



Research Article

Nitrogen isotope enrichment associated with skull shape variation in the Dwarf little fruit bat *Rhinophylla pumilio*

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Abstract

Intraspecific variation in phenotype and ecology within a population is an important component of eco-evolutionary dynamics, that influence species longevity, community structure and ecosystem function. We studied nitrogen enrichment variability in a population of the dwarf little fruit bat *Rhinophylla pumilio* in association to its variation in skull shape. In the studied population (Atlantic Forest in northern Espírito Santo, Brazil), *R. pumilio* has been recorded to feed not only on fruits, as in most of its range, but also nectar. So far, this has not been recorded anywhere else for this species. Our results have shown support for phenotypic specialisation within this population that facilitates a nectarivorous diet in some individuals, contributing to intra-population variation in this locality. Combining geometric morphometrics and stable isotope analysis, it was possible to show that skulls with longer rostra and more procumbent incisors were associated with nitrogen enrichment in the range of $\delta^{15}\text{N}$ levels observed in other nectarivorous species at the same locality. Because nectar is a low-protein food item, nectarivorous bats often incorporate other protein sources into their diet (like pollen and/or insects). In the case of *R. pumilio*, the use of nectar is likely opportunistic for those individuals with favourable phenotypes. However, there is no evidence so far that *R. pumilio* is able to digest pollen or eventually incorporate insects in the diet. The nitrogen enrichment observed can be caused by physiological stress due to a protein poor diet, causing a mixed incorporation of nitrogen from internal and external sources (from diet). This scenario could be an intermediate step in the transition to a more nectarivorous diet, and the pattern of within-population phenotype-ecology association might shed light into the early stages of ecological specialization in phytophagous bats.

Introduction

The origin and maintenance of variation are crucial tenets in evolutionary biology (Hallgrímsson and Hall, 2005). The coexistence of different phenotypes within the same population might allow for the exploitation of a more diversified niche (Van Valen, 1965) and could be a step in the direction of speciation and ecological specialization, as natural selection favours specific phenotypes in a spatially heterogeneous environment (Futuyma and Moreno, 1998; Bell, 2008; Martin and Pfennig, 2009). On the other hand, ecological and phenotypic variation among individuals within a species can have an effect on the eco-evolutionary dynamics (Bolnick et al., 2003, 2011; Schreiber et al., 2011; Snowberg et al., 2015), with consequences to species longevity and coexistence. The interaction between phenotype and ecology, therefore, provides a link to the process of natural selection and its effects that will emerge on higher levels of organization, such as the structure of communities and the functioning of ecosystems (Hendry, 2017).

Bats are a unique biological model for studying the association between morphological and ecological variation at different levels of organization, due to their ability to explore different food sources opportunistically, along with the factors that facilitate this plasticity (e.g. morphological disparity) (Monteiro and Nogueira, 2010; Santana et al., 2012). Bats show a range of different morphologies that are intimately associated with specific functional demands towards their feeding and foraging behaviours (e.g. bite force optimization for processing

hard food items) (Monteiro and Nogueira, 2011; Santana et al., 2012), hence presenting considerable inter- and intraspecific variation (Dumont, 2004; Dumont and O'Neal, 2004; Nogueira et al., 2005; Dumont et al., 2009; Nogueira et al., 2009; Monteiro and Nogueira, 2010).

Studies on bat feeding ecology have shown a large diversity of feeding habits, and a wide range of variation within dietary categories (Wetters et al., 2000; Dumont and O'Neal, 2004). Phytophagous bats are great examples to look at with a magnifying glass in this matter, because they present an almost continuous range from generalist to specialized diets and cranial shapes (Dumont, 2004; Monteiro and Nogueira, 2011). Although they often present opportunistic feeding behaviour (associated with temporal and spatial variation of food sources), a number of species also present strong preferences to specific food items (Voigt et al., 2009; Lima et al., 2016). This inter-biotic dynamics selected a range of specific morphologies through time, that are correlated to the consumption of liquid and soft to hard plant food items, that is, from nectar and soft fruits to harder fruits, leaves and seeds consumption (Dumont and O'Neal, 2004; Nogueira et al., 2005; Dumont et al., 2009; Nogueira et al., 2009; Santana et al., 2012).

In order to understand the different levels of interaction between phytophagous bats and plants, scientists work with a variety of methods for quantifying and identifying their diet (Voigt et al., 2009; Lima et al., 2016). More traditional approaches such as the analyses of faeces and stomach contents, active foraging observations in the field and the analyses of seed rain are crucial in this matter because they provide information on species-specific interactions and on the quantification of

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Figure 1 – An individual of *Rhinophylla pumilio*, captured with pollen by MN and IL at Reserva Natural Vale, municipality of Linhares, northern state of Espírito Santo, southeastern Brazil.

the abundance of specific items, that is, on bat food preferences (Fenton et al., 1998; Voigt et al., 2009; Ragusa-Netto and Santos, 2015; Lima et al., 2016). In addition, molecular methods are being used in order to achieve more detailed information on the taxonomy of food content, as species classifications are not always possible solely on morphological analyses of food items (Voigt et al., 2009). These methods are important to understand bat feeding ecology. However, they can only identify food items on bat diets over a short time-span, showing what bats consumed in the last hours prior to sampling (Roswag et al., 2014). Therefore, several studies have relied on stable isotopes in order to quantify the information on the nutrients that integrate into animal tissue over longer periods (Bolnick et al., 2003; Voigt and Matt, 2004; Rex et al., 2010; Frick et al., 2014; Roswag et al., 2014).

The incorporation of stable isotopes in studies of bat feeding ecology has provided important data on dietary specialisation (Rex et al., 2010), feeding plasticity (Rex et al., 2010; Frick et al., 2014), community assemblages (Monadjem et al., 2018) and trophic niche differentiation (Rex et al., 2011). However, when dealing with stable isotopic data, it is important to understand that other factors — abiotic (e.g. seasonal and geographical variation) and biotic factors (e.g. species metabolism rates, stress levels, sexual dimorphism and ontogeny) — can contribute to $\delta^{15}\text{N}$ variation in animal tissue, and bats are no exception to this issue (Voigt and Matt, 2004; Ben-David and Flaherty, 2012; Roswag et al., 2014). The careful combination of isotope analyses with other evidence and methodologies can provide advances in knowledge on mammal feeding ecology, especially in association with phenotypic variation (Walsh et al., 2016; Yue et al., 2020).

In the present study, we investigate the correlation patterns between nitrogen enrichment and skull morphology in the Dwarf little fruit bat *Rhinophylla pumilio* Peters, 1865, a small-sized bat (ca. 9 g) endemic to South America (Rinehart and Kunz, 2006). This species has been recorded as frugivorous along all its range (Reis and Peracchi, 1987; Gorchov et al., 1995; Delaval et al., 2005; Lima et al., 2016), but at Reserva Natural Vale (RNV), situated in the Atlantic Forest of northern Espírito Santo State, southeastern Brazil, it is also known to feed on the nectar of passion flower *Passiflora ovalis* (Buzato and Franco, 1992). In their study, Buzato and Franco (1992) reported that, in order to feed on the nectar, the bats push their snout into the flower to lap the nectar, while the top of its head touches the anthers and stig-

mata. While doing this, the pollen can get attached to the top of the bat's head. The pollen of *P. ovalis* is yellow, as the one recorded by two of the authors (MN and IL) in a captured *R. pumilio* in this location (Fig. 1), and as previously reported by Pedro and Passos (1995). This way, a more elongated rostrum could be key to acquire nectar more easily, like the one from *Glossophaga soricina* (Pallas, 1766), a nectar feeding bat also reported to visit the plant in the location (Buzato and Franco, 1992). We therefore focused on quantifying the skull morphology of *Rhinophylla pumilio* in this specific population and relating this variation to $\delta^{15}\text{N}$ enrichment within this population. We chose this approach because it allowed us to gather paired information on skull shape and long-term diet on individuals, using museum specimens where both the skull (morphology) and the hair (isotope data) were available.

Studies on protein intake on specialised nectarivorous species have shown that these animals often complement their low protein diet with insects or pollen, therefore often presenting higher $\delta^{15}\text{N}$ values than expected solely by a liquid diet (Herrera et al., 1998, 2002; Mancina and Herrera, 2010). If this is the case, higher values of nitrogen could be related to more nectarivorous individuals in *R. pumilio*, which in turn could be a source which is explored more regularly by individuals with specific phenotypes (Mancina and Balseiro, 2010). This way, our hypothesis is that skull shape variation within this population can potentially facilitate the consumption of nectar in this specific population of *R. pumilio*, in which case, nitrogen enrichment would be higher in individuals with specific phenotypes within the pool of skull shape variation. This would suggest that individual specialization is occurring within the population of *R. pumilio* in the forests of northern Espírito Santo.

Material and Methods

Concentration of $\delta^{15}\text{N}$ and skull shape variation in *R. pumilio* was investigated based on a sample of 26 adult specimens (17 males and 9 females; Supplementary Materials), all collected in the same locality where the nectar-feeding behaviour of this bat was first described (Reserva Natural Vale, Espírito Santo, Brazil, RNV) (Buzato and Franco, 1992). Interpretation of isotope concentration in animal tissue usually requires measures of the same isotopes in food items, but in the absence of these data we used hair samples from other phytophagous bats collected at the same site we sampled *R. pumilio* (RNV) to gain insight about the diet variation associated to nitrogen values (Mancina and Herrera, 2010).

Nitrogen enrichment was obtained from hair samples cut from the upper back of specimens of *R. pumilio* and other bat species, classified as frugivorous: *Artibeus lituratus* (n=1), *Chiroderma villosum* (n=2), and *Carollia perspicillata* (n=1), nectarivorous: *Anoura geoffroyi* (n=4) and *Glossophaga soricina* (n=7) or omnivorous *Phyllostomus discolor* (n=3) and *P. hastatus* (n=1) (see Supplementary Materials for more details on the analysed specimens). All specimens were collected in the same locality (RNV) and are prepared as skin and skull and deposited at Universidade Federal Rural do Rio de Janeiro, Rio de Janeiro, Brazil (ALP — Coleção Adriano Lúcio Peracchi). The isotopic composition of hair does not change after formation, and the isotopic turnover can represent several months due to seasonal molting (Voigt et al., 2003; York and Billings, 2009). Hair samples were chopped, cleaned with ultra-pure water, dried at 60 °C during 24 h, transferred to tin capsules (subsamples of ca. 0.4 mg), and then analyzed in a Thermo Finnigan Delta V Advantage mass spectrometer (IR-MS-Thermo Scientific) attached to a Flash 2000 element analyser (Organic elemental analyser — Thermo Scientific) in the Laboratório de Ciências Ambientais, Universidade Estadual do Norte Fluminense Darcy Ribeiro. Samples were analyzed with analytical blanks and urea analytical standard (IVA Analysentechnik-330802174; $\text{CH}_4\text{N}_2\text{O}$ Mw=60, N=46%) with certified isotopic composition ($\delta^{15}\text{N}=-0.73\text{‰}$). The analytical control was performed for each 10 samples using a certified isotopic standard (Elemental Microanalysis Protein Standard — OEA: $\delta^{15}\text{N}=+5.94\text{‰}$). The analytical reproducibility was based on triplicates for each 10 samples: $\pm 0.3\text{‰}$ for $\delta^{15}\text{N}$. The reference value

for $\delta^{15}\text{N}$ was the atmospheric nitrogen, and concentration of this isotope is expressed in parts per thousand (‰, exactness close to 95% and analytical precision of $\pm 0.2\text{‰}$). Box plots representing medians and quartiles of nitrogen values were used to explore the position of *R. pumilio* with respect to the ensembles listed above.

For skull shape, we used aligned two-dimensional coordinates of landmarks and semi-landmarks (Monteiro and dos Reis, 1999; Gunz and Mitteroecker, 2013) from dorsal, lateral, and ventral views of the cranium, and lateral view of the mandible as shape variables (Fig. 2, see Supplementary Materials for landmark descriptions). These images were obtained with a digital camera (Canon EOS Rebel XS, coupled with a Canon EF 100 mm f/2.8 Macro lens), and each specimen was set in a standardized position with respect to the camera lens. Landmarks and semi-landmarks were generated in the software tpsDig2 (Rohlf, 2015), and the sliding of the semi-landmarks and alignment of raw coordinates (via Generalized Procrustes Analysis) were performed in the package geomorph (Adams and Otárola-Castillo, 2013) in the R environment (R Core Team, 2020). Aligned coordinates in different cranium and mandible views were used as shape variables in subsequent analyses. In the case of dorsal and ventral views of the skull, where bilateral landmarks are present, only the symmetric component was used in the analyses.



Figure 2 – Pictures of the cranium in dorsal, ventral and lateral view and mandible in lateral view of a *Rhinophylla pumilio* specimen (ALP 10121) showing the position of landmarks (squares) and semilandmarks (circles).

Because sexual dimorphism can potentially impact both skull morphology and $\delta^{15}\text{N}$ ratios in bat species, we tested for differences between males and females on skull shape, size and $\delta^{15}\text{N}$ ratios using a randomised linear model with 9999 permutations with the function `lm.rpp` of the R package RRPP (Collyer and Adams, 2018). In the case of sexual dimorphism being detected in either the response variable ($\delta^{15}\text{N}$ ratios) or predictor (skull shape and size), we tested for the interaction of sex and the predictor variable in the $\delta^{15}\text{N}$ ratios of *R. pumilio*.

We investigated the possible role of skull shape on facilitating nectar consumption in *R. pumilio*, as indicated by the enrichment of nitrogen using a partial least squares (PLS) analysis with 9999 permutations (Rohlf and Corti, 2000). When dealing with two blocks of variables, this method will produce pairs of new (combined) variables showing successively maximum covariance between these blocks (Klingenberg, 2011). In our case, because only one block (shape) is represented by a matrix of variables, the aim is to find a linear combination (PLS axis) of the aligned coordinates that explains most covariation with the vector of nitrogen values. A separate analysis was performed for each skull view using a permutation resampling procedure (9999 permutations) to test for the null hypothesis of no association between the two blocks of variables (nitrogen isotope ratio and skull shape) (Klingenberg, 2011). Shape changes associated with variation in nitrogen isotope values are graphically represented using PLS singular vectors. In this graph, we plot the hypothetical shape in the negative and positive sides of the axis using a factor scale equivalent to three times the range of variation recorded along the axis.

Results

Nitrogen isotope composition mean values obtained for *R. pumilio* were slightly higher than those recorded for other syntopic frugivorous bats and lower than recorded for both nectarivorous and omnivorous species, although the ALP 5605 specimen presented values comparable to nectarivores (Fig. 3).

No evidence of sexual dimorphism was found in the lateral and dorsal views of the cranium or the mandible, neither in the $\delta^{15}\text{N}$ composition ($p > 0.05$). There is a significant effect of sex in the ventral view of the cranium in *R. pumilio* ($R^2 = 0.137$, $F_{1,22} = 3.477$, $p = 0.002$), but no interaction was found between cranium shape (represented in this model by the first principal component of shape in this view) and sex to explain $\delta^{15}\text{N}$ composition in this view ($R^2 = 0.125$, $F_{3,22} = 0.956$,

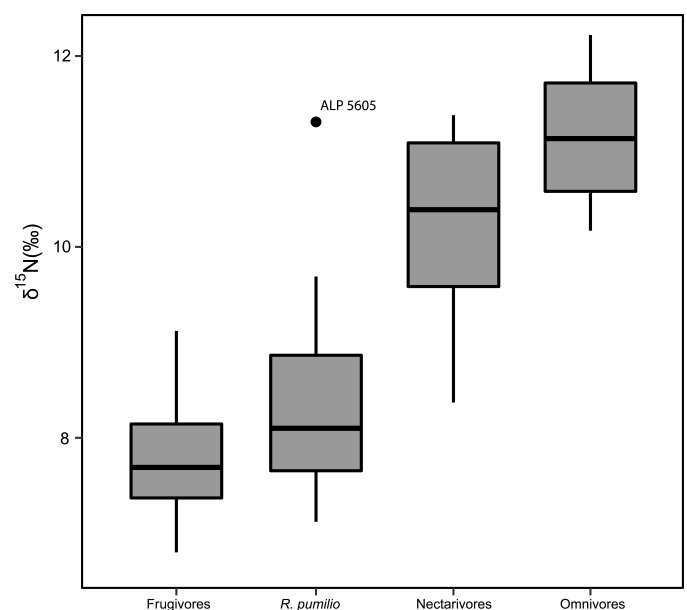


Figure 3 – Box plots of nitrogen isotope values for bat ensembles (Frugivores; Nectarivores; Omnivores) and *Rhinophylla pumilio* from southeastern Brazil. See Material and Methods for a list of the species in each ensemble and sample size. Specimen ALP 5605 is identified as an outlier with high $\delta^{15}\text{N}$ value.

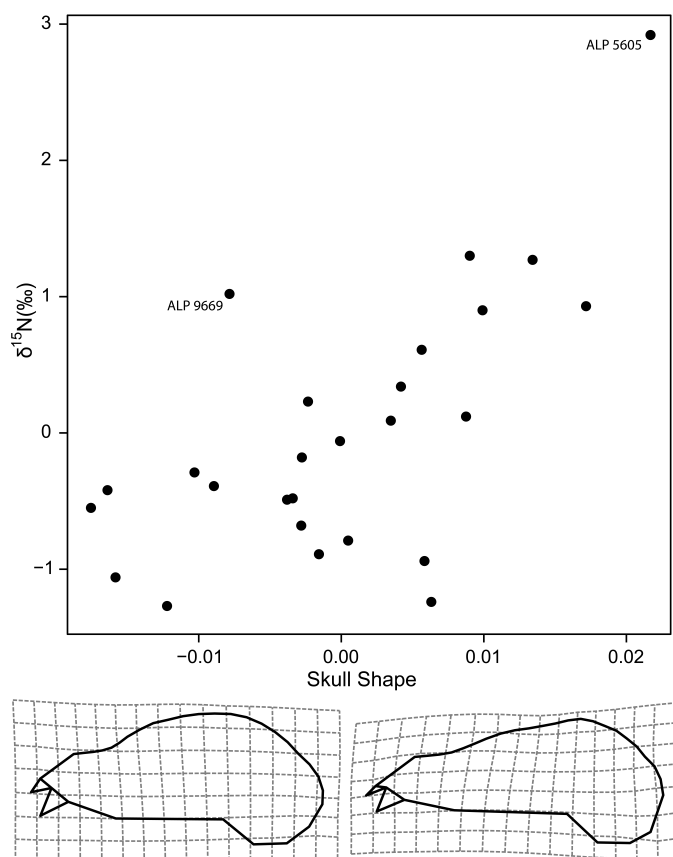


Figure 4 – Shape changes associated with positive values in the PLS shape vector of the lateral view of the cranium of *Rhinophylla pumilio* from southeastern Brazil. Shape magnification equal to three.

$p=0.420$). Therefore we can conclude that sexual dimorphism does not alter our subsequent results.

The PLS shape vector obtained for the lateral view of the cranium presented a correlation with the vector of nitrogen values that is higher than it could be expected based on chance alone ($N=26$, $r_{PLS}=0.662$, $p=0.042$). Shape vectors for other skull views did not show a nitrogen-shape trend (dorsal: $N=25$, $r_{PLS}=0.415$, $p=0.848$; ventral: $N=24$, $r_{PLS}=0.592$, $p=0.260$; mandible: $N=25$, $r_{PLS}=0.521$; $p=0.591$).

Shape changes described by the PLS shape vector of the lateral view of the cranium show that individuals with higher values of nitrogen isotopic composition are those with more elongated rostrum, lower braincase, and more procumbent incisors (Fig. 4). We noticed two outliers in the analyses, specimens ALP 9669 and ALP 5605, which are indicated at Fig. 4. These are true phenotypes within this population and are not related to measurement error. Still, we repeated the PLS analyses after removing these specimens to see if the $\delta^{15}\text{N}$ -shape deformation pattern remained. PLS of skull in lateral view without the two outliers had similar correlation r_{PLS} to the analyses with all individuals plus the $\delta^{15}\text{N}$ -shape deformation pattern described with the full data stands ($N=24$, $r_{PLS}=0.658$, $p=0.089$).

Discussion

The variation of $\delta^{15}\text{N}$ observed in the population of the dwarf little fruit bat *R. pumilio* at Reserva Natural Vale (RNV) is within the range of other phytophagous bats, showing intermediate values between frugivores and nectarivores. This species has been recorded as frugivorous over its distribution (Reis and Peracchi, 1987; Gorchov et al., 1995; Delaval et al., 2005; Lima et al., 2016), but $\delta^{15}\text{N}$ values for some of the specimens from RNV are more comparable to nectarivorous bat species from the same locality (Fig. 3). Nectar itself is known to be a protein poor food source, but a good source of carbohydrates (Mancina and Herrera, 2010; Frick et al., 2014). To cope with this problem, nectarivorous bats are known to compensate their deficient protein diet by

incorporating other sources of protein intake such as animal (insects) and plant (pollen) based protein sources (Herrera et al., 1998; Mancina et al., 2005; Mancina and Herrera, 2010). In RNV, *R. pumilio* has been recorded to feed on nectar (Buzato and Franco, 1992) and has been captured with pollen on the head (Fig. 1, also see Pedro and Passos, 1995). Nitrogen enrichment was associated with a specific phenotype in *R. pumilio*. Individuals that presented the highest values of $\delta^{15}\text{N}$ also presented a more elongated rostrum, lower braincase, and more procumbent incisors. This particular morphology might enhance the individual capability to access nectar from flowers, using their elongated rostrum and possibly tongue (Winter and von Helversen, 2003) to forage the nectar content inside the flowers. The question that stands is how these individuals are incorporating nitrogen from a protein poor diet.

Other phytophagous bats, such as the frugivorous *Sturnira lilum*, *Artibeus jamaicensis* and *Uroderma bilobatum* and the nectarivorous *Monophyllus redmani* and *Glossophaga soricina*, are widely known to use insects as an additional source of protein (Herrera et al., 2002; Mancina and Herrera, 2010). To our knowledge, insects have never been recorded as part of the diet in *R. pumilio*, even in RNV (Reis and Peracchi, 1987; Gorchov et al., 1995; Delaval et al., 2005; Rinehart and Kunz, 2006; Lima et al., 2016). However, the lack of records does not completely exclude the possibility that the nitrogen enriched individuals in our sample had been eating insects to compensate for the protein deficient diet. Another possibility would be the incorporation of plant protein, through the consumption of pollen while feeding of the nectar (Herrera et al., 1998; Mancina et al., 2005; Mancina and Herrera, 2010). Some nectarivorous bats are able to incorporate protein from pollen, but this requires a specialisation of their digestive tract to break the cellular wall from plant cells (Herrera et al., 1998; Mancina et al., 2005). Frick et al. (2014) have reported a case where the primarily insectivorous pallid bats incorporate nectar as a seasonal energy source. This shift, however did not cause depletion of nitrogen, as the nitrogen ratios remains stable between winter and summer. The authors argued that the nitrogen enriched values could have remained stable due to stress level increase (Frick et al., 2014). The most likely explanation, however, is the nitrogen enrichment due to a protein poor diet. In a controlled experiment with two nectar-feeding species, Voigt and Matt (2004) tested the effect of a nitrogen-poor diet into the incorporation of $\delta^{15}\text{N}$. They found that bats fed a nitrogen-poor regime, presented higher values of $\delta^{15}\text{N}$. They also argued that these unexpected results could have been caused by a mixed incorporation of nitrogen from internal (by recycling nitrogen from catabolized body substance) and external sources (from diet) (Voigt and Matt, 2004). In our study, the *R. pumilio* individuals that are more capable of incorporating nectar in their diets have access to an energy rich, but protein poor item, that might cause nutritional stress over a long period (Voigt and Matt, 2004). This scenario could be an intermediate step in the transition to a more nectarivorous diet, where further adaptation to increase protein content are not present, either by allowing for insectivory or the digestion of pollen.

We have also considered the possibility that the observed $\delta^{15}\text{N}$ variation was a result of other factors, such as temporal, spatial, ontogenetic and sexual variation (Rex et al., 2010). Other than temporal variation, all other factors have been controlled in this study by focusing solely on one site, only including adult specimens within our sample and by testing for sex differences in $\delta^{15}\text{N}$ values and skull shape variation. Temporal variation was not tested here, however, we did notice that the museum records were randomly scattered in the PLS plot with regard to year of collection. Even if there was temporal variation in resource abundance within our samples, we argue that this would not alter our interpretation of results because of the association between $\delta^{15}\text{N}$ values and skull morphology.

The sampled population shows considerable phenotypic variation, which facilitates diet plasticity, for some individuals are able to take advantage of the available nectar resource present at RNV (pers obs., Buzato and Franco, 1992; Pedro and Passos, 1995). The intra-population variation described here is a replicate in smaller scale of the well-know

intrinsic evolutionary trend of skull shape variation described for interspecific variation patterns of phytophagous bats (Dumont and O'Neal, 2004; Nogueira et al., 2009). Interspecific skull shape variation of phytophagous bats has been well described as being related to the hardness of consumed food items, ranging from shorter and wider skulls (see negative scores associated deformations at Fig. 4), with a stronger bite force, specialised to consume seeds to hard fruits to longer and thinner skulls, that are better equipped tools to access nectar (liquid diet) (positive scores, Fig. 4) (Dumont, 2004; Dumont and O'Neal, 2004; Nogueira et al., 2009). The phenotypic variance in *R. pumilio* emphasizes the importance of individual specialisation in bat populations.

Enhanced phenotypical and ecological variation in spatially heterogeneous environments are consistent with the tangled bank hypothesis (Lively and Morran, 2014). The forests in northern Espírito Santo State are among the richest in plant species, across the Atlantic Forest domain. They are formed by a mosaic of vegetation formations, from open areas to dense forest, due to soil characteristics (Rolim et al., 2016a). The *Passiflora* species that is used by *R. pumilio* as nectar source is found growing as vines at the edge of the forest and native fields (Rolim et al., 2016b), and the opportunity for nectar consumption is possibly linked to the spatial heterogeneity in the area. Individual specialisation has been described in bat populations and communities (Cryan et al., 2012; Alberdi et al., 2020; Kerches-Rogeri et al., 2020), but has not been often associated with morphological variation (de Oliveira et al., 2020). Generalist and abundant species (like *R. pumilio*), are commonly reported to have a great variety of phenotypes along with specific and individual preferences that are associated with this variation (Mancina and Balseiro, 2010; Mancina and Herrera, 2010; Rex et al., 2010; Monteiro et al., 2019; Kerches-Rogeri et al., 2020). Further studies are needed to understand the patterns of cranial and ecological variation at higher scales, with more individuals and populations over a large spatial range. Exploring individual specialisation and patterns within-population associations between phenotype and ecology might shed light into the evolution of ecological specialization of phytophagous bats. 🐼

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Supplemental information

Additional Supplemental Information may be found in the online version of this article:

Appendix S1 *Rhinophylla pumilio* specimens used for morphometric and stable isotopes analyses.

Appendix S2 Specimens used for isotopic ratio comparison in Fig. 2.

Appendix S3 Landmark descriptions in accordance to numbers at Fig. 1.