SALAMANDRA **57**(3): 353–370
15 August 2021 | ISSN 0036–3375

SALAMANDRA
German Journal of Herpetology

Reproductive biology and phylogenetic relationships of *Vitreorana baliomma* (Anura: Centrolenidae)

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Manuscript received: 15 December 2020
Accepted: 26 May 2021 by Jörn Köhler

Abstract. The behaviour, ecology, and phylogenetics of glassfrogs from the Atlantic Forest are poorly understood. To contribute to these three topics, we studied reproductive behaviours, microhabitat use, influence of environmental variables on the activity of adults, and clutch thickness, as well as the phylogenetic relationships of *V. baliomma*, a key centrolenid species from the northern Atlantic Forest. We sampled two streams in the south of the state of Bahia, Brazil, between November 2018 and April 2019, and measured relevant specimen and environmental variables. Our results include (i) the first observations of female-only egg-brooding in this species, (ii) the statistically significant dependence of reproductive activity on daily rainfall and high humidity, and (iii) that adults use different leaf face for clutch deposition and activity, with clutches being placed on both sides of leaves and at statistically significant lower heights and horizontal distances from water. Maximum likelihood phylogenetic analyses of DNA sequences of three mitochondrial and one nuclear gene (12, 16S, COI, and POMC, respectively) confirmed the phylogenetic position of *V. baliomma* within the genus. Although our results inferred *V. baliomma* as sister of *V. gorzulae* (a Guiana Shield species) and both as sister taxa of all other Atlantic Forest and Cerrado species, these relationships have low support.

Key words. Amphibia, Atlantic Forest, brooding behavior, glassfrogs, mating patterns, microhabitat use, parental care.

Introduction

Anurans have a large diversity of traits that are related to their reproductive biology (Duellman & Trueb 1994) and are one of the groups with the greatest variety of reproductive modes among vertebrates (Haddad & Prado 2005, Crump 2015). Their reproductive aspects provide important insights into their systematics, evolution, ecology, and may determine species conservation measures (Hoffmann et al. 2008, Guayasamin et al. 2009, Vargas-Salinas et al. 2014, Delia et al. 2017, Escalona-Sulbarán et al. 2019). However, information on reproductive biology is scarce or even nonexistent for many species (Wells 2007, Delia et al. 2017).

The family Centrolenidae is an interesting group due to its morphological, ecological, and behavioural characteristics (Ruiz-Carranza & Lynch 1991, Señaris & Ayarzagüena 2005, Cisneros-Heredia & McDiarmid 2007), as well as its phylogenetic and biogeographical complexity (Guayasamin et al. 2008, Castroviejo-Fisher et al. 2014). This family is restricted to the American Tropics, where its species inhabit forest-lined waterfalls and streams, perching on vegetation and laying their eggs on leaves, branches, mosses, or rocks overhanging the water (Cisneros-Heredia & McDiarmid 2007, Guayasamin et al. 2009, Twomey et al. 2014). After hatching, tadpoles drop into the water and continue their development buried in sand and debris on the bottom of water bodies (Kubicki 2007, Guayasamin et al. 2009).

While most of the few described species were known from cursory descriptions of their external morphology based on preserved material before the 1990's (e.g., Taylor 1949, Rivero 1968, Lynch & Duellman 1973, Duellman 1976, Rivero 1985), centrolenids are now emerging as a model system for ecological and evolutionary studies (e.g., Hutter et al. 2013, Delia et al. 2013, 2017, 2020, Castroviejo-Fisher et al. 2014, Escalona-Sulbarán et al. 2019, Barnett et al 2020). In fact, glassfrogs have become a recurrent model for studies addressing amphibian reproductive strategies, with focus on issues such as parental care (e.g., Hayes 1991, Vockenhuber et al. 2009, Delia et al. 2017, 2020, Schulte et al. 2020), acoustic communication (e.g., Wells & Schwartz 1982, Escalona-Sulbarán et al. 2019), reproductive activity and patterns (e.g., Greer & Wells 1980, Jacobson 1985, Basto-Riascos et al. 2017, Valencia-Aguilar et al. 2020a), and microhabitat selection (e.g., Valencia-Aguilar et al. 2012, Cabanzo-Olarte et al. 2013, Díaz-Ricaurte et al. 2019, Ortiz-Ross et al. 2020).

Parental care in glassfrogs was considered a rare behaviour, exclusive to males (McDiarmid 1978), but recent studies reported that this is in fact a widespread trait in this family, with an interesting evolutionary pattern in variation of time invested (brief or prolonged) and sex (male or female) of providing parental care (Bravo-Valencia & Delia 2016, Delia et al. 2017, 2020). Parental care increases the chances of survival or benefits the growth of offspring, thus being an important measure to maximize fitness (Trivers 1972, McDiarmid 1978, Clutton-Brock 1991). Brooding behaviour is one mode of egg care found in the Centrolenidae. It is performed by ventral contact, where the parent positions its body over the clutch (Hayes 1991, Guayasamin et al. 2009) in a quest to protect embryos from desiccation, predation, developmental abnormalities, and fungal infection (Hayes 1991, Crump 1995, Vockenhuber et al. 2009, Delia et al. 2013, 2017, Lehtinen et al. 2014, Bravo-Valencia & Delia 2016).

Reproductive site selection (call and oviposition site) is another important trait related to parental fitness and offspring survival (Resetarits 1996). Reproductive microhabitat has also been suggested to influence centrolenid call variability (Bosch & De la Riva 2004) – for example, it increases the effectiveness of acoustic signalling in *Hyalinobatrachium fleischmanni* (Greer & Wells 1980, Wells & Schwartz 1982) – and apparently plays an important role in the evolution of anuran sexual size dimorphism (Silva et al. 2020). Furthermore, the oviposition site is of great importance to offspring survivorship (Resetarits 1996). Females make choices from a complex composition of options that vary according to both biotic and abiotic cues, such as absence of predators and desiccation risk (Bernardo 1996, Rudolf & Rödel 2005, Touchon & Worley 2015). Oviposition site choice in centrolenids appears to protect embryos from the very same risks that favour parental attendance of eggs, with an evolutionary association between time spent on care, clutch structure, and oviposition site (Delia et al. 2020).

Within glassfrogs, the genus *Vitreorana*, with ten species distributed throughout the Cordillera de la Costa, Guiana Shield, Amazonia, Atlantic Forest, and Cerrado, has received less attention than other genera (Guayasamin et al. 2009, Pontes et al. 2014, Santana et al. 2015, Frost 2020). For example, *Vitreorana baliomma* is a small species (snout–vent length = 16.4–21.2 mm) described only based on morphological characteristics of preserved specimens (Pontes et al. 2014), and with its advertisement call quantitatively described just recently (Bang et al. 2020). It occurs in forests associated with streams and creeks in the northern portion of the Atlantic Forest of Brazil, from 100 to 600 m a.s.l. (Gouveia et al. 2012, Pontes et al. 2014). Individuals use marginal vegetation along streams, perching on leaves and trunks, ranging in height from 0.3 to 4 m (Gouveia et al. 2012). Gouveia et al. (2012), and Lisboa et al. (2019) presented notes about the reproduction of *V. baliomma*, indicating that females lay their eggs, preferably, but not exclusively, on the upper side of leaves, at 0.5 to 2.0 m high, although neither study detected signs of parental care.

Other fundamental aspects of its reproduction, such as microhabitat use or whether climatic conditions influence behavioural activity, are also unknown. This lack of data is problematic if we consider the phylogenetic position of *Vitreorana* as one of the earliest splitting clades within the tribe Cochranellini (Guayasamin et al. 2008, Castroviejo-Fisher et al. 2014, Twomey et al. 2014) and that the aforementioned characters are also unknown for most of the other *Vitreorana* species. For example, *V. ritae* is one of the species for which maternal care has been reported (formerly no care), nevertheless, just one specimen was observed (Delia et al. 2017), and we do not as yet know whether this behaviour is possibly more widespread in this genus.

The phylogenetic affinities of *V. baliomma* are speculative and even its placement within *Vitreorana* is somewhat tentative, being based upon the following morphological characters: parietal and urinary bladder peritonea transparent, pericardium and visceral peritoneum white, bones green in life, liver bulbous, and dorsal coloration in preservative lavender (Pontes et al. 2014). Besides *Vitreorana*, this combination of characters is also present, within the Centrolenidae, in species of the genera *Chimerella*, *Hyalinobatrachium,* and *Teratohyla*. Faced with this situation and in the absence of DNA sequence analyses, Pontes et al. (2014) opted for allocating this species to *Vitreorana.* Their decision was based on biogeographic affinities, because all known species of glassfrogs from the Atlantic Forest form a clade within *Vitreorana*. This Atlantic Forest clade is the sister taxon of the Amazonian and Guiana Shield species of the genus (Guayasamin et al. 2008, 2020, Castroviejo-Fisher et al. 2014, Twomey et al. 2014). However, all the Atlantic Forest species of *Vitreorana* have the synapomorphy of a white urinary bladder peritoneum (Guayasamin et al. 2009), which is conspicuously absent in all other species of *Vitreorana* (including *V. baliomma*), and casts doubts on the phylogenetic affinities of *V. baliomma*. The phylogenetic relationships of *V. baliomma* transcend the details of glassfrog systematics because the relationships between the Atlantic Forest and Amazonia-Guiana Shield clades constitute a key example of the biogeographic connection between these two regions in the late Miocene (e.g., Castroviejo-Fisher et al. 2014, Rodrigues et al. 2014, Prates et al. 2017).

In this study, we identified *Vitreorana baliomma* as a species of primary interest to overcome important knowledge gaps in the phylogenetic relationships (Darwinian shortfall) and biological traits (Raunkiæran shortfall) of glassfrogs (Hortal et al. 2015). Although modern technology may offer partial solutions to these shortfalls, fieldwork is still the most important means to fill these gaps (Wilson 2017). Considering the aforementioned relevance of *V. baliomma*, we conducted field expeditions to collect data to address the following issues: (1) describe the pattern of reproductive behaviours; (2) investigate the possible occurrence of parental care; (3) characterize the physical parameters of the sites used by adults for activity and depositing egg clutches; (4) characterize egg clutches; (5) evaluate the influence of abiotic variables on the number of active adult individuals; (6) evaluate the influence of microhabitat on clutch thickness; and (7) infer its phylogenetic relationships within Centrolenidae using DNA sequences and quantitative phylogenetic analyses.

Material and methods

Study sites

We collected data between November 2018 and April 2019, in two slow- to moderate-current streams with sandy/rocky beds: (1) Fazenda Santo Antônio (15°30'8.65" S, 39°22'33.14" W; 105 m a.s.l.), municipality of Mascote and (2) at 3 km of Cachoeira do Tijuípe (14°23'17.61" S, 39°2'37.89" W; 89 m a.s.l.) near BA001 – Ilhéus-Itacaré road, municipality of Uruçuca (hereafter Mascote and BA001, respectively). Both streams are located in Atlantic Forest in the south of the state of Bahia, Brazil. The stream in Mascote runs through a cabruca forest, which is an agroforestry system where some of the native forest serves to provide shade for cocoa trees (*Theobroma cocoa*). The BA001 locality constitutes a well-regenerated secondary forest. The sampled area lies in the conservation unit Área de Proteção Ambiental (APA) Costa de Itacaré and within the buffer zone of the Parque Estadual Serra do Conduru (PESC). We collected all the data used for our reproductive behaviour studies from a 150-m transect in Mascote and a 100-m one in BA001, where we observed the greatest aggregation of individuals. In southern Bahia, the climate is characterized as being hot and humid, without a dry season (Köppen 1936). Mean annual rainfall is between 1500–1700 mm, mean annual temperature is 23–24°C, and relative humidity is ≥ 80% (Roeder 1975). To better understand the geographic distribution of the species, we reviewed the literature and the Museu de Zoologia da Universidade Estadual de Santa Cruz (MZUESC) scientific collection from which we here provide an updated distribution map for this species (Fig. 1).

Fieldwork

We sampled at night, between 17:00 and 03:00 h, with sporadic diurnal visits to verify the permanence of adults near clutches, which totalled 32 hours of sampling within 13 days. We measured temperature and humidity at the beginning and end of each sampling period (period of time from arrival until leaving the stream), using a digital thermo-hygrometer Instrutherm HT-260 (accuracy ± 2.5°C; ± 3.5% RH). For each sampling period, we used as a descriptive variable the sum of the start and end values divided by two. Also, we measured daily precipitation for Mascote using a 70-mm pluviometer fixed at 5 cm atop a wooden stake, placed in a locale without canopy cover. We installed the pluviometer just before sampling (between 16:30 and 16:50 h) and read it every 24 hours. For BA001, we used daily precipitation values (00:00–01:00 to 23:00–00:00 h) from the Instituto Nacional de Meteorologia (INMET – https://portal.inmet.gov.br/), as recorded by the Ilhéus automated weather station (14°39'31.69" S, 39°10'53.18" W; 80 m a.s.l.), located 33 km from our sampling site.

We conducted visual encounter surveys for adults and clutches and auditory searches for calling males. We marked encounter sites of adults and clutches with uniquely coded flagging tape. When possible, we differentiated males from females by observing the vocal sac, present only in males, and by the presence of oocytes in the oviduct, visible through the translucent belly of reproductively active females. We collected at random representative voucher specimens of adults (females and males), embryos, and tadpoles. We euthanized specimens in the lab with a topical dose of the anaesthetic benzocaine (50 mg/g) and fixed them in 10% formalin. After 24 h, we preserved adults in 70% ethanol. We removed samples of thigh muscle tissue from some specimens and preserved them in 96% ethanol for DNA analyses. We deposited specimens and tissue samples in the Amphibian Collection of the Museu de Ciências e Tecnologia da Pontifícia Universidade Católica do Rio Grande do Sul (MCP).

Behavioural observations

We opportunistically monitored pairs in amplexus until oviposition and recorded other intraspecific interaction events throughout the duration of amplexus. We used a headlamp with a red-light filter to minimize disturbance (Vargas-Salinas et al. 2014, Touzot et al. 2020). We visited egg clutches during the following day and night to evaluate the presence of adults and their potential interactions with egg clutches after oviposition. We recorded the number of males heard vocalizing and the number of individuals visually detected (males and females) during each sampling period to assess if the measured abiotic variables influenced the species' activity (see Statistical analyses). We recorded these data across the 13 days of sampling, with a frequency of at least one-night monthly in one of the localities and duration of sampling periods between 2–5 h.

Microhabitat data

We recorded the following variables for each encounter site of adults and clutches: (1) vertical distance to substrate, (2) horizontal distance from streambed, (3) type of structure used as perch (i.e., leaf, branch, moss, rock), (4) perch size, (5) face (i.e., upper or lower), and (6) type of leaf surface (i.e., smooth or hairy), (7) position relative to the streambed (i.e., streambed, bank of stream, outside the stream flood area), (8) side to the stream flow (i.e., left or right), and (9) canopy cover. We measured the height of the exact encounter site above the substrate, and the horizontal distance of the location of the adult, pair, or clutch on the perch to the streambed margin. We quantified both variables using a 5-m millimetric tape. We quantified perch size using a 2-m millimetric tape to measure the length (L) and width (W) of the leaf and calculated the elliptic area (A) with the formula

$$A = \frac{L}{2} \times \frac{W}{2} \times 2\pi.$$

We quantified canopy cover using photos taken during daytime visits, with a fisheye lens attached to a smartphone camera. We took all photos with the phone positioned 5 cm above the centre of the encounter site and levelled at 180°. For each photo, we calculated the percentage of canopy-free pixels using the software Gap Light Analyzer (Frazer et al. 1999). We compared adult and clutch data to test if there is a difference between the microhabitats used by adults and oviposition sites (see Statistical analyses).

Egg clutch characterization

We recorded the diameter and thickness of the entire egg capsule, the number of eggs, stage of development, and embryo coloration of each observed clutch. We measured length (L) and width (W) using a digital calliper (accuracy ± 0.02 mm), and then calculated the diameter (D) by using the formula

$$D = 2\sqrt{\frac{A}{\pi}},$$

by which we defined elliptic area (A) for clutches, too. To measure clutch thickness, we used small wooden rods scaled in millimetres (mm). We used Gosner (1960) to identify embryonic stages. Moreover, we tested if the char-

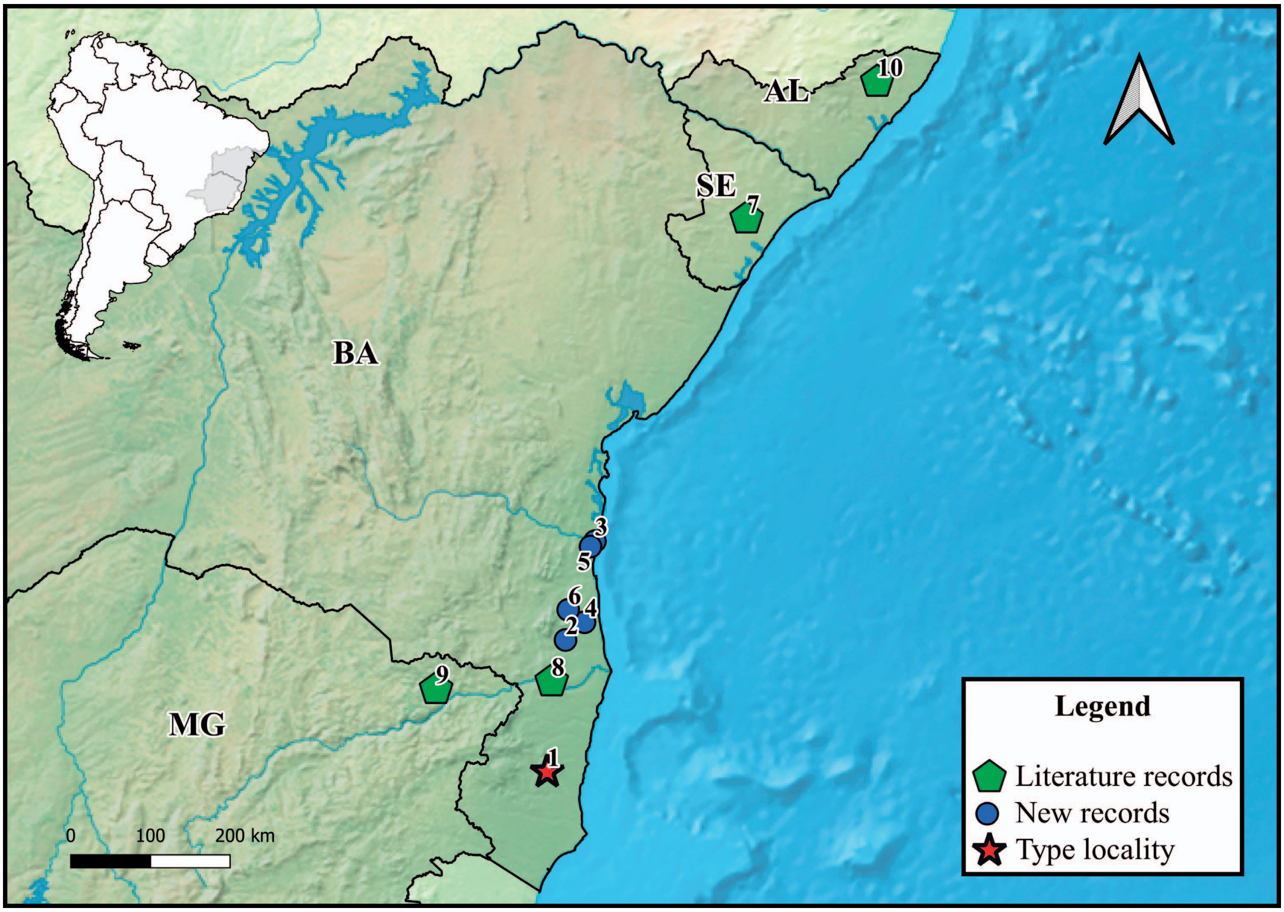

Figure 1. Map of northeastern Brazil with symbols indicating known localities of *Vitreorana baliomma*. 1 – Fazenda Novo Pau Brasil, municipality of Itamaraju; 2 – Fazenda Santo Antônio, municipality of Mascote; 3 – BA001 (Ilhéus-Itacaré road), municipality of Uruçuca; 4 – RPPN Ararauna, municipality of Una; 5 – Parque Estadual Serra do Conduru, municipality of Uruçuca; 6 – Parque Nacional (PARNA) da Serra das Lontras, municipality of Arataca; 7 – PARNA Serra de Itabaiana, municipality of Areia Branca; 8 – Fazenda Palmeiras, municipality of Itapebi; 9 – Fazenda Limoeiro, municipality of Almenara; 10 – Murici Ecological Station, municipality of Murici. Brazilian states included are: Alagoas (AL), Bahia (BA), Minas Gerais (MG) and Sergipe (SE). We collected reproductive behaviour data from localities 2 and 3, while sequenced tissues are from 2, 3, 4 and 5.

acteristics of clutch structure or oviposition site are related to clutch thickness (see Statistical analyses). We assigned clutches to *V. baliomma* based on the following criteria and observations: 1) clutches, egg capsules, and embryos were similar in structure, colour, size, and position site to those laid by females directly observed by us; 2) despite our field efforts, we did not find or hear other species of glassfrogs in the area around the sampled streams; 3) egg clutches of other known sympatric anuran species that lay their eggs on leaves (e.g., *Dendropsophus* spp. and *Phyllomedusa* spp.) are clearly different from those of *V. baliomma*. For example, females of *Phyllomedusa* spp. build a nest with the leaves; 4) we incubated one clutch in the laboratory and observed its development from oviposition to hatching, to identify the developmental stages.

Molecular methods and phylogenetic analyses

We extracted genomic DNA from ethanol-preserved muscle tissue using the Wizard Genomic DNA Purification Kit (Promega, Madison-WI, USA) as per manufacturer's instructions. We performed polymerase chain reactions (PCR) in 25-µL volumes with 2.0 µL of genomic DNA to amplify ~ 646 bp of the COI mitochondrial gene (T3-AnF1 and T7-AnR1; Lyra et al. 2017), ~576 bp of the 16S rRNA gene (primers 16Sar and 16Sbr; Palumbi et al. 1991), 562 bp of the 12S rRNA gene (primers t-Phe-frog and 12S-frogR; Wiens et al. 2005), and ~492 bp of POMC (primers POMC-1 and POMC-2; Wiens et al. 2005). We checked amplifications in agarose gels (1–2%), sequenced fragments in both directions, and used Sequencher v4.1.4 (Gene Codes Co., Ann Arbor-MI, USA) to assemble and edit the resultant chromatograms.

Additionally, we downloaded homologous sequences of other *Vitreorana* spp. available from GenBank (as of 30 December 2019). As outgroups, we used homologous sequences from GenBank of one or two terminals of all other centrolenid genera. We rooted all trees with sequences of *Allophryne ruthveni*, which forms part of the sister group of Centrolenidae (Guayasamin et al. 2008, 2009). For some terminals, if genetic distances were <1%, we assigned sequences from different specimens to a single composite terminal (Supplementary Table S1). We did this to reduce the numbers of missing entries per terminal and search space during phylogenetic analyses (Wilkinson 1995, Kearney 2002, Brower 2018). Our final dataset included a total of 21 species and 185 terminals (12 for outgroup and 173 for ingroup). Specimens and DNA sequences used in the phylogenetic analysis are listed in Supplementary Table S1.

We individually aligned sequences of each marker using MUSCLE (Edgar 2004) in AliView (Larsson 2014) under default parameters. We combined the alignments mentioned above into a single matrix using SequenceMatrix 1.8 (Vaidya et al. 2011). Phylogenetic analysis was based on the maximum likelihood (ML) criterion. We determined the most appropriate model of nucleotide substitution and data partition using ModelFinder (Kalyaanamoorthy et al. 2017) in IQ-TREE. We divided the dataset by gene and, for COI and POMC, by codon positions. We performed 500 independent searches in IQ-TREE (Minh et al. 2020) under default settings and using the models and partitions derived from the results of ModelFinder (Chernomor et al. 2016). We assessed clade support with 1000 bootstrap (BS) pseudoreplicates using IQ-TREE with the same aforementioned settings. We used the SumTrees package in DendroPy v4.4.0 (Sukumaran & Holder 2010) to add BS frequencies to the best ML tree.

Statistical analyses

We performed all analyses in R 4.0.2 (R Core Team, 2020) and RStudio 1.3.1073 (RStudio Team, 2020) code editor, with packages lme4 (Bates et al. 2015) for the Linear Mixed Model (LMM) and Generalized Linear Mixed Model (GLMM) analyses, car (Fox & Weisberg 2019) to obtain the significance of models using likelihood ratio test, AICcmodavg (Mazerolle 2020) for model selection based on AICc and ggplot2 (Wickham 2016), and gridExtra (Auguie 2017) to generate the figures. Other functions used are those present in the R base package. We considered results as statistically significant if P-values were ≤ 0.05.

Prior to analysis, we scaled and centred (function: scale) temperature, humidity and precipitation to standardize explanatory variables and logarithm-transformed (function: log) leaf size values to decrease data amplitude. We ran Shapiro-Wilk tests (function: shapiro.test) to test the normality of dependent variables for models and quantitative variables for comparison of microhabitat use. We found that the nightly number of calling males ($W = 0.83706$, $p = 0.01945$), sampled individuals ($W = 0.66706$, $p = 0.0002649$), horizontal distance ($W = 0.55034$, $p < 0.001$), leaf size ($W = 0.94141$, $p = 0.02917$), and canopy openness ($W = 0.73687$, $p < 0.001$) all followed a non-normal distribution. Clutch thickness ($W = 0.97026$, $p = 0.8026$) and vertical height ($W = 0.97734$, $p = 0.5457$) instead followed a normal distribution.

Consequently, we performed a GLMM analysis (function: glmer) to test if average temperature and humidity, and daily rainfall are predictors of the number of calling males and sampled individuals. We used the sampling locality as a random variable, while considering different abundances in each area, in both analyses.

To test if the variables summarizing clutch structure (i.e., number of eggs, diameter, and developmental stage) are predictors of clutch thickness, we performed a Linear Model (LM) analysis (function: lm). After that, we performed an LMM analysis (function: lmer) to test if microhabitat variables are predictors of clutch thickness, and selected just vertical height, leaf size, and leaf surface as independent variables to prevent overparameterized models based on our sample size. We added developmental stage as a random effect to test if microhabitat variables are predictors of clutch thickness in LMM, given that it is an intrinsic feature of clutches (see Results).

We selected additive or simple models using the Akaike information criterion (AIC; Akaike 1973). To generate AICc comparable values for models, we performed the function aictab. For each dependent variable, we fitted a simple model to each independent variable, an additive model with independent variables without interaction, and an additive model with independent variables interacting between them. After selection, we used the analysis of variance (ANOVA; function: Anova) as a likelihood ratio test to quantify the significance of variables in models. We fitted LMM and GLMM with the likelihood function and Poisson distribution, respectively.

To assess if there were differences between the microhabitat used by adults and oviposition sites, we performed independent 2-group Student's t-Tests (function: t.test) for variables with normal distributions, and Mann-Whitney U Tests (function: wilcox.test) for variables with non-normal distributions, and beyond these, Pearson's Chi-squared Tests (function: chisq.test) for categorical variables (i.e., leaf surface, position at stream, stream side).

Results

We found a total of 24 individuals (14 males, six females, and four unsexed individuals) and 19 clutches (we disregarded one clutch with hatched embryos for its number of eggs, diameter, and thickness). During sampling, temperature varied from 23.7–29.1°C, humidity from 77–93.2%, and daily rainfall was between 0 and 53 mm. The region of the study area experienced abnormally low rainfall between January and March 2019 compared to the previous 10 years.

Reproductive behaviour

We observed four mating events, one in its entirety (including courtship, amplexus, oviposition, associated behaviours, and female egg brooding), two without preceding courtship, and one without courtship, oviposition, and brooding. On the days of our observations, temperatures varied from 24.3–26°C, humidity from 90.7–93.2%, and daily rainfall from 14–18.4 mm. Our description is based mainly on a pair encountered on 11 April 2019, in which we could observe all behaviours from the beginning to the end. At approximately 22:15 h, we discovered a female positioned ~ 30 cm away from a male vocalizing from a bush. The male was calling from the upper side of a leaf ~ 100 cm above the ground. The female moved across the foliage, circling the male's calling site. At 22:25 h, the female leaped to the leaf positioned in front of the calling male. The male, still calling, turned to face the female, which then leaped on to the same leaf. The male then climbed on the female's back and initiated an axillary amplexus. The amplectant pair moved over the bush by the female leaping to leaves above and below the initial encounter site (Fig. 2A) and in the process switching between the upper and lower faces of the leaves (Fig. 2B). While on a leaf, the pair constantly performed 180 and 360° rotations (Fig. 2C). After 102 minutes in amplexus, the female deposited a clutch on the upper surface of a leaf. Right after oviposition, the female moved backwards, positioning her body above the clutch in a brooding posture. The male released the female just after she had laid her clutch, leaped to a leaf approximately 10 cm above the oviposition site, and began to vocalize again. The female remained positioned above the clutch for another 110 minutes, raising the posterior part of her body at intervals of 1 to 10 minutes (Fig. 2D). At 01:58 h, the female left the clutch, remained next to it for a few seconds more, then leaped to other leaves and left the oviposition site.

In the other three mating events, we observed the same behaviours as exhibited by the pair described above, with the following differences. In one case, at 21:10 h, also on 11 April 2019, we found the individuals already in amplexus (continued for a further 25 min), with the female depositing the clutch on the lower face of the leaf and brooding the eggs. In the third mating event, we found individuals in amplexus at 23:14 h, on 14 April 2019. While in amplexus, the female performed movements with its front limbs, rubbing them on her face. After 56 min in amplexus, it started to rain and the pair became more active, moving about more frequently in the vegetation. At 01:08 h, while attempting to leap to an adjacent leaf, the pair fell into a spider web. They remained in amplexus as the female tried to break free from the web, unsuccessfully. After 60 min observing the individuals in the spider web, we collected and placed them in a transparent plastic bag with damp leaves from the surrounding vegetation. On the next day, we found an egg clutch deposited inside the collection bag, attached to the upper side of a leaf, with the female not brooding the eggs. Finally, in the fourth event on 3 December 2018, we collected an amplectant pair that we placed in a transparent plastic bag for direct observations. The collected female showed the same behaviours as the females observed in nature, with the amplexus lasting ~ 100 min. Clutches observed after oviposition and care had a mean of 14 eggs (13–16), measured 1.57 cm in diameter (1.30–2.09), and 4.5 mm in thickness (3–6). Female egg brooding lasted for a mean of 110 mins (range = 99–120 mins, N = 3).

Although we observed some males calling during courtship and amplexus, we were not able to record them. To the ear, the calls emitted during these events appeared identical to the advertisement call. We checked the clutches of the aforementioned events for two days following oviposition. We did not observe individuals close to them, neither at day nor at night. On the second night, we found that all eggs from all these clutches were missing, purportedly predated. We did not observe aggressive behaviours between conspecific males or predation events involving individuals or clutches.

In the analysis of abiotic variables affecting reproductive activity, we selected the additive model without interaction for number of calling males and simple models for number of sampled individuals. The number of calling males was positively correlated with humidity ($X^2 = 10.6828$, $P = 0.0010813$, $N = 13$) and precipitation ($X^2 = 13.8005$, $P =$

<0.001, N = 13) and negatively correlated with temperature (X^2 = 6.8499, P = 0.0088646, N = 13) (Fig. 3). The number of sampled individuals was positively correlated with humidity (X^2 = 8.2314, P = 0.004117, N = 13) (Fig. 4) and not related to precipitation (X^2 = 1.4078, P = 0.2354, N = 13) or temperature (X^2 = 3.1656, P = 0.07521, N = 13).

Microhabitats of adults and oviposition sites

We found all adults on the upper faces of green smooth leaves (N = 24). The sizes of used leaves ranged from 6.6 × 2.2 to 39.9 × 8.5 cm. Most adults used the left side of streams (N = 23), and were perched on leaves positioned above the streambed (N = 14) (Table 1). We found all clutches deposited on green smooth leaves (N = 19), ranging in size from 6.0 × 2.4 to 61.3 × 28.6 cm, more frequently on the upper face (N= 15) and on the left side of the stream (N = 14), above the streambed (N = 15) (Table 1).

The microhabitats used by adults and oviposition sites were significantly different in vertical height (t = 4.2009, P = <0.001, N = 43) and horizontal distance (W = 300, P = 0.02703, N = 43), but not in leaf size (W = 188, P = 0.334, N = 43), canopy openness (W = 96, P = 0.3602, N = 32)

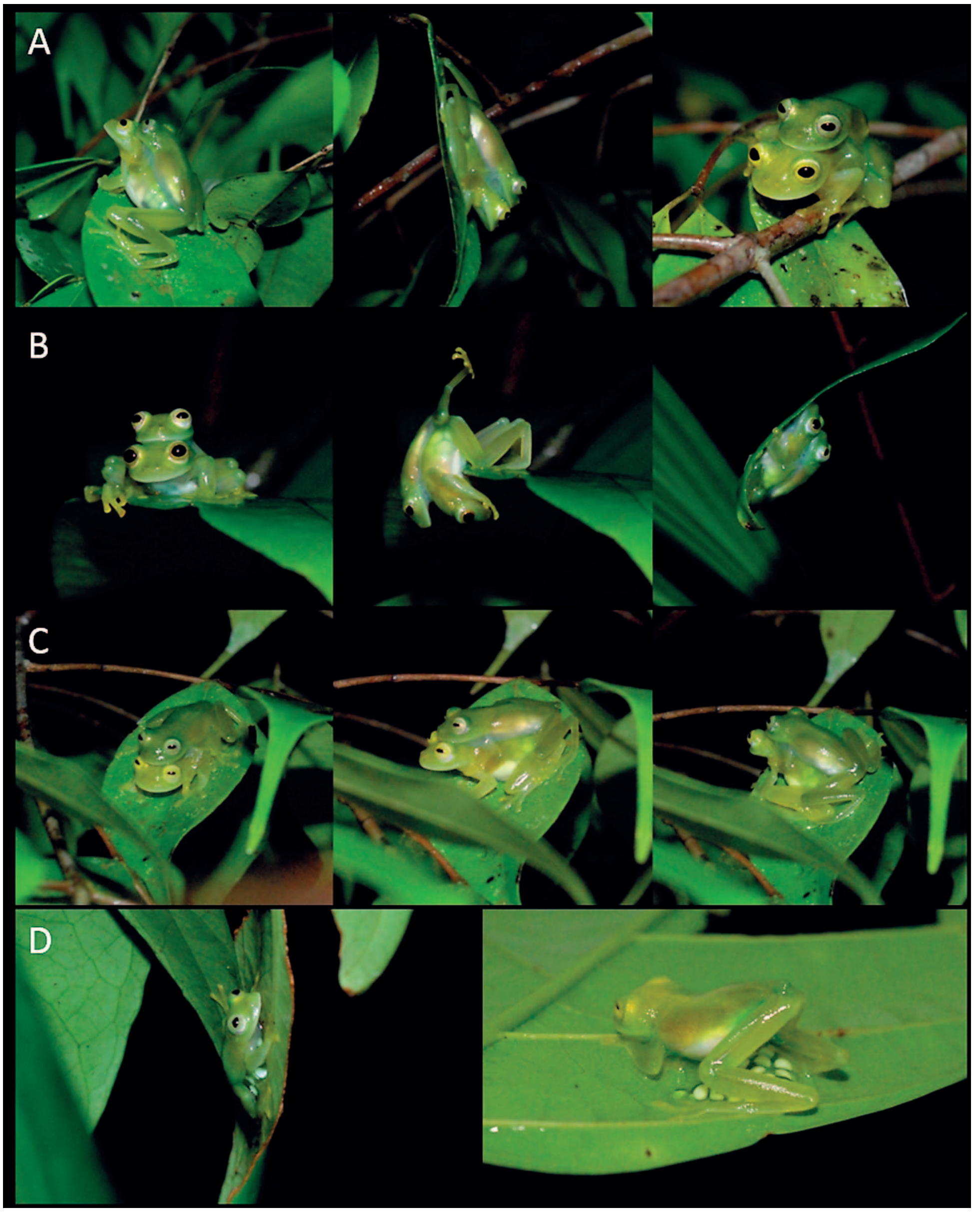

Figure 2. Reproductive behaviour in *Vitreorana baliomma*. While in amplexus, pairs move over leaves and branches (A), change the leaf face (B), and perform 360 and 180° rotations on leaves (C). After oviposition, the female will brood her egg clutch, elevating the posterior part of her body (D). Photographs depict various pairs.

Table 1. Variables of microhabitats used by adults and oviposition sites of Vitreorana baliomma at two streams in the Atlantic Forest in the south of the state of Bahia, Brazil. * 20 adults measured, ** 12 clutches measured.

	Height (cm)	Horizontal distance (cm)	Leaf surface		Leaf area (cm^2)	Stream side			Position at stream			Canopy openness (%)
			Upper (%)	Lower (%)		Left (%)	Right (%)	Center (%)	Streambed (%)	Bank (%)	Outside bank (%)	
Adults (N=24)	142±43.5 (60.7–231.4)	12.3±21 (0–72)	100	0	121±76.2 (11.4–266.2)	95	5	0	62	33	5	10.5±3.11 (3.72–15.24)*
Clutches (N=19)	79±52 (6–219)	3.2±12.9 (0–58)	79	21	236±350.5 (11.3–1376.2)	74	21	5	79	21	0	13.75±8.13 (6.58–37.89)**

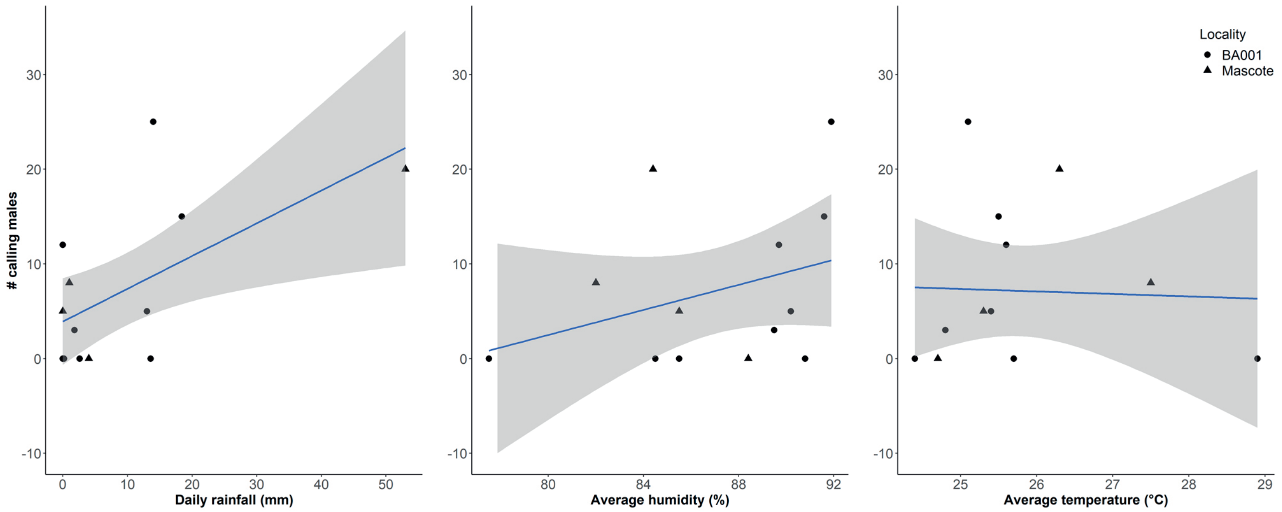

Figure 3. Correlation between the number of calling males of *Vitreorana baliomma* and daily rainfall (χ^2 = 13.8005, P < 0.001), humidity (χ^2 = 10.6828, P = 0.0010813), and temperature (χ^2 = 6.8499, P = 0.0088646). The grey area represents the 95-% confidence interval.

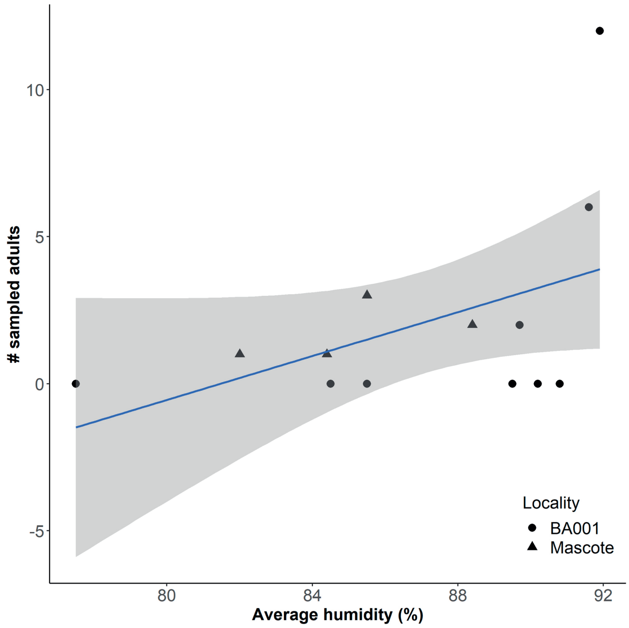

Figure 4. Correlation between number of sampled adults of *Vitreorana baliomma* and humidity (χ^2 = 8.2314, P = 0.004117). The grey area represents the 95-% confidence interval.

(Fig. 5), position at stream (X^2 = 2.4087, P = 0.2999, N = 43), stream side (X^2 = 4.4682, P = 0.1071, N = 43), and leaf surface (X^2 = 3.355, P = 0.067, N = 43) (Fig. 6).

Clutch characterization

Mode clutch size was 14 ± 4 eggs (7–22). The mean diameter and thickness of egg capsules were 2.49 ± 0.75 cm^2 (1.31–4.32) and 6.7 ± 2.8 mm (2–13), respectively (N = 18). Clutches had embryos between stages >10 and 25 according to Gosner (1960), where >10 was the stage immediately after oviposition and 25 the stage of hatching embryos. We found two differently aged clutches on the same leaf (stages 19 and 21). All clutches exhibited a jelly-rich matrix surrounding the embryos. At stages 10–16, we observed yellowish cream coloured embryos (slightly greenish immediately after deposition) (Fig. 7A). At stages 19–22, we observed whitish or slightly greyish embryos, with a yellowish cream vitellus (Fig. 7B). Late-stage embryos (23–25) were light brown to grey with reddish hues and a reduced yellowish cream vitellus (Fig. 7C). In early-stage clutches, eggs were in a monolayer array (Fig. 7A). However, this changed over time (Figs 7B, C), with most clutches increasing in thickness during development.

We selected simple models for the analysis of both clutch structure and microhabitat affecting clutch thickness. Clutch thickness was positively correlated with developmental stage (F = 8.6555, P = 0.009568, N = 18) (Fig. 8). However, clutch thickness was not related to the number of eggs in the clutch (F = 2.2403, P = 0.1539, N = 18) or clutch diameter (F = 0.756, P = 0.3974, N = 18). As for the effects of microhabitat data, clutch thickness was positively correlated with leaf size (χ^2 = 5.1464, P = 0.02329, N = 18) (Fig. 8), whereas vertical height (χ^2 = 0.2118, P = 0.6453, N = 18) and leaf surface (χ^2 = 1.4638, P = 0.2263, N = 18) did not influence clutch thickness.

Phylogenetic analyses

Our final dataset included 3176 characters (12S: 984 aligned positions, 18% of terminals; 16S: 883, 21%; COI: 705, 89%; POMC: 604, 69%). ModelFinder selected the following data partitions and models of nucleotide evolution: TIM2+FU+I+G4 for 12S and 16S, TIM2e+I+G4 for COI first codon and POMC first codon, TN+FU+I for COI second codon and POMC second and third codons, and TN+FU+G4 for COI third codon. ML searches found a single most likely tree (Ln = -18153.711; Fig. 9), which recovered a monophyletic *Vitreorana* (BS = 80) within

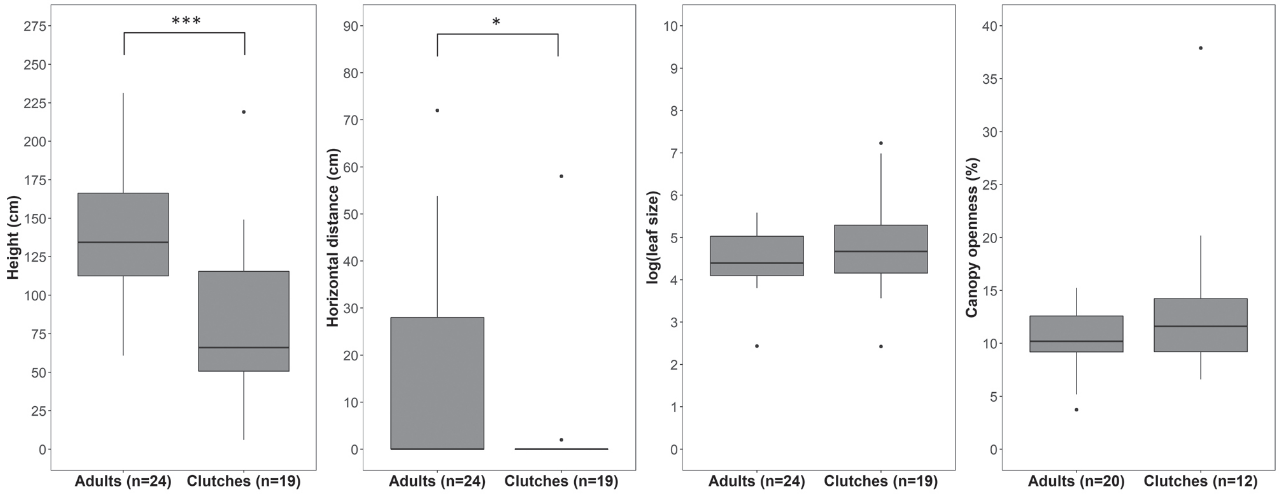

Figure 5. Comparison between quantitative variables of the microhabitat used by adults and oviposition sites. Median (horizontal line), quartiles (boxes), extremes (vertical lines), and outliers (points) are shown. Significant values are pointed out as * P < 0.05 and *** P < 0.001.

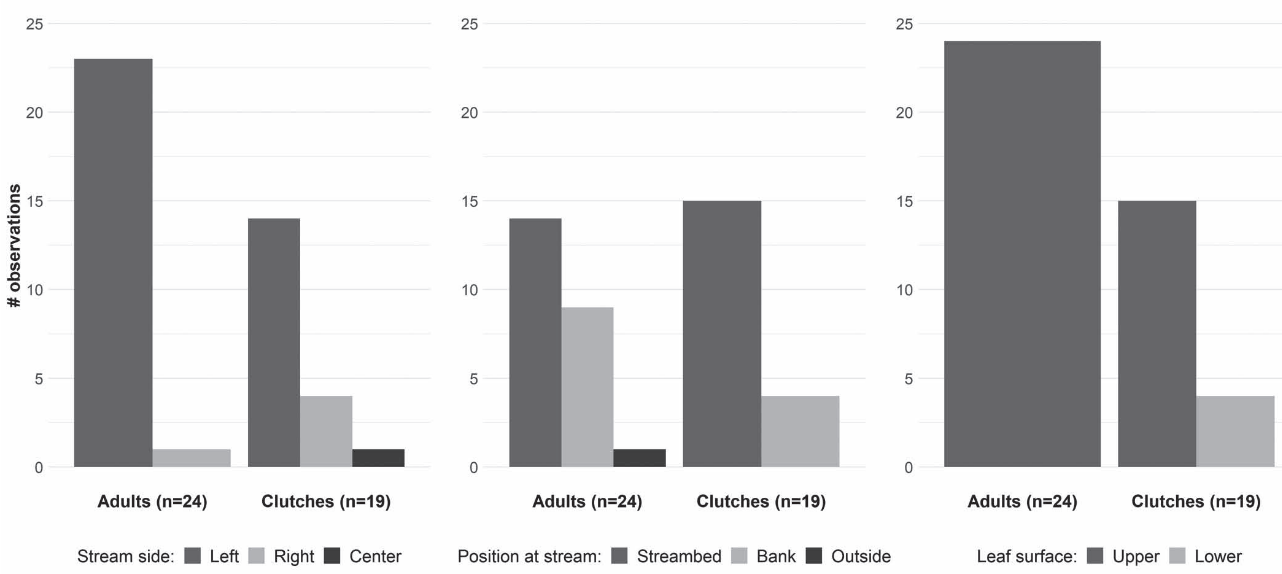

Figure 6. Comparison between qualitative variables of the microhabitat used by adults and oviposition sites.

Centroleninae (BS = 70). All species of *Vitreorana* with more than one sampled specimen are monophyletic except for *V. uranoscopa*, which is paraphyletic with regard to *V. franciscana*. Within *Vitreorana*, the clade formed by the Guiana Shield/Amazonian species *V. helenae* and *V. ritae* (BS = 100) is sister to all other *Vitreorana* species (BS < 50). The two Cordillera de la Costa species (*V. antisthenesi* and *V. castroviejoi*) form a clade (BS = 100) sister to all the other species. The Atlantic Forest and Cerrado species of *Vitreorana* are not monophyletic because *V. baliomma* is sister to *V. gorzulae* from the Guiana Shield (BS < 50).

Discussion

Reproductive behaviour and parental care

The briefness of female brooding after amplexus is probably key to understanding why this behaviour went unnoticed in the family Centrolenidae until now. Through detailed field studies and careful observations during oviposition, our study was the first to detected parental care in an Atlantic Forest glassfrog, broadening the knowledge about the reproductive biology for the group. *Vitreorana baliomma* females perform brief brooding just after oviposition. Although we have not quantified the effects of this behaviour on embryo survival, it is likely to have a similar impact to what Delia et al. (2017) found for *Cochranella granulosa* and *Teratohyla pulverata*, two species of closely related genera (Guayasamin et al. 2009), where brief brooding functions to hydrate eggs, protecting embryos from dehydration and early predation. Thereafter, clutches will depend on water from rain and ambient humidity to maintain hydration levels and clutch thickness (Delia et al. 2017, 2020). This discovery in *V. baliomma* reinforces the pattern of parental care evolution hypothesized for centrolenids (Delia et al. 2017) and suggests that the behaviour may be widespread in *Vitreorana* and Cochranellini species. Nevertheless, more studies and experiments are necessary to determine how brooding impact the clutches.

During brooding, we observed females elevating the posterior part of their body. This movement may serve to trap humidity (considering that the belly of anurans has a greater absorption capacity), reposition the body above the clutch, or aid egg fertilization. Other centrolenid species with brief maternal care brooded for similar periods of time. For example, the female of *V. ritae* brooded for 80 min (Delia et al. 2017). However, variation is large, from 10 to 180 min, in species of the genera *Centrolene*, *Espadarana*, *Nymphargus*, and *Teratohyla* (Jacobson 1985, Guevara-Molina & Vargas-Salinas 2014, Diaz-Ricaurte et al. 2016, Arcila-Pérez et al. 2017, Basto-Riascos et al. 2017, Rios-Soto et al. 2017). Some species rotated their

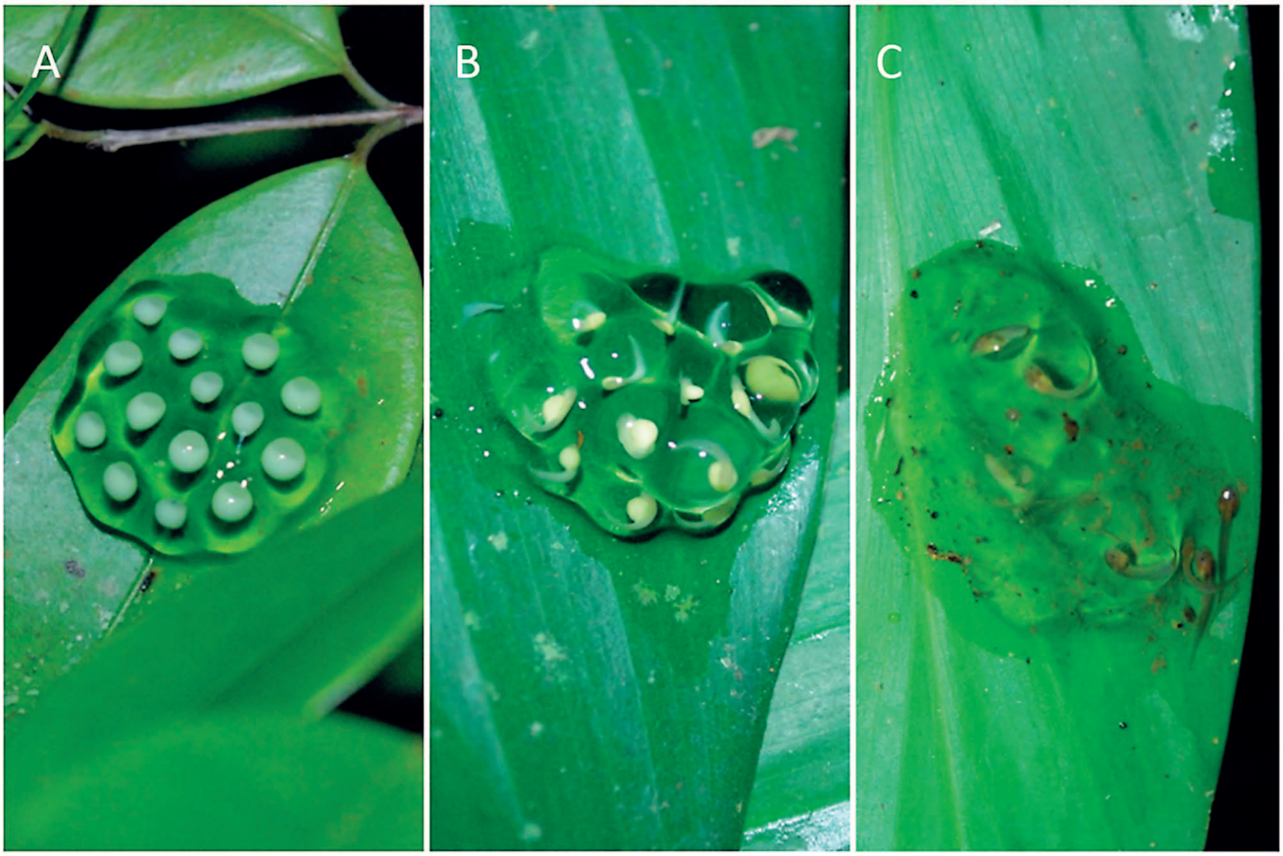

Figure 7. Egg clutches of *Vitreorana baliomma* at early (A), intermediate (B), and late (C) stages of development.

eggs while caring, but ours is the first record of this body elevation behaviour.

Our discovery of female brooding behaviour in *V. baliomma* contrasts with the only two studies (Gouveia et al. 2012, Lisboa et al. 2019) that provided data on reproductive aspects of *V. baliomma*, but did not document parental care in this species. Lisboa et al. (2019) did not observe oviposition events, which significantly reduces the chances of observing female brooding behaviour. Gouveia et al. (2012), based on a single amplectant pair, described that the female stayed near the clutch until dawn, a behaviour we did not observe, without mentioning interactions between the female and her egg clutch. This could indicate that *V. baliomma* regulates its maternal care based on environmental factors such as ambient moisture, daily rainfall, or both. This would be similar to the variation in paternal care observed in *Hyalinobatrachium* (Vockenhuber et al. 2008; Delia et al. 2013) or attributed to some degree of behavioural plasticity (Wells 1981). Another possibility is that maternal brooding is subject to variation between certain populations, which means that this character could have a phylogeographic signal function, possibly with a genetic component influencing this behaviour (Bubac et al. 2020). Finally, we cannot rule out that these authors disturbed the females when taking measurements of the eggs immediately after oviposition as stated in Gouveia et al. (2012: 18). Individuals engaged in caring are normally more risk-tolerant, as females staying near their clutches will surely potentially attract the attention of predators (Bravo-Valencia & Delia 2016, Valencia-Aguilar et al. 2020b). It is important to test if *V. baliomma* exhibits variation in parental care and, if so, which variables could be associated with it.

The general sequence of observed mating behaviours is shared with all other species of glassfrogs, with variation being associated with details regarding aspects such as call and oviposition sites, caring sex, and extension and type of parental care (Greer & Wells 1980, Jacobson 1985, Vockenhuber et al. 2008, Vargas-Salinas et al. 2014, Arcila-Pérez et al. 2017, Basto-Riascos et al. 2017, Rios-Soto et al. 2017, Díaz-Ricaurte et al. 2019). The behaviours observed during the amplexus suggest that the female selects the best place for depositing the egg clutch. While in amplexus, the female does not only move through the foliage of the plant in which the amplexus started, but also through that of other plants nearby and frequently switches between leaf faces. This face-changing behaviour explains why we found 21% of clutches on the undersides of leaves. This could be random variation, but we argue that it could also result from a trade-off, where eggs on the upper face will be exposed to rain and so can benefit from a non-parental source of hydration, while those on the lower face may be better protected from embryo loss.

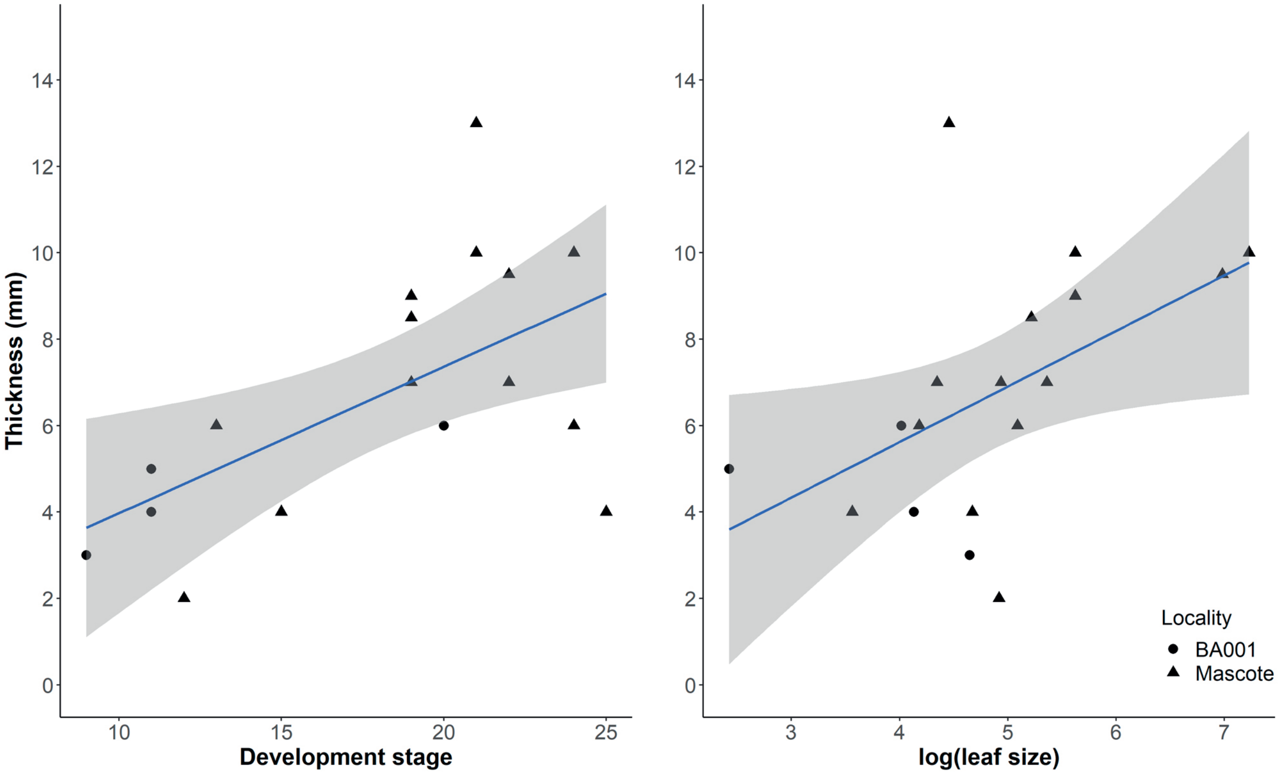

Figure 8. Correlation between clutch thickness of *Vitreorana baliomma* and developmental stage (F = 8.6555, P = 0.009568) and leaf size (χ^2 = 5.1464, P = 0.02329). The grey area represents the 95-% confidence interval.

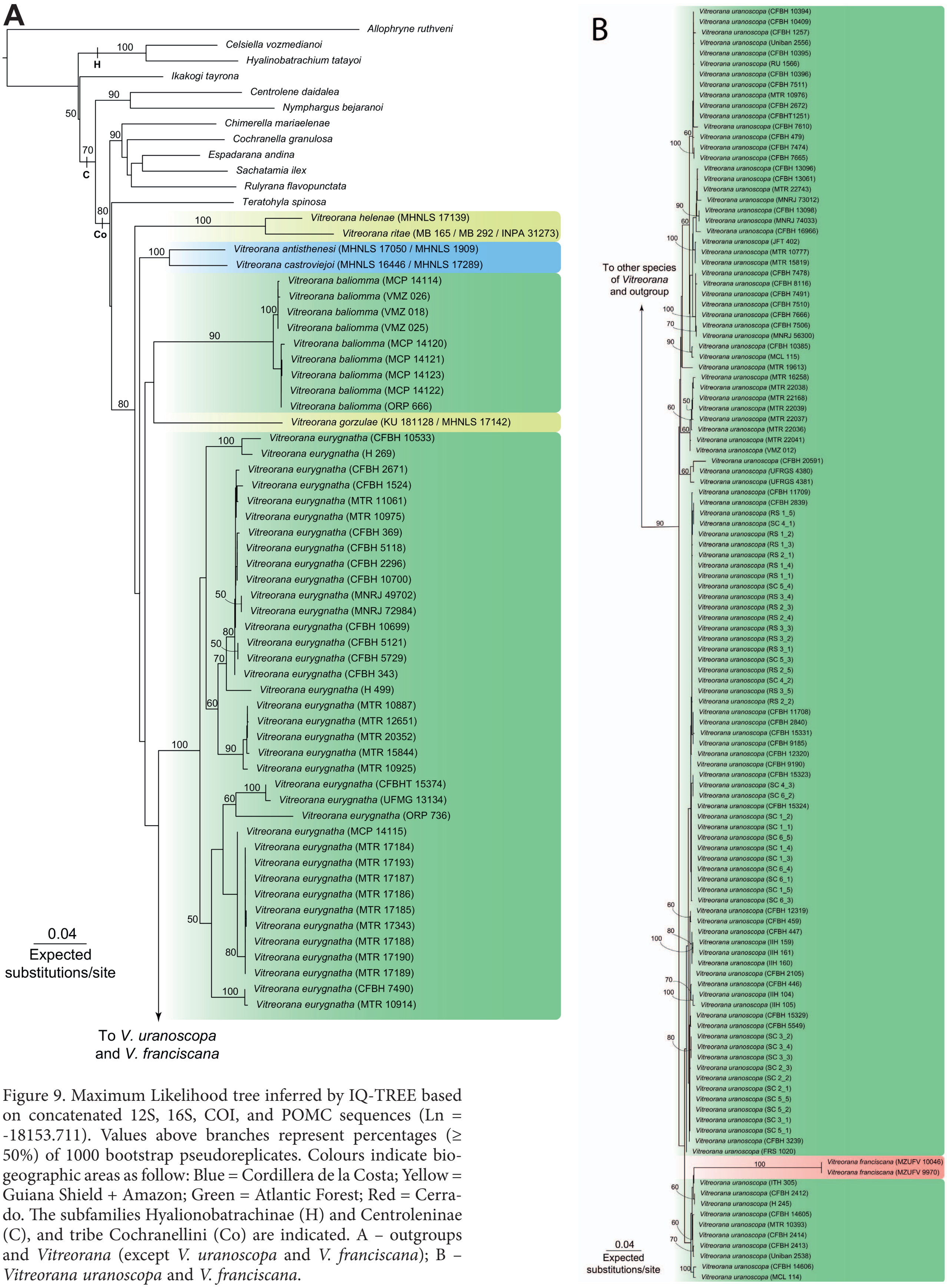

Figure 9. Maximum Likelihood tree inferred by IQ-TREE based on concatenated 12S, 16S, COI, and POMC sequences (Ln = -18153.711). Values above branches represent percentages (≥ 50%) of 1000 bootstrap pseudoreplicates. Colours indicate biogeographic areas as follow: Blue = Cordillera de la Costa; Yellow = Guiana Shield + Amazon; Green = Atlantic Forest; Red = Cerrado. The subfamilies Hyalionobatrachinae (H) and Centroleninae (C), and tribe Cochranellini (Co) are indicated. A – outgroups and *Vitreorana* (except *V. uranoscopa* and *V. franciscana*); B – *Vitreorana uranoscopa* and *V. franciscana*.

Amplectant pairs moved about for more than an hour before oviposition. This is compatible with the observation that species with maternal care move across greater distances, investing more time in amplexus than those with paternal care (Jacobson 1985). This might be related to territoriality, inasmuch as in species with paternal care, males might choose a place that is good for both signalling for mates and embryonic development. As these sites are probably available only to a limited extent, males are expected to display more aggressive territorial behaviours (McDiarmid & Adler 1974, Restrepo & Naranjo 1999, Cardozo-Urdaneta & Señaris 2012, but see Noronha & Rodrigues 2018) and do not move far away from their territory. On the other hand, in species with maternal care, males might choose the best site to enhance their acoustic (or other) signals, while females must choose a good place for embryo development (Touchon 2012).

As expected, we found a positive relationship between average relative humidity and daily rainfall with the number of calling males. This relationship points to the importance of precipitation for breeding activity in *V. baliomma*. Notwithstanding, we found a negative relationship between average temperature and the number of calling males. This probably results from an inverse correlation of temperature and daily rainfall. Sampled adults presented a relationship with relative humidity too, with more individuals found at times of higher humidity. Despite our sample period having a relatively low frequency and a non-standardized duration of sampling, our results were compatible with other studies analyzing the reproductive activity in centrolenids. Precipitation is a good predictor of calling activity in the Centrolenidae (Vockenhuber et al. 2008, Basto-Riascos et al. 2017, Rios-Soto et al. 2017), and *Vitreorana* species call, almost exclusively, during and after rainy days (Cardoso & Haddad 1992, Canelas & Bertoluci 2007). Also, activity of male and female centrolenids tends to be higher in conditions of more rainfall and lower temperature (Basto-Riascos et al. 2017, Rios-Soto et al. 2017). Vocalizing is energetically demanding for anurans, causing a significant increase in their metabolic rate (Gerhardt 1994), so that it is expected that species call just when the environmental conditions are good for doing so. In fact, during the unusually long dry periods (i.e., more than three consecutive weeks of almost zero precipitation) during our sampling, the entire population became inactive, and no males were heard, or individuals observed.

Microhabitats of adults and oviposition sites

Our results on variables associated with the oviposition site indicate a preference for the upper sides of large smooth green leaves, relatively close to the substrate, above water, and on the left side of streams. Meanwhile, adults also appear to prefer the upper side of smooth green leaves, above water, and on the left side of streams, but in contrast to oviposition sites exhibit a preference for greater heights above the ground, larger horizontal distances from water and, apparently, smaller leaves. Analyses comparing the microhabitats of adults and clutch deposition sites revealed significant differences only for vertical height and horizontal distance. However, our sample size is too limited for egg clutches to comprehensively confirm patterns of microhabitat use, especially for the preference of stream side, leaf size, and canopy openness. Oviposition sites located at lower heights can result in higher survival probabilities (Jacobson 1985), perhaps due to higher humidity levels closer to the ground (Cardoso & Haddad 1992), which could decrease dehydration. Furthermore, these sites could benefit tadpole hatching by decreasing the drop distance to the water and the influence of wind. On the other hand, because most adults that we observed were calling males, we hypothesize that their greater perch height, compared to clutches, enhances the dissemination of acoustic signalling (Greer & Wells 1980, but see Valencia-Aguilar et al. 2012, Vargas-Salinas et al. 2014).

We found all clutches either directly above the streambed or, less frequently, on the stream bank (in the area that will be flooded after strong rainfalls). Considering that tadpoles need to fall into the water after hatching to continue their development, oviposition sites above streams should be strongly favoured by females (Cardozo-Urdaneta & Señaris 2012). Meanwhile, we argue that the selection of males' calling sites distance from the water is less stringent, allowing males to vocalize from places outside the flooding area, which are more densely covered by vegetation and so have a reduced predation risk. Male choice for greater heights could be a response too for places more covered, once dense vegetation reduces propagation of calls (Wells & Schwartz 1982, Vockenhuber et al. 2008).

Although adult microhabitat preferences and oviposition site requirements do not significantly differ as to leaf size used, clutches were more commonly found on larger leaves. The use of large leaves might be associated with male territoriality and prolonged care in some *Hyalinobatrachium* species, because males can then attend many clutches at the same time (Vockenhuber et al. 2008, Valencia-Aguilar et al. 2012). We have not observed male territoriality and prolonged care in *V. baliomma*, but our linear model shows a correlation of leaf size with clutch thickness. Globular clutches (like in species of *Cochranella*, *Espadarana*, *Nymphargus,* and *Vitreorana*) can retain larger amounts of water, requiring an initial bout of maternal brooding and subsequent rainfall to maintain hydration levels (Delia et al. 2020). We hypothesize that larger leaves may accumulate more water on their surfaces, aiding in clutch hydration. Thus, water on leaves after rainfall might help to ensure embryo survival.

Clutches and adults did not significantly differ in occupying leaf face, although adults were found just on the upper side and clutches on both faces. Species with brief parental care show tendencies to deposit clutches on the upper faces of leaves or other exposed locations (Delia et al. 2020). Although oviposition strategies may be flexible with females adjusting their strategies according to ecological conditions (Marsh & Borrell 2001), we found that *V. baliomma* females appear to have a preference for depositing their clutches on the upper side, but do not do so exclusively. As previously discussed, oviposition on both the upper and lower sides of leaves may be related to a trade-off between hydration from rainwater on the upper side and protection from predators and the washing-away of eggs by heavy rainfall and/or shielding from UV light on the lower side (Blaustein & Belden 2003, Vonesh 2005). Leaf-side use for oviposition is considered a taxonomically informative trait in the Centrolenidae (e.g., Ruiz-Carranza & Lynch 1991, Guayasamin et al. 2009). However, field observations indicate that this character is more intraspecifically variable than previously thought (Delia et al. 2010, Bravo-Valencia & Delia 2016, Arcila-Perez et al. 2017, Maynard et al. 2020). Taking this variation into account may have an important impact on our understanding of the evolution of this strategy.

We suspect that a smooth leaf surface facilitates that tadpoles more readily slide and fall into the stream below, while the apparent preference of the left side of the stream could be related to slope level, which, by pure chance, was similar in the two sampled streams, causing the left side to receive more water. We did not observe a major difference in the vegetation structure between the both stream sides. It is likely that, as females do not afford their clutches prolonged care, the selective pressures might act in a way that leads to selecting oviposition sites at which clutches will be better protected from dehydration. Yet, oviposition site choice is a complex process, where females have to consider the maximization of embryo survival, maximization of maternal survival, optimization of offspring phenotype, proximity to suitable habitat for offspring, maintenance of natal philopatry, or, in an indirect manner, mate choice (Refsnider & Janzen 2010). It is therefore crucial to test the hypotheses for decisions that rule oviposition site selection, the impact of female choice (i.e., upper vs. lower side of leaves, large vs. small leaves) on offspring survival and include observations of other populations and other *Vitreorana* species to improve our understanding of microhabitat use in this group.

Egg clutches

Egg and embryo colours, number of eggs, and early stages of clutches of *V. baliomma* are similar to the descriptions of Gouveia et al. (2012), although we noted temporal modifications to the egg clutches during their development. In their early stages, clutches are in a monolayer array; however, throughout embryogenesis, they take on a more complex tridimensional structure due to increased egg and egg-jelly hydration. We also found a relationship between developmental stage and clutch thickness. As is the case with clutch structure, this results from the addition of environmental water, as jelly-rich clutches are fast to absorb water, store much water, and take more time to dehydrate (Delia et al. 2013, 2020).

Phylogenetic relationships

We here present the first phylogenetic analysis including data of *Vitreorana baliomma*. Maximum likelihood analysis corroborates the monophyly of *Vitreorana*, with moderate bootstrap support. The phylogenetic relationships within *Vitreorana* are different in several important aspects from previous well-sampled phylogenies of the genus. Our results indicate that the position of *V. franciscana* within the genus is particularly problematic. This species is just represented by DNA sequences of POMC (Santana et al. 2015), a nuclear protein coding gene without sufficient variation to resolve the relationships between the species of *Vitreorana*. This sampling bias most likely explains the inferred phylogenetic position of our two samples of *V. franciscana*, which are nested within specimens of *V. uranoscopa* on a conspicuously long branch. Thus, an important next step to improve our knowledge of the evolutionary relationships of *Vitreorana* requires gathering more phylogenetically informative DNA sequences and genes, especially for *V. franciscana*, such as mitochondrial genes and faster-evolving nuclear markers. The sister relationship of *V. baliomma* and *V. gorzulae* is remarkable. From a morphological perspective, both species share completely transparent parietal and urinary bladder peritonea, white peritonea covering the heart, bulbous liver, stomach, and intestines, green bones in life, and dorsum lavender in preservative (Castroviejo-Fisher et al. 2008, 2009, Guayasamin et al. 2009, Pontes et al. 2014). Considering that all other species of Atlantic Forest and Cerrado *Vitreorana* (i.e., *V. eurygnatha*, *V. franciscana*, *V. parvula*, and *V. uranoscopa*) share the presence of white pigment on the urinary bladder peritoneum, which is absent from all other known glassfrogs, the recovered phylogenetic position of *V. baliomma* supports this character as an unambiguous and non-reversed synapomorphy of all other Atlantic Forest species. From a biogeographic perspective, the sister relationship of *V. baliomma* and *V. gorzulae* indicates a scenario of multiple dispersal/vicariant events between Amazonia and the Atlantic Forest, which deviates from the single dispersal into the Atlantic Forest from Amazonia proposed by Castroviejo-Fisher et al. (2014). We highlight that this new scenario should be tested with the addition of more characters and other potentially closely related species from the Guiana Shield such as "*Cochranella*" *duidaeana* and "*Cochranella*" *riveroi*.

Acknowledgements

We thank everyone who assisted in the execution of this work, whether with help in the field or analyses, especially K. Ramos and D. Grundmann. We are thankful to J. Delia for all the help with the development of this work and the extremely useful tips and suggestions in data collection and statistical analysis. We also thank C. S. Fontana, C. P. A. Prado, and P. L. V. Peloso for their suggestions that helped to improve earlier versions of the manuscript, and the chief editor for final contributions. Specimen collection was authorized under Instituto Chico

Mendes de Conservação da Biodiversidade (ICMBio) licences (SISBIO numbers 64810-2, 13708-2 and 41199-3). This study was completed while benefiting from scholarships from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil) to VMZ (131808/2018-4), IRD (151218/2021-8) and SC-F (314142/2020-7), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil) to OR-P (88887.343060/2019-00). Funding was provided by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (PROTAX-project 440615/2015-1) and the Fundação de Amparo à Pesquisa do Estado da Bahia (FAPESB) (PTX0002/2016).

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Supplementary data

The following data are available online:

Supplementary document 1. List of species, voucher codes, localities, and GenBank accession numbers of the sequences analyzed in this study.