

# Trophic ecology of two amphibian species in patches and core forest of Atlantic Forest: A dietary and isotopic approach

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**Abstract** Species richness and abundance, as well as trophic relationships, are affected by habitat configuration. Smaller habitat patches suffer greater external interference, being more susceptible to diversity loss and could also receive more trophic subsidies from outside or matrix, which can favour the maintenance of populations even in small patches. Natural mosaics of forest patches in a grassland matrix in southern Brazil are good places to analyse such a process. Our objective was to analyse the effect of habitat patching on trophic relationships based on the evaluation of two forest amphibian species (*Physalaemus lisei* and *P. carrizorum*) and compare those from habitat patches *versus* core/contiguous habitat. We compared isotopic carbon ( $\delta^{13}\text{C}$ ) and nitrogen ratios ( $\delta^{15}\text{N}$ ) in two anuran species as well the variation in their diet at different forest patch sizes. We chose carbon isotopes since they act as a proxy for inferences on the original habitat from which the matter flows towards the anurans, while nitrogen reveals their trophic level. Our results revealed that trophic sources for these amphibians derives predominantly from primary forest productivity. However, *Physalaemus lisei*  $\delta^{13}\text{C}$  values indicated that the contribution of grassland trophic sources in the diet was higher in frogs from forests patches (<100 ha) than in those from core forest habitat. In smaller patches, *P. lisei* consumed predominantly ants, which was the most abundant prey on habitat. On the other hand, in the core forest, where isopods were the most abundant prey, most of its consumption was beetles, spiders and ants. In contrast, *P. carrizorum* showed no significant changes in isotopic ratios or diet across habitats. Isotopic data suggested that both species occupied lower trophic levels in smaller forests than in core forests. Our data showed a tendency towards the increase of grassland contribution and decrease of amphibian trophic position in patched habitats in comparison to continuous habitat.

**Key words:** anuran, isotopes, nutrient flow, productivity, trophic web.

## INTRODUCTION

The species richness and abundance that an ecosystem can shelter are directly related to habitat size (Hanski & Gilpin, 1997; Brook *et al.* 2000; Ewers & Didham 2006). Patchy habitats are more likely to experience interference coming from outside such as wind, quick temperature changes and biological invasions, which may impose restrictions on the survival of patch species (Saunders *et al.* 1991; Matte *et al.* 2015). Consequently, small patches may be more susceptible to diversity impoverishment processes, such as local extinctions (Lande 1993; Brook *et al.* 2002). At the same time, the landscape heterogeneity in a patched habitat improve the opportunity for colonisation and interchanges of individuals between

different patches increasing regional diversity (Dutra *et al.* 2009; Kwet *et al.* 2010). In addition, if the rise or maintenance of those patches are based on intermediate levels of disturbance, their local species diversity could be maximised (Catford *et al.* 2012). We must consider to how those processes operate in natural mosaic landscapes where patches are formed by natural processes and remain stable for decades. We could also inquire that mosaic configuration of habitat has effects that are not limited to species composition (diversity). In fact, they extend to different ecological aspects of a community, including its trophic relationships. Such effects are well known and widely studied in anthropogenically fragmented mosaic landscapes (Baker 1995; Fahrig 1997; Fahrig 2003; Becker *et al.* 2007; Ribeiro *et al.* 2009). However, little is known about the effect of habitat size in natural mosaic landscapes.

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Studies suggest that patch size affects trophic interactions among organisms in food webs (Valladares *et al.* 2006; Gray *et al.* 2007). Those interactions could be expressed in terms of changes in the number of ramifications and the number of trophic levels in food webs across patches of different sizes (Holt *et al.* 1999; May *et al.* 2007). In addition, the smaller a patch is, the more prone it is to incorporate nutrients from external habitats into its food webs, which is observed by the increasing contribution of nutrients from the matrix to trophic webs in smaller patches (Mackenzie *et al.* 2002). Nutrient (subsidies) flow has been extensively investigated between aquatic and terrestrial ecosystems. Such studies have used stable isotope analyses of producers and consumers to assess trophic relationships (Polis & Hurd 1996; Polis *et al.* 1997; Oliveira *et al.* 2014; Claudino *et al.* 2015). Mosaic landscapes offer an excellent opportunity to study nutrient exchanges between different habitats. However, studies based on stable isotope evaluation on terrestrial environments are still incipient, especially in the Neotropical region.

Naturally formed mosaic landscapes can be found on the southern edge of the Atlantic Forest (Fig. 1; Matte *et al.* 2015). In this region, forest patches are immersed in a high-altitude grassland matrix called the “Campos de Cima da Serra” (Matte *et al.* 2015). This landscape formation is the result of forest expansion into the grassland that has been occurring for at least 1000 years (Behling *et al.* 2004; Oliveira & Pillar, 2004; Matte *et al.* 2015).

Amphibians are excellent model organisms for the evaluation of trophic web in Campos de Cima da Serra habitats because they are locally abundant (Kwet *et al.* 2010) and act as both predators and potential prey to many other species (Duellman & Trueb 1986; Simon & Toft 1991). The role of amphibians as vectors of nutrients between habitats has already been documented in limnic/terrestrial systems (Huckembeck *et al.* 2014). Thus, amphibians are important links in the energy flow of food chains (Whiles *et al.* 2006).

Given that habitat size can influence both the capacity of communities to be trophically self-sufficient and nutrient flow from adjacent habitats, our objective was to compare the trophic relationships of two amphibian species in natural forest patches of different sizes with a core forest. All evaluated forests are dispersed in a grassland matrix. We intended to evaluate trophic relationships based on both carbon and nitrogen isotope ratios and gut content analysis.

Our working hypothesis is based on the idea that small forest patches will be more prone to incorporate nutrients from external habitats into their food webs. As a consequence, an increasing contribution of nutrients from the matrix to trophic/nutrient

networks will be observed in small patches than in core forest. As a result, we expected that: (i) at core forest, anurans will present greater contribution of carbon/nitrogen derived from the forest in their tissues; (ii) at forest patches, anurans will present a significant increase of the contribution of carbon/nitrogen derived from grassland in their tissues.

## MATERIAL AND METHODS

### Study site

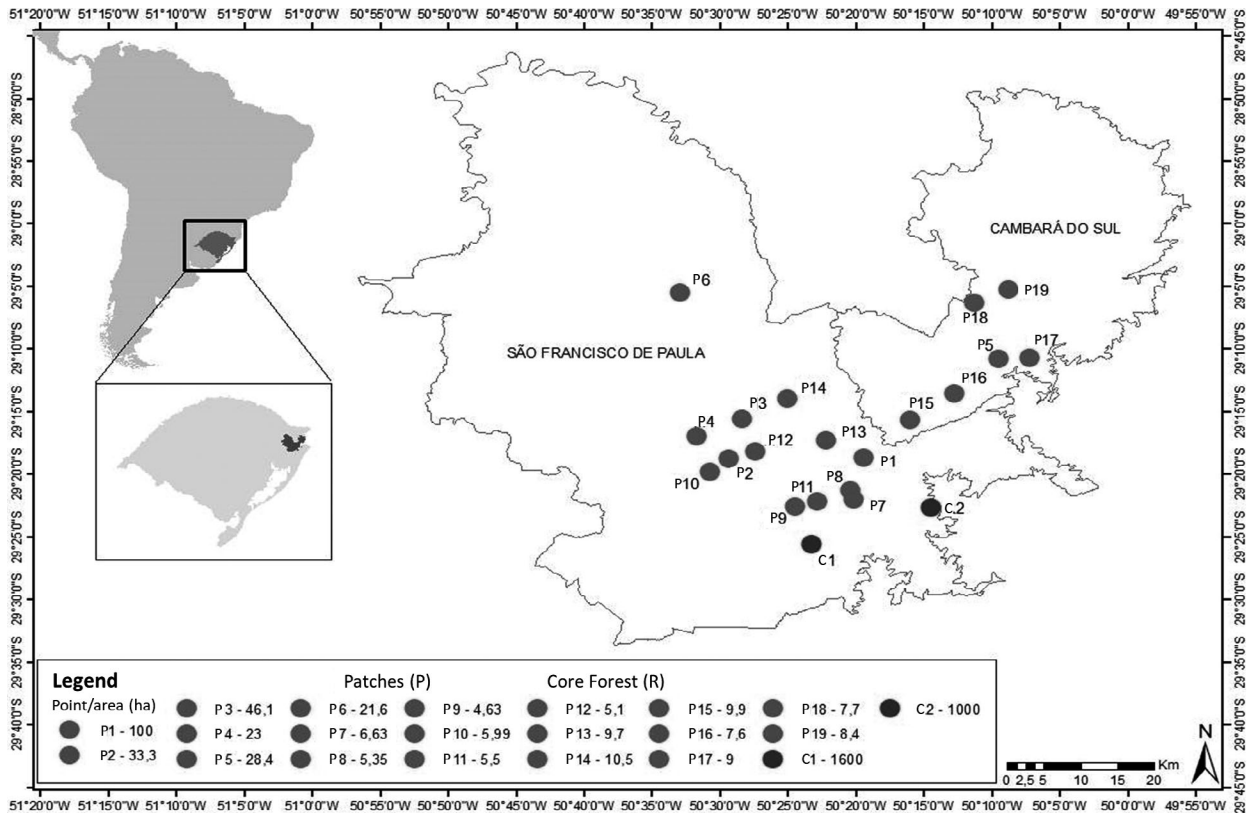
We conducted the study in forest areas of Brazil's extreme south between 28°46'S, 49°59'W and 29°28'S, 50°48'W, in the municipalities of São Francisco de Paula and Camará do Sul (Fig. 1). The area is within the Atlantic Forest domain and is formed by a mosaic landscape of natural grasslands (high altitude grasslands) associated with forest patches. The vegetation phytophysiology known as Mixed Ombrophilous Forest predominates in the sampled habitat, which is dominated by *Araucaria angustifolia* (Bertol.) Kuntze (Araucaria Forest). The region's climate is temperate, with an average annual temperature of 14.4°C and average annual rainfall of 2162 mm evenly distributed over the year (Maluf 2000).

We selected forest patches of different sizes (areas) as sample units (Fig. 1). Forest size was estimated based on recent satellite images obtained from Google Earth and on-site visits, so that all patches were located in a natural grassland matrix (no adjacent agriculture). The selected forest patches were, at least, two kilometres apart and showed well-defined boundaries with abrupt forest/grassland interface transitions. Due to this standardisation, we did not record a continuous variation among size classes. Therefore, selected sample units were grouped into two categories: patches (between 4 and 100 ha;  $N = 19$ ) and core forests (between 1000 and 1600 ha;  $N = 2$ ; Fig. 1). Core forests corresponded to the forests with the greatest continuous forest cover in the study area.

### Fieldwork and data collection

Fieldwork was conducted monthly (7 day/month) between September and December in 2014, 2015 and 2016, the most favourable period for collecting the species. Frogs were found through active search during the day. We used two amphibian species belonging to Leptodactylidae family as models to evaluate the trophic web in forest areas: *Physalaemus lisei* Braun and Braun 1977 and *Physalaemus carrizorum* Cardozo and Pereyra, 2018. The first species occurs exclusively in southern Brazilian forests (Kwet *et al.* 2010). *Physalaemus carrizorum* shows a larger distribution area than *P. lisei*, occurring from southeastern Brazil to Uruguay and Argentina (Langone 1994), in forests and rural habitats (Kwet *et al.* 2010). Its body size and diet are similar to that in *P. lisei* (Borges-Martins *et al.* 2007; Moser *et al.* 2017).

We choose *P. lisei* and *P. carrizorum* as target species because, based on parallel surveys, they are the most



**Fig 1.** Map of Mixed Ombrophilous Forest patches and Core Forest. The map shows the 21 Mixed Ombrophilous Forest points (sites) sampled, divided into two categories (core forests and patches).

abundant terrestrial frogs in patches and core forest. The sampling design focused on “target” species is a current approach in the isotopic evaluation of trophic ecology (Huckembeck *et al.* 2014). Those surveys were performed by systematised sampling effort during two years in which *P. lisei* corresponded to 70% of the anuran captures (Oliveira, unpubl. data, 2017). Furthermore, the species of this study are also suitable due to their small body size and low dispersion, reducing ability to move between forest patches and the grassland matrix.

#### Stable isotopes evaluation

We used carbon stable isotope analyses ( $\delta^{13}\text{C}$ ) to infer the nutritional contribution of the primary producers from different habitats (grassland or forest) to amphibians. We also used nitrogen isotopes ( $\delta^{15}\text{N}$ ) to assess the trophic level of amphibians. Primary producers that have been indirectly incorporated into amphibian tissues can be identified, despite the latter being carnivorous (Huckembeck *et al.* 2014). Carbon fixed by plants is incorporated into the food chain by herbivores (e.g. arthropods). Its subsequent transfer to other trophic levels (when anurans prey on arthropods) allows track and estimate the origin of carbon from tissue samples of secondary or higher trophic level consumers (Fry 2006). Carbon isotopic ratios vary according to the plant’s type of photosynthetic metabolism (e.g. C3 or C4; Martinelli *et al.* 2009). In our study area, forest

dominant plants have C3 photosynthetic systems and lower carbon isotopic ratios (average de  $\delta^{13}\text{C} = -28\text{‰}$ ), whereas grassland matrix dominant plants have C4 photosynthesis and higher isotopic ratios (average de  $\delta^{13}\text{C} = -12\text{‰}$ ; Oliveira, unpubl. data, 2017).

We obtained samples of forest and grassland matrix plants from leaf tissue. We sampled the most abundant plant species in terms of biomass in both forest and grassland. We collected *Araucaria angustifolia* (Brazilian pine), *Myrcia* sp. (Guamirim) and *Vriesea* sp. (bromeliad) as primary forest representatives while the Poaceae set (grasses) (Table 1) as the grassland representative. Grasses were identified only at the family level (Poaceae) due to their similar isotopic ratios. The collected plants were kept on ice until placed in a freezer in the laboratory.

Plants and amphibians were collected simultaneously in each sampling campaign. Regarding amphibians, specimens were collected by hand, anaesthetised with xylocaine in lethal doses for euthanasia and kept on ice in the same way as plants. Afterwards, this biological material was taken to the laboratory and kept frozen until processing. Collections were carried out under SISBIO #45861-1 and approved by the Ethics Committee #PPCEUA07.2016. The total number of individual are presented in supplementary material (Appendix S1).

At the laboratory, the biological material was first defrosted and washed in deionised water for residue removal. Tissue samples from leaves and amphibian

**Table 1.** Isotopic ratios

Basal sources / amphibians	Forest size categories			
	Core		Patches	
	$\delta^{13}\text{C} \pm \delta^{13}\text{CSD}$	$\delta^{15}\text{N} \pm \delta^{15}\text{NSD}$	$\delta^{13}\text{C} \pm \delta^{13}\text{CSD}$	$\delta^{15}\text{N} \pm \delta^{15}\text{NSD}$
Brazilian pine <i>Araucaria angustifolia</i>	$-28.24 \pm 0.50$	$-0.86 \pm 1.27$	$-28.94 \pm 0.64$	$2.23 \pm 1.11$
Guamirim	$-31.55 \pm 0.96$	$0.69 \pm 2.20$	$-31.58 \pm 0.88$	$2.22 \pm 1.49$
<i>Myrcia</i> sp.				
Bromeliad	$-27.04 \pm 0.12$	$-2.67 \pm 0.66$	$-28.30 \pm 0.63$	$-0.18 \pm 1.32$
<i>Vriesea</i> sp.				
Average forest sources	$-29.32 \pm 2.04$	$-0.60 \pm 2.01$	$-29.69 \pm 1.51$	$0.94 \pm 1.73$
Poaceae (grassland)	$-12.55 \pm 0.98$	$0.87 \pm 1.34$	$-12.55 \pm 0.98$	$0.87 \pm 1.34$
<i>Physalaemus lisei</i>	$-24.55 \pm 0.57$	$7.00 \pm 0.54$	$-23.52 \pm 1.05$	$6.43 \pm 1.14$
<i>Physalaemus carrizorum</i>	$-23.82 \pm 0.87$	$7.02 \pm 0.62$	$-23.92 \pm 0.77$	$6.75 \pm 0.55$

Means and standard deviations of isotopic ratios from samples of food chain basal source (primary producers) of forest and grassland and of two anurans species of a subtropical Atlantic forest in Brazil's extreme south.

femoral muscles were extracted. Each sample was washed with deionised water, individually placed in a petri dish and dried in an oven at 60°C for 48 h. Samples were individually ground using a mortar and pestle and stored in micro-centrifuge tubes. Sub-samples containing 1 to 2 mg were placed in ultra-pure tin capsules (Costech) and sent to the Isotopic Ecology Laboratory, University of São Paulo, Piracicaba, São Paulo, Brazil, for isotopic ratio determination ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ). The isotopic ratio of carbon ( $\delta^{13}\text{C}$ ) and nitrogen ( $\delta^{15}\text{N}$ ) in the samples was measured via mass spectrometry (Continuous-flow-Isotope Ratio Mass Spectrometry – CF-IRMS) using a Carlo Erba elemental analyser (CHN 1110) coupled to a Delta Plus mass spectrometer from Thermo Scientific. Isotopic ratio results were expressed in delta notation ( $\delta$ ) in which  $\delta^{13}\text{C}$  or  $\delta^{15}\text{N} = [(\text{R}_{\text{sample}} / \text{R}_{\text{standard}}) - 1] * 1000$ , and  $\text{R} = {}^{13}\text{C}/{}^{12}\text{C}$  or  ${}^{14}\text{N}/{}^{15}\text{N}$  in parts per thousand (‰). Pee Dee Belemnite (PDB) was used as the carbon standard material and atmospheric nitrogen as the nitrogen standard.

#### Diet evaluation

We assessed the amphibian diet through gastrointestinal content analysis. All individuals used in the isotopic analysis were also evaluated in terms of diet. Additional individuals (used only for diet description; *P. lisei* = 306; *P. carrizorum* = 218) underwent the same capture and euthanasia procedures described for stable isotope analysis. Amphibians used to access dietary information were kept in ice containers immediately after capture to decrease the activity of gastrointestinal content digestion. Specimens were dissected and gastrointestinal contents were removed and stored in 70% ethanol in vials. We screened the contents using a stereomicroscope. Food items were identified to order or family level (lower taxonomic level possible depending on the prey fragmentation degree), with the support of guides and taxonomic keys. After identification, we quantified categories by estimating their volume. The collected material was macerated and spread evenly across a Petri dish, maintaining a regular height of 1 mm (Moser

*et al.* 2020). Subsequently, the occupied area was assessed and used to calculate volume (Hellawell & Abel 1971). The gastrointestinal content of each animal was considered as an individual sample. For each food category, we calculated the number (*N*), volume (*V*) and frequency of occurrence (FO) in absolute and percentage terms.

We also estimated prey availability by sampling leaf litter. We collected leaf litter from amphibian plots, packed it in plastic bags, and sorted it on the same day. Screening was performed using the time-limited screening method. Invertebrates were placed in Falcon tubes containing 70% ethanol for later identification.

#### Data analysis

##### Stable isotopes

We assessed isotopic carbon and nitrogen ratios of 55 *Physalaemus lisei* (nine obtained from core forests and 46 from patches) and 15 *P. carrizorum* (five from core forests and 10 from patches). We tested normality of the isotopic dataset using the Shapiro–Wilk test. We verified the variance of the data through ANOVA when the data were normal. If the data did not reach normality even after logarithmic transformation or performing gamma distribution of data, we used the Mann–Whitney test (*U*) to compare pairs or Kruskal–Wallis test to compare groups.

We applied mixing models to estimate the relative contributions of basal sources (primary producers) from different origins (forest and grassland) to anurans. We used Stable Isotope Mixing Models package in R (SIMMR, Version 0.4.5), which uses a Bayesian statistical framework to estimate the parameters (Parnell 2016). A Bayesian approach allows the incorporation of uncertainty in trophic discrimination factors (TDF), sources and estimated mixtures (Parnell *et al.* 2010). We fitted these models using the Markov Chain Monte Carlo (MCMC) method, which generates simulations of source contributions to consumer tissue.

Trophic discrimination factors values represent the difference between isotopic values of the consumer and potential sources (Post 2002). Because there are no specific TDF estimates for the anurans evaluated in this study, we compiled different values used in studies with amphibians (Supplementary material, Appendix S1). As the pattern did not vary, in the results presented in the mixing models and other analyses that require TDF, we used average TDF values of  $1.3 \pm 0.30$  for  $\delta^{13}\text{C}$  and  $2.9 \pm 0.32$  for  $\delta^{15}\text{N}$  (McCutchan *et al.* 2003). We multiplied the TDF values by the average trophic position (TP) of each anuran species in order to take isotope fractionation along the food chain into account (Phillips *et al.* 2014).

To estimate anuran trophic positions (TP) and to identify possible variations in trophic chain levels between forest patches and core forests, we applied the following equation:  $\text{TP} = \lambda + (\delta^{15}\text{N}_{\text{consumer}} - \delta^{15}\text{N}_{\text{baseline}})/\text{TDF}$ .  $\lambda$  is the trophic level of the baseline source (Vander Zanden *et al.* 1997),  $\delta^{15}\text{N}_{\text{consumer}}$  is the nitrogen isotopic ratio of each consumer,  $\delta^{15}\text{N}_{\text{baseline}}$  is the mean  $\delta^{15}\text{N}$  of the baseline (i.e. primary producers from forest and grassland), and TDF is the trophic discrimination factor of nitrogen per trophic level (2.9‰; McCutchan *et al.* 2003). The difference between the trophic position occupied by each species was tested by ANOVA (Zar 1999).

The isotopic niche of each anuran species from core forests and patches were quantified from standard ellipse areas (SEA, expressed in ‰<sup>2</sup>) using the Stable Isotope Bayesian Ellipses calculated by SIBER (Jackson *et al.* 2011) in the SIAR package in R (Parnell *et al.* 2010). We corrected standard ellipse area (SEA<sub>C</sub>) for small sample sizes. SEA<sub>C</sub> is a bivariate measure of the distribution of individuals in trophic space, where each ellipse encloses 40% of the data regardless of sample size. We also calculated the Bayesian approximation of the standard ellipse area (SEA<sub>B</sub>). SEA<sub>C</sub> and SEA<sub>B</sub> calculation allowed us to measure the trophic niche and indicate the degree of niche overlap among species, reported as a percentage of each SEA<sub>C</sub> (Jackson *et al.* 2011, Jackson *et al.* 2012).

### Diet

We assessed the importance of each prey in the diet by calculating the Index of Relative Importance (IRI):  $\text{IRI} = (N + P) \text{FO}$ . The percentage of prey items that belonged to that prey was represented by  $N$ , while  $P$  is the prey volume percentage, and  $F$  indicates the prey occurrence frequency percentage (Pinkas *et al.* 1971; Krebs 1999). The higher the IRI value of a prey type, the greater its importance in the diet. The index was calculated in percentages to facilitate comparisons between groups.

We estimated the selectivity level of the diet by calculating the Jacobs Electivity Index ( $D$ ). This index evaluates the presence of each prey category in relation to its availability in the environment by means of the following formula:  $D = (R-P)/R + P-2$ . Rk.Pk (Jacobs 1974), where “k” is a given prey category and “R” and “P” are the proportion of this category in the diet and the environment, respectively. The  $D$  value varies from  $-1$  to  $+1$ , where positive values higher than  $0.2$  ( $D > 0.2$ ) indicate that a particular prey is selected by the anuran (preferred prey) (Hayward *et al.* 2011).

We defined trophic niche size by calculating the Standardized Levins Trophic Niche Amplitude Index ( $B_{\text{sta}}$ ) (Krebs 1999), which allows comparisons in food specialisation between species. The index is limited to a scale of 0 to 1 according to the following equation:  $B_{\text{sta}} = (B-1)/(n-1)$ , in which  $n$  represents the number of resources (prey types) recorded, and  $B = 1/\sum p_i^2$ , where  $p$  represents the individual proportion of a given resource  $i$  (prey type) found in the diet. Values near zero are assigned to specialised diets, while those closer to 1, to generalist diets.

## RESULTS

### Stable isotope

The range in  $\delta^{13}\text{C}$  values of basal sources was similar between the localities ( $-32.53\text{‰}$  to  $-11.88\text{‰}$  in core forests; and  $-33.11\text{‰}$  to  $-11.36\text{‰}$  in patches; Table 1). No significant difference was found in  $\delta^{13}\text{C}$  in the primary producers of forest origin (C3) between core forests and patches ( $U = 363.5$ ;  $P = 0.25$ ), neither for producers of grassland origin (C4) ( $U = 93.5$ ;  $P = 0.39$ ). In  $\delta^{15}\text{N}$  values, the range of basal sources was greater in core forests ( $-3.34\text{‰}$  to  $2.89\text{‰}$ ) than patches ( $-1.87\text{‰}$  to  $6.24\text{‰}$ ; Table 1). We found significant difference in  $\delta^{15}\text{N}$  in the primary producers of forest origin (C3) between core forests and patches ( $F_{1,73} = 24.82$ ;  $P < 0.01$ ). On the other hand, no significant difference was found for producers of grassland origin (C4) ( $F_{1,30} = 1.76$ ;  $P = 0.19$ ).

For anurans, the range in  $\delta^{13}\text{C}$  values in *P. carrizorum* was greater in patches ( $-25.63\text{‰}$  to  $-22.13\text{‰}$ ) than core forest ( $-24.74\text{‰}$  to  $-22.29\text{‰}$ ), without showing significant difference between both locality ( $F_{1,13} = 0.11$ ;  $P = 0.74$ ). In *P. lisei*, the range was even bigger in patches ( $-25.11\text{‰}$  to  $-18.58\text{‰}$ ) than in core forest ( $-25.32\text{‰}$  to  $-23.58\text{‰}$ ; Table 1), with a significant difference between both localities ( $U = 64$ ;  $P < 0.01$ ). The range in  $\delta^{15}\text{N}$  values in *P. carrizorum* was greater in patches ( $6.07\text{‰}$  to  $8.16\text{‰}$ ) than core forest ( $6.37\text{‰}$  to  $7.69\text{‰}$ ), without showing significant difference between both localities ( $F_{1,13} = 0.35$ ;  $P = 0.55$ ). In *P. lisei*, the range was even bigger in patches ( $3.84\text{‰}$  to  $9.33\text{‰}$ ) than in core forest ( $5.91\text{‰}$  to  $7.59\text{‰}$ ; Table 1), however, there was no significant difference ( $U = 114$ ;  $P = 0.15$ ). When analysing the existence of variance between both species in both localities, a significant difference was found only between *P. lisei* from core forests and patches ( $H = 11.74$ ;  $P < 0.01$ ).

In general, both species presented carbon values closer to basal source values of forests (C3 plants) than to those of grasslands (C4 plants; Table 1). This pattern was reinforced by mixing models, which also indicated

a greater contribution of forest sources than grassland sources to sustain both amphibian species, regardless of locality (forests or patches; Fig. 2 and 3). The percent contribution of forest sources to the diet of *P. lisei* was 94.1% in core forests and 83.9% in patches, while the percent contribution of grassland sources was, on average, 5.9% in core forest and 16.1% in patches (Fig. 2). For *P. carrizorum*, the contribution of forest sources was, on average, 90.3% in core forest and 87.7% in patches, while the contribution of grassland sources was, on average, 9.7% in core forest and 12.3% in patches (Fig. 3). Other results of the mixing models carried out with different TDF values are found in the supplementary material (Table S1).

Isotopic ellipses of both species showed lower amplitudes for individuals captured in core forests than for those from patches. This difference was more intense for *P. lisei* (core forest =  $1.04\%_{oo}^2$ ; patches =  $5.10\%_{oo}^2$ ) than for *P. carrizorum* ( $1.94\%_{oo}^2$  and  $2.32\%_{oo}^2$ , respectively). In addition, patch ellipses highly overlapped with those of core forests for *P. carrizorum*, whereas there was no overlap for *P. lisei* (Fig. 4).

We detected difference in the trophic position of frogs from forest patches versus core forest. Both species presented lower trophic positions in patches than core forests. The positions of *P. carrizorum* and *P. lisei* from core forests were the same, with average values of  $3.36 \pm 0.21$  and  $3.36 \pm 0.19$ , respectively. Trophic positions estimated in patches for *P. carrizorum*

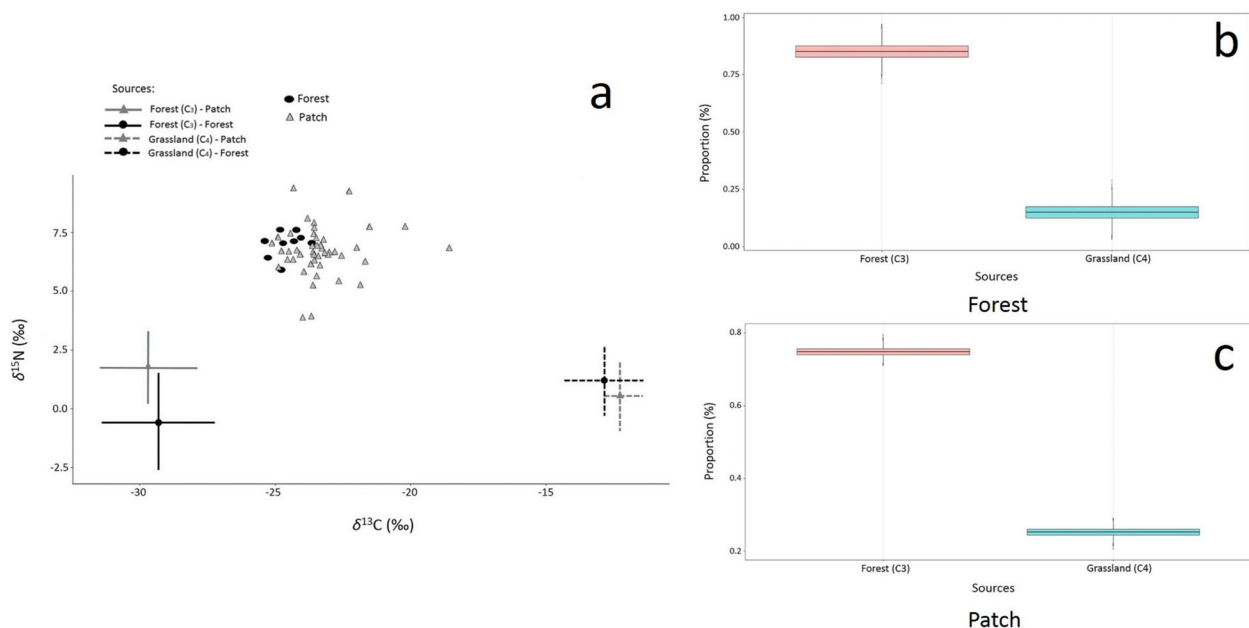
presented the average value of  $2.86 \pm 0.23$ , and for *P. lisei* presented  $2.79 \pm 0.36$ . The difference in trophic position for both species was significant according to locality (*P. carrizorum*:  $F_{1,13} = 17.61$ ;  $P < 0.01$ ; *P. lisei*:  $F_{1,53} = 21.13$ ;  $P < 0.01$ ).

### Prey consumption analysis

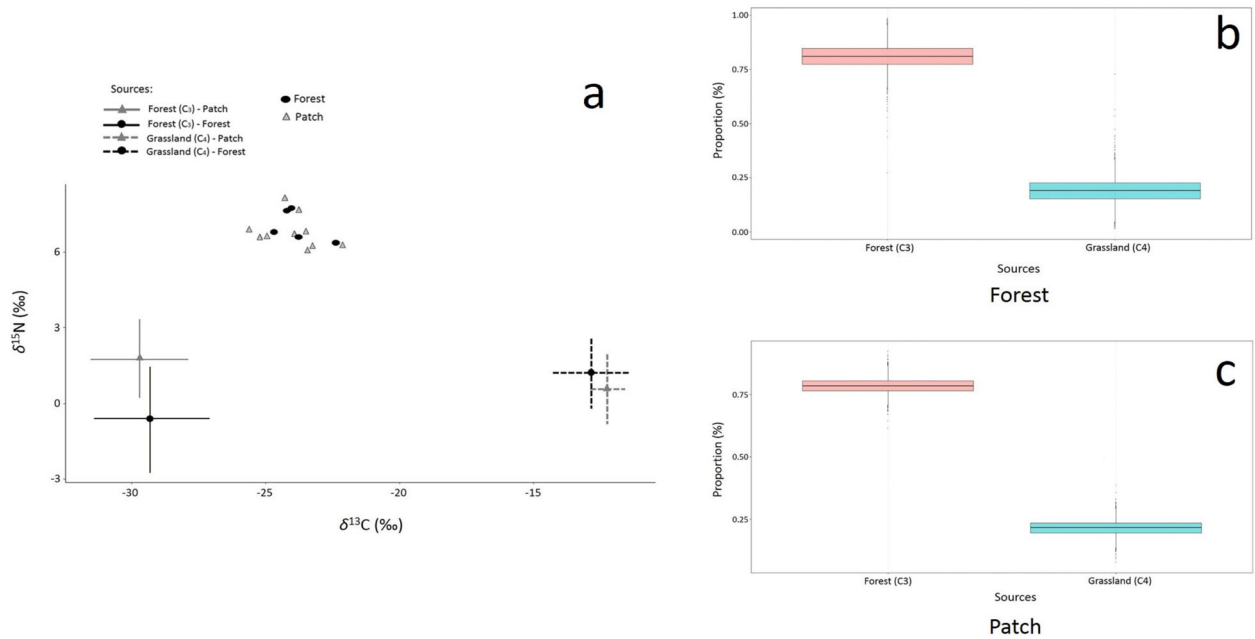
Only the diet of *P. lisei* showed differences in composition when comparing habitats. On patches, *P. lisei* consumed predominantly Formicidae (IRI% = 45.7), whereas in core forests, the relevance of this family was reduced (IRI% = 22.4), becoming similar to that of Coleoptera (IRI% = 25.4) and Araneae (IRI% = 24.4; Table 2). In contrast, *P. carrizorum* primarily consumed Formicidae both in patches (IRI% = 44.5) and core forests (IRI% = 52; Table 2).

Prey availability varied between habitats. The most abundant prey items in core forests were Isopods (N% = 41.8%), followed by Araneae (18.2%) and Formicidae (17.3%) (Table 2). In patches, the most abundant prey was Formicidae (41.1%), Isopoda (21.3%) and Araneae (13.6%) (Table 2). The Jacobs Electivity Index (D) indicated some level of prey selectivity (positive D values)

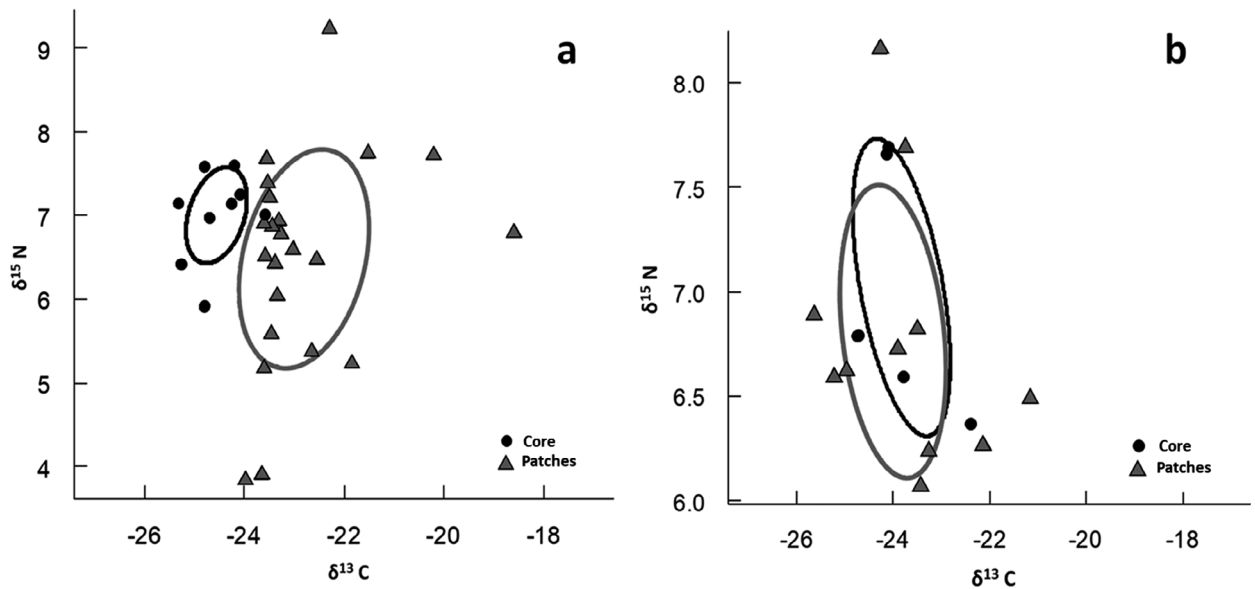
Both species, *P. lisei* and *P. carrizorum*, showed a preference for consuming Coleoptera in patches ( $D = 0.45$  and  $0.44$ , respectively) as well as in core



**Fig. 2.** (a) Individual  $\delta^{13}C$  and  $\delta^{15}N$  values for *Physalaemus lisei* according to habitat (black circle = forest; grey triangle = patch) and the mean ( $\pm$ SD) of primary sources from forest (C<sub>3</sub>) and grassland (C<sub>4</sub>) according to habitat (black circle = forest; grey triangle = patch). (b) Comparison of primary source contributions to anuran biomass in the forest and; (c) patch (95% credible interval values from the SIMMR Bayesian isotopic mixing model).



**Fig 3.** (a) Individual  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values for *Physalaemus carrizorum* according to habitat (black circle = forest; grey triangle = patch) and the mean ( $\pm\text{SD}$ ) of primary sources from forest ( $C_3$ ) and grassland ( $C_4$ ) according to habitat (black circle = forest; grey triangle = patch). (b) Comparison of primary source contributions to anuran biomass in the forest and; (c) patch (95% credible interval values from the SIMMR Bayesian isotopic mixing model).



**Fig 4.** Isotopic niche. Isotopic niche of two anuran species in different categories of forest patches. (a) *Physalaemus lisei*, black ellipse – forest patches ( $1.04 \text{‰}^2$ ); grey ellipse – forest patches ( $5.10 \text{‰}^2$ ); (b) *P. carrizorum*, black ellipse – core forests ( $1.94 \text{‰}^2$ ); grey ellipse – forest patch ( $2.32 \text{‰}^2$ ) (black circle = core; grey triangle = patch).

forests ( $D = 0.64, 0.63$ ).  $D$  values also showed preferential predation of ants by *P. carrizorum* exclusively in core forests ( $D = 0.52$ ).

The trophic niche amplitude for *P. lisei* was higher in core forests ( $B_{\text{sta}} = 0.28$ ) than in forest patches ( $B_{\text{sta}} = 0.16$ ). The pattern was more discrete for

**Table 2.** Index of relative importance (IRI), trophic niche amplitude and prey availability

Prey categories	Prey consumed (IRI%)				Prey availability (%)	
	<i>P. lisei</i>		<i>P. carrizorum</i>		Core	Patches
	Core ( <i>N</i> = 60)	Patches ( <i>N</i> = 301)	Core ( <i>N</i> = 33)	Patches ( <i>N</i> = 200)		
Coleoptera	25.4	8.9	21.9	9.1	4.5	3.4
Araneae	24.4	18.3	25.4	17.5	18.2	13.6
Formicidae	22.4	45.7	44.5	52	17.3	41.1
Isopoda	17.5	22.7	2.5	17	41.8	21.3
Hemiptera	5.8	0.2	0.5	0.5	0	0.3
Acari	1.2	0.8	1.2	0.3	0	1.9
Dermaptera	0.4	0.4	1.6	1	1.8	3.7
Mollusca	0.1	0.2	0.01	0.6	0	0.8
Hymenoptera	0.3	0.3	0.3	0.5	0.9	0.4
Lepidoptera-Larva	0.1	0.07	0.2	0.2	2.7	0.7
Diplopoda	1.5	0.5	0.4	0.002	0.9	0.9
Coleoptera-Larva	0.1	0.2	0.4	0.2	0	1.4
Chilopoda	0.2	0.6	0.03	0.03	0.9	1.3
Diptera	0.3	0.7	0.2	1.1	0	0.3
Arachnida	0	0.0008	0	0	0	1
Plecoptera	0.03	0.005	0.01	0	0	0.4
Ephemeroptera	0	0.001	0	0	0	0
Lepidoptera	0.04	0.02	0	0.007	0	0.1
Amphipoda	0.02	0	0.04	0	0	0
Isoptera	0.02	0	0	0	0	0
Blattodea	0.009	0.2	0.03	0.07	3.6	3.8
Opiliones	0	0.0003	0.02	0	0	0
Collembola	0	0.001	0	0.01	0	0
Escorpiones	0	0	0	0.003	0	0
Dermaptera-Larva	0.007	0.0003	0	0.04	0	0
Diptera-Larva	0.008	0.06	0.05	0.002	0.9	2
Odonata	0	0.001	0	0	0	0
Orthoptera	0.07	0.05	0.4	0.004	0	0
Neuroptera	0	0	0.03	0	0	0
B <sub>sta</sub>	0.28	0.16	0.18	0.16	-	-

Index of Relative Importance (IRI) of prey categories found in stomach contents and Levins Standardized Trophic Niche Amplitude (B<sub>sta</sub>) of *Physalaemus lisei* and *P. carrizorum*, as well as prey availability (%) in forests (core and patches) of a sub-tropical Atlantic forest in Brazil's extreme south.

*P. carrizorum* (core forests: B<sub>sta</sub> = 0.18; patches: B<sub>sta</sub> = 0.16; Table 2).

## DISCUSSION

Our results strongly suggest that anurans in small patches of forest are more prone to receive a trophic contribution to their diet from outside habitat (grassland matrix) than those that live in core forest. Forest basal source contribution was greater than grassland contribution to sustain both amphibian species in all sample units. The result agrees with the high primary productivity expected for forests, whose generated biomass would have a predominant role in supporting associated food webs (Melillo *et al.* 1993; Clark *et al.* 2001). However, grassland contribution to

tissue formation in both species, although inferior relative to forest contribution, reached values close to 40% of the total contribution. This indicates considerable representativeness of the grassland matrix in biodiversity maintenance in the ecosystem, as already mentioned by Matte *et al.* (2015). Comparing amphibian samples from core forest and patches, *Physalaemus lisei* presented a significantly higher carbon level enrichment in patches. This result suggests that patches receive a greater contribution from grassland, leading to a greater contribution to the trophic maintenance of *P. lisei* in smaller habitats (patches). The result agrees with our premise that, in forests with smaller areas, the participation of grassland sources in the food web sustenance tends to increase. However, the increase in the contribution of grassland sources was substantial only for *P. lisei*,



indicating that the two studied species respond differently to variations in habitat size, even though they are ecologically and phylogenetically close. We must address some attention to the effect of the use of a unique discrimination factors for both species. Discrimination factor introduces in the analysis the expected change in isotope values between prey and consumer (Post 2002). This values can vary according to the tissue and to the species evaluated. Unfortunately, we lack specific discrimination values for our species, as well as for any other Brazilian anuran species.

The species' isotopic niche reinforces the pattern of primary source contribution in response to habitat size. While the isotopic niche of *P. carrizorum* showed only a small variation, *P. lisei* showed a larger niche area in forest patches than in core forests. The trophic web of *P. lisei* may be supported by a more varied primary producer combination in forest patches due to an association of grassland and forest contribution. The result suggests that, in smaller habitats, the matrix may exert a greater influence on the maintenance of some species (Wilcove *et al.* 1986; Polis *et al.* 1997). However, trophic niche (estimated by diet) showed a narrower amplitude in forest patches for *P. lisei* than in core forests. Many studies argue that a narrow trophic niche results from specialised diets (Levins 1968; Pianka 1973; Da Rosa *et al.* 2002; Araújo *et al.* 2014; Oliveira *et al.* 2015). Other anuran species, despite having narrow niches, were considered generalist regarding their feeding behaviour (Duré *et al.* 2009; Batista *et al.* 2011; Oliveira *et al.* 2014). Thus, in this study, the expressive increase in ant availability in patches may have become advantageous enough for anurans to prioritise the consumption of ants, narrowing their trophic niche. Although the variation was lower for *P. carrizorum*, the explanation seems to be the same.

Many interspecific differences may be related to several aspects, such as the biology of both species (e.g. diet composition, foraging mode and sensitivity to habitats changes) and habitat variations (e.g. prey availability). Intra and interspecific isotopic variations have already been attributed to opportunistic feeding behaviour in response to prey availability (Gillespie 2013) and differences in species biology (Arribas *et al.* 2015). Nevertheless, other studies reveal that variations in the trophic niche are related to changes in prey availability and opportunistic behaviour, possibly due to optimal foraging (Oliveira 2014; Oliveira *et al.* 2015).

Although there is a gap in the knowledge of the spatial ecology of neotropical amphibians, species larger than *Physalaemus* show, in general, low mobility (Tozetti & Toledo 2005; Oliveira *et al.* 2016). Moreover, the grassland matrix is unfavourable for amphibian displacement due to the degree of

exposure to predators and desiccation risk (Rothermel & Semlitsch 2002; Lion *et al.* 2014). Thus, it is unlikely that anurans are moving into the grassland. Therefore, the input of grassland carbon in anuran tissues would depend on biological vectors (prey; Polis *et al.* 1997) moving in the grassland to forest direction.

Although both species feed on common and abundant prey in both habitats (Baretta 2007), we believe that the responses to nutrient inputs and niche variations may be in the species' diet. In fact, we observed a greater level of "prey selection" in *P. lisei* than in *P. carrizorum*. In addition, *P. carrizorum* seems to be more selective in core forests than in patches. We observed an expressive increase in ant availability in patches, but ants were preferentially consumed only by *P. carrizorum* in core forests (Table 2). We could assume that ants are the main vectors of grassland nutrients, leading to trophic niche reduction, isotopic niche enlargement, and an increase in grassland contribution to the trophic web of *P. lisei*. However, we are not comfortable to conducting a deep discussion about this observation since we did not evaluate the isotopic ratios of prey. At the same time, we encourage new studies evaluating the role of ants on anuran trophic webs. Ants are considered an abundant prey group with great displacement capacity (Baretta 2007). They are generally resistant to disturbances and have a high ability to colonise altered habitats (Mitchell *et al.* 2002; Dauber *et al.* 2006), which makes them capable of exploiting forest edges and even grassland resources. We must highlight that not only prey but also physical elements, such as water, could act as carbon vectors when transporting organic matter from the grassland to the forest (Polis *et al.* 1997). However, these elements would act similarly in all sampled units and, for this reason, we do not believe that they are responsible for the detected differences.

Patterns evidenced in *P. lisei* were not observed in *P. carrizorum*, possibly because the diet of the latter did not vary too much between habitats as it feeds considerably on ants in both patches and reference forests. Such observation is reinforced by the high isotopic ellipse overlap between patches and core forests exclusively for *P. carrizorum*. It is possible that species are responding differently due to different levels of specialisation and sensitivity to habitat changes (Vázquez & Simberloff 2002). In addition, a higher taxonomic resolution of consumed ants could reveal differences in consumed guild types.

Both species showed a decline in trophic position in forest patches compared to core forest, which suggests a possible reduction of a trophic web component (Holt *et al.* 1999; Fahrig 2003; Martinson & Fagan 2014). Some authors associate the event to a process of biological impoverishment of food webs

(Lovejoy *et al.* 1986; Camargo & Kapos 1995; Turner and Corlett, 1996; Vander Zanden *et al.* 1997). A species' trophic position can be altered by the addition or loss of other species occupying lower trophic levels (Anderson & Cabana 2007). Thus, the dominance of Formicidae in the anuran diet in forest patches should be a reasonable cause to justify the decline in the amphibian trophic position and the reduction of the trophic web components. This hypothesis makes sense only if consumed ants occupy lowers trophic levels. However, ant isotopic values vary considerably both within and among colonies even for a particular species (Tillberg *et al.* 2006).

The importance of different prey (IRI) varied among species and habitats. Despite the great importance of Coleoptera, Araneae, Formicidae and Isopoda, only Coleoptera and Formicidae presented any level of preferential consumption by anurans. Moreover, the order Araneae, which is composed of predatory arthropods, showed minor dietary importance in patches. Additionally, there was an increase in the importance of the order Isopoda (herbivores). In other words, both species decreased consumption of secondary consumers and began to feed more on primary consumers in forest patches, which may have contributed to their decrease in trophic position. Vander Zanden *et al.* (1997) also found variations in the trophic position of different populations of the same species in distinct sites due to an increase or loss in species number in the sites. Other evidence that the increase in the importance of Formicidae may explain the reduction of the anuran trophic position is the fact that ants show poorly enriched nitrogen isotopic ratios in general (Davidson *et al.* 2003). It is worth noting that patch producers were more enriched in nitrogen than those of core forests. The pattern may be related to nitrogen sources of anthropogenic origin, such as ashes from field burnings (Galloway *et al.* 2004). Nitrogen enrichment could generate imbalances in community composition and functionality (Vitousek *et al.* 1997; Frey *et al.* 2004).

Our results revealed that forest basal sources show a greater contribution to sustain both amphibian species rather than grassland sources. This reinforces that the high primary productivity of evaluated forests is pivotal for the maintenance of the anuran trophic web. This seem to be true even for small forests patches (smaller than 9 ha). This pointed out the key role of forest remnants in the maintenance of the trophic web in which the studied anurans participate. At the same time, the increased contribution of grasslands to the trophic web of small forests patches highlight the potential of interference of matrix over forest fragments food web. Our data also reinforce the strategy of estimate the incorporation of grassland-generated matter using the isotopic carbon signature of consumers. However, in some respects,

species responded differently to the examined variables. For *P. lisei*, there was an increase in the contribution of grassland sources to the trophic web at forest patches. Also, we recorded alterations in the species' niche amplitude, both in trophic and isotopic niches. Trophic niche amplitude was lower in forest patches, while isotopic niche, which was narrow in core forests, showed a considerable amplitude increase. Although the carbon variation was not evident for *P. carrizorum*, the result for *P. lisei* suggests that larger forests are more self-sufficient than small forest patches. However, grassland sources play a considerable role in sustaining food webs in forest formations. It is possible that ants are primarily responsible for the detected variation in isotopic ratios, acting as carbon vectors. Our data showed a tendency towards the increase in grassland contribution and decrease in amphibian trophic position as forest habitats reduce in area.

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## AUTHOR CONTRIBUTIONS

**Mateus Oliveira:** Conceptualization (equal); Data curation (lead); Formal analysis (lead); Investigation (lead); Project administration (lead); Writing-original draft (lead). **Camila Moser:** Data curation (equal); Investigation (equal); Methodology (equal); Writing-review & editing (equal). **Marluci Rebelato:** Formal analysis (equal); Writing-review & editing (equal). **Plinio B. Camargo:** Methodology (equal); Resources (equal); Software (equal). **alexandro tozetti:** Conceptualization (equal); Methodology (equal); Project administration (equal); Supervision (lead); Writing-review & editing (leads).

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## SUPPORTING INFORMATION

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**Appendix S1** Mean and standard deviation (SD) of estimated percent contributions of basal sources (primary producers) to anurans biomass using different values for the trophic discrimination factor (TDF). Results estimated by Bayesian isotopic mixing model using SIMMR. TDFs: (1) McCutchan *et al.* 2003; (2) Post, 2002; (3) Vanderklift and Ponsard, 2003; (4) Cloyd *et al.* 2015; (5) Sebastián *et al.* 2015.