

Reproductive traits of sympatric populations of *Physalaemus albonotatus* (Steindachner, 1864) and *P. santafecinus* Barrio, 1965 (Anura: Leptodactylidae)

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Abstract. *Physalaemus albonotatus* and *Physalaemus santafecinus* lay their eggs in foam nests that float on the water surface and may do so syntopically. However, details of this behavior remain poorly studied, and other reproductive traits are still unknown. Therefore, the present study aimed to describe and compare the reproductive traits of *P. albonotatus* (*Pa*) and *P. santafecinus* (*Ps*) sympatric populations from the floodplain of the Middle Paraná River (Santa Fe, Argentina). For each species, we characterized the spatial arrangement of the foam nests in the water bodies and their morphology. We estimated the size of the clutches and described egg color and chemical composition: glycogen, lipids, and proteins proportions. We described the anatomo-morphological changes during the embryonic stages of development. Finally, we estimated the hatching time and evaluated the hatching success *in situ*. The results show a high overlap in reproductive traits between the two species, without statistical differences in distance to the nearest coastline (*Pa* = 211.33 ± 152.72 mm, n = 6; *Ps* = 132.25 ± 176.50 mm, n = 8) and water depth under foam nest (*Pa* = 176.00 ± 109.03 mm, n = 6; *Ps* = 130.63 ± 133.86 mm, n = 8), the macromolecular composition of the eggs (Glycogen: *Pa* = 3.69 ± 1.30 mm, n = 5; *Ps* = 6.11 ± 2.30 mm, n = 8; Lipids: *Pa* = 19.48 ± 4.09 mm, n = 5; *Ps* = 15.61 ± 3.60 mm, n = 8; Proteins: *Pa* = 24.10 ± 8.66 mm, n = 2; *Ps* = 5.24 ± 3.05 mm, n = 6), embryonic development, and hatching success (*Pa* = 0.93 ± 0.04, n = 4; *Ps* = 0.96 ± 0.02, n = 4). We recorded differences in the diameter (*Pa* = 43.97 ± 6.95 mm, n = 12; *Ps* = 55.77 ± 5.84 mm, n = 11) and height of the foam nest (*Pa* = 30.01 ± 5.94 mm, n = 12; *Ps* = 36.40 ± 7.94 mm, n = 11), the number of eggs per clutch (*Pa* = 498 ± 160, n = 25; *Ps* = 1,241 ± 556, n = 22) and hatching time (*Pa* = 39.07 ± 3.95 h, n = 7; *Ps* = 25.07 ± 3.58 h, n = 7). Divergence in these reproductive traits would facilitate the syntopic coexistence of the studied foam frogs.

Keywords: amphibians, clutches, eggs, embryonic development, foam nest.

Introduction

Reproductive mode is defined by Salthe & Duellman (1973) as a combination of developmental and oviposition factors, including oviposition site characteristics, egg and clutch characteristics, duration of development, offspring stages, and size, and, in certain cases, the presence of parental care. So far, 71 different reproductive modes in amphibians have been described, encompassing a different combination of traits (Duellman & Trueb 1994, Haddad & Prado 2005, Iskandar et al. 2014, Seshadri et al. 2015, Nunes de Almeida et al. 2021). In Argentinean leptodactylids, two reproductive modes are recognized involving laying eggs in foam nests, with nests placed in or out of the water (Lavilla 2018), and rapidly hatching eggs (from a few hours to 2 days) (Downie 1993, Zaracho et al. 2005, Valetti et al. 2014). Foam nests are considered an adaptation to environments with unpredictable rains, as the foam protects the eggs and tadpoles against desiccation (Heyer 1969, Duellman & Trueb 1986) and predators (Downie 1990, Menin & Giaretta 2003). The nine species of the genus *Physalaemus* Fitzinger 1826 (Anura: Leptodactylidae) found in Argentina lay their eggs in a foam nest that floats on the surface of the water; the embryonic development and the first stages of the typical larva take place in the nest without parental care (Duellman & Trueb 1994, Lavilla 2018). Additionally, embryonic

development in species with oviposition strongly depends on the egg's macromolecular composition (e.g., carbohydrates, lipids, and proteins) for its nutritional requirements (Lubzens et al. 2010, Li & Zhang 2017). Still, few studies have analyzed these parameters in amphibian eggs (e.g., Wallace & Selman 1990, O'Brien et al. 2010).

The studied species were *Physalaemus albonotatus* (Steindachner 1864), and *P. santafecinus* Barrio 1965. Both species are semi-terrestrial, with small body size (snout to vent length: 28–34 mm) and explosive pattern reproductive activity concentrated in the spring–summer (September to March) (Zaracho et al. 2005, Ghirardi & López 2020). *Physalaemus albonotatus* is widely distributed in Brazil, Bolivia, Paraguay, and Argentina. In Argentina, this species inhabits the provinces of Misiones, Formosa, Chaco, Corrientes, Entre Ríos, and Santa Fe (Vaira et al. 2012). The distribution of *P. santafecinus* is smaller, encompassing a small portion of southern Paraguay and parts of the Argentinian provinces of Chaco, Corrientes, Formosa, and Santa Fe (Lavilla et al. 2002, Brusquetti et al. 2009, Vaira et al. 2012).

More information is available on the reproductive biology of *P. albonotatus* (Perotti 1994, Rodrigues et al. 2004, Zaracho et al. 2004, 2005, Prado & Haddad 2005, Schaefer & Kehr 2010, Gómez et al. 2016, Pupin et al. 2018, Cajade et al. 2020) than *P. santafecinus* (Cajade et al. 2012, 2020). To

further understand the mechanisms facilitating the syntopic coexistence of these two species, our goal was to fill knowledge gaps, mainly on *P. santafecinus*, and compare reproductive traits between species. Thus, we describe and assess differences in foam nests morphology (diameter and height), foam nests' spatial arrangement (depth under the nest and distance to the nearest coastline), number and color of eggs, embryonic development, egg's macromolecular composition, and hatching time and success, in sympatric populations of *P. albonotatus* and *P. santafecinus* in the floodplain of the Paraná Medio River (Santa Fe, Argentina).

Material and methods

Field observations and data collection were carried out during December 2014, March 2015, January, February, March, October, and December 2016 (Table 1) in three water bodies from the floodplain of the Middle Paraná River, Santa Fe province, Argentina: Site 1 (31°38'27.8" S, 60°40'20.5" W, datum WGS84), Site 2 (31°36'18.2" S, 60°36'00.7" W, datum WGS84); Site 3 (31°33'54.4" S, 60°31'15.3" W, datum WGS84) (Fig. 1). The three water bodies were temporary ponds of approximately 3000–3500 m², with a maximum depth of

28–35 cm, surrounded mostly by Poaceae vegetation. Rainfall data for the week before each field observation were provided by the Centro de Informaciones Meteorológicas (FICH-UNL) (Lewis & Goldingay 2005) (Table 1).

Table 1. Date, sampling site, number of clutches collected for *Physalaemus albonotatus* (*Pa*) and *P. santafecinus* (*Ps*), and accumulated rainfall during the seven days before sampling.

Sampling date	Site	<i>Pa</i>	<i>Ps</i>	Rainfall (mm)
11 December 2014	2	2	4	61.50
4 March 2015	2	0	2	193.00
23 March 2015	1	5	0	14.50
14 January 2016	2	6	3	20.50
10 February 2016	2	3	3	96.50
3 March 2016	2	3	0	103.00
17 October 2016	3	0	2	52.00
24 December 2016	3	6	0	82.00
26 December 2016	3	4	4	160.75
27 December 2016	3	0	8	160.75

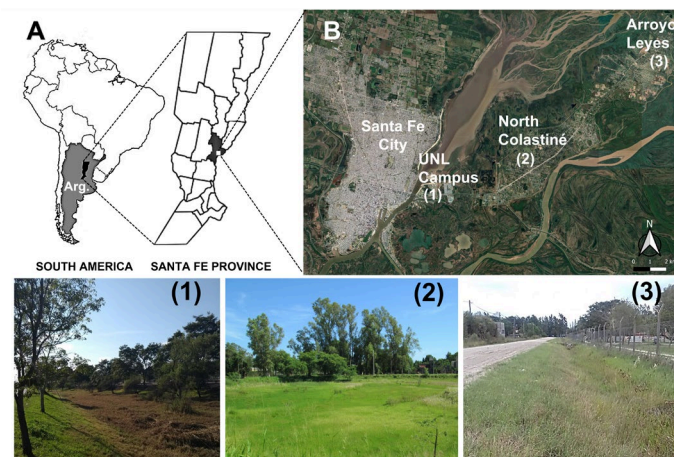


Figure 1. Studied sites. (A) Geographical representation of the study sites in the continent, country and province. (B) Satellite image with the localization of three studied sites (Google Earth). (1) Site 1, located between the University City of UNL and National Route 168, in Santa Fe city. (2) Site 2, located in the North Colastiné residential neighborhood of Santa Fe city. (3) Site 3, located in the locality Arroyo Leyes.

Foam nest size and spatial arrangement

In every sampling date, we randomly selected 1 or 2 foam nests from the found species and measured the nest's maximum diameter and height *in situ* (*P. albonotatus* $n = 12$, *P. santafecinus* $n = 11$) with a digital vernier caliper (precision 0.05 mm) and the distance to the nearest coastline and the water depth under foam nest with a measuring tape (precision 1 mm). We collected foam nests from both species (*P. albonotatus* $n = 25$; *P. santafecinus* $n = 22$) and fixed them *in situ* in 4% formalin. In the laboratory, we gently separated and counted each egg from the nests under a stereomicroscope. Following the German Reichs-Ausschuß für Lieferbedingungen color standard code (RAL), we registered the eggs' color while being illuminated with a 450lm lamp at a distance of 20 cm. Also, we compiled published data on the number of eggs per nest from different populations of both species. The height and diameter of the foam nest, the water depth under the nest, the distance to the nearest coastline, and the number of eggs (i.e., response numerical variables) were compared between the species (explanatory categorical variable with two levels) using Gaussian-based linear models (LMs). Residual plots (quantile-quantile plots, residual vs. fitted values), Shapiro-Wilk, and Levene's test were used to test homoscedasticity

and normality of residuals of LMs. Because the LMs that included water depth under the nest and the distance to the nearest coastline as response variables presented heteroscedasticity of residuals, we assessed the significance of these LMs using a permutation-based version of the Wald-type statistic (WTPS) since this analysis does not require normally distributed data or variance homogeneity (Friedrich et al. 2017). These statistical analyses were performed using the package GFD (Friedrich et al. 2017) in R 4.1.1 (R Core Team 2020). For all analyses, we used a significance level (alpha) of 0.05. Values were expressed as means ± 1 standard deviation (SD).

Eggs macromolecular composition

To determine the macromolecular composition of eggs, we separated 15 eggs (stages 8–15; Gosner 1960) from six fresh clutches (a total of 90 eggs) of *P. albonotatus* and eight fresh clutches (a total of 120 eggs) of *P. santafecinus*. Eggs were carefully separated from the foam and frozen at -18 °C. Eggs' glycogen, lipids, and proteins were quantified following the protocols of Seifter et al. (1950), Folch et al. (1957), and Lowry et al. (1951). Glycogen (*P. albonotatus*, $n = 6$; *P. santafecinus*, $n = 8$) and lipids (*P. albonotatus*, $n = 6$; *P. santafecinus*, $n = 8$) were compared between species using LMs (see Foam nest size and spatial

arrangement). Proteins were quantified in only two samples for *P. albonotatus* and six samples for *P. santafecinus*; thus, differences between species were not statistically assessed.

Embryonic development

To study changes in embryonic phases, we searched from 21:00 h for three fresh clutches from each species, laid during the same night at Site 3, thus exposed to comparable environmental and microhabitat conditions to reduce external differences in embryonic development. In the field, we extracted 10 eggs from each sampled clutch immediately after the foam nest was constructed and then every three hours until the eggs hatched. The samples were fixed *in situ* in 4% formalin. Embryos were studied and photographed in the laboratory using a digital camera (Canon EOS Rebel T2i) mounted on a stereoscopic microscope (Leica L2). Embryonic changes were interpreted and described following Gosner (1960) and Gómez et al. (2016).

Hatching time and success

We selected foam nests of each species built during the same night at Site 3 to study hatching time (*P. albonotatus*, $n = 7$; *P. santafecinus*, $n = 7$) and success (*P. albonotatus*, $n = 4$; *P. santafecinus*, $n = 4$). Immediately after construction, each foam nest was placed inside a closed plastic container (size: 170 × 220 mm side, 50 mm depth), filled $\frac{3}{4}$ with pond water and placed back in the same location where it was found (Fig. 2). Every three hours, the nests were checked, and the live and/or dead larvae in the container were recorded; these larvae were extracted and fixed in 4% formalin for later processing. Once the nest was completely dismantled and larvae were no longer recorded, the remaining foam with unhatched eggs were collected. We calculated the proportion of hatched and unhatched eggs per clutch. The hatching time and success were compared between species using LMs (see Foam nest size and spatial arrangement).

Results

Both species reproduced during spring and summer, following accumulated rainfall above 14.50 mm (14.50 to 193.00 mm) during the previous week (Table 1). We registered aggregations of nests intentionally built close together (communal clutches) of both species in site 2 and site 3 (Fig. 3).

We found a significant difference in foam nest height (H) ($F = 4.82$; $p = 0.03$; $df = 1$), with *P. albonotatus* foam nests being lower. Also, we found a significant difference in foam nest diameter ($F = 19.21$; $p < 0.001$; $df = 1$), with *P. albonotatus* foam nests having a smaller diameter (Table 2). However, no significant differences were found between water depth under the foam nest (WTPS = 0.48; $p = 0.49$) nor in distance to the nearest coastline (WTPS = 0.80; $p = 0.39$) (Table 2).

We found significant differences between species in the number of eggs per clutch (WTPS = 9.01; $p = 0.005$). Clutches of *P. albonotatus* (mean = 498 ± 160 , range = 184–840, $n = 25$) were smaller than clutches of *P. santafecinus* (mean = $1,241 \pm 556$, range = 593–2,781, $n = 22$) (Table 3). Published data shows that the number of eggs per laying is highly variable among populations in both species (Table 3). We also noticed differences in the egg color, with *P. albonotatus* eggs being pure white (RAL 9010) and those of *P. santafecinus* ivory (RAL 1014).



Figure 2. Foam nest used to evaluate hatching time and hatching success.



Figure 3. Communal clutch of *Physalaemus albonotatus*. Approximately 38 foam nests laid in a temporary pond.

Table 2. Height, diameter, depth and distance to the nearest coastline of the foam nests of *Physalaemus albonotatus* (*Pa*) and *P. santafecinus* (*Ps*). *X*: mean; SD: standard deviation.

Species	Nest characteristics	n	X (mm)	Range (mm)	SD (mm)
<i>Pa</i>	Height	12	30.01	21–38	5.94
	Diameter	12	43.97	35–57	6.95
	Depth	6	176.00	86–320	109.03
	Nearest coastline	6	211.33	0–410	152.72
<i>Ps</i>	Height	11	36.40	24–47	7.94
	Diameter	11	55.77	45–65	5.84
	Depth	8	130.63	10–400	133.86
	Nearest coastline	8	132.25	0–500	176.50

Eggs macromolecular composition

We found no significant differences between species in egg content of glycogen and lipids (WTPS = 0.57, $p = 0.33$, $n = 6$; WTPS = 0.04, $p = 0.33$, $n = 8$, respectively) (Table 4). The proteins values were very variable (17.98 μg and 30.23 μg proteins/egg for *P. albonotatus* and 1.03 μg , 3.26 μg , 5.41 μg , 4.42 μg , 8.22 μg and 9.13 μg proteins/egg for *P. santafecinus*) (Table 4).

Embryonic development

The first samples of eggs used to describe embryonic development were taken 2 to 3 hours after being laid by the parent frogs. The earliest foam nests for *P. albonotatus* were recorded at 22:15 h, 22:30 h, and 22:35 h and had dimensions (height × diameter, mm) of 35 × 41, 24 × 40, and 35 × 39, respectively. When initially observed, embryos were in stages 8–11 (Gosner 1960).

The earliest foam nests of *P. santafecinus* were recorded at 21:45 h, 22:10 h, and 22:35 h and had dimensions (height × diameter, mm) of 43 × 56, 30 × 53.5, 26 × 45. The three clutches had embryos between stages 12 and 13 (Gosner 1960).

The anatomo-morphological changes of the embryonic

phases through both species were observed and are described as follows:

Embryonic development of *Physalaemus albonotatus* (Fig. 4)

Stage 8. Easily distinguishable blastomeres (B), no synchronization in the division planes.

Stage 9. No differentiation in blastomeres boundaries. The embryo begins to have a smooth surface. It corresponds to a late blastula stage.

Stage 10. The cells begin to invaginate and form the blastopore dorsal lip (DL).

Stage 11. Cell invagination continues. The formation of the ventral lip around the blastopore gives rise to a yolk plug (YP) of significant size.

Table 3. Coordinates of sampled sites and numbers of eggs and nests (in brackets) for *Physalaemus albonotatus* (*Pa*) and *P. santafecinus* (*Ps*). * The authors do not provide the number of nests used to count the eggs. ** In the original work, the values appear as means and 1 standard deviation.

<i>Pa</i>	<i>Ps</i>	Coordinates	Source
184–840 (25)	593–2,781 (22)	31°33'S, 60°31'W	This work
320–1,163*	461–3,165*	27°25'S, 58°44'W	Cajade et al. (2020)
242–850 (6)		27°29'S, 58°45'W	Schaefer & Kehr (2010)
435–616 (3)		27°28'S, 58°46'W	Zaracho et al. (2005)
246–1,562 (19)		19°34'S, 57°00'W	Prado & Haddad (2005)
1474 (SD = 418)** (29)		20°40'S, 56°45'W	Rodrigues et al. (2004)

Table 4. Chemical composition of eggs evaluated for glycogen (nmol glucose/egg), lipid (nmol triolein/egg), and proteins (µg proteins/egg) content for *Physalaemus albonotatus* (*Pa*) and *P. santafecinus* (*Ps*). X: mean; SD: standard deviation.

Species	Chemical composition	n	X	Range	SD
<i>Pa</i>	Glycogen	5	3.69	2.43–5.84	1.30
	Lipids	5	19.48	13.05–23.72	4.09
	Proteins	2	24.10	17.98–30.23	8.66
<i>Ps</i>	Glycogen	8	6.11	3.46–9.91	2.30
	Lipids	8	15.61	9.66–19.95	3.60
	Proteins	6	5.24	1.03–9.13	3.05

Embryonic development of *P. albonotatus* and *P. santafecinus* (Figs. 4, 5):

Stage 12. The yolk plug decreases in size, and the egg begins to lose its spherical shape. It corresponds to a late gastrula stage.

Stage 13. The egg loses its spherical shape and begins to acquire an oblong shape. There is a groove that belongs to the incipient formation of the neural plate (NP). Stage 13 is early, and the blastopore (Bp) caused by gastrulation is observed (Fig. 5).

Stage 14. Oblong embryo. Neural folds (NF) forming a wide neural groove indicate an early stage. The neural groove (NG) is narrow, so stage 14 is advanced.

Stage 15. Fused neural folds. In figure 5, an advanced

stage 15 is observed, the embryo elongates and arches sharply, and three regions begin to differentiate (R1, R2, R3).

Stage 16. The embryo continues to elongate. Three regions are observed: cephalic region (CeR), abdominal region (AR) (large and globose due to the presence of vitellus), caudal region (CaR) (small and thin). In the first region, the stomodeum (S) outline is shown (advanced stage 16, Fig. 5).

Stage 17. Notably distinguishable regions: in the cephalic region, there is an invagination pertaining to stomodeum. In addition, a bulge on each side of that region will cause external gills (G). The abdominal region acquires a larger size and globose shape, and the caudal region becomes elongated and thin about the previous stage. It is possible to differentiate the somites (So) in the dorsal middle part of the embryo.

Stage 18. Two pairs of bumps in the cephalic region correspond to the outline of adhesive papillae (AP) (lower ventral cephalic region) and external gills (lower lateral cephalic region). The abdomen remains globose and large. Increases the size of the caudal region in which the dorsal and ventral fins are gently hinted. The somites are still observed.

Stage 19. Poorly developed external gills in the cephalic region, distinguishable as a bifurcated protuberance. The stomodeum continues to be observed, and the adhesive papillae become more evident. There is scattered pigmentation on the back. The tail developed (long and thin)

with the dorsal and ventral fins (VTF) (Fig. 5).

Stage 20. Increased pigmentation throughout the back. More developed external gills are observed in two pairs on each side of the cephalic region, one inferior with three filaments (IG1, IG2, and IG3) and another superior with two filaments (SG1 and SG2). The pair of adhesive papillae is easily visualized.

Stage 21. The body acquires an elongated and thin shape. Eyes (E) are more pigmented than the body; the gills change shape and size (several shorter branches). The abdominal

region gets thinner. The fins are translucent and larger (DTF, VTF) (observable in Fig. 5).

Hatching time and hatching success

A significant difference was found between species in time to hatch (WTPS = 24.07; $p = 0.018$). *Physalaemus albonotatus* had a mean hatching time of 39.07 ± 3.95 h (range=33.50–43.00 h; $n = 7$), while the mean hatching time of *P. santafecinus* was 25.07 ± 3.58 h (range=22.33–31.25 h; $n=7$) (Fig. 6).

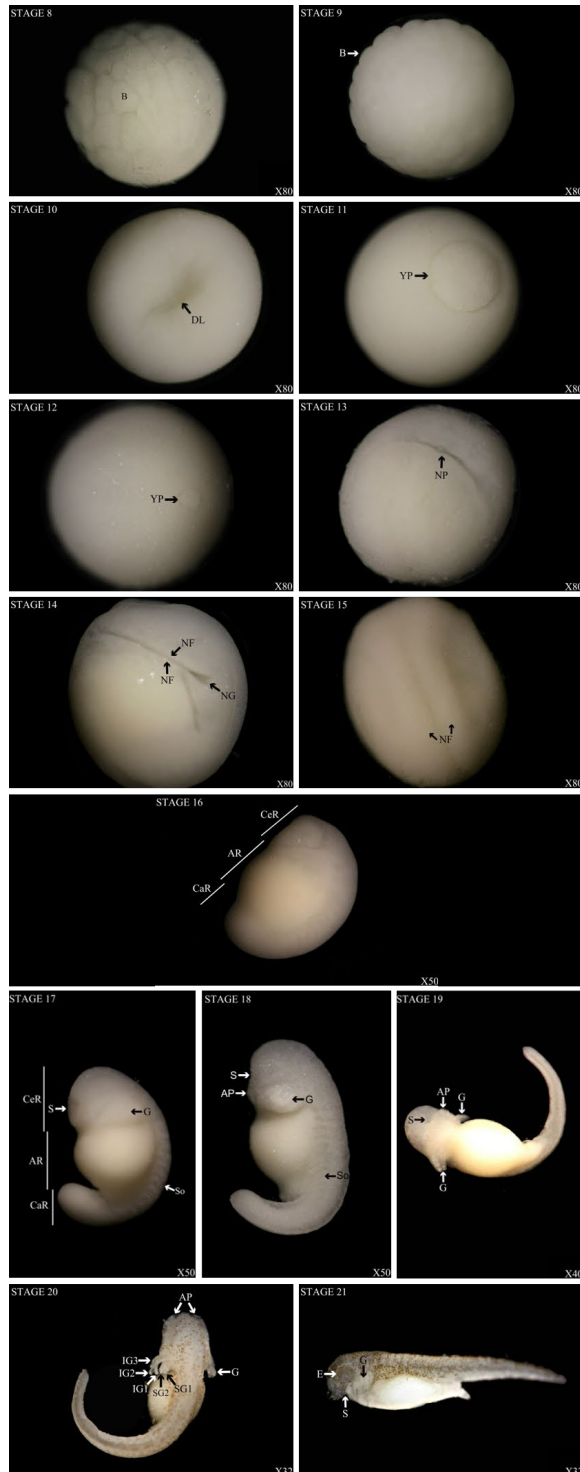


Figure 4. Details on embryonic development of *P. albonotatus*.

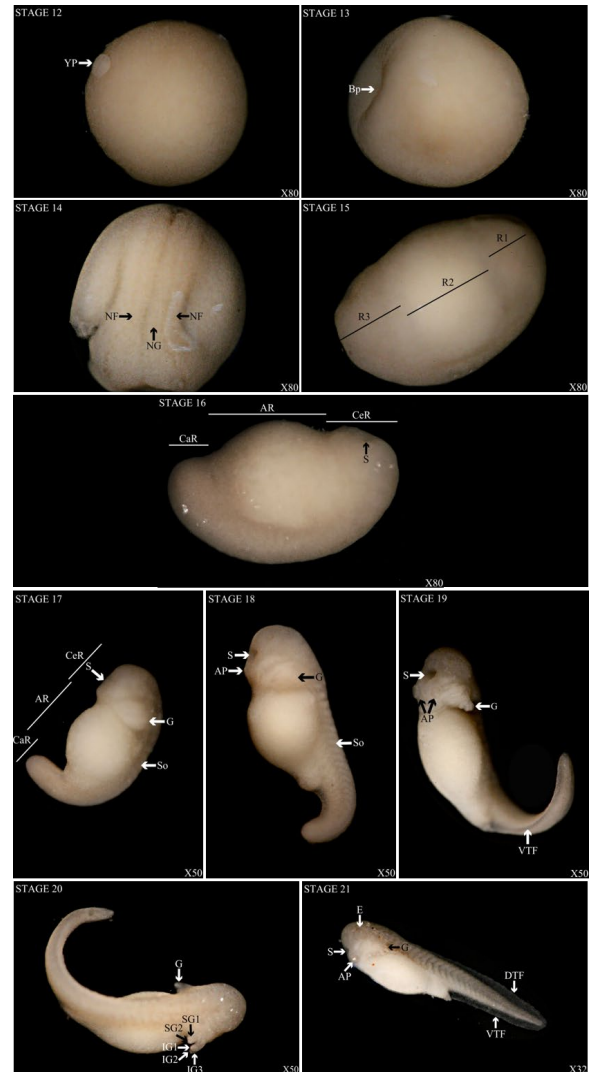


Figure 5. Details on embryonic development of *P. santafecinus*.

The proportion of hatched eggs in *P. albonotatus* clutches was 0.93 (SD = 0.04; $n = 4$), while that of *P. santafecinus* was 0.96 (SD = 0.02; $n = 4$) (Fig. 6). No significant differences were found between species in hatching success (WTPS = 1.607; $p = 0.258$).

During the 43 hours that the study on time and hatching success lasted, air temperatures were 20.2 °C to 33.6 °C, the sky was mostly cloudy, the relative humidity was 80% to 99%, and the atmospheric pressure was 1001.5–1014.2 hPa. The water temperature at the nest site ranged from 24.8 °C to 32 °C during the day.

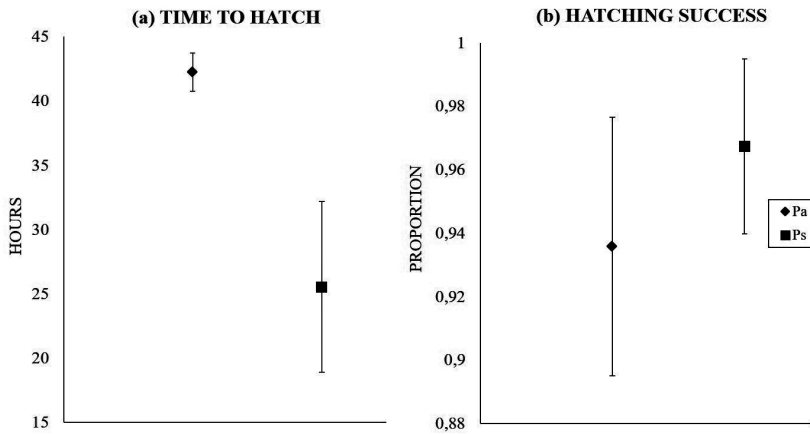


Figure 6. Mean hatching time of eggs per clutch (a) and success of eggs per clutch (b) of *P. albonotatus* (Pa) and *P. santafecinus* (Ps).

Discussion

We confirmed a major overlap in the reproductive characteristics of the two studied species, which is not surprising considering they are congeners that inhabit the same habitats within their area of sympatry. Still, *P. albonotatus* and *P. santafecinus* differed in the diameter and height of the foam nest, the number of eggs per clutch, and hatching time, possibly as a way of decreasing interspecific competition when they reproduce in syntopy.

Physalaemus albonotatus and *P. santafecinus* overlapped in the reproductive microhabitat. Both laid eggs at the same water depth under the nest and the same distance to the coastline. Both species lay their eggs in shallow temporary water bodies or the shallow areas of semi-permanent water bodies (< 400 mm depth), flooded after spring and summer rainfall, in zones vegetated by emerging rooted macrophytes (Schaefer & Kehr 2010, Cajade et al. 2020). Using shallow temporary or semi-permanent ponds as reproduction microhabitats is a conserved trait, extended in Leiuperinae (Duré et al. 2004, Rodríguez 2004, Giarretta & Facure 2009, Hartmann et al. 2010). Syntopy at the microhabitat level, together with the temporal (seasonal) overlap of reproductive activity, imply a potential competition in this dimension of the ecological niche (Pianka 1986). However, they differ in other aspects, such as foam nest morphology and clutch size.

The height, diameter, and number of eggs per nest of *Physalaemus santafecinus* were greater than the ones of *P. albonotatus*. The significant differences found between species in foam nest size (height and diameter), also described by Cajade et al. (2020), are probably related to the differences in the number of eggs they lay. Several studies show that larger eggs or more numerous eggs per clutch come from larger females (Perotti 1994, 1997, Wells 2007, Camargo et al. 2008, Teixeira & Ferreira 2010, Guayara Barragán & Bernal 2012, Liedtke et al. 2014). However, females of both species have similar body sizes (Ghirardi & López 2020) and the same diameter of the eggs (~0.9 mm: Zaracho et al. 2005). Thus, the reproductive effort of *P. santafecinus* would be comparatively greater than that of *P. albonotatus*. This would be a particularly interesting

characteristic since it is the only strategy that would differentiate the reproduction between the two species and contribute to ecological niche segregation.

The number of eggs per nest in both species was highly variable. At lower latitudes, Cajade et al. (2020) recorded between 300 and 400 more eggs per nest in both species than our observed eggs. For *P. albonotatus*, Zaracho et al. (2005) and Schaefer & Kehr (2010), working at the same latitude as Cajade et al. (2020), report small differences between the studies that could be due to the number of nests analyzed. In even lower latitudes than the previously mentioned works, Rodríguez et al. (2004) and Prado & Haddad (2005) registered almost twice as many eggs per nest as our study (see Table 3). The variations found between the different studies may be due to intrinsic characteristics of the populations and individuals, such as being composed of females of different ages and sizes, where young and small females lay fewer eggs than older and larger females and, therefore, have different reproductive output (Salthe & Duellman 1973, Perotti 1997, Sousa & Ávila 2015). Another factor that may influence the differences is the number of oviposition events that females had during the reproductive season, with each consecutive reproductive event during the same season resulting in fewer eggs laid (Howard 1978). The differences in the number of eggs could also be explained due to the asynchronous maturation of the oocytes that would lead to different availability of mature eggs in each reproduction (Davidson & Hough 1969). Also, extrinsic characteristics of the population, such as geographic (latitudinal) or abiotic (temperature and rainfall) factors, are known to influence the number of eggs per laying (Morrison & Hero 2003). An idea for future assessments is that each female evaluates the possibility of the eggs' survival (considering the temperatures and rainfall) and "bets" a certain amount in each reproduction.

In contrast, a characteristic that shows a high overlap in reproductive characteristics between the species is the communal clutches. Communal clutches are a reproductive strategy present by both species. Different studies described communal clutches in species of the subfamily Leiuperinae. This behavior has been hypothesized as a reproductive strategy to (i) increase the protection against desiccation of

the embryos and decrease the predation on tadpoles (Giaretta & Menin 2004, Zina 2006, De Lacerda et al. 2010); (ii) anchor the clutches when the terrain is steep and sparse of vegetation (Giaretta & Facure 2006), although this would not be the case for here studied populations that inhabit water bodies without runoff; or, (iii) it can also be the involuntary result of massive aggregations of reproductive adults (Giaretta & Facure 2006). Within the genus *Physalaemus*, communal clutches have been recorded in *P. nattereri* (Steindachner 1863), *P. cuvieri* Fitzinger, 1826, *P. gracilis* (Boulenger, 1883), *P. biligonigerus* (Cope, 1861), *P. aff. olfersii* (Lichtenstein & Martens 1856) (Barreto & Andrade 1995, Giaretta & Menin 2004, Giaretta & Facure 2006, Zina 2006, De Lacerda et al. 2010, Villamil & Maneyro 2014). This behavior was also recorded in the genera *Pleurodema* (*P. guayanae*: Barrio 1964) and *Engystomops* (*E. pustulosus* (Cope 1864): Valetti et al. 2014), both from the subfamily Leiuperinae. Furthermore, we recorded a communal clutch composed of foam nests of the two species studied, a poorly documented event (Giaretta & Menin 2004, Cajade et al. 2020).

When analyzing the macromolecular composition of eggs, we found a similar composition of glycogen and lipids between species. However, as we had few samples with very variable protein values, we cannot conclude about this variable. Anyway, being a little-studied trait, our results serve as a basis for future studies and comparisons (e.g., with less related species). Considering the macromolecular composition similitude of the eggs between species, the observed differences in the embryo development timing should result from other factors rather than macromolecular composition (Lubzens et al. 2010, Li & Zhang 2017).

The embryos of the foam nests were initially at stage 8 for *P. albonotatus* and at stage 12 for *P. santafecinus*. Both were seen at the same time; therefore, fertilization in *P. santafecinus* occurred sometime earlier than in *P. albonotatus* (Cajade et al. 2020), or the development of the first stages of their embryos is faster. For both species, the embryos developed approximately one stage every three hours. Embryos belonging to a predominant stage were found in each sample obtained. However, eggs at one lower or higher stage than the most frequent were also observed in each sample, so it would be interesting to assess which factors are influencing the individual embryonic development of the eggs within the same nest, generating this asynchrony. From stage 16, the embryos curve over the yolk (Grosso et al. 2019). At stage 19, small anterodorsal brown spots appear that increase with each stage (Grosso et al. 2019). The characteristics observed at each stage for both species coincide with those described by Gosner (1960) and Gómez et al. (2016). In both species, embryos hatch from the egg at stage 21, with long gills and operculum at the base (Grosso et al. 2019). This stage corresponds to the second stage belonging to the prometamorphic larval stage, according to Gómez et al. (2016). According to Gosner (1960), the embryos hatch between stages 17 and 20, and from stage 21 and before stage 25, the transition to a feeding and free-

swimming tadpole occurs.

Interestingly, although embryonic development is similar in both species, we found differences in hatching time. The time to hatching was greater in *P. albonotatus* than in *P. santafecinus*. Considering that the studies were carried out simultaneously in the same pond for both species and, as we mentioned earlier, the size of the eggs does not differ between species; therefore, this difference should be attributed to the intrinsic genetic characteristics of species. Hatching is the first ontogenetic switch point in the lives of most animals. Hatchlings leave the protection and constraints of the egg, gaining mobility and access to external resources, but often suffer high mortality levels (Wilbur 1980, Gosselin & Qian 1997), creating a strong opportunity for natural selection (Arnold 1986). Hatching plasticity linked to the time of embryonic development and timing of hatching may occur in response to physical conditions, predators, and pathogens (Martin 1999, Warkentin 2007, Gomez Mestre et al. 2008). It may be for intra- or interspecific resource competition of hatchlings. The differences could indicate a strategy by *P. santafecinus* to release their larvae sooner in the water body so they could take advantage of the resources before the tadpoles of *P. albonotatus* do, moreover considering the similarity in ecomorphology of their tadpoles (Altig & Johnston 1989). Another possibility would be that hatching asynchrony decreases interspecific competition during the larval stage since morphologically similar but differently sized larvae would occupy different niches (for example, consuming prey of various sizes) (Santos et al. 2015, Protazio et al. 2019). These hypotheses must be tested by evaluating niche overlap and competition between tadpoles of each species. Shortening the egg phase could also be a strategy to reduce hazards from these stages, like predation, nanoparticles, parasitism, pond desiccation, and food availability, among other several threats to embryonic stages (Vargas & Gutiérrez 2005, Lind et al. 2008, Enriquez Urzelai et al. 2013, Fan et al. 2016, Nogueira Costa et al. 2016, Spence et al. 2016, Bach et al. 2018, Groffen et al. 2019, Hudgens & Harbert 2019, Úbeda et al. 2019). Among these threats, aquatic moulds from studied environments are known to increase the physiological stress and mortality of *P. albonotatus* embryos (Ghirardi et al. 2018). However, we did not observe infections in the studied clutches and registered a high hatching success. Vonesh & De la Cruz (2002) and Halliday (2008) showed that larval stages are more sensitive to predation, desiccation, and other common amphibian threats than egg stages. Moreover, a significant portion of the eggs that did not hatch (*P. albonotatus* = 7%, *P. santafecinus* = 4%) may correspond to unfertilized eggs or unviable embryos.

Amphibians are the vertebrate group with the highest proportion of vulnerable and declining species (Stuart et al. 2004, Lötters et al. 2009, Kiesecker 2011, IUCN 2022), knowing their basic biology is essential for their conservation. This work provides valuable data that help to fill the knowledge gap on the reproductive biology of *P. albonotatus* and *P. santafecinus*.

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