Lat. Am. J. Aquat. Res., 46(2): 346-354, 2018
DOI: 10.3856/vol46-issue2-fulltext-10

Research Article

Mirroring-in nature? Comparison of kinship analysis in clutches of the endangered giant Amazon River turtle, *Podocnemis expansa* (Chelonia: Podocnemididae) in both captivity and natural habitat

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ABSTRACT. The incidence of multiple paternities in two populations of *Podocnemis expansa* of Brazilian Amazon both rose in captivity and in natural habitat, was verified by using five microsatellite loci. Three hundred twenty-seven offspring from two different sampling sites were genotyped. The analysis revealed a 100% rate of multiple paternities in both populations. The Mendelian distribution of the alleles found in each nest was used to determine the number of contributing males. It was estimated that at least ten males contributed to each brood in captivity and nine contributed to each brood in the wild. These values are the highest ever recorded for the number of contributing *P. expansa* males. The findings have considerable implications regarding the conservation of this species, given that multiple paternities is important to the maintenance of genetic variability and has important consequences in increasing the effective size of a population in comparison to single paternities.

Keywords: *Podocnemis expansa*, multiple paternities, molecular analysis, chelonian, polyandry, wild population.

INTRODUCTION

Many studies performed on reproductive behavior report multiple paternities in different species of chelonians based on molecular analysis. Multiple paternities has been detected in different species of sea turtles: *Caretta caretta* (Bollmer *et al.*, 1999; Moore & Ball, 2002; Zbinden *et al.*, 2007), *Chelonia mydas* (Fitzsimmons, 1998; Ireland *et al.*, 2003; Lee & Hays, 2004), *Lepidochelys olivacea* (Kichler *et al.*, 1999; Hoekert *et al.*, 2002; Jensen *et al.*, 2006) and *Natator depressus* (Theissinger *et al.*, 2009), and also in freshwater turtles such as *Chrysemys picta* (Mctaggart, 2000; Pearse *et al.*, 2001), *Emydoidea blandigii* (Refsnider, 2009) and *Emys orbicularis* (Roques *et al.*, 2006).

For species of the genus *Podocnemis*, previous studies have demonstrated multiple paternities in *P. expansa* (Valenzuela, 2000; Pearse *et al.*, 2006), *P. unifilis* (Fantin, 2008), *P. sextuberculata* (Fantin, 2008; Pereira *et al.*, 2011) and *P. erytrocephala* (Fantin *et al.*, 2010). Many researchers have also observed cases of multiple paternities and polyandry in different species of vertebrates and invertebrates (Refsnider, 2009).

The turtles of the genus *Podocnemis* are known to exhibit polyandrous behavior (Lee & Hays, 2004) and its females can store sperm from multiple mating episodes in the internal cavity for long periods of time (Pearse *et al.,* 2001). Polyandrous behavior, which explains the occurrence of multiple paternities, is attributed to different factors, such as indirect genetic benefits to the offspring, increasing in genetic variability (Pearse *et al.*, 2001, 2006), a reduction in the likelihood of inbreeding and a reduction of costs resulting from reproductive failure due to genetic incompatibility (Stockley *et al.*, 1993). However, some researchers report that promiscuous female mating behavior could also lead to a greater exposure to disease (Thrall *et al.*, 2000), an increased risk of predation (Rowe, 1994), increased energy expenditure and physical harm (Watson *et al.*, 1998). Thus, the persistence of this type of mating must be compensated by

Corresponding editor: Sergio Palma

benefits to the offspring (Lee & Hays, 2004; Wright *et al.*, 2013).

The Arrau River turtle *Podocnemis expansa* is widely distributed along the major tributaries of the Orinoco and Essequibo rivers as well as drainages of the Amazon River in Colombia, Venezuela, Guiana, northeastern Peru, eastern Ecuador, northern Bolivia and northern Brazil (Vogt, 2008). This species can exceed 90 kg in body weight and 80 cm in carapace length (Pritchard & Trebbau, 1984), which makes it the largest turtle of the suborder Pleurodira and the largest freshwater chelonian in South America (Vogt, 2008). Due to its large size, *P. expansa* is heavily exploited for food and trade for the consumption and direct sale of its meat, viscera, and eggs, or for the use of its carapace in craftwork (Cantarelli, 2006). For these reasons, the giant Amazon River turtle is currently classified as low risk/dependent on conservation on the Red List of Threatened Species of the International Union for Conservation of Nature and Natural Resources (IUCN, 2011).

As a conservation measure for these species, farming activities were permitted with the issuing of Ministerial Directive 142/92 in 1992, which encourages the creation of farms dedicated to raising these turtles for economic purposes. The law allows the farming of species as a food source in the Amazon region in order to reduce the pressure placed on the turtles in the wild. The Brazilian Environmental Agency IBAMA provides approximately 4000 hatchlings to registered breeders with the commitment to raise them until adulthood and breed them in captivity. However, the lack of technical support to governmental agencies and breeders regarding the biology of the species and the dynamics of chelonian populations has led to the development of turtle farming activities without appropriate scientific methods (Andrade, 2004).

Multiple paternities in *P. expansa* offspring was first observed in the wild by Valenzuela (2000) in nests at the Caquetá River in Colombia, and subsequently by Pearse *et al*. (2006) at the Orinoco River in Venezuela. However, no evidence of reproductive behavior has been documented for populations raised in captivity until this current study. Multiple mating is believed to occur with greater frequency in captivity than in the wild, given that cloistered specimens are more likely to mate. As *P. expansa* requires a long period prior to sexual maturity, which occurs between 11 and 15 years of age among females and seven years of age among males (IBAMA, 1989), polyandry may be an important strategy for maintaining genetic diversity among descendants.

The conservation and determination of genetic diversity of *P. expansa* require an understanding of the reproductive behavior of this species. Thus, the aim of the present study was to determine the kinship degree among offspring in the same litter in populations found both in captivity and the wild to investigate the occurrence of multiple paternities in these two environments and compare the findings, using microsatellite markers. In the Amazon River basin, the current alternative for the conservation of this species in nature is to encourage its production in captivity. Therefore, the information presented herein, along with other data regarding the biology of the species, can contribute to the establishment of better conservation and management strategies for *P. expansa*.

MATERIALS AND METHODS

Blood samples were collected from *P. expansa* hatchlings raised both in captivity and in the wild. Individuals in captive were sampled on the São Francisco Farm (3°14'44.65"S, 60°36'26.76"W) located at km 71 of the Manoel Urbano Roadway (AM-070) in the municipality of Manacapuru, the State of Amazonas, Brazil. A total of 191 hatchlings were distributed in different nests as follows: N1:37, N2:37, N3:36, N4:29, N5:30, N6:22. Individuals in natural habitat were sampled at the Abufari, Sororoca and Juruá tablelands (P. Andrade, *comm. pers.*) along the Juruá River in the community of Manarian (5°28'15"S, 67°28'31"W), municipality of Carauari, the State of Amazonas, Brazil. 136 captive hatchlings were distributed in five nests (N7:30, N8:30, N9:23, N10:32, N11:21 (Fig. 1).

Sampling was performed by the team of the Yellow-Spotted Amazon River Turtle Project. Sampling in the wild occurred in the following manner: after females laid the eggs, each nest was surrounded by a screen to protect and control the hatching and marked with a wooden stake on which the nest number was printed. Blood was collected as soon as the hatchlings emerged. For such, the femoral vein was punctured with a 1 mL syringe for the collection of 100 µL of blood, which was stored in 2 mL tubes containing 500 µL of absolute ethanol and stored at 4°C. The hatchlings were then released at the origin site. No blood was taken from mothers.

Genomic DNA was extracted using cetyltrimethylammonium bromide (Doyle & Doyle, 1987). Five microsatellite loci were used for DNA amplification: two of them described by Fantin *et al.* (2007) and three others described by Valenzuela (2000) (Table 1).

DNA amplification was performed using the economic polymerase chain reaction (PCR) protocol described by Schuelke (2000) with a final reaction volume of 13.5 µL: 4.8 µL of H_2O, 1.5 µL of $MgCl_2$ (25

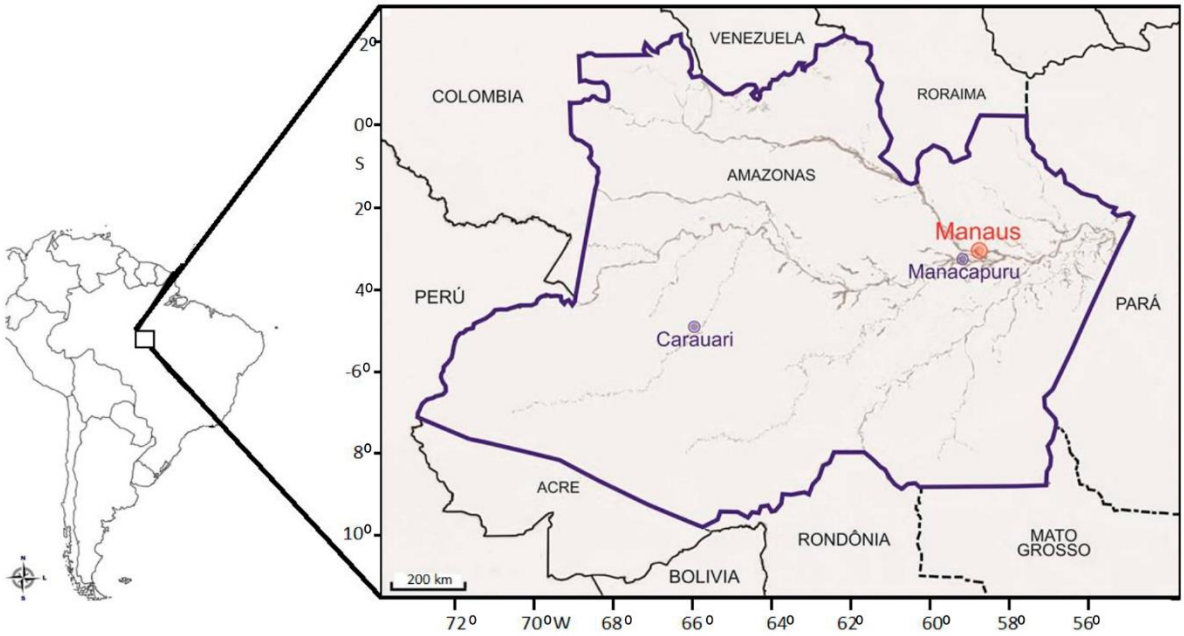

Figure 1. Map showing two sampling sites of nests with *P. expansa* hatchlings.

Table 1. Characteristics of microsatellite loci used for *P. expansa* paternities analysis.

Locus	Repetition	Temperature (°C)	Reference
Puni_1D12	$(GA)_{10}$	55	Fantin *et al.* (2007)
Puni_1E1	$(CT)_9tt(CT)_7$	64	Fantin *et al.* (2007)
PE344	$(AG)_{13}$	50	Valenzuela (2000)
PE519	$(CT)_7(CA)_8(CG)_2(CA)_8$	56	Valenzuela (2000)
PE1075	$(AC)_{11}$	54	Valenzuela (2000)

mM), 1.5 µL of dNTP mix (0.2 µM of each dNTP), 1.5 µL of PCR buffer, 0.75 µL of the M13 forward primer (0.2 µM), 0.75 µL of the M13 primer (TET) (0.2 µM), 1.5 µL of the reverse primer (0.2 µM), 0.2 µL of *Taq* polymerase enzyme (2 U/ul) and 1.0 µL of DNA. Thermal cycling conditions were performed as follows: initial denaturing temperature of 94ºC for 2 min, followed by 25 cycles at 94ºC for 50 s, 55 to 64ºC (depending on the specific hybridization temperature of each primer) for 50 s and 72ºC for 1 min, followed by 20 cycles at 94ºC for 40 s, 53ºC for 35 s and 72ºC for 40 s for hybridization of the M13 primer and final extension at 72ºC for 20 min. The amplified product was submitted to electrophoresis in 1% agarose gel for the verification of PCR efficiency. The PCR products were diluted in distilled water at a proportion of 1:100 and the ROX pUC-19 size marker (90, 105, 131, 151, 182, 201, 254, 306, 337, 362, 425, 486, 509 and 560) modified from DeWoody *et al.* (2004) was added. Genotyping was performed in the ABI 3130xl automatic DNA sequencer. The analysis of alleles for each locus was performed using the GeneMarker V2.2.0 software.

The allelic frequency of each locus analyzed in the population was calculated using the Arlequin 3.1 program (Excoffier *et al.*, 2005). The probability of genetic identity (I) and exclusion of paternities (Q) was calculated based on Paetkau *et al.* (1995) and Weir (1996) based on the frequencies determined for all five microsatellite loci. Combined probability (genetic identity [CI] and exclusion of paternities [CQ]) was determined in the same fashion. "I" is a parameter used to calculate the possibility of two unrelated individuals having the same genotype within a population and Q is based on the exclusion of different males involved in the mating process, but that can be statistically distinguished. The method allows the exclusion of randomly chosen males in a given population. If Q values are close to 1, the exclusion of a male as a father of the brood is 100% reliable.

Possible genotyping errors due to null alleles in the microsatellites of diploid populations were identified

by the Micro-Checker software (Oosterhout *et al.*, 2004), which estimates the frequency of these alleles for the microsatellite loci studied.

Using the simple allele count method (Myers & Zamudio, 2004), which presupposes Mendelian distribution of the alleles in the offspring, the presence of five alleles per locus among the hatchlings in each nest is considered indicative of multiple paternities if no maternal allele is known (two maternal alleles, two alleles from one male and one allele from a second male). Analysis of paternities was also based on the inference of maternal genotypes, which was identified by the presence of homozygous hatchlings per locus within a nest. One maternal allele can be inferred when a hatchling is homozygous for a given locus (AA) and the complete maternal genotype can be inferred when the hatchlings are homozygous for two different alleles (AA and BB; maternal genotype = AB). When a maternal allele can be inferred, multiple paternities is estimated for a nest if the analysis of a locus indicates the presence of four alleles. If two maternal alleles can be detected, the presence of three alleles in the locus analyzed indicates multiple paternities in the nest.

The multi-locus analysis was also performed using Kinalyzer software (Berger-Wolf *et al.*, 2007), which allows the inference of groups of siblings using the genotype of co-dominant markers, such as microsatellites. From the analysis of Mendelian inheritance and the inferences of combinations under the presupposition of parsimony, an algorithm is formulated, which then correctly reconstructs the smallest group of siblings under such restrictions. This method does not depend on prior genotypic knowledge regarding the population studied and is, therefore, appropriate for reconstructing groups of siblings in each nest.

RESULTS

Characterization of microsatellite loci

All five loci analyzed proved to be quite polymorphic for the kinship analysis in *P. expansa*. Locus PE519 was the most polymorphic, exhibiting 32 different alleles in the samples of individuals in captivity and 29 different alleles for the samples taken in the wild. The mean number of alleles was 26.4 (range: 19 to 31) among hatchlings in captivity and 22.4 (range: 15 to 29) among hatchlings in the wild (Table 2).

The combined probability of genetic identity was low (captivity: CI = 1.08×10^{-6}; wild: CI = 2.85×10^{-6}), which demonstrates the discrimination power of these loci regarding two unrelated individuals exhibiting the same genotype. In contrast, the probability of exclusion of paternities was high (captivity: CQ = 0.9999; wild: CQ = 0.9999), indicating 99.99% probability of the efficient detection of multiple paternities using these loci (Table 2).

Paternity in natural habitat

All 191 hatchings from the six *P. expansa* nests on São Francisco Farm in the municipality of Manacapuru (State of Amazonas, Brazil) were genotyped. The sex ratio at this site was 1.6♀:1♂, with approximately 500 turtles in the reproductive phase (Garcez, 2009). The minimum number of alleles was five (Puni_1E1) in Nest 5 and the maximum number of 22 alleles (PE344 and PE1075) found in Nests 2 and 5. Using the simple allele count method (Myers & Zamudio, 2004), multiple paternities was found in all six nests analyzed, with a maximum of 10 males contributing to Nests 2 and 5. Determination of maternal genotype was not performed, as more than two homozygous hatchlings were found for different alleles at all loci.

Based on all five loci analyzed, Kynalyser software allowed the estimation of the number of groups of siblings in each nest, indicating multiple paternities in all nests of this population. The evidence revealed that at least five males contributed to offspring in Nest 6 (PE519 and PE1075) and at least ten males contributed to Nests 2 (PE519 and PE344) and 5 (PE1075) (Table 3).

Paternity in captivity

A total of 136 *P. expansa* hatchlings from five nests sampled in the Juruá River in the municipality of Carauari (state of Amazonas, Brazil) were genotyped. The observed sex ratio at this site was 9♀:1♂ (P. Andrade, *comm. pers.*). The analysis of loci based on the simple allele count (Myers & Zamudio, 2004) indicated multiple paternities in the five nests analyzed. The minimum number of alleles was five (PE519) in Nest 11 and the maximum was 20 (PE1075) in Nest 8. It was not possible to infer the maternal genotype due to the presence of more than two hatchlings that were homozygous for different alleles.

Based on the five loci analyzed, the Kynalyser software allowed the estimation of the number of groups of siblings in each nest, indicating multiple paternities in all nests analyzed in this population. The evidence revealed that at least six males contributed to the offspring in Nest 11 (PE344 and PE1075) and at least nine males contributed to Nest 8 (PE1075) (Table 3).

DISCUSSION

This is the first report of multiple paternities in *P. expansa* raised in captivity. This study also supports the

Table 2. Characterization of five microsatellite loci in *P. expansa* in captivity and in nature. A: number of alleles; Ho: observed heterozygosity; He: expected heterozygosity; I: the probability of genetic identity; Q: the probability of exclusion of paternities; CI: the combined probability of genetic identity; CQ: combine probability of exclusion for five loci.

Samples from captivity					
Locus	A	Ho	He	I	Q
Puni_1D12	19	1.00000	0.91625	0.0139	0.8308
Puni_1E1	28	0.62778	0.93545	0.0085	0.8610
PE344	22	0.67045	0.93229	0.0088	0.8610
PE519	32	0.61081	0.90364	0.0168	0.8174
PE1075	31	0.62295	0.94542	0.0062	0.8871
Mean	26.4	0.70639	0.92661	CI = $1.08x10^{-6}$	CQ = 0.999935
Samples from nature					
Locus	A	Ho	He	I	Q
Puni_1D12	20	1.00000	0.93025	0.0100	0.8567
Puni_1E1	15	0.76774	0.90356	0.0183	0.8059
PE344	23	0.63580	0.93202	0.0095	0.8602
PE519	29	0.76316	0.89999	0.0190	0.8084
PE1075	25	0.59748	0.93494	0.0087	0.8665
Mean	22.4	0.752836	0.920152	CI = $2.85x10^{-6}$	CQ = 0.999900

Table 3. Evidence of *P. expansa* multiple paternities in nests in captivity and in nature. Number of alleles per locus; *Minimum number of fathers inferred; Kynaliser inference of the number of groups of siblings estimated for each locus, indicating the presence of multiple paternities in all nests studied. MP: multiple paternities.

Captivity									
Nest	Nº of hatchlings	Number of alleles per locus					Kynaliser		Result
		Puni 1D12	Puni 1E1	PE344	PE519	PE1075	Nº of inferred fathers*	Group of inferred siblings	
N1	37	15	18	16	14	8	8	16	MP
N2	37	14	16	22	21	19	10	17	MP
N3	36	15	13	14	13	17	8	14	MP
N4	29	9	8	10	16	18	8	12	MP
N5	30	12	5	13	11	22	10	13	MP
N6	22	10	10	8	11	11	5	8	MP

Nature									
Nest	Nº of hatchlings	Number of alleles per locus					Kynaliser		Result
		Puni 1D12	Puni 1E1	PE344	PE519	PE1075	Nº of inferred fathers*	Group of inferred siblings	
N7	30	13	8	12	11	17	8	13	MP
N8	30	16	11	14	13	20	9	12	MP
N9	23	16	12	13	12	9	7	10	MP
N10	32	17	8	14	18	17	8	13	MP
N11	21	10	9	13	5	13	6	9	MP

occurrence of this mating behavior previously observed by Valenzuela (2000) and Pearse *et al.* (2006) for wild populations along the Caquetá River (Colombia) and on Playita Island (Venezuela). Valenzuela (2000) found a 100% occurrence rate of multiple paternities in two *P. expansa* nests using eight microsatellite loci. The analysis allowed the estimation of the contribution of at least two males in one nest and three in the other one. Using a sample of 32 nests, Pearse *et al.* (2006) analyzed seven microsatellite loci and found only a 10.3% contribution rate of more than one male per brood. Despite the larger sample size in comparison to the study conducted by Valenzuela (2000), the sample sizes among nests studied by Pearse *et al.* (2006) were considerable heterogeneous, with nests ranging from 9 to 76 hatchlings. These authors found single paternities

in nests with a smaller number of hatchlings and multiple paternities in those with a larger number of hatchlings. According to Pearse *et al.* (2002), the higher the number of nests and the more homogenous sample sizes among nests, the higher the probability of detecting the occurrence of this latter reproductive behavior. Thus, these factors should be observed in a multiple mating study in order to obtain more reliable data about the rates of multiple paternities found in a population.

In the present study, a total of 11 nests were sampled (n = 6 in captivity and n = 5 in the wild), with a mean of 31.8 hatchlings per nest in captivity and 27.5 per nest in the wild. The rate of 100% multiple paternities found demonstrates the same mating behavior under both conditions. The number of microsatellite loci used was sufficient and with considerable discriminatory power to determine the type of paternities in *P. expansa*, as showed by the values regarding the probability of two unrelated individuals exhibiting the same genotype (CI = 1.08×10^{-6} for samples taken from specimens in captivity and 2.85×10^{-6} for samples taken from specimens in the wild) as well as high probability of paternities exclusion (CQ = 99.9% in both situations), indicating the power of the five loci used to detect multiple paternities (Table 2). Based on the simple allele count, 10 males were estimated to be contributors for Nests 2 and 5 in captivity and nine were estimated to be contributors for Nest 2 in the wild. If a DNA sample had been taken from the female (mother) of each nest, the genotype would allow a better analysis of the allele count for each locus, as it would be possible to estimate which alleles the fathers contributed to the brood.

A good number of alleles were found in each population studied for four loci (Puni_1E1, PE344, PE519, and PE1075). However, observed heterozygosity was lower than expected heterozygosity, which may be explained by the excess of homozygotes and null alleles in the loci: only one locus (Puni_1D12) showed higher observed heterozygosity than expected heterozygosity. An individual detected as homozygous may be heterozygous for the null allele. The fact that such alleles are generally identifiable with the aid of software programs makes them a considerable problem for the kinship analysis (Jones & Ardren, 2003).

According to Chesser & Baker (1996), multiple paternities greatly influences the effective size of a population than single paternities in some organisms, especially endangered species, such as *P. expansa,* with a greater frequency of multiple paternities enhancing the ability to maintain or increase inter-population and intra-population genetic variability. The genetic findings described by Pearse *et al.* (2006) demonstrate that natural populations of *P. expansa* have recently undergone a reduction in effective population size. The incentive for farming the species in captivity is a good measure for taking the pressure off natural populations hunted by local human communities and allowing an opportunity for the recovery of genetic diversity. The present data demonstrate that individuals in captivity have the genetic potential for controlled management and that the effects of inbreeding in this situation are minimized by the characteristics of promiscuity. Moreover, breeding farms should be regarded as potential sources of individuals for the repopulation of intensely suffering areas from population bottlenecks. However, the genetic differences found in each river basin studied by Pearse *et al.* (2006) should be taken into consideration.

The advantages of multiple paternities reside in indirect genetic benefits for the offspring (Pearse *et al.*, 2001), a reduction in the likelihood of inbreeding (Stockley *et al.*, 1993) and an increase in genetic variability in the population (Pearse *et al.*, 2006). The high frequency of multiple paternities suggests that polyandry is a common reproductive strategy among females of the genus *Podocnemis*, as reported by Valenzuela (2000), Pearse *et al.* (2006) and Fantin *et al.* (2008, 2010). The polyandrous behavior of females may avoid energy expenditures resulting from reproductive failures due to genetic incompatibility (Stockley *et al.*, 1993), thereby allowing females to avoid wasting eggs and embryos. Moreover, this behavior implies direct benefits to the health of the offspring (Lee & Hays, 2004). Females do not derive genetic benefits from this behavior. The real benefit is for the offspring, which inherit "good genes" (Jennions & Petrie, 2000). The choice of females to mate with different males and the capacity to store sperm from several mating events for long periods of time (Pearse *et al.*, 2002) increases the odds of multiple paternities and results in the competition of spermatozoids from different males, thereby increasing the chance of the offspring acquiring good genes (Pearse *et al.*, 2002; Theissinger *et al.*, 2009).

Polyandry, together with the capacity to store sperm, allows the genetic variability of the descendants to be temporarily unlinked to the availability of males for mating. As individuals remain relatively close to each other in captivity, it is possible that multiple paternities are common in this environment. The sex ratio in captivity was 1.6♀:1♂ (Garcez, 2009) and multiple paternities occurred even with a smaller number of males. The same was true in the wild, as the sex ratio in the community of Manarian was approximately 9♀:1♂ in 2011 (P. Andrade, *comm. pers.*) and multiple paternities was also detected, although individuals are more geographically distant from each other than what

occurs in captivity. As the same result was found in both situations, one may infer that different populations exposed to different environments may exhibit the same type of reproductive behavior.

As satisfactory genetic variability rates were found in the individuals raised in captivity and in its natural habitat, it might be a good alternative for farmed individuals to be introduced into a threatened population to minimize the occurrence of inbreeding and the reduction of genetic diversity. The molecular analysis provides additional information on the distribution of a species, which can contribute to the reintroduction of a species in another location since it is necessary to have ecological knowledge on the distribution of a species before its reintroduction (Frankham *et al.*, 2008).

The present results suggest that *P. expansa* females in captivity can store the sperm of up to ten males, whereas those in the wild can store the sperm of nine males (Table 3). These findings only constitute an estimate of the sperm from different males that females can store. According to Olsson & Madsen (1998), the storage of sperm in captivity is well established, as female turtles isolated from males continue to produce offspring for long periods of time and a female can carry the genetic material of a male for a long time even if the male dies.

The present findings directly contribute to knowledge regarding the reproductive behavior of *P. expansa*. However, further paternities studies should be conducted to evaluate the occurrence of multiple paternities at different nesting sites to determine the frequency of multiple mating events and the extent to which ecological differences among populations exert an influence on the behavior of species. As each population has a differentiated type of reproductive system, knowledge on the mating behavior of *P. expansa* both in captivity and the wild can assist in the development of adequate conservation strategies and constitutes additional biological information for the proper management of this species on breeding farms.

Polyandry and multiple paternities are important reproductive strategies with potential implications for the conservation of species as well as the establishment of adequate management practices. In the present study, multiple paternities was found in populations both raised in captivity and the wild, which suggests that this kind of mating behavior is common in *P. expansa* and may ensure the maintenance of genetic variability as well as increasing the effective size of the populations. The sample size and number of microsatellite loci used in this study were efficient for the detection of multiple paternities in this species. These results contribute to knowledge on the reproductive behavior of *P. expansa* in captive and wild populations.

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Received: 19 September 2016; Accepted: 2 November 2017

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