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Natural nests incubated in two different soil types lead to an overall balanced sex ratio in *Podocnemis unifilis* hatchlings on the lower Purus River, Brazil

JOSÉ ERICKSON^{1,2}, CAMILA KURZMANN FAGUNDES³, MARCELA DOS SANTOS MAGALHÃES⁴,
LUCAS CASTANHOLA DIAS⁵, RICHARD CARL VOGT⁶, IZENI PIRES FARIAS² & JANSEN ZUANON⁶

¹Instituto Nacional de Pesquisas da Amazônia. Programa de Pós-Graduação em Biologia de Água Doce e Pesca Interior, Av. André Araújo, 2936 CEP: 69067-375 Manaus, AM, Brazil

²Universidade Federal do Amazonas. Departamento de Genética. Laboratório de Evolução e Genética Animal, Av. Rodrigo Octávio, 6200 CEP: 69080-900 Manaus, AM, Brazil

³Wildlife Conservation Society. Brazil Program, Av. Rodrigo Octávio, 6200 CEP: 69080-900 Manaus, AM, Brazil

⁴Universidade Federal do Amazonas. Departamento de Morfologia, Av. Rodrigo Octávio, 6200 CEP: 69080-900 Manaus, AM, Brazil

⁵Instituto Nacional de Pesquisas da Amazônia. Laboratório Temático de Microscopia e Nanotecnologia, Av. André Araújo, 2936 CEP: 69067-375 Manaus, AM, Brazil

⁶Instituto Nacional de Pesquisas da Amazônia. Coordenação de Biodiversidade, Av. André Araújo, 2936 CEP: 69067-375 Manaus, AM, Brazil

Corresponding author: JOSÉ ERICKSON, e-mail: erickson.herpeto@gmail.com

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Abstract. Temperature-dependent sex determination occurs in many species of turtles. Because substrates differ in their heat retention capacities, a relatively small change in ambient temperature can exert great influence on sex ratios of hatchlings from clutches deposited in different substrates even within the same geographical area. Since *Podocnemis unifilis* is one of the Amazonian turtle species (Podocnemididae) with high behavioural plasticity in the use of nesting sites, we tested the influence of soil types on hatchling sex ratios in the Piagaçu Purus Sustainable Development Reserve along the lower Purus River, Brazil. During the 2013 and 2014 nesting seasons, we evaluated 26 nests on a sand bank ($n = 14$) and clay banks ($n = 12$). Approximately half of the hatchlings that emerged from each nest were sexed via gonad histological analysis ($n = 341$). We found a higher incidence of females in nests from the sandy substrate and more males in nests from the clayey substrate ($\chi^2 = 40.466$, $df = 1$, $P < 0.0001$). Ratios of 1 male to 2.28 females were recorded from sand bank nests, and 1.9 males to 1 female from clay banks. Overall, however, the hatchling sex ratio approximated to 1:1. Due to the gradual increase in global temperatures, it has been suggested for species with Temperature Sex Determination (TSD) that some turtle populations become increasingly female-dominated. However, it is likely that the use of different nesting substrates may help to mitigate the effects of global warming on hatchling sex ratio, especially in species with greater behavioural plasticity, such as *P. unifilis*.

Key words. Behavioural plasticity, freshwater turtles, gonad, hatchlings, incubation temperature, sex determination, Testudines, Yellow-spotted Amazon River Turtle.

Introduction

The ecological and evolutionary consequences of the mechanisms of sex determination are key to understanding the life histories of turtles (VALENZUELA & ADAMS 2011). Two distinct sexual differentiation mechanisms are known: Genotypic Sex Determination – GSD (BULL 1980), where sex is determined genetically, and Temperature Sex Determination – TSD, where the sex is determined by incubation temperature (BULL & VOGT 1979, EWERT & NEL-

SON 1991). Most turtles studied exhibit the TSD pattern, producing males at lower incubation temperatures and females at higher temperatures (VALENZUELA & LANCE 2004). However, in some species, females are produced at very low and very high temperatures, and males at intermediate temperatures (BULL & VOGT 1979, EWERT & NELSON 1991). The influence of temperature on sexual differentiation occurs only during the second third of embryogenesis (Thermosensitive Period – TSP: VOGT & BULL 1982, MROSOVSKY & PIEAU 1991). More precisely, the determi-

nation of embryo sex in TSD turtles depends on the cumulative effect of temperature during the TSP, which may vary according to the amplitude of thermal variation during this phase (VOGT & BULL 1982, MROSOVSKY & PIEAU 1991). Changes of as little as 1°C in ambient temperature (YOUNG et al. 2004, GEORGES 2013), or small changes in the fluctuations of the means of high and low temperatures can be sufficient to alter the sex ratio produced during the TSP (NEUWALD & VALENZUELA 2011).

Both regional and local climatic conditions can directly affect nest temperatures and influence several aspects of turtle reproductive biology such as embryogenesis, sex determination, incubation success, and hatchling survival (FUENTES et al. 2010, REFSNIDER et al. 2013, ERICKSON et al. 2020). Although the climatic temperature is the basic element of sex determination, the microclimate produced by the nest substrate clearly affects these characteristics as well (HAYS et al. 2001, FERREIRA-JÚNIOR et al. 2007, ERICKSON et al. 2020). Sandy soils retain more heat than clayey soils since sand compaction can reduce gas exchange between the egg chamber and the atmosphere, alter nest temperature and impact on embryo growth (ACKERMAN & PRANGE 1972). For species that lay eggs exclusively on sand banks, global warming has led to a significant increase in the proportion of female hatchlings (GODLEY et al. 2001, JENSEN et al. 2018). On the other hand, turtle species that lay eggs in spatially separated habitats that differ in their thermal and soil properties tend to be less vulnerable to climate changes (FERREIRA-JÚNIOR et al. 2007, HAWKES et al. 2007, NEUWALD & VALENZUELA 2011). Behavioural plasticity in nesting site choice (PIKE 2013, ERICKSON et al. 2020) may be essential for the persistence of populations (GEORGES 2013), since changes in the selection of nesting sites may also occur rather spontaneously (e.g., within the lifespans of individuals) and directly influence hatchling sex ratios (HAYS et al. 2001, FUENTES et al. 2010).

The Yellow-spotted Amazon River turtle (*Podocnemis unifilis*) is a TSD species with one of the widest geographic distribution ranges among freshwater turtles (Turtle Taxonomy Working Group 2017) and provides a good example of adaptation to environmentally induced natural variations (ERICKSON et al. 2020). This aquatic species nests in a variety of substrates with different geomorphological characteristics (sand banks with a wide variation in grain size, clay banks, savannah areas, and, rarely, on banks of floating macrophytes (FERREIRA-JÚNIOR et al. 2007, VOGT 2008, ERICKSON et al. 2020). Studies on the nesting ecology of *P. unifilis* are available for both sand and clay banks (FACHÍN-TERÁN & VON MÜLHEN 2003, ESCALONA et al. 2009, ARRAES et al. 2014, ERICKSON et al. 2020). However, despite the influence that soil type has on the sex determination in TSD species, no study has thus far compared the hatchling sex ratios of *P. unifilis* incubated in different nesting substrates in the same geographical area.

Considering the generalist nesting behaviour exhibited by *P. unifilis*, we compared hatchling sex ratios in naturally incubated nests in two different types of nesting substrates (i.e., clayey soil and sandy soil) during two subsequent

nesting seasons in a population on the lower Purus River, Amazonas, Brazil. We hypothesized that hatchling sex ratios differed according to the incubation substrate, with a higher incidence of females among hatchlings from nests on a sand bank (beach), and a higher number of males in nests on clay banks.

Material and methods

Study site and fieldwork procedures

This study was carried out in the Piagaçu Purus Sustainable Development Reserve (PPSDR; 4°03'–5°25' S, 61°40'–63°30' W), a protected area of 834.245 ha in the Purus River basin, Brazilian Amazon (Fig. 1) between the end of the low-water season and the beginning of the high-water season (September–December), corresponding to the nesting period of *P. unifilis* (VOGT 2008). The climate in the region belongs to group Af (humid or superhumid tropical climate) with a monthly mean temperature above 18°C and annual rainfall between 2,000 and 2,600 mm (PEEL et al. 2007). The landscape in the Amazon basin lowlands is heavily influenced by seasonal fluctuations in water levels (JUNK et al. 1989). The water level in the lower Purus River can vary up to 12 m between the dry and flood seasons (GOULDING et al. 2003) and generates a mosaic of connected environments, including seasonally flooded forests, floodplain lakes, sand banks (e.g., beaches), and clay banks.

We sampled two nesting sites about 80 km apart during the nesting seasons of 2013 and 2014. The first nesting area was located between the Caua-Cuiuanã and Itapurú sectors of the reserve in an area of clay banks with irregular topography (04°15'28.1" S, 61°55'52.9" W, Fig. 2A). The area has abundant grass cover, with variable declivity and extension along streams, canals, and lakes connected to the Purus River (ERICKSON & BACCARO 2016). The second nesting site was located at the Ayapúa sector on an approximately 250 m stretch of sand bank (04°26'030" S, 62°17'42.7" W) with sparse vegetation cover during the low-water season (Fig. 2B).

Since *P. unifilis* usually nests at night (VOGT 2008), we searched for fresh nests in the studied areas in the first hours after dawn throughout the two nesting seasons. The nests were located from the tracks left on the substrate by the nesting females. We monitored and sampled a total of 26 nests in 2013 and 2014 nesting seasons: 12 nests on the clay banks and 14 nests on the sand bank. Each nest was individually marked with a numbered stake. We inserted a data logger (HOBO Pendant Temp/Light Logger, accuracy ± 0.47°C at 25°C, size 58 × 33 × 23 mm) in each nest during the nesting season of 2013 to measure and record temperature changes every hour throughout the incubation period; the data loggers were positioned between the eggs (ca. 8 cm depth). Nests were protected with plastic screens and monitored weekly throughout the incubation period. For this *P. unifilis* population in the PPSDR, the mean incubation period (i.e., until emergence of the first hatchling) is significantly longer in nests on clay banks (approx-

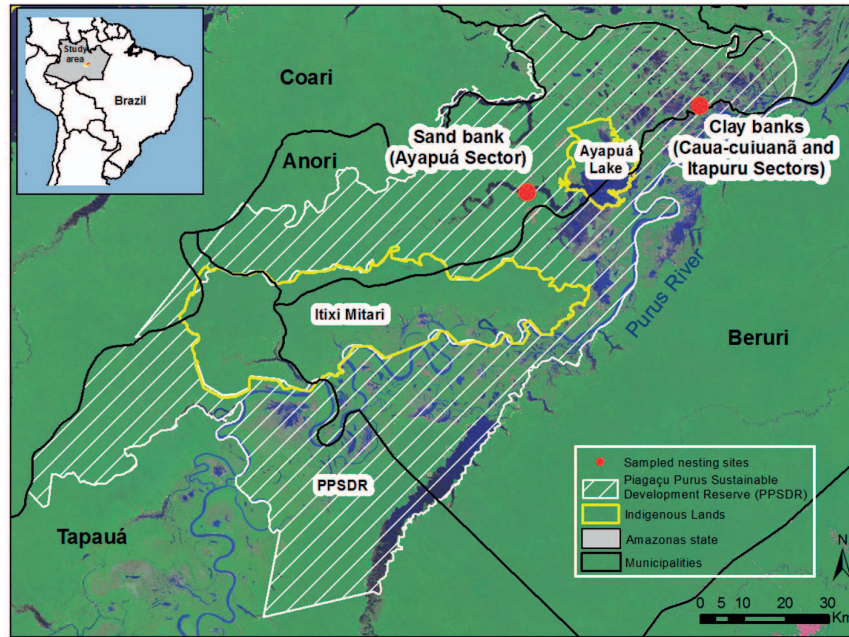


Figure 1. Study area showing the two sampled nesting sites of *Podocnemis unifilis* in the Piagaçu Purus Sustainable Development Reserve (PPSDR), located in the border area between the municipalities of Anori, Beruri, Coari, and Tapauá, state of Amazonas, Brazil.

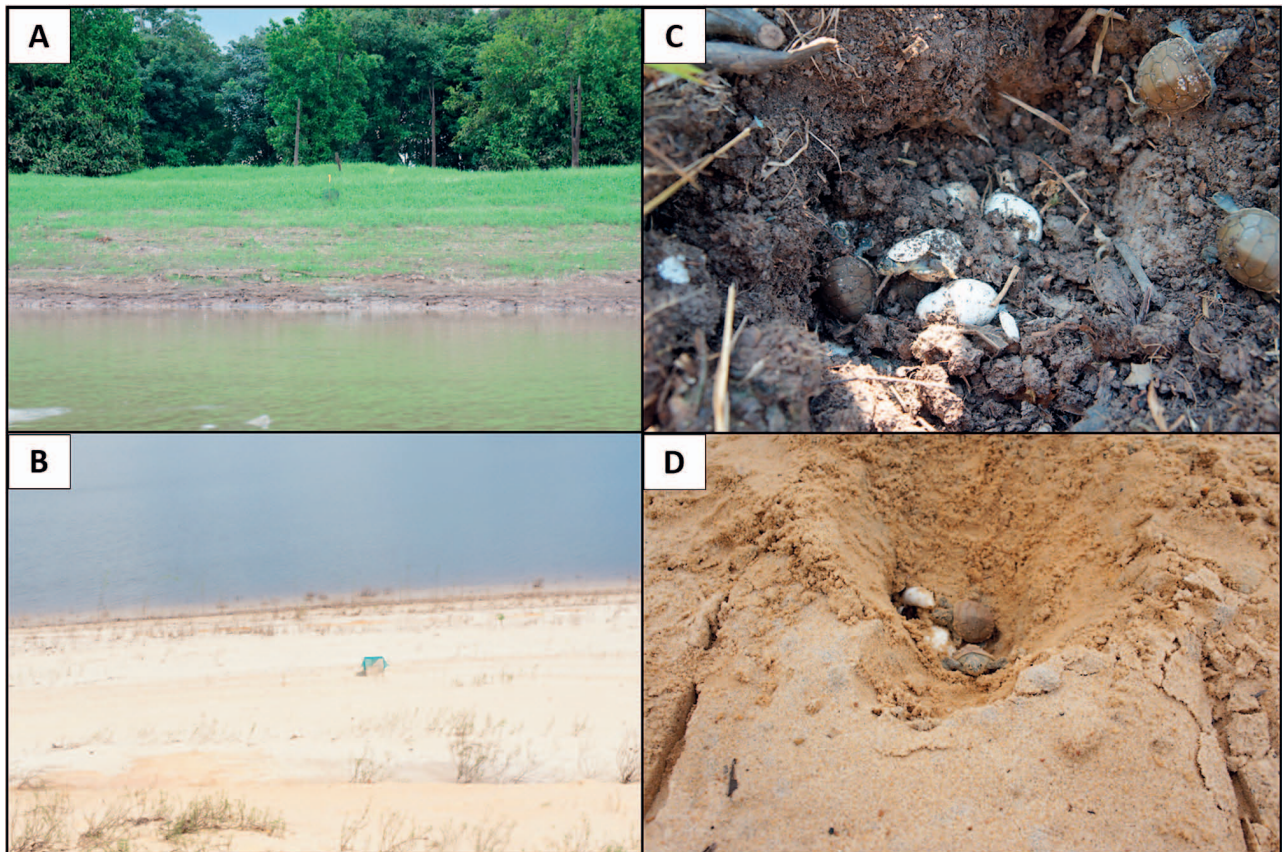


Figure 2. Characterization of nesting sites and nests of *Podocnemis unifilis* sampled in the Piagaçu Purus Sustainable Development Reserve (PPSDR), Amazonas, Brazil. A) Clay banks covered with low vegetation (grasses) in the Caua-Cuiuanã and Itapuru sectors; B) sand bank in the Ayapua sector; C) emergence of hatchlings of *P. unifilis* from a nest on a clay bank; and D) and on a sand bank.

mately 77 days, Fig. 2C) than in those on the sand bank (62 days, Fig. 2D) (ERICKSON et al. 2020). Sibling hatchlings of *P. unifilis* typically emerge over a period of several hours or days. Nests were periodically checked for emerging individuals near the expected time of emergence.

Sexing of hatchlings by histological analysis

We randomly sampled approximately half of the hatchlings emerging from each monitored nest at the two nesting sites. The sampled hatchlings were euthanized on site (Licenses ICMBio: 38782-3; 50930-1) with a lethal intramuscular injection of propofol (CFBio n.148/2012). Once death was confirmed, we removed the plastron and the remaining yolk sac to fix the reproductive organs in 10% neutral buffered formalin for 24 h (MAIA 1979). After this, the gonads were individually preserved in 70% ethanol, and stored in labelled containers. The sex of these hatchlings were identified by means of histological analysis of their gonads (MALVASIO et al. 2012). Samples were processed and analyzed histologically in the Thematic Laboratory of Nanotechnology and Electronic Microscopy at INPA, Manaus, Amazonas, Brazil. Female individuals presented a well-developed cortex with germ cells and inner medulla formed by connective tissue (Fig. 3A). We considered individuals to be males when they had a simple cubic epithelium and tubular structures representing the development of seminiferous tubules (Fig. 3B).

Statistical analysis

We used one-way analysis of variance (ANOVA) to compare the daily means of nest temperatures in both study areas during the incubation period. Two-factor contingency table analysis were used to calculate the proportion of

male and female hatchlings from each incubation substrate separately (i.e., clay banks and sand bank). To evaluate the distribution of values shown in Table 1 and to test our hypothesis that hatchling sex ratios differed according to the incubation substrate, we applied a Pearson's Chi-squared Test to the observed frequencies according to a multinomial probability distribution. We used a two-way ANOVA to evaluate whether the overall proportion of males and females generated in both substrates and study years differed from each other. All statistical analysis were performed using R 3.3.2 (R Development Core Team 2018).

Results

We analyzed histological preparations of the gonads of 341 *P. unifilis* individuals from 26 nests monitored at two nesting sites, 12 nests from the clay banks and 14 nests from the sand bank (Table 1). Nest temperatures varied between 24.35 and 52.73°C. The daily mean nest temperatures ranged from 25 to 42°C and were higher on the sand bank than on the clay banks ($F = 28.33$, $df = 1$, $P < 0.0003$). The daily amplitude of nest temperature variation was also higher on the sand bank (1.8–11.5°C) than on the clay banks (0.5–4.3°C). The proportion of male relative to female hatchlings differed between the two substrates. The male to female sex ratio was 1:2.28 ($n = 164$) for the nests incubated on the sand bank, and 1.90:1 ($n = 177$) on the clay banks (Fig. 4). We found a predominance of females emerging from the nests on the sand bank, and of males inform those on the clay banks ($\chi^2 = 40.466$, $df = 1$, $P < 0.0001$). Considering all sampled nests in both substrate types, 166 individuals were males and 175 were females, equal to a sex ratio of 1:1.05. Sex ratios did not differ between nesting seasons (2013 = 1.05:1; 2014 = 1:1.14), and the overall sex ratios for both substrate types did not deviate from 1:1 ($F = 0.566$, $df = 1$, $P = 0.456$).

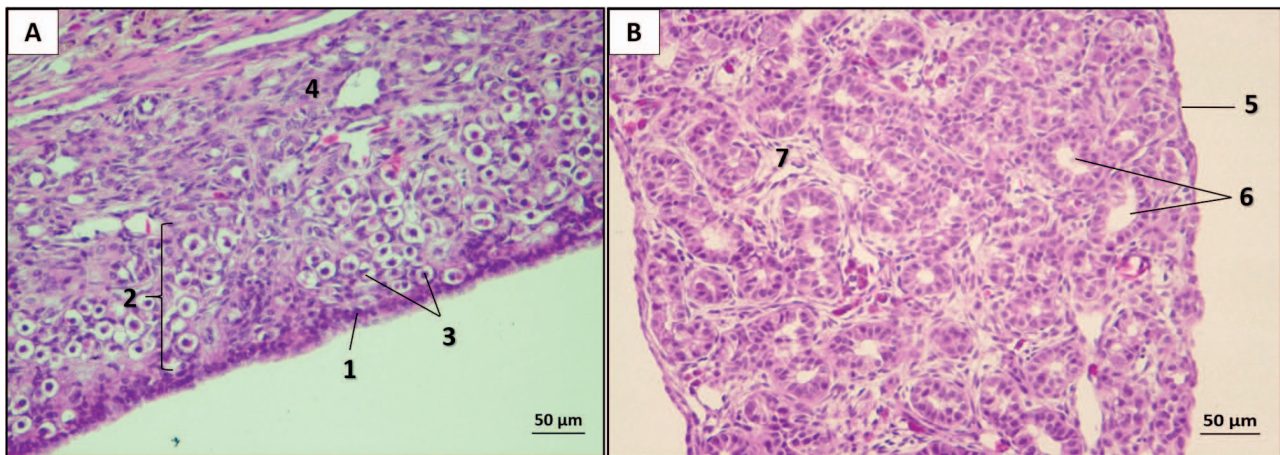


Figure 3. Photomicrography of the gonads of *Podocnemis unifilis* hatchlings. A) Female gonad: 1 – Germinative epithelium of ovary coating, 2 – Cortex, 3 – Germ cells, 4 – Medular region; B) Male gonad: 5 – Testicle-coating epithelium, 6 – Seminiferous tubules, 7 – Interstitial tissue.

Sex ratio balanced in nests of the Yellow-spotted Amazon River turtle

Table 1. Number of males and females and the proportion of each sex of *Podocnemis unifilis* hatchlings from nesting sites with different substrates in the Piagaçu Purus Sustainable Development Reserve (PPSDR), Amazonas, Brazil. *N° is the number of hatchlings sampled for histological analysis.

Sand bank						Clay banks					
Year	Nest	Clutch size	*N°	Males (%)	Females (%)	Year	Nest	Clutch size	*N°	Males (%)	Females (%)
2013	N1	18	9	2(0.23)	7(0.77)	2013					
	N2	28	14	6(0.43)	8(0.57)		N1	28	14	9(0.64)	5(0.36)
	N3	28	14	0(0)	14(1)		N2	24	12	12(1)	0(0)
	N4	22	11	3(0.28)	8(0.72)		N3	30	15	10(0.66)	5(0.34)
	N5	24	12	4(0.34)	8(0.66)		N4	18	9	6(0.66)	3(0.34)
	N6	24	12	7(0.58)	5(0.42)		N5	14	7	5(0.61)	2(0.39)
	N7	10	5	1(0.2)	4(0.6)		N6	32	16	12(0.75)	4(0.25)
2014	N8	30	15	5(0.34)	10(0.66)	2014	N7	44	22	5(0.23)	17(0.77)
	N9	6	3	0(0)	3(1)		N8	20	10	3(0.3)	7(0.7)
	N10	20	10	3(0.3)	7(0.7)		N9	38	19	12(0.63)	7(0.37)
	N11	22	11	6(0.55)	5(0.45)		N10	44	22	19(0.86)	3(0.14)
	N12	46	23	4(0.18)	19(0.82)		N11	28	14	11(0.78)	3(0.22)
	N13	34	17	5(0.3)	12(0.7)		N12	34	17	12(0.7)	5(0.30)
	N14	16	8	4(0.5)	4(0.5)						
Total		328	164	50(0.3)	114(0.7)	Total		354	177	116(0.66)	61(0.34)

Discussion

Sex ratio and nesting substrate

The relationship between sex ratio and the types of nesting substrate has been investigated only for a very small number of freshwater turtles (e.g., *Carettochelys insculpta*: DOODY et al. 2004, *Trachemys scripta*: SIFUENTES-ROMERO et al. 2017). Particularly for Amazonian species, such

as *P. unifilis* and *P. expansa*, studies that provide information about the sex ratio of hatchlings have been mainly conducted on nests deposited in sandy substrates and those incubated under captive conditions (SOUZA & VOGT 1994, PÁEZ & BOCK 1998, BONACH et al. 2011, MALVASIO et al. 2012, PIGNATI et al. 2013), or focused on nesting areas with clayey substrates (FACHÍN-TERÁN & VON MÜLHEN 2003, ESCALONA et al. 2009, ARRAES et al. 2014). To the best of our current knowledge, the present study is the first to compare the sex ratio of *P. unifilis* hatchlings from nests laid in different substrate types under natural conditions and for more than one nesting season in the same geographical area. We have demonstrated that the type of nesting substrate influences the proportion of male and female *P. unifilis* hatchlings. Our results highlight the importance of sampling nesting areas with different types of substrate to verify the sex ratio of hatchlings from one region, and to infer the overall proportion of males and females that hatch in a given population.

Our study focused on the importance of soil type on nest temperature and therefore on the sex ratios of hatchlings. As we expected, the environmental conditions offered by the substrate resulted in nest temperatures that determined the sex of *P. unifilis* hatchlings. The proportion of females was higher than that of males in nests incubated on the sand bank, where we recorded higher daily mean temperatures and higher daily amplitudes of nest temperature. Sand banks have a higher incidence of solar irradiation, and due to their higher permeability (MROSOVSKY & PIEAU 1991, HAYS et al. 2001) facilitate the passage of mineral salts and water to the deeper layers of the subsoil (LEPSCH 2011, SANTOS et al. 2013). Besides its limiting effect on plant cover growth and its possible shadowing of

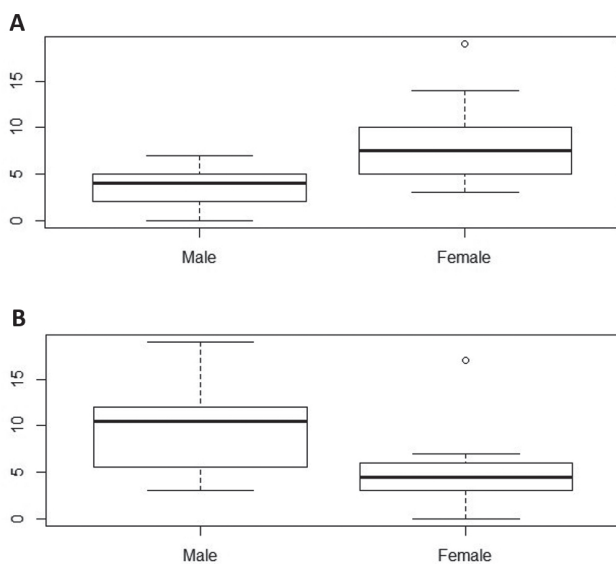


Figure 4. Numbers of males and females from nests sampled on A) the sand bank and on B) the clay banks monitored in the Piagaçu Purus Sustainable Development Reserve (PPSDR), Amazonas, Brazil.

the nests, sand offers an increased substrate temperature (HAYS et al. 2001), and as a consequence decreases incubation time (ERICKSON et al. 2020). On the other hand, the grains of clayey soils are smaller than those of sand, retain less heat (LEPSCH 2011, SANTOS et al. 2013), and prolong incubation periods (PIGNATI et al. 2013, ERICKSON et al. 2020). These characteristics led to a higher number of male hatchlings emerging from this substrate than from nests observed on the sand bank.

Studies of TSD turtle species usually find higher proportion of female hatchlings (VOGT & BULL 1984, HAWKES et al. 2007, PATINO-MARTINEZ et al. 2012). This pattern is also confirmed for *Podocnemis*, however, most studies on this genus so far were conducted on alluvial substrates and sand banks (MALVASIO et al. 2012, PIGNATI et al. 2013), which have higher soil temperatures than other types of nesting substrates. However, PIGNATI et al. (2013) observed that sex ratio differed between the 2007 and 2009 nesting seasons in sandy soil on the lower Amazon River, northeast of Santarém, municipality of Pará state, Brazil, due to environmental factors. Nests in more humid, shaded areas on sand and on clay banks can decrease the number of females or produce hatchlings at a balanced sex ratio (REFSNIDER et al. 2013).

Climate change and trends

The evolution of sex determination from GSD to TSD in turtles seems to have occurred independently in different lineages (VALENZUELA & ADAMS 2011) and may be related to the ability of turtles to adapt to the range of climate changes that have occurred during their evolutionary history (FUENTES et al. 2010). The increase of global temperature is often linked to the feminization of TSD turtle hatchlings, particularly in nesting areas located in open sandy substrates (HAWKES et al. 2007, PATINO-MARTINEZ et al. 2012). In the Purus River region, we found no inter-annual differences in the overall proportion of male and female hatchlings (considering both nesting sites), which indicates that the utilisation of different nesting substrates may counterbalance the possible effects of temperature on the sex ratio of the local population of *P. unifilis*. For another population of the species, it was verified that the sex ratio varied between years, with more females hatching in 2007 and more males in 2009 (PIGNATI et al. 2013). However, this study was conducted on sand banks where the alluvial substrate was rich in organic matter. Our results reinforce the need for further long-term investigations to properly record effective changes in the sex ratio of natural populations of turtles and its relationship with environmental variables. In particular, the selection of reproductive patterns over several generations is likely to mitigate the effects of climate change on turtle populations by narrowing the sex ratio bias of hatchlings (HAWKES et al. 2007, NEUWALD & VALENZUELA 2011). Turtles have persisted during dramatic periods of global warming in the past (HAWKES et al. 2007), but current climatic changes are oc-

curring much faster due to the human activities (BROHAN et al. 2006), which creates uncertainty as to whether turtles are able to adapt their physiological characteristics at the same speed (HAMANN et al. 2007).

Conservation and management

Differences in the proportion of male and female hatchlings may not necessarily have negative impacts on populations, since different mortality rates between age categories may result in a different adult sex ratio (WIBBELS et al. 1991, DELGADO et al. 2010). FACHÍN-TÉLAN & VOGT (2004) observed that the sex ratio of adult *P. unifilis* in the Guaporé River in the Brazilian state of Rondônia was 9.8 males to 1 female ($n = 613$), and attributed these differences not only to fewer females than males being produced in different nesting substrates, but also to the preferential exploitation of females by hunters. Moreover, the biological characteristics of some turtle species might be able to ensure the viability of populations with a female-biased sex ratios, at least along short to moderately long time scales. Viable populations of marine turtles are known to have a proportion of one male to two or three females (FUENTES et al. 2010). *Podocnemis unifilis* is a polyandrous species (FANTIN et al. 2008), and females of some turtle species can store viable sperm for many years (GIST & CONGDON 1998, PEARSE & AVISE 2001). Thus, populations with more females are likely to be stable.

Differences found in the life histories of *P. unifilis* reflect flexible responses largely linked to the diversity of the habitats this species utilises for nesting (ERICKSON et al. 2020). In our study, we have demonstrated that this characteristic is important for maintaining a balanced sex ratio amongst hatchlings in the lower Purus River basin. TSD turtles species that nest in different types of substrate are exposed to different environmental conditions (ERICKSON et al. 2020) and, therefore, are supposedly less impacted by increasing global temperatures (JANZEN & MORJAN 2001, KAMEL & MROSOVSKY 2005), at least in not too drastic climatic scenarios. Despite the behavioural plasticity observed, *P. unifilis* is still highly impacted by the illegal trade in its eggs and adults, with conservation measures for populations of *P. unifilis* being concentrated on (or limited to) the monitoring and management of nesting sites on sand banks. Thus, strategies to conserve this species should also include nesting areas predominated by clay banks (ERICKSON et al. 2020).

Lastly, it is important to highlight the need for in-depth and long-term studies to understand the sex determination process in nests naturally incubated under different habitat conditions and in different soil types in various geographical areas, as well as the ability of *P. unifilis* to adapt to different and changing environmental circumstances. The majority of studies investigating sexual determination in TSD turtles have been conducted on nests incubating at constant temperatures (VALENZUELA 2001, YOUNG et al. 2004, GEORGES 2013), whereas nests in natural conditions experience complex temporal temperature fluctuations (JAN-

ZEN 1994, SHINE & HARLOW 1996). These pieces of information are, therefore, essential for effective conservation planning for threatened populations of *P. unifilis*, as well as other TSD turtle species in the face of current global climate changes.

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