.*, 2004).

Parentage Analysis. We used two tests to estimate the efficiency of the markers in detecting multiple paternities in 'arapaima': the probability of genetic identity for each locus (I) and the probability of paternity exclusion for each locus (Q). Additionally, we estimated the joint probability of genetic identity – IC (Paetkau *et al.*, 1995) and the joint probability of paternity exclusion method – QC (Weir, 1996) for each test, respectively.

For the parentage analysis we implemented three methods. First, we used a simple method based in the Mendelian genetics of counting the number of observed alleles, and comparing it to the expected number of alleles in each nest (Myers & Zamudio, 2004). The maximum number of expected alleles in each nest under the assumption of monogamy is four, unless one observes homozygous progeny (Fitzsimmons, 1998; Valenzuela, 2000). Each homozygous genotype indicates that alleles are shared between parents, and therefore the number of expected alleles decrease to three with one homozygous genotype, and to two with two homozygous genotypes.

Considering that the counting method does not consider the combination of all alleles across the different *loci*, and is therefore likely to underestimate the number of sires contributing to the brood, we carried out a second analysis using the program KINALYZER (Berger-Wolf *et al.*, 2007)

which analyzes all *loci* simultaneously making use of a minimum 2-allele set cover approach based on Mendelian properties and parsimoniously finds the smallest number of sibling groups (Berger-Wolf *et al.*, 2007). The inference of full sibling groups (groups of individuals sharing the same two parents) is possible even with no *a priori* knowledge of parental genotypes, as this program performs a heuristic analysis based on allelic inheritance in diploid organisms – every offspring inherits one allele at each *locus* from each of its parents.

We also use the program ML-Relate (Kalinowski *et al.*, 2006) to estimate the relationships among individuals of the same clutch. This program uses maximum likelihood estimates of relatedness to classify pairs of individuals as unrelated, half-sibs, full-sibs, or parent-offspring relationships (Jones *et al.*, 2010).

Results

Statistical power and genetic diversity parameters. The microsatellite *loci* had high statistical power to discriminate between individuals and exclude paternity. The probability of genetic identity (I) varied from 0.09657709 (CTm7 in Ita2) to 0.50743744 (Cam13 in FSA) while the joint probability of genetic identity (IC) ranged from 3.5 x 10^{-6} (FSA2) to less than 1 x 10^{-9} (FSA2), demonstrating the high discriminatory power of these *loci*. On the other hand, the probability of paternity exclusion (Q), varied from 0.15687672 (CTm8 in FSA) to 0.56049347 (CTm7 in Ita2) while the joint probability of exclusion (QC) ranged from 0.975539087 (FSA1) to 0.99958039 (SMI) again demonstrating the high discriminatory power of these *loci* (Table 1). In other words, the set of markers used for this analysis of the relationship among the 'arapaima' offspring proved to be very robust for the analyses performed.

Table 1. Main genetic pattern for each *loci* analysed in each brood of *Arapaima gigas*. A = number of alleles, Ho = observed heterozygosity, I = probability of genetic identity, Q = probability of paternity exclusion, IC = Joint probability of genetic identity at all *loci*, QC = Joint probability of paternal exclusion at all *loci*. Ita1 = Itacoatiara 1, Ita2 = Itacoatiara 2, FSA1 = Fazenda Santo Antônio 1, FSA2 = Fazenda Santo Antônio 2, SMI = São Miguel Island.

Locus	Brood	A	Ho	I	Q
	Ita1	3	0.67	0.20344499	0.38345932
	Ita2	4	0.71	0.20017117	0.39057430
CAm2	FSA1	3	0.96	0.21937219	0.36396933
	FSA2	3	0.52	0.33972743	0.33277032
	SMI	7	1.00	0.04600000	0.84882000
	Ita1	3	0.90	0.19287109	0.39761353
	Ita2	3	0.42	0.22394993	0.36119489
CAm13	FSA1	4	0.36	0.50743744	0.16072177
	FSA2	4	0.61	0.24703503	0.45878767
	SMI	5	1.00	0.09720000	0.71690000
	Ita1	3	1.00	0.21234609	0.37251904
	Ita2	3	1.00	0.23607452	0.34785121
CAm15	FSA1	3	1.00	0.21296736	0.37185680
	FSA2	3	0.94	0.23984910	0.45613258
	SMI	3	0.60	0.28540000	0.41243400
	Ita1	2	0.55	0.44100234	0.17949980
	Ita2	3	0.54	0.42060755	0.19638795
CAm16	FSA1	2	0.60	0.40453216	0.19922232
	FSA2	3	1.00	0.33462692	0.33601261
	SMI	3	0.80	0.28540000	0.41243400
	Ita1	7	0.39	0.23398590	0.35992771
	Ita2	7	0.58	0.13866669	0.47938601
CAm20	FSA1	1	monomorphic	-	-
	FSA2	2	0.53	0.44997352	0.24355883
	SMI		0.80	0.14040000	0.65007600
	Ita1	2	0.45	0.48493984	0.15917168
	Ita2	3	0.42	0.35978129	0.23695979
CTm3	FSA1	4	0.80	0.18546624	0.40934769
	FSA2	2	0.20	0.68860000	0.14387400
	SMI	2	0.80	0.38560000	0.27494400
	Ita1	2	0.60	0.42460000	0.18795000
	Ita2	2	0.46	0.48059870	0.16097246
CTm4	FSA1	2	0.48	0.47001856	0.16576512
	FSA2	3	0.55	0.35408359	0.35804720
	SMI	1	monomorphic	1	monomorphic
	Ita1	4	1.00	0.11743594	0.51773691
	Ita2	5	1.00	0.10322760	0.54627331
CTm5	FSA1	4	1.00	0.10962528	0.53270936
	FSA2	7	0.81	0.06602096	0.79509603
	SMI	4	1.00	0.11580000	0.66544200
	Ita1	4	1.00	0.11697253	0.51867430
	Ita2	5	1.00	0.09657709	0.56049347
CTm7	FSA1	4	1.00	0.11063136	0.53073344
	FSA2	5	0.50	0.16963593	0.59538968
	SMI	1	1.00	0.11580000	0.66544200
	Ita1	2	0.45	0.48493984	0.15917168
	Ita2	2	0.63	0.41757202	0.19191183
CTm8	FSA1	2	0.44	0.49027936	0.15687672
	FSA2	2	0.60	0.42460000	0.25506600
	SMI	1	monomorphic	1	monomorphic
All loci	Brood	A	Ho	IC=	QC=
	Ita1	3.2	0.70	0.00000118	0.98000000
	Ita2	3.7	0.71	0.00000044	0.98887951
	FSA1	2.9	0.74	0.00000000	0.97553909
	FSA2	3.4	0.66	0.00000351	0.99665175
	SMI	3.5	0.70	0.00000026	0.99958039

The average genetic diversity for all the *loci* in each locality varied from 0.486 (FSA) to 0.583 (Itacoatiara 2) (Table 2). Wright's endogamy index (F_{IS}) was low and not significant in any of the broods. Of the ten microsatellite *loci* genotyped, the *loci* CTm4 and CTm8 had the lowest number of alleles in all the broods (Table 1). *Locus* Cam20 was monomorphic in the FSA brood, and CTm4 and CTm8 were monomorphic in the SMI brood. In most cases, the remaining *loci* showed a considerable degree of polymorphism.

Parentage Analysis. The control analyzes of the three pairs and their offspring sampled from FSA showed that all the juveniles were offspring of their hypothetical parents. There were no new alleles detected, and all *loci* and alleles followed correct Mendelian inheritance patterns (results not shown). Monogamy was, therefore, detected in the experimental set-up where monogamy was the only expected system of mating, given that the adult couple was isolated from all other adults.

Considering the set of alleles found in the groups of young in each nest, and assuming absence of information on the parents, we began with a simple analysis based on the patterns of Mendelian inheritance. Considering that each offspring possesses 50% of the maternal and 50% of the paternal genome, and each parent can be heterozygous at any given *locus*, the total number of alleles at a *locus* observed in each brood can be at most four. The presence of five or more alleles in a brood suggests a possible extra contribution in addition to the parents (two maternal alleles, two paternal alleles, and the additional allele(s) from an extra-pair individual). Out of the *loci* analyzed in this study, five presented five or more alleles in the broods analyzed: Ita1, *locus* CAm20 (7 alleles); Ita2, *loci* CAm20 (7 alleles), CTm5 (5 alleles), CTm7 (5 alleles); FSA2, *loci* CTm5 (7 alleles), CTm7 (5 alleles); SMI, *loci* CAm2 (7 alleles), CAm13 (5 alleles) (Table 3). The FSA1 brood had a maximum of four alleles at each *locus*.

Table 2. Main genetic pattern for each brood analysed in *Arapaima gigas*. Note: H_O= observed heterozygosity, H_E= expected heterozygosity, F_{IS} = fixation index.

Broods	N	Gene Diversity	Average number of alleles	H_O-H_E (Mean)	F_{IS} (P>0.05)
Itacoatiara 1	20	0.528±0.288	3.2	0.701-0.558	-0.30705
Itacoatiara 2	24	0.583±0.314	3.7	0.713-0.583	-0.22887
Fazenda Santo Antônio 1	25	0.486±0.267	2.9	0.664-0.496	-0.35982
Fazenda Santo Antônio 2	20	0.446±0.249	3.4	0.662-0.528	-0.36886
São Miguel Island	5	0.591±0.348	4.1	0.875-0.738	-0.29327

Table 3. Alleles frequencies (allele size/frequency) and number of homozygotes (allele size/number of homozygote individuals) observed in each brood of *Arapaima gigas*. The number of sib-groups inferred in Kinalyzer is also listed for each brood. N = number of offspring. NHm = number of homozygote individuals.

Itacoatiara 1 (N=20) – Kinalyzer Sibgroup = 3									
CAm2	CAm13	CAm15	CAm16	CAm20	CTm3	CTm4	CTm5	CTm7	CTm8
Alleles	Alleles	Alleles	Alleles	Alleles	Alleles	Alleles	Alleles	Alleles	Alleles
298/0.278	305/0.375	231/0.500	256/0.725	264/0.056	296/0.225	280/0.300	260/0.175	281/0.184	277/0.775
312/0.472	319/0.375	247/0.225	258/0.275	266/0.667	300/0.775	286/0.700	274/0.225	295/0.211	279/0.225
322/0.250	339/0.250	249/0.275		274/0.028			280/0.275	301/0.289	
				276/0.083			284/0.325	305/0.316	
				278/0.056					
				284/0.055					
				288/0.055					
allele/NHm				allele/NHm	allele/NHm	allele/NHm			allele/NHm
312/5				266/11	300/11	280/8			277/11
298/1									

Itacoatiara 2 (N=24) – Kinalyzer Sibgroup = 4									
CAm2	CAm13	CAm15	CAm16	CAm20	CTm3	CTm4	CTm5	CTm7	CTm8
Alleles	Alleles	Alleles	Alleles	Alleles	Alleles	Alleles	Alleles	Alleles	Alleles
298/0.271	305/0.479	231/0.500	254/0.021	262/0.021	296/0.292	280/0.229	260/0.250	281/0.250	277/0.687
310/0.042	319/0.167	247/0.354	256/0.729	264/0.187	298/0.042	286/0.771	274/0.292	293/0.042	279/0.313
312/0.521	339/0.354	249/0.146	258/0.250	266/0.500	300/0.666		280/0.208	295/0.292	

Itacoatiara 2 (N=24) – Kinalyzer Sibgroup = 4									
CAm2	CAm13	CAm15	CAm16	CAm20	CTm3	CTm4	CTm5	CTm7	CTm8
322/0.166				268/0.104			282/0.021	301/0.208	
				270/0.146			284/0.229	305/0.208	
				276/0.021					
				278/0.021					
allele/NHm	allele/NHm		allele/NHm	allele/NHm	allele/NHm	allele/NHm			allele/NHm
312/7	305/5		256/11	266/10	300/11	286/13			277/9
					298/1				
					296/2				

Fazenda Santo Antônio 1 (N=25) – Kinalyzer Sibgroup = 2									
CAm2	CAm13	CAm15	CAm16	CAm20	CTm3	CTm4	CTm5	CTm7	CTm8
Alleles	Alleles	Alleles	Alleles	Alleles	Alleles	Alleles	Alleles	Alleles	Alleles
298/0.522	305/0.020	231/0.280	254/0.660	266/1	286/0.220	280/0.240	256/0.260	277/0.260	277/0.780
316/0.217	319/0.820	239/0.220	256/0.340		296/0.340	286/0.760	268/0.240	289/0.240	279/0.220
326/0.261	321/0.140	247/0.500			298/0.020		276/0.260	297/0.280	
	339/0.020				300/0.420		280/0.240	301/0.220	
allele/NHm	allele/NHm		allele/NHm	allele/NHm	allele/NHm	allele/NHm			allele/NHm
298/1	319/17		254/9	266/25	300/5	286/13			277/14

Fazenda Santo Antônio 2 (N=20) – Kinalyzer Sibgroup = 4									
CAm2	CAm13	CAm15	CAm16	CAm20	CTm3	CTm4	CTm5	CTm7	CTm8
Alleles	Alleles	Alleles	Alleles	Alleles	Alleles	Alleles	Alleles	Alleles	Alleles
300/0.029	305/0.417	229/0.132	244/0.500	266/0.263	300/0.900	280/0.075	256/0.125	277/0.225	277/0.700
314/0.559	321/0.028	231/0.447	246/0.471	270/0.737	302/0.100	286/0.700	260/0.188	289/0.025	279/0.300
324/0.412	325/0.083	247/0.421	248/0.029			288/0.225	266/0.031	297/0.100	
	331/0.472						268/0.031	299/0.125	
							276/0.125	301/0.525	
							278/0.219		
							280/0.281		
allele/NHm	allele/NHm			allele/NHm	allele/NHm	allele/NHm	allele/NHm	allele/NHm	allele/NHm
314/2	305/4			270/9	300/16	286/9	280/3	277/2	277/8
	331/3							299/1	
								301/7	

São Miguel Island (N= 5) – Kinalyzer Sibgroup = 3									
CAm2	CAm13	CAm15	CAm16	CAm20	CTm3	CTm4	CTm5	CTm7	CTm8
Alleles	Alleles	Alleles	Alleles	Alleles	Alleles	Alleles	Alleles	Alleles	Alleles
0.200/312	0.300/305	0.600/231	0.300/256	0.100/242	0.600/300	1.00/278	0.200/260	0.200/279	1.00/277
0.100/314	0.300/319	0.100/245	0.600/258	0.100/246	0.400/302		0.200/268	0.200/287	
0.100/316	0.200/329	0.300/247	0.100/260	0.500/264			0.300/276	0.300/295	
0.100/322	0.100/331			0.100/266			0.300/280	0.300/299	
0.100/324	0.100/333			0.200/268					
0.200/326									
0.200/328									
		allele/NHm	allele/NHm	allele/NHm	allele/NHm				
		231/1	258/1	264/1	300/1				

The presence of homozygous genotypes in the offspring is also important in inferring of how many adult individuals could be contributing genetically to each group of offspring (Table 3), mainly in cases where there occur three or four alleles per *locus*. For example, a shared parental allele may be inferred when an offspring is homozygous for a given locus (*e.g.*, offspring genotype = AA; parental genotype = A–) which leads to the expectation of only three alleles being observed in a brood. Similarly, the complete parental genotype may be inferred, when the offspring are homozygous for two different alleles (*e.g.*, offspring genotypes = AA and BB; parental genotype = AB) which leads to the expectation of only two alleles being observed in a brood. Additional alleles will have to have been contributed via extra-pair mating events. For example, upon analysis of the locus CAm2 in the brood Ita1, we observe three alleles (298, 312, 322) and two types of homozygotes: five offspring 312/312 and one offspring 298/298. Based on the presence of these homozygotes, we can infer that the genotype of the parents is 298/312, and that the appearance of allele 322 observed in the brood took place by extra-pair contribution of at least one individual 322/-. This individual may have been a homozygote (322/322), a heterozygote sharing the second allele with the other same sex parent (322/312 or 322/298), or a heterozygote where the second allele was not passed on or not detected in our sample. Using this analysis, extra-pair contribution was also detected in the broods Ita2, FSA1 and FSA2 (Table 3).

In summary, extra-pair contribution was observed in all broods. In the broods Ita1, Ita2, FSA2 and SMI the presence of five or more alleles was inferred, while extra alleles were inferred in the broods Ita1, Ita2, FSA1, and FSA2.

The presence of multiple sib-groups in all five broods was inferred in the program KINALYZER. At least two sib-groups per brood were inferred (Table 3). Analyses in ML-Relate also indicate the presence of multiple sib-groups in each group resulting from the contribution of multiple unrelated individuals (results not shown). The ML-Relate analysis thus indicated that in each group more than one female and more than one male contributed to each brood. All analyses therefore suggest that *Arapaima gigas* does not have a monogamous system of mating.

Discussion

The analysis of microsatellite data provides powerful information on the relatedness and parentage of individuals, including fishes (Avise *et al.*, 2002). Various authors have discussed the diverse reproductive behaviors and tactics found in fishes through genetic assessments of parentage (Taborsky, 2001, 2008; Wilson & Ferguson, 2002; Hain & Neff, 2007), and their findings have revolutionized the study of reproductive behavior, revealing that individuals of many species engage in extra-pair copulations, including parasitic spawning behavior between satellite and territorial males such as sneaking, egg piracy and female mimicry of cooperative breeding between satellite and territorial males.

The results obtained in this study suggest that *Arapaima gigas* broods have contributions from multiple unrelated males and females. Therefore, we can reject the hypothesis that *Arapaima gigas* is a monogamous species in natural situations. Given that multiple male and female individuals contribute to each brood, the spawning behavior of *Arapaima gigas* is likely to be complex. Since males are the primary caregivers and guardians of the brood, males may chose to mate with multiple females, and the contribution of additional males to the brood would then likely to be result of parasitic behavior of satellite males (Roldán & Soler, 2011). Another, not necessarily exclusive possibility, is the existence of alloparental care, where other pairs spawn in the nest of the caregiver male (Wisenden, 1999). Alloparental care is thus defined as care of unrelated young.

There are many reasons for why a male would choose to mate with multiple females (Ridley, 1978). A male may choose to mate with an additional female or females, if they are likely to increase his fitness. The male does not need to give up the current brood to mate again, and actually may even become more attractive for females if already with offspring (Ridley, 1978). Given that parental care in majority of fishes including *Arapaima gigas* takes form of protecting against predators rather provisioning of offspring (Wisenden, 1999), the increase in size in the clutch has minimal effect on parental investment. Thus, it may be advantageous for the male to solicit multiple matings, thus increasing the total number of offspring, and hedging his bets, and consequently increasing his fitness through increasing his chances of a larger portion of his offspring surviving. Alloparental care may also increase reproductive fitness. While alloparental care appears to be disadvantageous (Wisenden, 1999), this behavior may have some advantages for the male caregiver. One of the possible advantages of alloparental care is the dilution of predation (Wisenden, 1999): if predation pressure is high, having unrelated fry mixed with the male's offspring lessens the chance of his young being preyed upon. The percentage of the offspring not related to the male caregiver will exist as an equilibrium between investment into unrelated offspring and probability of predation of one owns offspring. However, since in fishes parental care is usually only a protection against predators, and involves no investment in provisioning of offspring (McKaye, 1981), an increase in the size of a brood may not be much of a disadvantage for caregivers, since increase in brood size increases energetic investment only marginally (McKaye, 1981). Unrelated fry in the male's clutch may also be the result of parental-care parasitism, but may have similar indirect benefits as alloparental care. Parental-care parasitism is defined as an interaction in which an individual (extra-pair)

obtains reproductive benefits while reducing or completely eliminating its own costs of parenting by exploiting any type of offspring care provided by other individuals (Roldán & Soler, 2011), such as the protection to offspring provided by the male caregiver in *Arapaima gigas*.

The results observed here correspond mainly to an artificial environment (breeding station) or semi-artificial (dammed reservoir) that do not possess many of the same characteristics as the natural environment. However, contributions of multiple males and females to a brood were also observed in a natural situation in the São Miguel Island (SMI) brood. Therefore multiple paternity is likely a general phenomenon of the reproductive system of *Arapaima gigas*.

We believe these results will stimulate more complex studies of the mating system of the 'arapaima' and will encourage fish farmers to consider possible alternative reproductive tactics for the management in captivity. From a management perspective, this behavior may be exploited to maintain genetic diversity in captive and as well in wild populations of *Arapaima gigas*. Polygamous mating systems maintain genetic diversity and heterozygosity of populations, and thus may be exploited in the genetic management of captive populations and breeding stocks. The discovery of polygamous mating system may also eliminate the perceived need and the associated cost of isolating reproducing couples from the rest of the adults.

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