# BREEDING BIOLOGY AND MATING BEHAVIOR OF *Pithecopus gonzagai* (Anura: Phyllomedusidae) FROM THE BRAZILIAN SEMIARID

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*Abstract.—Pithecopus gonzagai* (no common name) is a recently described phyllomedusid in northeastern Brazil. Given its recent description, many aspects of its biology are completely unknown. Here we describe the reproductive behavior of *P. gonzagai* through the characterization of oviposition sites, the process of egg deposition, and the behavior of individuals in amplexus, and we evaluate the occurrence of assortative mating. We collected data in February-May of 2017 in the municipality of Groaíras, Ceará state, northeastern Brazil, in an area of Caatinga *sensu stricto*. Oviposition occurred predominantly in leaves of Euphorbiaceae (53.8%, n = 14) and Poaceae (23.08%, n = 6). The height of the selected plants averaged 102.3 cm, the leaves selected for oviposition duration averaged 48.5 min (n = 12), and clutch size averaged 102.6 eggs (n = 12), with a mean proportion of 96.2% of eggs fertilized. Males in amplexus interacted acoustically with neighbors. We also recorded cases of multiple males in amplexus and reactions of amplected males to the intrusions by rivals. Body mass and length of males in amplexus (n = 7) did not differ significantly from those of non-amplected males (n = 11). We found, however, a positive relationship between the mass of males and the mass of females paired in amplexus (n = 12), which suggests assortative mating. Our results expand the knowledge on the life history of frogs in the genus *Pithecopus* and the family Phyllomedusidae.

Key Words.—acoustic interactions; assortative mating; mating success; oviposition sites; reproductive behavior

*Resumo.—Pithecopus gonzagai* é um filomedusídeo recentemente descrito no nordeste do Brasil. Dado sua recente descrição, muitos aspectos de sua biologia são completamente desconhecidos. Aqui descrevemos o comportamento reprodutivo de *P. gonzagai*, através da caracterização dos sítios de oviposição, do processo de deposição dos ovos, do comportamento de indivíduos em amplexo, além de avaliar a ocorrência de acasalamento assortativo na espécie. Coletamos os dados entre fevereiro e maio de 2017, no município de Groaíras, Ceará, Brasil, em uma área de Caatinga *sensu stricto*. As oviposturas foram depositadas predominantemente em plantas das famílias Euphorbiaceae (53,8%, n = 14) e Poaceae (23,08%, n = 6). A altura média das plantas selecionadas para ovipostura foi de 102,3 cm, as folhas selecionadas estavam a uma distância média de 69,1 cm da superfície da água e a profundidade média da água sob as oviposturas foi de 16,6 cm. O tempo médio para deposição dos ovos foi de 48,5 min (n = 12) e as desovas foram constituídas em média por 102,6 ovos (n = 12), com uma proporção média de 96,22% dos ovos fertilizados. Machos em amplexo interagiram acusticamente com vizinhos. Registramos também casos de amplexos múltiplos e as reações dos machos amplexados à invasão por machos rivais. A massa e o tamanho corpóreo de machos em amplexo (n = 7) não diferiram significativamente daqueles não-amplexados (n = 11). Entretanto, encontramos uma relação positiva entre a massa de machos e fêmeas acasalados (n = 12). Nossos resultados ampliam o conhecimento sobre a história de vida do gênero *Pithecopus* e da família Phyllomedusidae.

Palavras-chave.--acasalamento assortativo; comportamento reprodutivo; interações acústicas; sítios de oviposição; sucesso de acasalamento

#### INTRODUCTION

In anurans, the selection of mating partners can occur randomly, regardless of phenotypic attributes of mates (random mating; e.g., Székely et al. 2018), or consider individual traits of reproductive partners (non-random mating; e.g., Fan et al. 2013). Among non-random mating species, the main mechanisms related to partner acquisition are the contests between rival males for the access to females (intrasexual selection) and the specific mate choice by females (intersexual selection; Darwin 1871; Hosken and House 2011). Female anurans usually select males based on characteristics of their acoustic signals (e.g., call intensity and call duration) or their body attributes (e.g., mass and body length; Morris 1989; Tárano and Herrera 2003; Wogel et al. 2004). When phenotypic attributes of sexual partners tend to be similar (e.g., size of females positively related to the size of males), it is assumed to be an assortative mating system (reviewed in Jiang et al. 2013; Moura et al. 2021). Notwithstanding, the great variability observed in the presence and intensity of assortative mating among anurans still prevents the detection of consistent patterns in this taxon (Vojar et al. 2015; Green 2019). Besides partner selection, the reproductive success of the couple is also influenced by other behavioral aspects, such as the selection of suitable sites for oviposition (Silva and Giaretta 2008).

Oviposition in anurans consists of females depositing their eggs for fertilization by males. Currently, 71 types of reproductive modes are recorded for anurans that differ, among other criteria, by the type of substrate and microhabitat in which oviposition occurs (Nunes-de-Almeida et al. 2021). Although most anurans lay eggs directly in water (Wells 2007), in some species the eggs are placed on the back of the female, and many other species deposit their clutches in terrestrial environments (Haddad and Prado 2005; Pombal and Haddad 2007). For anurans with terrestrial reproduction, the selection of suitable reproductive sites is an important step for reproductive success because the risk of egg desiccation is higher in comparison with aquatic oviposition (García et al. 2013). This choice of specific microhabitats occurs through the selection of biotic and abiotic characteristics related to the greater suitability for the development of eggs and the survival of embryos (Silva and Giaretta 2008).

Phyllomedusidae species are known for laying their eggs on leaves above water bodies (Neckel-Oliveira and Wachlevski 2004; Oliveira 2017). After eclosion (hatching), the tadpoles fall into the water, where they conclude their development (Caramaschi and Cruz 2002). Despite the higher risk of desiccation out of water, laying eggs out of water is a strategy that can reduce the risk of predation by aquatic predators (Touchon and Worley 2015). Although some studies have investigated the characteristics of phyllomedusid oviposition sites (Neckel-Oliveira and Wachlevski 2004; Venâncio and Melo-Sampaio 2010; Dias et al. 2014; Oliveira 2017), few studies have addressed these reproductive aspects in highly seasonal environments (e.g., García et al. 2013). An example of a highly seasonal environment is the Caatinga, a domain located in the semiarid region of Brazil (the latter often referred to as the Brazilian Semiarid; Silva et al. 2017).

Pithecopus gonzagai (no common name) is a recently described phyllomedusid distributed to the north of the São Francisco River in northeastern Brazil (Andrade et al. 2020). Like its sister species Pithecopus nordestinus (no common name) from which it was split, this treefrog is arboricolous, feeding and breeding in the vegetation on the margins of temporary water bodies (Andrade et al. 2020). This is a primarily nocturnal species, but during its reproductive period it can be also found in activity in the early daylight hours of the morning (Brasileiro et al. 2020). Given its recent description, many aspects of its biology are completely unknown. The few exceptions concern information from some populations attributed to P. nordestinus that are actually related to P. gonzagai, such as descriptions and characterization of release and aggressive calls (Mângia et al. 2019; Brasileiro et al. 2021) and behaviors of fighting and selection of resting microhabitats (Brasileiro et al. 2020; Wachlevski and Specifically considering reproductive Passos 2021). behavior, it is only known that the species lays its clutches on leaves over lentic water bodies, from which exotrophic tadpoles hatch (reproductive mode 24 sensu Haddad and Prado 2005; reproductive mode 37 sensu Nunes-de-Almeida et al. 2021). Thus, in the present study, we describe in detail unknown aspects of the reproductive biology of P. gonzagai: the characteristics of oviposition sites, the process of deposition and fertilization rate of eggs, the behavior between pairs in amplexus and against intruders, and the relationships of body attributes between amplected males and females. Besides expanding the knowledge on mating behavior of a poorly known Pithecopus species, this information will enable better comparisons among congeners, contributing to understanding the variation in the breeding biology of phyllomedusids as a whole.

#### MATERIALS AND METHODS

We collected the data on 32 non-consecutive days between February and May (rainy season) of 2017 in the municipality of Groaíras, Ceará state, northeastern Brazil. The average air temperature in the region ranges from 26° to 28° C, the climate is referred to as semiarid hot tropical, and the average annual rainfall is approximately 900 mm with precipitation concentrated between January and April (IPECE [Instituto de Pesquisa e Estratégia Econômica do Ceará]. 2016. Perfil Básico Municipal - Groaíras 2016. Available from https://www.ipece.ce.gov.br/wp-content/uploads/ sites/45/2018/09/Groairas\_2016.pdf [Accessed 22 October 2018]). We collected data at two dams (reservoirs) and two temporary ponds at the localities of Itamaracá (3°52'04.2"S, 40°22'11.9"W and 3°51'49.7"S, 40°22'16.5"W) and Lagoa do Peixe (3°56'19.5"S, 40°23'39.2"W and 3°56'18.2"S, 40°23'17.3"W), both in the municipality of Groaíras.

The studied areas are located in a well-recognized ecological system (hereafter Caatinga) comprising a unique morphoclimatic and phytogeographical domain that lies in the semiarid region of Brazil (Silva et al. 2017). The Caatinga domain is characterized by marked seasonality of rainfall and high annual evapotranspiration, which results in hydric shortfall during most of the year (Coe and Sousa 2014). The term domain is applied due to the ecological and evolutionary heterogeneity of this region, which encompasses several types of vegetation other than the Caatinga *stricto sensu* (Queiroz et al. 2017).

We analyzed the oviposition behavior (from its beginning to completion) by the focal animal sampling method, making observations of each pair of amplected individuals in the field. We undertook most of the sampling between 1800 and 2400 but recorded some observations between 0525 and 0730 (see details in Brasileiro et al. 2020). We recorded the duration (in minutes) of the oviposition process and the time that the females remained at the location of oviposition after the males left. To characterize oviposition sites, we used a measuring tape (0.1 cm resolution) to determine the height above the ground level, the distance from nests to the surface of the water, and the depth of the water immediately below the clutches. We collected samples of the plants where the clutches were placed and identified them to the lowest possible taxonomic level. We collected the leaves with the nests in the field, placed them individually in plastic bags with water from the collection site to receive the tadpoles after hatching in the nest, and transferred them to the laboratory (6 km away from the study area). We removed the nests from the field site at least 5 d after oviposition to reduce possible effects of translocation on early embryonic development.

In the laboratory, we maintained clutches individually in closed plastic bags to ensure protection against predation, to maintain relatively constant humidity levels, and to allow the accurate monitoring of fertilization success. We counted the total number of eggs and the number of fertilized eggs for each oviposition, considering as fertilized eggs those from which tadpoles hatched or that exhibited some evidence of developmental progress (see Wogel et al. 2005; Fan et al. 2013). We monitored the nests daily and, on the day of eclosion, we counted the hatched tadpoles and released them in the exact sites where we collected the respective nests.

We investigated the behavior of pairs in amplexus by the focal observation of individuals. We identified the calls made by amplected males by audio/video recording (camera model DSC HX200V, Sony, Minato, Tokyo, Japan) and the respective counting of emitted calls. For classification of call types, we followed the parameters described by Mângia et al. (2019), Andrade et al. (2020), and Brasileiro et al. (2021). The advertisement call of P. gonzagai consists in a single pulsed note, with average duration of 28.5 ms, and dominant frequency varying from 1969 to 2391 Hz (Andrade et al. 2020). The aggressive call consists in a single multi-pulsed note with amplitude modulation, presenting quite variable duration between contexts out of fights and during physical clashes, but with a nearly invariant dominant frequency averaging 2126 Hz (Brasileiro et al. 2021). The uneasiness call consists of a call series of single pulsed notes with no amplitude and frequency modulation, with average duration of 36 ms and dominant frequency of 1508 Hz (Mângia et al. 2019). To compare morphological characteristics of amplected and non-amplected males, we recorded the mass (resolution at 0.01 g) and the snout-vent length (SVL; 0.1 mm resolution) of the individuals in amplexus, as well of other non-amplected males within a 50-cm radius from the amplected pair. After these measurements, we made photos of the flank region of all individuals, where they exhibit natural markings, enabling individual identification to avoid pseudoreplication (e.g., Oliveira et al. 2012; Lima-Araujo et al. 2021). To evaluate the relationship between body mass and length of couples in amplexus, we measured the mass and SVL of amplected males and females. We released all sampled individuals in the same place where they were found after taking the measurements, except for six adult specimens collected as voucher specimens that we deposited in the Herpetological Collection of the Universidade Federal do Ceará (CHUFC A8619-A8624; collection license no. 13587 from Instituto Chico Mendes de Conservação da Biodiversidade).

Before the analyses we evaluated the data regarding the assumptions of normality (Shapiro-Wilk test) and homoscedasticity (Levene test). When requirements were met, we used parametric tests; otherwise, we used non-parametric tests. We examined differences in body mass and length between amplected and nonamplected males using Student's *t*-tests. We evaluated the relationship of body length and mass between mates using Pearson's (*r*) and Spearman's (*rho*) correlations, respectively. Additionally, we also provided the specific parameters of the Linear Regression between body mass of males and females that was statistically significant. We also evaluated the relationship of female body length and mass with the clutch size using Pearson's and Spearman's correlations, respectively. We conducted all statistical analyses in R v.4.1.0 (R Core Team 2021). For all tests,  $\alpha = 0.05$ .

#### RESULTS

We gathered information on 27 clutches of P. gonzagai, all deposited on leaves of plants at the margins of temporary water bodies. The descriptive statistics follow as: mean  $\pm$  standard deviation (range, sample size). We identified 26 of 27 plants used for oviposition. Plants belonged to the taxa Euphorbiaceae: Sacatrapo (Caperonia palustris; 53.8%, n = 14); Poaceae: grasses (23.08%, n = 6); Malvaceae: Pyramid Flower (Melochia pyramidata; 11.5%, n = 3); Fabaceae: Sickelpod (Senna obtusifolia; 4.4%, n = 1); Convolvulaceae: Hairy Wood Rose (Merremia aegyptia; 4.4%, n = 1) and morning glory (Ipomoea sp.; 4.4%, n = 1), with an average height of  $102.3 \pm 28.8$  cm (56–160 cm, n = 20). The leaves selected for oviposition were at  $69.1 \pm 28.5$  cm (22–14 cm, n = 20) above the water surface, and the depth of water below the clutches was  $16.6 \pm 10.5$  cm (3–46 cm, n = 20). Most clutches (81.48%, n = 22) were deposited on only one leaf; however, one female joined two Poaceae leaves to place a single clutch (Fig. 1), and two other females placed part of the clutch on a leaf and a subset of eggs on another leaf, representing the partition of a single clutch onto separate leaves. In one of these cases in which we were able to access the nest, most

eggs (114 eggs, 93% of the clutch) were laid in the first leaf and nine eggs (7% of the clutch) were laid in the second one.

Throughout the night, we observed non-amplected females (n = 9) either standing over the vegetation (n = 6) or manipulating leaves with hindlimbs (n=3) with movements similar to those used during spawning (described below). Most (97.5%, n=39) of the monitored pairs were already in amplexus at the beginning of the observation, except for one, which was observed from the beginning of the approach between individuals. On this occasion, a male approached the female without vocalizing and jumped onto her back while the calling male in that area was involved in an agonistic interaction with another rival male. In all the amplexus events, the females walked with the males on their back and chose a leaf for oviposition. Oviposition behavior was always performed with movement of the pair from the distal portion of the leaf towards the proximal portion of the leaf, on its adaxial surface (Fig. 2). Females released the eggs in gelatinous capsules by abdominal contractions. Females closed the leaves with their posterior limbs, always orienting toward the proximal part of the leaf. The oviposition duration averaged  $48.5 \pm 17.6 \min (30 - 100)$ 82 min, n = 12). After spawning, the males were the first to leave the oviposition sites. After the males left, the females remained near the clutches for  $3.0 \pm 1.2$  min (1-5 min, n = 11) performing abdominal movements. The average number of eggs per clutch was  $102.6 \pm 22.7$ (75-138, n = 12). There was no significant relationship between the clutch size and female body length (t =0.298, P = 0.775; n = 9) or body mass (t = 76.64; P = 0.339; n = 9). The percentage of fertilized eggs per



**FIGURE 1.** *Pithecopus gonzagai* (no common name) nests on Poaceae leaves in Groaíras municipality, Ceará state, northeastern Brazil. (A) Nest formed by a single leaf (inside the folded leaf, indicated by arrow). (B) Nest formed by the joining of two Poaceae leaves (conspicuous white eggs, indicated by arrow). (Photographed by Ana Brasileiro).



**FIGURE 2.** Amplexus by *Pithecopus gonzagai* (no common name) from Groaíras municipality, Ceará state, northeastern Brazil. (A) Single amplexus. Note the direction of egg deposition from the distal towards the proximal region of leaves. (B) Multiple amplexus showing the interference of four extra-pair males on an amplected pair. (Photographed by Ana Brasileiro).

nest averaged  $96.2 \pm 1.93\%$  (91.8-98.4%, n = 10). In a particular case, in which a female was amplected by two males, the female laid part of the clutch in one leaf while mounted by both males (114 eggs, of which 80.7% were fertilized) and another batch of eggs in a second leaf with a sole remaining amplected male (nine eggs, none fertilized). In this event, the overall percentage of fertilized eggs was 74.8% (92 of 123 eggs).

We monitored the acoustic behavior of 11 males in amplexus, totaling 41.28 min of recordings and averaging 2.43 min per individual. Most males (82%) emitted advertisement calls (40.89  $\pm$  30.14; 5-82 emissions, n = 9) and aggressive calls (14.89  $\pm$  10.94; 1-35 emissions, n = 9), but a single male emitted only advertisement calls and another male emitted only aggressive calls. The repetition rate of advertisement calls was  $9.54 \pm 4.99$  call/min and that of aggressive calls was  $4.60 \pm 3.98$  call/min. In addition to these vocalizations, during amplexus six males emitted uneasiness calls (sensu Brasileiro et al. 2020) when the amplected pair was moving or appeared to be in danger of falling from the vegetation. Amplected males had a SVL of  $32.8 \pm 0.1$  mm (31.5-34.6 mm, n = 7) and body mass of  $1.54 \pm 0.17$  g (1.39–1.9 g, n = 7), while those non-amplected had SVL of  $32.5 \pm 0.15$  mm (29.6–34.8 mm, n = 11) and body mass of  $1.55 \pm 0.12$  g (1.34-1.73g, n = 11). There were no significant differences in SVL (t = 0.65; df = 18.0; P = 0.520) or in mass (t = 0.46; df =17.9; P = 0.651) between amplected and non-amplected males.

During the period prior to oviposition, some couples in amplexus were approached by other males (20%, n = 8). In four cases, when males in amplexus were touched by others, they emitted the uneasiness calls (*sensu* Brasileiro et al. 2020). Amplexus invasion attempts (n = 8) were predominantly performed by only a single male (87.5%, n = 7). In general, the invaders were greeted with kicks from the amplected males and failed to dislodge the amplected male. In two cases, invading males obtained success in amplexus with the couple (multiple amplexus), one of them involving four invading males simultaneously (Fig. 2). In a single event the invader succeeded in replacing the initially amplected male.

The body length of amplected males was  $32 \pm 1.4$  mm (28.5–35.5 mm, n = 45) and that of females was  $36.2 \pm 1.7$  mm (32.5–40.4 mm, n = 45). The ratio between the body length of males and females was  $0.88 \pm 0.06$  mm (0.77–1.02 mm, n = 45). There was no correlation between body length of males and females in amplexus (t = 0.454, P = 0.652; n = 45; Fig. 3). The body mass of amplected males was  $1.65 \pm 0.2$  g (1.39-2.09 g, n = 12), and that of amplected females prior to oviposition was  $3.59 \pm 0.5$  g (2.65-4.36 g, n = 12). The ratio between the body mass of males and females was  $0.49 \pm 0.06$  mm (0.40-0.58 mm, n = 12). The relationship of body mass of males and females in amplexus was significant ( $r^2 = 0.37$ ;  $F_{1.10} = 5.99$ ; P = 0.034; Fig. 3).

#### DISCUSSION

Most (53.8%) of *P. gonzagai* egg clutches we found were deposited in Euphorbiaceae plants. This plant family was also the most used for oviposition



**FIGURE 3.** Body size relationships between males and females in amplexus of *Pithecopus gonzagai* (no common name) from Groaíras municipality, Ceará state, northeastern Brazil, showing (A) the relationship of body length (snout-vent length) and (B) body mass.

by P. nordestinus populations from the Caatinga sensu stricto (89%) and from the Atlantic Forest (50%; Caldas et al. 2016). Faraulo et al. (2019) reported that Euphorbiaceae and Poaceae plants were the most used as vocalization sites by males of P. nordestinus and that these results suggest that males of this species could select vocalization sites with similar traits to those used as oviposition sites by females. Spatially overlapping vocalization and oviposition sites was already recorded in other Pithecopus species, such as Large-headed Leaf Frog (P. megacephalus; Oliveira et al. 2012) and Rohde's Leaf Frog (P. rohdei; Wogel et al. 2006). In this way, it is possible that the selection of specific sites for oviposition, primarily attributed to females (see Silva and Giaretta 2008), may also be influenced by the choice of vocalization sites by males (e.g., Lantyer-Silva et al. 2018). Pithecopus gonzagai uses predominantly plants with trichomes on the leaves (Caperonia palustris) to perform oviposition (https://floradobrasil.jbrj.gov. br/FB35701). Plants with trichomes (physic nut, Jatropha spp.) were also selected predominantly by two populations of P. nordestinus (Caldas et al. 2016). Faraulo et al. (2019) suggested that the presence of trichomes in the leaves could be an important factor for the choice of reproductive sites by P. nordestinus by facilitating the attachment of clutches.

In our study, *P. gonzagai* egg clutches were always placed in leaves above the water at the edges of temporary ponds, a behavior already reported for this species (Ferreira-Silva et al. 2016; Caldas et al. 2019). The selection of suitable places for oviposition is essential for species with arboreal nests, mainly in highly seasonal environments as the semiarid region of Brazil. The height chosen for oviposition is important to avoid flooding the nest if the water level rises, while the depth of water below the clutches assures that tadpoles fall into the water after eclosion (i.e., the deeper the water,

the less likely the pond will dry up), as was suggested for the Painted-belly Leaf Frog (*Phyllomedusa sauvagii*; García et al. 2013). For instance, Dias et al. (2017) reported Southern Walking Leaf Frog (*Phyllomedusa iheringii*) tadpole mortality in a case where the nest was not placed above the water surface. Although Caldas et al. (2019) did not provide information on nest location, they found that water depth did not influence the occurrence of tadpoles in *P. nordestinus*. Nevertheless, these authors raised the possibility that greater depths compromise the establishment of tadpoles in this species due to interspecific competition. So, beyond avoiding extremes, it remains unclear which factors drive the selection of places for egg deposition in *Pithecopus*, and how.

Females of P. gonzagai usually used a single leaf for oviposition, but in one case, a female used two Poaceae leaves. Until now, it was believed that the oviposition on a single leaf would be the rule among species from the Pithecopus hypochondrialis group (e.g., Pithecopus megacephalus, Oliveira et al. 2012; Reticulate Leaf Frog, Pithecopus ayeaye, Oliveira 2017; Pithecopus rohdei, Wogel et al. 2005). It is also known, however, that several large-bodied phyllomedusids may use two or more leaves to build their nests (e.g., Red-rimmed Leaf Frog, Phyllomedusa boliviana, Vaira 2001; Brownbelly Leaf Frog, Phyllomedusa tarsius, Neckel-Oliveira and Wachlevski 2004). Thus, although unexpected and relatively unusual, this oviposition record involving two leaves by P. gonzagai reveals that the ability to manipulate multiple leaves to build the nests was not necessarily lost in the genus Pithecopus, and it may circumstantially occur, as in other Phyllomedusidae species.

The size of *P. gonzagai* clutches observed in our study  $(102.6 \pm 22.7 \text{ eggs})$  was greater than those reported for two *P. nordestinus* populations from the State of Sergipe,

one in a Caatinga *sensu stricto* area ( $86 \pm 32$  eggs) and another in an Atlantic Forest region  $(91 \pm 12 \text{ eggs}; \text{Caldas})$ et al. 2016), but smaller than that reported for another P. nordestinus population in the State of Bahia (123.8  $\pm$  27.3 eggs; Faraulo et al. 2019). Despite *P. gonzagai* having smaller body mass and length compared to P. nordestinus (Andrade et al. 2020), the contrasting results do not allow us to suggest the occurrence of consistent differences in the clutch size between the species for two reasons. First, in species with more than one oviposition per reproductive season, the clutches tend to be progressively smaller (e.g., Camurugi and Juncá 2013). Second, females of Pithecopus are able to lay their eggs separately by dividing clutches in multiple nests or through the retention of mature eggs in the ovaries just after oviposition (e.g., Faraulo et al. 2019). In fact, we observed a female of P. gonzagai in amplexus twice in the same breeding season (indicating multiple reproductive events) as well as two females dividing their eggs into distinct nests (indicating subdivision of clutches). Furthermore, the occurrence of multiple ovipositions in a single reproductive season may also contribute to explain the observed absence of correlation between female body size (length and mass) and clutch size in P. gonzagai. Thus, regardless of any differences in clutch size, our findings highlight more similarities than differences between the reproductive biology of P. nordestinus and P. gonzagai.

The vocal activity of P. gonzagai males in amplexus consisted predominantly of advertisement calls, but amplected males also issued calls not associated with reproduction. The calling behavior of males during amplexus is relatively well reported among phyllomedusids, as in Pithecopus ayeaye (Oliveira 2017), P. azureus (no common name; Costa et al. 2010), Giant Monkey Frog (Phyllomedusa bicolor; Venâncio and Melo-Sampaio 2010), and P. boliviana (Vaira 2001). On the other hand, the possible roles of nonreproductive vocalizations during amplexus are still not well explained in most species. For instance, the previously called Territorial Call II of P. nordestinus (Vilaça et al. 2011; emitted by six males during this study) has already been considered with three distinct functions: (1) Distress Call, issued when manipulated (Vilaça et al. 2011); (2) Release Call, issued when amplected or touched by other males (Mângia et al. 2019); and (3) Uneasiness Call, issued when the individual is uncomfortable (Brasileiro et al. 2020). It is noteworthy that these last two applications, in fact, must be attributed to P. gonzagai, as they were reported for populations that currently are considered part of this species. During our observations, P. gonzagai males in amplexus emitted the call deemed Territorial Call II in two contexts: when the amplected male was touched by another male in an attempt to invade the amplexus

(condition consistent with a Release Call - *sensu* Toledo et al. 2014; Mângia et al. 2019), and when the amplected pair was unbalanced in the vegetation in situations of imminent falling. Although the Uneasiness Call category was originally proposed for the calls issued by males in submission posture after losing physical clashes against rivals (Brasileiro et al. 2020), these authors suggest that this call reflects a general situation of annoyance or discomfort. In this way, because we demonstrate that this call was used in a variety of other unpleasant contexts, we support the usage of Uneasiness Call (*sensu* Brasileiro et al. 2020) as a more appropriate category to classify this call type of *P. gonzagai* (and consequently in *P. nordestinus* too).

The invasion of the amplexus by other males, as we observed in P. gonzagai, seems to be very common among phyllomedusids. Indeed, the occurrence of multiple amplexus has been recorded for other species of Pithecopus (P. aveave, Oliveira 2017; P. azureus, Dias et al. 2012; P. megacephalus, Oliveira et al. 2012; P. rohdei, Wogel et al. 2005). In general, the invasion of amplexus is usually recorded in species with short reproductive periods and in high density choruses, in which the strategy to actively seek females can bring benefits complementary to the emission of acoustic signals (Wells 1977). Polyandry can be beneficial to female reproductive success for some animal groups through factors such as increased egg fertilization rates and increased genetic diversity of the offspring (Yasui 1998; Barbosa et al. 2012). In other cases, however, polvandry can reduce reproductive success, especially when there is epigamic selection or males adopting alternative reproductive strategies. For instance, Byrne and Roberts (1998) found for the anuran Quacking Frog (Crinia georgiana) that the fertilization rate of eggs was smaller when mating involved more than one male in comparison to matings with a single male. In addition, amplected pairs of the Olive Frog (Nidinara adenopleura) disturbed by other males had prolonged amplexus and reduced fertilization rates (Chuang et al. 2013). The fertilization rate of the eggs of a female P. gonzagai that amplected with two males was 21.42% lower than the average recorded for females that amplected with only one male. Based on the foregoing, despite the extensive occurrence of multiple amplexus in the genus, it is possible that this behavior in phyllomedusids is not substantially advantageous for the fitness of females and males (except to the invader). Additional research is required to test this hypothesis among other phyllomedusids.

There was no difference between the body length and mass of amplected and non-amplected males in *P. gonzagai*, similar to the results previously reported for other Phyllomedusidae treefrogs (*Pithecopus rohdei*, Wogel et al. 2005; *P. megacephalus*, Oliveira et al. 2012; P. ayeave, Oliveira 2017). These results together with a small body length at sexual maturity and low investment in gonads observed in males of Pithecopus (Borges et al. 2018; Faraulo et al. 2019) support the idea suggested for *P. nordestinus* that the recruitment of relatively young and, consequently, small males can be advantageous in highly seasonal environments as in the Caatinga sensu stricto (Faraulo et al. 2019). On the other hand, we observed a positive correlation between the body mass of males and females in amplexus, suggesting the occurrence of assortative mating in P. gonzagai. A recent meta-analysis (Green 2019) revealed that there is little support for the occurrence of true assortative mating between anurans. Green (2019) highlights that the anuran species that often display correlations between body attributes usually have disputes between males for acquisition or maintenance of mates (scramble strategy), as occurs in P. gonzagai. The scramble strategy would benefit large males generating what is called apparent assortative mating (sensu Arnqvist et al. 1996) or a pattern of what is disproportionate mating (sensu Green 2019). In addition, Green (2019) argued that as the body size is usually a highly variable trait in anurans, being sensitive to several environmental and ecological factors, it is possible that this morphological attribute does not have enough selective value to act as an assortative trait. In fact, the available evidence for phyllomedusids does not suggest the occurrence of assortative mating regarding body length (e.g., Phyllomedusa boliviana, Pithecous rohdei, and Phyllomedusa iheringii; Vaira 2001: Wogel et al. 2005: Dias et al. 2017) nor body mass (Pithecous rohdei; Wogel et al. 2005). In this way, as we detected a high correlation only between the body mass of amplected males and females of P. gonzagai (but not for body length), it remains uncertain if the observed pattern was derived from a true assortative mating mechanism or just a disproportionate mating system.

Our findings revealed several unknown aspects of P. gonzagai reproductive biology, many of which showed high similarities with those known to its sister species, P. nordestinus. This probably comes from their relatively recent evolutionary divergence from the proximate common ancestor, estimated for the Plio-Pleistocene transition (Bruschi et al. 2019; Andrade et al. 2020). In fact, the high level of phenotypic (morphological and acoustic traits) conservation among species from P. hypochondrialis group is well known (Bruschi et al. 2013; Haga et al. 2017), and our results contribute to expanding this notion in relation to reproductive traits. Thus, rather than revealing differences between phylogenetically closely related species, we contribute to the understanding of variations in mating behavior in the genus Pithecopus and, in this way, expand the knowledge of the life-history patterns of Phyllomedusidae as a whole.

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### Herpetological Conservation and Biology



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