

Trade-off between soluble protein production and nutritional storage in Bromeliaceae

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• **Background and Aims** Bromeliads are able to occupy some of the most nutrient-poor environments especially because they possess absorptive leaf trichomes, leaves organized in rosettes, distinct photosynthetic pathways [C_3 , Crassulacean acid metabolism (CAM) or facultative C_3 -CAM], and may present an epiphytic habit. The more derived features related to these traits are described for the Tillandsioideae subfamily. In this context, the aims of this study were to evaluate how terrestrial predators contribute to the nutrition and performance of bromeliad species, subfamilies and ecophysiological types, whether these species differ in their ecophysiological traits and whether the physiological outcomes are consistent among subfamilies and types (e.g. presence/absence of tank, soil/tank/atmosphere source of nutrients, trichomes/roots access to nutrients).

• **Methods** Isotopic (^{15}N -enriched predator faeces) and physiological methods (analyses of plant protein, amino acids, growth, leaf mass per area and total N incorporated) in greenhouse experiments were used to investigate the ecophysiological contrasts between Tillandsioideae and Bromelioideae, and among ecophysiological types when a predatory anuran contributes to their nutrition.

• **Key Results** It was observed that Bromelioideae had higher concentrations of soluble protein and only one species grew more (*Ananas bracteatus*), while Tillandsioideae showed higher concentrations of total amino acids, asparagine and did not grow. The ecophysiological types that showed similar protein contents also had similar growth. Additionally, an ordination analysis showed that the subfamilies and ecophysiological types were discrepant considering the results of the total nitrogen incorporated from predators, soluble protein and asparagine concentrations, relative growth rate and leaf mass per area.

• **Conclusions** Bromeliad subfamilies showed a trade-off between two strategies: Tillandsioideae stored nitrogen into amino acids possibly for transamination reactions during nutritional stress and did not grow, whereas Bromelioideae used nitrogen for soluble protein production for immediate utilization, possibly for fast growth. These results highlight that Bromeliaceae evolution may be directly associated with the ability to stock nutrients.

Key words: Bromelioideae, Tillandsioideae, ecophysiological types, trichomes, phytotelmata, amino acids, asparagine, nitrogen flux, stable isotopes.

INTRODUCTION

Bromeliaceae is considered a monophyletic family and comprises 58 genera and 3346 Neotropical species within eight subfamilies (Crayn *et al.*, 2004; Bremer *et al.*, 2009; Givnish *et al.*, 2011; Escobedo-Sarti *et al.*, 2013). Among flowering plants, Bromeliaceae are remarkable considering their vegetative features that allow them to occur in many habitats, such as tropical forests, dry savannas, rocky fields and semi-arid regions, from sea level to mountainous areas (Benzing, 1986, 2000; Crayn *et al.*, 2004; Gitaí *et al.*, 2005). Bromeliads are capable of occurring in resource-poor environments because they possess a number of traits that allow efficient uptake and use of water and nutrients, including foliar trichomes (i.e. epidermal cells that absorb water and nutrients) and the improvement of their absorptive capacity, the presence of

phytotelmata (i.e. tanks with water) and the diversification of carbon metabolism (Benzing and Burt, 1970; Benzing, 2000; Givnish *et al.*, 2014).

Bromeliad subfamilies (i.e. Brocchinioideae, Lindmanioideae, Hechtioideae, Navioideae, Pitcairnioideae, Puyoideae, Bromelioideae and Tillandsioideae) have contrasting growth forms, morphological and physiological features, and distinct flower morphology, features that may explain their different habitat preferences (Benzing and Burt, 1970; Givnish *et al.*, 2011). Benzing (2000) divided the family into five ecophysiological types according to their sources of nutrients (i.e. soil, tank or atmosphere) and their traits to access them (i.e. foliar trichomes and/or roots). Bromelioideae is a monophyletic subfamily with some exclusively terrestrial genera included in the ecophysiological type II (e.g. *Ananas* and *Bromelia*); they do not form phytotelmata, have non-specialized trichomes in

absorbing complex nitrogen compounds (e.g. amino acids) and bear well-developed roots responsible for water and nutrient acquisition from soil (Benzing and Burt, 1970; Benzing, 2000; Endres and Mercier, 2003). Moreover, other Bromelioideae genera are type III (e.g. *Aechmea*, *Neoregelia* and *Quesnelia*), are terrestrial or epiphytic, and have phytotelmata, specialized trichomes and roots responsible for fixing the plant into the substrate (Benzing and Burt, 1970; Benzing, 2000). Furthermore, the Tillandsioideae subfamily is monophyletic and shares the most derived morphological features of the family (Benzing et al., 1985; Benzing, 2000). All species of this subfamily are epiphytic and dependent on their leaves to absorb nutrients; some of them are type IV and have phytotelmata (e.g. *Vriesea*) while others are known as atmospheric epiphytes, included in the ecophysiological type V (e.g. some *Tillandsia*), which absorb nutrients from the atmosphere, representing the most derived response of Bromeliaceae to multiple environmental stresses (Benzing, 1986, 2000; Martin, 1994). Benzing (2000) described the ecophysiological types III and IV as a tank-absorbing trichome group with anchorage and conditionally absorptive roots; however, type II is predominantly CAM (i.e. performs Crassulacean acid metabolism) while type IV is C₃.

Epiphytic bromeliads obtain nutrients mostly from the deposition of canopy leaves, the atmosphere and interaction with animals. Since these nutrient sources are intermittent, epiphytic bromeliads may store them to use during nutritional stress conditions instead of using them for growth (Laube and Zotz, 2003). On the other hand, terrestrial bromeliads may utilize nutrients to grow as their roots are in constant contact with soil (Laube and Zotz, 2003). Indeed, Benzing (1983) reported that atmospheric bromeliads of the Tillandsioideae family did not grow with added fertilizer, suggesting that these plants have a slow-growing strategy, an adaptive response to oligotrophic environments (Aerts and Chapin, 2000). In more recent experiments, Endres and Mercier (2001a, b, 2003) showed that the epiphytic *Vriesea gigantea* (Tillandsioideae) presented higher levels of total free amino acids on leaves and accumulated asparagine after the addition of ammonium compared with the terrestrial tankless *Ananas comosus* (Bromelioideae). In contrast, the addition of this nutrient had little effect on the amino acid profile of *A. comosus*. The authors commented that the production of amino acids, especially asparagine, might be associated with temporary nutrient storage. Furthermore, these studies suggest differences in the allocation of nutrients into amino acids or growth between epiphytes and terrestrial bromeliads, but we still do not know whether this mechanism is consistent among other species from the two subfamilies or among bromeliad ecophysiological types (see Benzing, 2000).

Bromeliad leaves are organized in rosettes that allow the development of terrestrial and aquatic ecosystems (e.g. in tank bromeliads) where many organisms can live and drive bromeliad functioning (Benzing, 2000; Ngai and Srivastava, 2006; Romero et al., 2006, 2010; Gonçalves et al., 2014). These organisms are usually divided into functional groups, such as micro-organisms that mineralize organic compounds retained by bromeliad rosettes, detritivores (e.g. Chironomidae and Scirtidae) that are responsible for debris decomposition, and aquatic (e.g. Zygoptera and Tabanidae) and terrestrial

predators (e.g. spiders and anurans) that can control bromeliad food chains (Romero et al., 2006, 2010; Romero and Srivastava, 2010). Additionally, aquatic and terrestrial predators can directly improve bromeliad nutrition through their faeces, prey carcasses and other detritus from their activities (Benzing, 2000; Ngai and Srivastava, 2006; Romero et al., 2006, 2010; Gonçalves et al., 2011, 2014, 2016; Leroy et al., 2016). However, preliminary evidence suggests that the contribution of terrestrial predators to the mineral nutrition of bromeliads can vary among different subfamilies and plant life forms. For example, bromeliads with developed trichomes and with poorly developed root systems are more dependent on leaves to obtain water and nutrients, and those with epiphytic habit and/or with a tank may derive more nutrients from terrestrial predators (Benzing, 2000; Gonçalves et al., 2011; Leroy et al., 2016).

Many species of predatory anurans can use bromeliads temporarily or may depend on these plants for their survival and reproduction in tropical forests (Silva et al., 1989; Endres and Mercier, 2001a, b; Romero et al., 2010). Faeces of anurans and other predators are rich in nitrogen (i.e. urea and guanina; Lehninger et al., 1993; Duellman and Trueb, 1994; Romero et al., 2006) and can be an important nutrient source for bromeliad species in nutrient-poor habitats. Distinct morphological and physiological features among bromeliads can also affect the relative contribution of animal-derived nutrient sources to bromeliad nutrition. Although all Tillandsioideae species were described to have the more derived traits within bromeliads (i.e. developed trichomes, dependence on leaves to obtain water and nutrients, a poorly developed root system, epiphytism and amino acid storage), to the best of our knowledge there are no controlled experiments showing that these traits benefit Tillandsioideae more than other bromeliad species. In this study, we conducted an experiment using isotopic and physiological methods to evaluate (1) how predators (i.e. the anuran *Dendropsophus nanus*) contribute to the nutrition and performance of several Bromelioideae and Tillandsioideae species of distinct ecophysiological types; (2) whether these subfamilies and types differ in ecophysiological traits; and (3) whether the physiological outcomes are consistent among species within each subfamily and type.

MATERIALS AND METHODS

Organisms and experiment design

The Bromelioideae species *Ananas bracteatus*, *Quesnelia arvensis*, *Aechmea blanchetiana* and *Neoregelia cruenta* (all CAM), and the Tillandsioideae species *Vriesea gigantea*, *Vriesea bituminosa* and *Tillandsia cyanea* (all C₃) were chosen to evaluate how terrestrial predators contribute to the nutrition and physiological attributes of bromeliads with distinct ecophysiological traits. *Ananas bracteatus*, an ecophysiological type II, is a terrestrial tankless species that occurs in Colombia, Brazil, Paraguay and Argentina (Benzing, 2000). *Quesnelia arvensis*, *Aechmea blanchetiana* and *Neoregelia cruenta* are type III, are terrestrial or epiphytic, have phytotelmata and are found in restingas and outcrops of Brazil (Benzing, 2000; Romero and Vasconcellos-Neto, 2004; Romero, 2006). *Vriesea gigantea* and *V. bituminosa* are type IV, are epiphytic, have

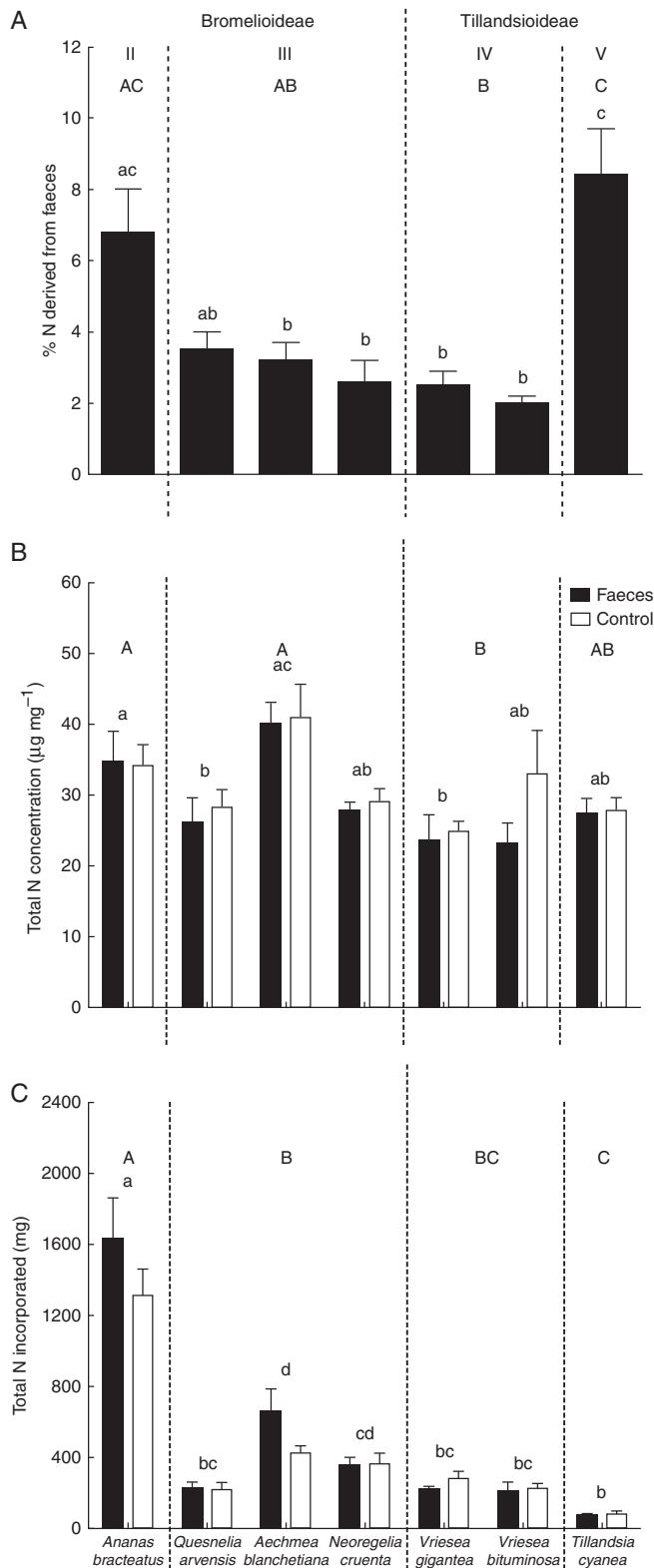


FIG. 1. (A) The percentage of nitrogen in the Bromelioideae species *Ananas bracteatus* (ecophysiological type II), *Quesnelia arvensis*, *Aechmea blanchetiana* and *Neoregelia cruenta* (type III), and in the Tillandsioideae species *Vriesea gigantea* and *Vriesea bituminosa* (type IV) and *Tillandsia cyanea* (type V) derived from *Dendropsophus nanus* faeces. Values were obtained from the two-source mixing models equations (see the Materials and Methods for details).

phytotelmata and occur in Brazil, but *V. bituminosa* also occurs in Venezuela (Benzing, 2000; Endres and Mercier, 2001a, b; Romero, 2006). *Tillandsia cyanea* is type V, and an epiphytic tankless species that occur in coastal rainforests of Ecuador (Gilmartin, 1977; Benzing, 2000).

All bromeliad species were bought at Veiga Arquitetura e Paisagismo® (CEASA, Campinas, São Paulo state, Brazil) and were similar in number of leaves and size to minimize the effects of N dilution [number of leaves between 11 and 36 units, mean \pm s.e.: 22.4 ± 0.7 leaves among species, one-way analysis of variance (ANOVA) of number of leaves among species: $P = 0.098$; the longest leaf length between 10 and 15 cm, mean \pm s.e.: 12.6 ± 0.1 cm of leaf length, one-way ANOVA of leaf length among species: $P = 0.155$]. Bromeliads were grown in a homogeneous soil mixture of a bark of *Pinus* sp., vermiculite and peat. To test the contribution of predatory anurans to the nutrition and performance of bromeliad subfamilies and types, we performed a greenhouse experiment from February to April 2009, lasting 70 d, at the campus of UNESP University in São José do Rio Preto (São Paulo state, Brazil). All plants were kept in a net greenhouse (1 mm mesh diameter) and were watered with a limited amount of water with an automatic irrigation system by a fine spray using three sprinklers, each with a release of 8 L h^{-1} that was activated for 15 min every 2 h, just enough to avoid excessive desiccation. The greenhouse net impeded the contact of bromeliads with any metazoans as we did not observe any organism in bromeliads throughout the experiment.

The experiment had two treatments that were applied to each species of bromeliad: (1) ^{15}N -labelled anuran faeces ($n = 6$ bromeliads) and (2) no faeces (control; $n = 6$). Control and treatment bromeliads were kept in the same greenhouse, but placed in separate stands 2 m apart. The anuran *Dendropsophus nanus* (Hylidae) is a small predator and was used as a model organism. Fifty *D. nanus* males were collected in the field (Nova Itapirema, São Paulo state) and kept in glass flasks of approx. 7 cm diameter and 10 cm in height in the laboratory at UNESP. Each anuran was fed every 2 d with 20 ^{15}N -enriched fruit flies (*Drosophila melanogaster*), an interval sufficient for the anuran to capture all the flies and produce faeces (mean \pm s.e. of dry weight of faeces: $97 \pm 15 \text{ mg}$). At 2 d intervals, anuran faeces were stored in polypropylene tubes for subsequent application to experimental bromeliads every 2 d (35 applications). The ^{15}N -enriched anuran faeces were carefully applied with tweezers to the central part of the rosettes at the base of the leaves. The flies were cultured from eggs in a medium of ^{15}N -labelled yeast. Details of the laboratory procedure for yeast and fly enrichment can be found in Romero *et al.* (2006).

Isotopic analyses

Two of the youngest leaves of each bromeliad rosette were randomly collected at the end of the experiment for isotopic

(B) Values of total nitrogen concentration of each species after treatments with predator faeces and controls. (C) Total nitrogen incorporated by leaves of each bromeliad species after the two treatments. Bars indicate the s.e.m. Different lower case letters indicate statistical differences among species, and upper case letters indicate statistical differences among ecophysiological types (ANOVA/Tukey HSD post-hoc test, $\alpha = 0.05$).

TABLE 1. Average $\delta^{15}\text{N}$ values of *Drosophila melanogaster* flies, *Dendropsophus nanus anuranus* and their enriched faeces, and values of leaves of *Ananas bracteatus*, *Quesnelia arvensis*, *Aechmea blanchetiana*, *Neoregelia cruenta*, *Vriesea gigantea*, *Vriesea bituminosa* and *Tillandsia cyanea* that received anuran faeces and controls

Treatment	$\delta^{15}\text{N}$ (s.e.m.)	<i>n</i>
<i>Drosophila melanogaster</i>		
Natural abundance	3.07 (0.19)	2
Enriched	1530.51 (76.95)	5
Anuran (adult male)		
Natural abundance	12.64 (0.37)	2
Enriched	542.04 (82.58)	5
Anuran faeces		
Natural abundance	8.97 (0.21)	3
Enriched	1046.47 (50.10)	5
<i>A. bracteatus</i>		
Faeces	70.43 (11.79)	6
Control	10.68 (0.31)	6
<i>Q. arvensis</i>		
Faeces	52.64 (4.62)	6
Control	8.33 (0.31)	6
<i>A. blanchetiana</i>		
Faeces	46.42 (4.63)	6
Control	11.56 (0.26)	6
<i>N. cruenta</i>		
Faeces	46.59 (5.72)	6
Control	9.21 (0.31)	6
<i>V. gigantea</i>		
Faeces	38.86 (3.85)	6
Control	9.38 (0.44)	6
<i>V. bituminosa</i>		
Faeces	30.33 (1.39)	6
Control	8.75 (0.38)	6
<i>T. cyanea</i>		
Faeces	84.57 (11.98)	6
Control	9.02 (0.34)	6

n, number of replicates.

analyses. The leaves were homogenized together and were dried at 60 °C for 48 h, crushed to obtain a fine powder and stored dry in polypropylene tubes until analysis. The total N concentration ($\mu\text{g mg}^{-1}$ dry leaf tissue) of bromeliad leaves and the $\delta^{15}\text{N}$ of flies, faeces and bromeliads were determined with an isotope ratio mass spectrometer (20–20 mass spectrometer; PDZ Europa, Sandbach, UK) after sample combustion to N_2 at 1000 °C by an on-line elemental analyser (PDZ Europa ANCA-GSL) in the Stable Isotope Facility at the University of California, Davis. The nitrogen fraction in bromeliads that received faeces (f_A) was calculated using mixing model equations with two sources of nitrogen (i.e. soil and faeces) and one single isotopic signature (e.g. $\delta^{15}\text{N}$; see Phillips and Gregg, 2001). The ^{15}N fractioning during its assimilation and the metabolic process of plants was calculated according to the equation of McCutchan *et al.* (2003): $f_A = (\delta_M - \delta_B - \Delta\delta^{15}\text{N}) / (\delta_A - \delta_B)$. The f_A is the proportionate contribution of labelled faeces absorbed by bromeliads (%), δ_M is the isotope ratio of bromeliads that received faeces, δ_A and δ_B are the isotope ratios of potential nitrogen sources (faeces and soil, respectively) and $\Delta\delta^{15}\text{N}$ is the trophic shift for nitrogen between diet (e.g. faeces or soil) and consumer (e.g. bromeliads). The values of $\Delta\delta^{15}\text{N}$ used were $+3.3 \pm 0.26$ [(mean \pm s.e.); Caut *et al.*, 2009].

Analyses of plant protein, total amino acids and asparagine

To determine if the nitrogen derived from predators had some influence on soluble protein concentration, two leaves from the intermediate part of each bromeliad rosette were randomly collected, were cut together into small pieces (1 cm²), and 1 g of these leaves was frozen in liquid nitrogen and homogenized with 3 mL of ultra-pure water. The homogenate was centrifuged at 12 000 rpm (g) for 10 min, and the supernatant (15 μL) was used to measure the protein concentration (Bradford, 1976). The absorbance was measured using a spectrophotometer at 595 nm, and a standard curve was obtained with bovine serum albumin. Total amino acid and asparagine concentrations were analysed by reverse phase HPLC with *o*-phthalaldehyde (OPA) derivatives as described previously in Puiatti and Sodek (1999).

Bromeliad growth, leaf mass per area and total N incorporated

To determine if the nitrogen derived from predators affected bromeliad growth, two leaves from the intermediate part of each bromeliad rosette but different from those used in physiological analysis were randomly labelled and their lengths were measured individually at the beginning and end of the experiment. The bromeliad leaf length was directly related to the leaf biomass (linear regressions: *A. bracteatus*, $r^2 = 0.69$, $P < 0.001$; *N. cruenta*, $r^2 = 0.68$, $P < 0.001$; *Q. arvensis*, $r^2 = 0.73$, $P < 0.001$; *A. blanchetiana*, $r^2 = 0.59$, $P = 0.006$; *V. bituminosa*, $r^2 = 0.75$, $P < 0.001$; *V. gigantea*, $r^2 = 0.68$, $P < 0.001$; *T. cyanea*, $r^2 = 0.69$, $P = 0.003$). Bromeliad leaves showed continuous growth during the experiment and their relative growth rate [RGR; $\ln(\text{cm}) \text{d}^{-1}$] was calculated using the following equation: $\text{RGR} = [\ln(L_{\text{final}}) - \ln(L_{\text{initial}})] / (t_2 - t_1)$. The $\ln(L_{\text{final}})$ and $\ln(L_{\text{initial}})$ are, respectively, the natural logarithm of the foliar final length and the natural logarithm of the foliar initial length, with $t_2 - t_1$ being the number on days between the initial and final measurements.

To estimate the bromeliad leaf dry mass per area (LMA, g m^{-2}), three leaves of the fourth inner node from each bromeliad rosette of the control treatment (different from leaves used for physiological analyses) had their central portion cut into 5 cm² pieces, dried at 60 °C for 48 h and the weight of each piece was obtained individually. LMA was taken only from control bromeliads to avoid the measurement of an enhancement of leaf mass when bromeliads received ^{15}N -enriched anuran faeces. For each experimental bromeliad, the total N incorporated during the experiment was calculated by multiplying the total nitrogen concentration of bromeliad leaves with their RGR and their LMA. This measure shows the allocation of N in the construction of leaf structures, either to increase the weight per unit of leaf area.

Statistical analyses

Comparisons of total N concentration, total N incorporated, concentrations of soluble protein, amino acids and asparagine, RGR and LMA between subfamilies and among ecophysiological types were performed using linear mixed models [LME; subfamilies or ecophysiological types and treatments (faeces

TABLE 2. Linear mixed models (LME) summarizing the effects of treatments (*Dendropsophus nanus faeces* and control) on the total nitrogen concentration, total nitrogen incorporated, soluble protein, total amino acids and asparagine concentrations, relative growth rate of leaves and leaf mass per area (LMA) of Bromeliaceae subfamilies and ecophysiological types (*Bromelioideae*, type II, *Ananas bracteatus*; type III, *Quesnelia arvensis*, *Aechmea blanchetiana* and *Neoregelia cruenta*; *Tillandsioideae*, type IV, *Vriesea gigantea* and *Vriesea bituminosa*; type V, *Tillandsia cyanea*)

Source of variation	Treatments			Subfamilies			Treatments vs. subfamilies		
	d.f.	F	P	d.f.	F	P	d.f.	F	P
Total N concentration	75	1.42	0.235	5	3.44	0.122	75	0.71	0.399
Total N incorporated	61	0.27	0.603	5	2.51	0.173	61	4.14	0.046
Soluble protein	74	10.08	0.002	5	0.11	0.748	74	12.13	<0.001
Total amino acids	75	151.16	<0.001	5	41.71	0.001	75	189.16	<0.001
Asparagine	75	65.01	<0.001	5	96.70	<0.001	75	87.05	<0.001
Relative growth rate	75	6.88	0.010	5	2.74	0.158	75	2.62	0.109
LMA	–	–	–	5	1.37	0.293	–	–	–

	Treatments			Types			Treatments vs. types		
	d.f.	F	P	d.f.	F	P	d.f.	F	P
Total N concentration	73	1.06	0.305	3	1.34	0.407	73	0.45	0.715
Total N incorporated	59	0.88	0.350	3	31.40	0.009	59	1.47	0.229
Soluble protein	73	9.16	0.003	3	5.31	0.101	73	22.7	<0.001
Total amino acids	73	186.7	<0.001	3	37.5	0.007	73	84.8	<0.001
Asparagine	73	63.4	<0.001	3	49.2	0.004	73	28.4	<0.001
Relative growth rate	73	17.4	<0.001	3	23.9	0.013	73	1.4	0.229
LMA	–	–	–	3	16.9	0.021	–	–	–

addition) were fixed effects and bromeliad species were a random effect]. We checked the normality and heteroscedasticity of residuals with standard diagnostic plots and metrics. Comparisons among bromeliad species were performed using nested ANOVA (species nested within subfamilies or ecophysiological types, [Supplementary Data Tables S1 and S2](#)) and Tukey HSD post-hoc tests were used for pair-wise comparisons. Statistical differences between treatments within each bromeliad species were performed using the *t*-test. The percentage of nitrogen in leaves of bromeliad species derived from predator faeces was compared using ANOVA and Tukey HSD post-hoc tests for pair-wise comparisons. We performed a *t*-test to compare the percentage of nitrogen derived from faeces between the two subfamilies and ANOVA to compare the percentage of nitrogen derived from faeces among ecophysiological types. Non-metric multidimensional scaling (NMDS) plot ordination was constructed considering the results of the total nitrogen incorporated, soluble protein and asparagine concentrations, RGR and LMA of leaves of each bromeliad species after receiving faeces in order to show the discrepancy between subfamilies and ecophysiological types. All statistical analyses were conducted with the statistical platform R (R Core Team, 2014).

RESULTS

The amount of nitrogen derived from anuran faeces did not differ between subfamilies (d.f. = 2, *t* = 0.22, *P* = 0.845), but was variable among bromeliad species ($F_{6,41} = 10.07$, *P* < 0.001; [Fig. 1A](#); [Table S1](#)) and bromeliad ecophysiological types ($F_{3,23} = 8.64$, *P* < 0.001; [Fig. 1A](#); [Table S2](#)). *Tillandsia cyanea* (type V, mean ± s.e., 8.4 ± 1.3 %) and *Ananas bracteatus* (type II, 6.8 ± 1.2 %) derived a similar amount of nitrogen from predators and were the species that

derived more nitrogen ([Table 1](#); [Fig. 1A](#)). *Quesnelia arvensis* (type III, 3.5 ± 0.5 %), *Aechmea blanchetiana* (type III, 3.2 ± 0.5 %), *Neoregelia cruenta* (type III, 2.6 ± 0.6 %), *Vriesea gigantea* (type IV, 2.5 ± 0.4 %) and *Vriesea bituminosa* (type IV, 2 ± 0.2 %) derived a similar amount of nitrogen from predators, but those from the ecophysiological type IV differed from those from type V, while type III was similar to type II ([Table 1](#); [Fig. 1A](#)). Despite predators having contributed to bromeliad nutrition, total nitrogen concentration and total nitrogen incorporated by bromeliad leaves did not differ between the subfamilies (total N concentration, $F_{1,75} = 3.44$, *P* = 0.122; N incorporated, $F_{1,61} = 2.53$, *P* = 0.172; [Table 2](#); [Fig. 1B, C](#)). The ecophysiological types showed a similar total nitrogen concentration, but differed in the total nitrogen incorporated (total N concentration, $F_{3,73} = 1.34$, *P* = 0.407; N incorporated, $F_{3,59} = 31.40$, *P* = 0.009; [Table 2](#), [Fig. 1B, C](#)). Nevertheless, comparisons among species and ecophysiological types revealed that *A. bracteatus* (type II) incorporated the greatest amount of nitrogen, while *T. cyanea* (type V) incorporated the lowest amount ([Fig. 1C](#); [Tables S1 and S2](#)). The addition of faeces did not alter the total N concentration or the N incorporated within each species ([Fig. 1B, C](#); [Table S1](#)).

Soluble protein concentration on leaves did not differ between subfamilies or ecophysiological types (subfamilies, $F_{1,74} = 0.11$, *P* = 0.748; types, $F_{3,73} = 5.31$, *P* = 0.101) especially because types III and V showed similar soluble protein concentration ([Table 2](#); [Fig. 2A](#)). *Ananas bracteatus*, *Q. arvensis* and *A. blanchetiana* (Bromelioideae) had more soluble protein after receiving predator faeces than Tillandsioideae species (treatments, $F_{1,74} = 10.08$, *P* = 0.002; subfamilies vs. treatments, $F_{1,74} = 12.13$, *P* < 0.001; [Table 2](#); [Fig. 2A](#); [Table S1](#)). Species also differed in their soluble protein content as *A. bracteatus* and *V. bituminosa* had the highest concentrations while *N.*

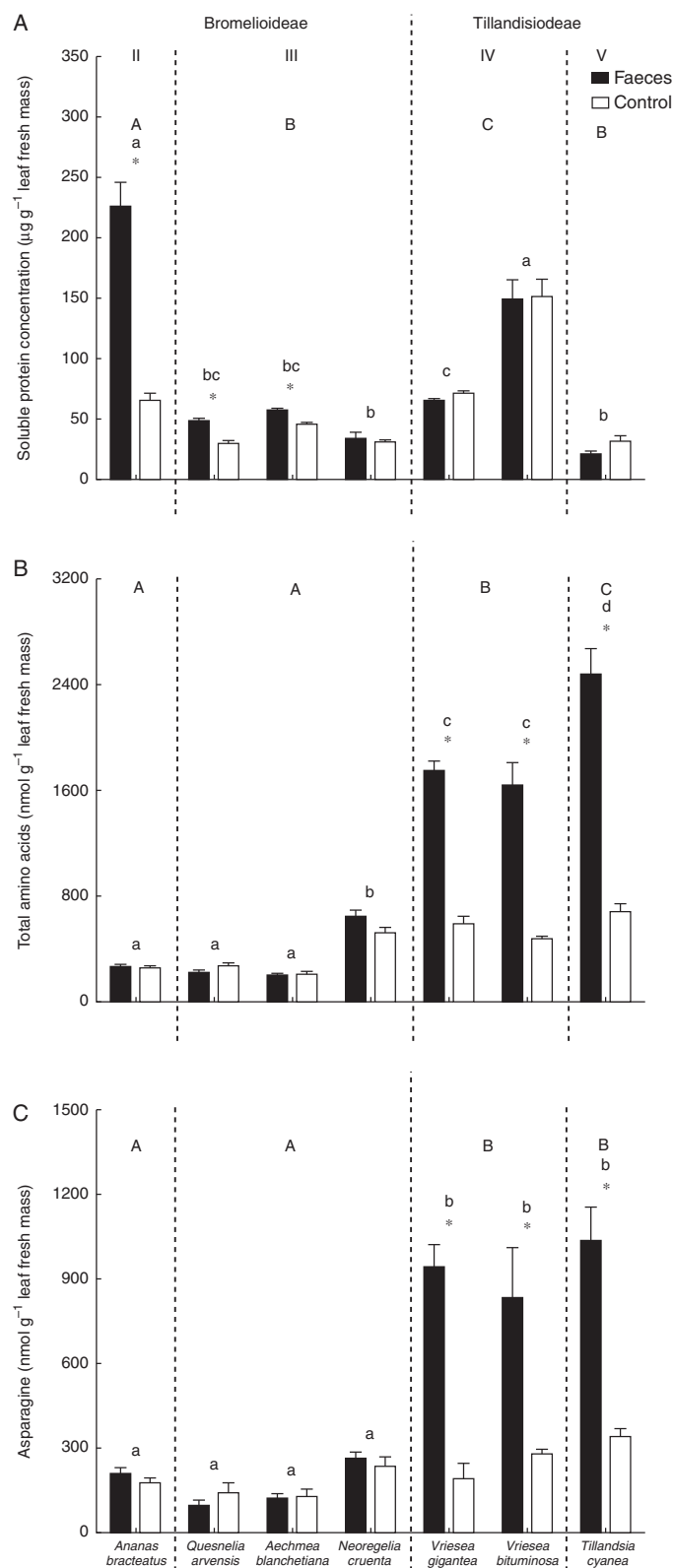


Fig. 2. (A) Soluble protein concentration for the Bromelioideae species *Ananas bracteatus* (ecophysiological type II), *Quesnelia arvensis*, *Aechmea blanchetiana* and *Neoregelia cruenta* (type III), and in the Tillandsioideae species *Vriesea gigantea* and *Vriesea bituminosa* (type IV) and *Tillandsia cyanea* (type V) after treatments with *Dendropsophus nanus* faeces and controls. (B) Total amino acid concentration for each species of bromeliads after the two

cruenta and *T. cyanea* had the lowest concentrations (Fig. 2A; Table S1). On the other hand, total amino acid and asparagine concentrations were higher in Tillandsioideae if compared with Bromelioideae (amino acids, $F_{1,75} = 41.71$, $P = 0.001$; asparagine, $F_{1,75} = 96.70$, $P < 0.001$), thus they were high in the ecophysiological types IV and V (Table 2; Fig. 2B, C). Despite the fact that types IV and V had a similar amount of asparagine, type V showed more amino acid content than type IV (Fig. 2B, C). Additionally, only Tillandsioideae species had higher contents of amino acids and asparagine after receiving predator faeces (Fig. 2B, C; Table S1).

The RGR did not differ between subfamilies, but differed among ecophysiological types (subfamilies, $F_{1,75} = 2.74$, $P = 0.158$; types, $F_{3,73} = 23.9$, $P = 0.013$; Table 2; Fig. 3). However, types III and V showed a similar RGR (Fig. 3). *Ananas bracteatus* grew more than other species and was the only species that had a higher growth rate after receiving predator faeces (treatment, $F_{1,75} = 6.88$, $P = 0.010$; Fig. 3; Table S1). The LMA did not differ between subfamilies, but differed among ecophysiological types (subfamilies, $F_{1,28} = 1.37$, $P = 0.293$; types, $F_{3,28} = 16.9$, $P = 0.021$; Table 2; Fig. 4). The tankless species *T. cyanea* (type V) and *A. bracteatus* (type II) had the highest LMA, while tank species from types III and IV had the lowest leaf mass per area (Fig. 4; Table S1). The NMDS ordination plot reinforces that Bromelioideae and Tillandsioideae subfamilies and the ecophysiological types are discrepant in the use and allocation of nitrogen (Fig. 5).

DISCUSSION

Our results indicate a trade-off between soluble protein production and nutritional storage in Bromeliaceae, as the bromeliad subfamilies and ecophysiological types showed contrasting strategies in nitrogen use. Apparently, Tillandsioideae species have the strategy to store nutrients, and this may be an evolutionary trait selected to live in oligotrophic environments. Furthermore, Bromelioideae species seem to produce proteins and may use them for growth since their developed roots may access nutrients more easily. We observed that the ecophysiological types with similar soluble protein production showed similar growth. In addition, one of the features that stood out in bromeliads was the presence or absence of the tank, as species without a tank derived more nitrogen from predators and showed the highest leaf biomass.

Bromelioideae had increased the concentration of soluble proteins after receiving predator faeces, and only one Bromelioideae species grew more (i.e. *Ananas bracteatus*). In contrast, Tillandsioideae showed high concentrations of amino acids and asparagine but did not grow after receiving faeces. Apparently, a trade-off in protein production or accumulating nitrogen may exist among Bromeliaceae subfamilies: while

treatments. (C) Total asparagine concentration for each species of bromeliads after the two treatments. Bars indicate the s.e.m. Asterisks (*) indicate statistical differences between treatments within each bromeliad species (*t*-test, $\alpha = 0.05$), different lower case letters indicate statistical differences among species, and upper case letters indicate statistical differences among ecophysiological types (ANOVA/Tukey HSD post-hoc test, $\alpha = 0.05$).

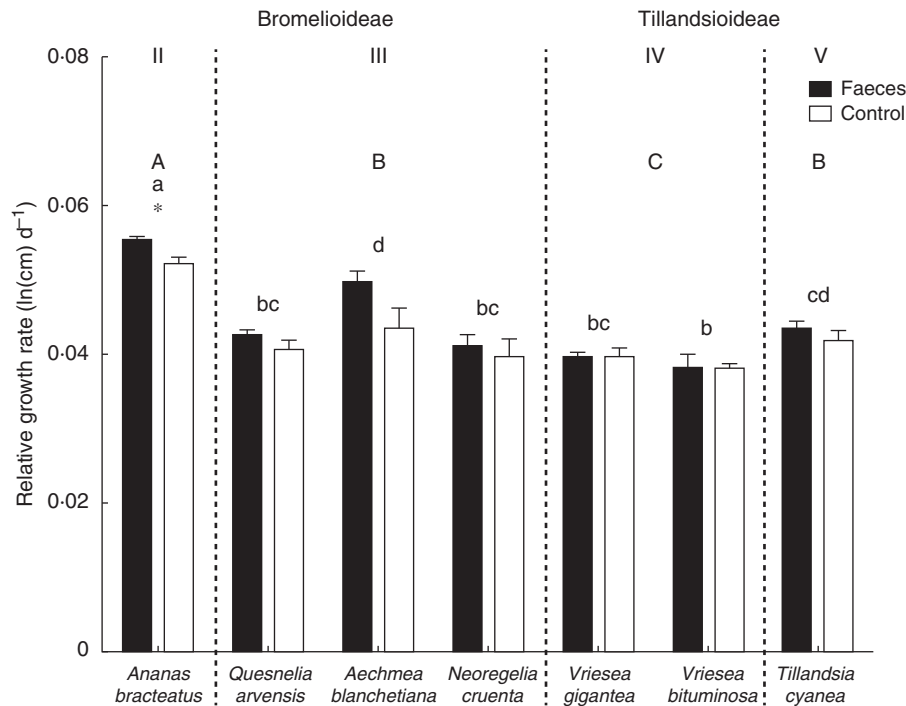


FIG. 3. Relative growth rate of the leaves of the Bromelioideae species *Ananas bracteatus* (ecophysiological type II), *Quesnelia arvensis*, *Aechmea blanchetiana* and *Neoregelia cruenta* (type III), and in the Tillandsioideae species *Vriesea gigantea* and *Vriesea bituminosa* (type IV) and *Tillandsia cyanea* (type V) after treatments with *Dendropsophus nanus* faeces and controls. Asterisks (*) indicate statistical differences between treatments within each bromeliad species (*t*-test, $\alpha = 0.05$), different lower case letters indicate statistical differences among species, and upper case letters indicate statistical differences among ecophysiological types (ANOVA/Tukey HSD post-hoc test, $\alpha = 0.05$).

Bromelioideae species allocate nitrogen to soluble proteins that may be converted into growth but have a lower ability to store nitrogen, Tillandsioideae species accumulate nitrogen in amino acids, probably keeping the nitrogen stored under conditions of nutritional stress. Soluble proteins are a source of nitrogen circulating on the leaves of bromeliads that can be used at any time, while asparagine appears to be a sink of nitrogen that can be converted into other amino acids by transamination reactions (Endres and Mercier, 2001a, b, 2003). Additionally, we observed that the ecophysiological types that showed similar soluble protein content also had similar growth (i.e. types III and V). Other studies showed a similar pattern between these two subfamilies, as a Bromelioideae species (i.e. *Ananas comosus*) had a lower concentration of asparagine in leaves while a Tillandsioideae species (i.e. *Vriesea gigantea*) accumulated this amino acid (Endres and Mercier, 2003). In addition, Romero *et al.* (2010) showed that *V. bituminosa* (Tillandsioideae) may have accumulated amino acids after receiving frog faeces since its soluble protein concentration decreased by a half. Nitrogen storage appears to be an adaptive response of Tillandsioideae to survive in oligotrophic environments and should be even more intense in atmospheric epiphytes since these plants depend on nutrients that are in low concentrations in the atmosphere (Benzing, 2000).

Bromeliad leaves are considered the most essential vegetative organs due to their ability to absorb nutrients through trichomes (Benzing, 2000). This ability was particularly important to *T. cyanea* and *A. bracteatus*, since they derived more nitrogen from predators through their leaves if compared

with other species. These species have no phytotelmata and, therefore, have no external reservoir to accumulate nutrients and water. Thus, since nutrients come into contact with trichomes of tankless species, the mechanism of efficient nutrient acquisition by leaves could have been selected during Bromeliaceae evolution to avoid nutrient losses. On the other hand, regardless of the subfamily and ecophysiological types, bromeliads with a tank obtained less nitrogen from predators than species without phytotelmata, possibly because of the dilution of nutrients by the water and the occurrence of a diversity of micro-organisms on phytotelmata. The tank water may dilute nutrients that should be concentrated at the base of leaves, where there is a larger amount of trichomes (Takahashi *et al.*, 2007). Thus, water reduces the contact of nutrients with the base of leaves, increasing nutrient contact with apical parts where the number of trichomes is gradually replaced by stomata. Additionally, bromeliads with phytotelmata might possess a more favourable environment for the occurrence of a greater diversity of micro-organisms, algae and metazoans compared with bromeliads without a tank. In fact, numerous micro-organisms can live in the bromeliad phyllosphere, where mineralization provides nutrients that can be absorbed easily by trichomes (Inselsbacher *et al.*, 2007; Gonçalves *et al.*, 2014). These micro-organisms and algae can immobilize amino acids and/or ammonium and compete for nutrients with bromeliads (Benzing, 2000; Inselsbacher *et al.*, 2007). Additionally, some insects can immobilize nutrients from the tank in their bodies and take them out of the bromeliad system when they emerge

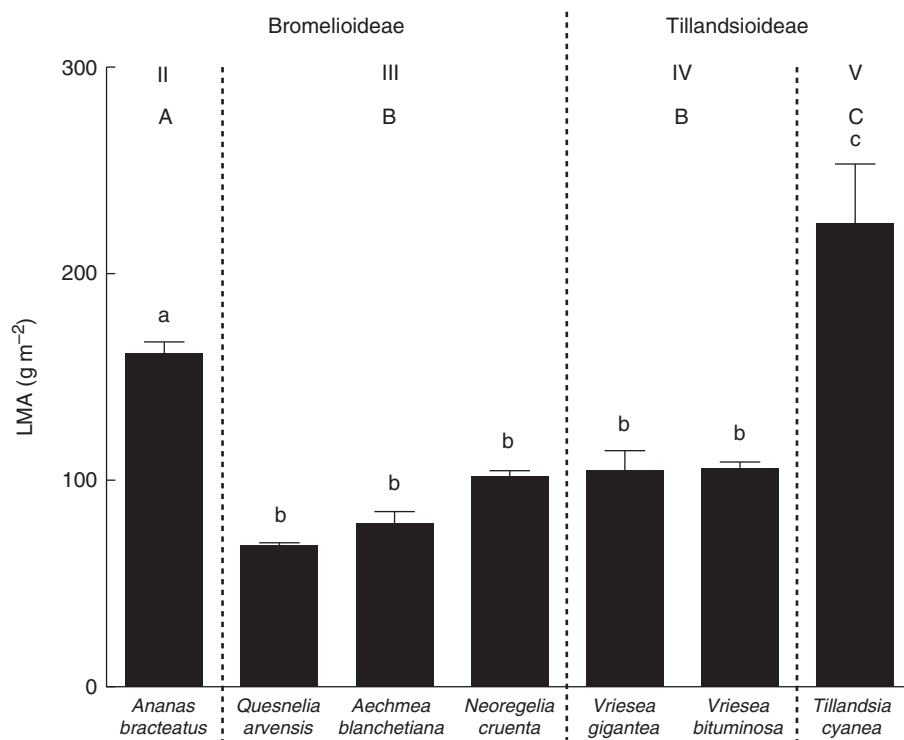


FIG. 4. Leaf mass per area (LMA) of the leaves of the Bromelioideae species *Ananas bracteatus* (ecophysiological type II), *Quesnelia arvensis*, *Aechmea blanchetiana* and *Neoregelia cruenta* (type III), and in the Tillandsioideae species *Vriesea gigantea* and *Vriesea bituminosa* (type IV) and *Tillandsia cyanea* (type V). Different lower case letters indicate statistical differences among species, and upper case letters indicate statistical differences among ecophysiological types (ANOVA/Tukey HSD post-hoc test, $\alpha = 0.05$).

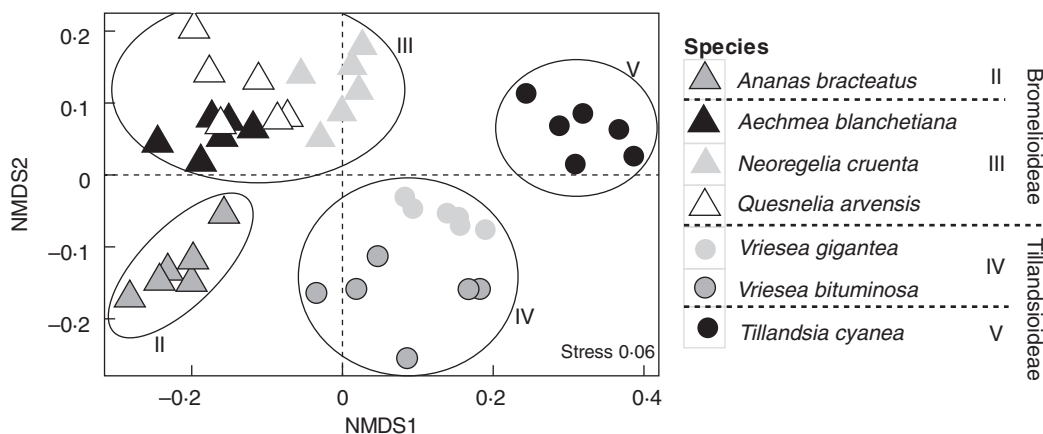


FIG. 5. Non-metric multidimensional scaling (NMDS) plot derived from the total nitrogen incorporated, soluble protein and asparagine concentrations, relative growth rate and leaf mass per area of leaves of each bromeliad species after receiving *Dendropsophus nanus* faeces. Triangles indicate Bromelioideae and circles indicate the Tillandsioideae subfamily. Roman letters indicate the ecophysiological types of each bromeliad species (see the Materials and Methods for details).

as adults (Ngai and Srivastava, 2006). Therefore, bromeliads with phytotelmata may be competing more with microorganisms, algae and some metazoans compared with *T. cyanea* and *A. bracteatus*.

Tillandsia cyanea derived 20 % more nitrogen from predators than *A. bracteatus*, possibly because Tillandsioideae species, especially that from ecophysiological type V, possess more developed trichomes and in a greater abundance with

numerous mitochondria that assist them during the absorption of nutrients (Sakai and Sanford, 1980; Benzing, 1986, 2000). Leaf nutrient uptake is significant for *T. cyanea* because it occupies habitats with scarce rainfall periods where water and nutrients must be absorbed quickly and in a greater amount when available (Benzing et al., 1976). In contrast, *A. bracteatus* is in contact with soil nutrients and incorporated the largest amount of nitrogen among the species tested, which possibly

allowed its greatest growth. These results may be related to the greater amount of nitrogen that *A. bracteatus* derived from predators, the allocation of this nutrient in the production of soluble proteins, and its use in vegetative development. On the other hand, *T. cyanea* have obtained as much nitrogen from predators as *A. bracteatus*; however, *T. cyanea* showed lower growth and accumulated asparagine as did other species of Tillandsioideae.

The two tankless bromeliads, *T. cyanea* and *A. bracteatus*, also showed the highest values of LMA. This result may be linked to the allocation of nutrients to the construction of leaf tissues since building leaves with high LMA requires a greater investment (Wright *et al.*, 2004). However, it is possible that these species have shown a greater LMA for different reasons, i.e. while *T. cyanea* must have more sclerenchyma tissues to increase its leaf life span, *A. bracteatus* should have more chlorenchyma, allowing a greater photosynthetic activity and growth (d'Eeckenbrugge and Leal, 2002). Future studies may further investigate the differences between photosynthetic and mechanical tissues in bromeliads with similar LMA but with distinct ecophysiological strategies. High values of LMA allow continuous operation of the functions performed by leaves and are associated with long leaf life span, extent and abundance of air spaces and water-storing tissues (i.e. hydrenchyma) (Rascio *et al.*, 1990; Witkowski and Lamont, 1991; Wright *et al.*, 2002, 2004; Freschi *et al.*, 2010). Since the species with high LMA did not have phytotelmata and species with low LMA had a tank with water, high LMA may be related to water storage. Thus, we can observe two distinct strategies concerning water storage that are clearly unrelated to the bromeliad subfamilies or ecophysiological types: those bromeliads that may store water in the hydrenchyma with high LMA, and those that store water outside the leaves (i.e. in phytotelmata) with low LMA.

In this study, we demonstrate that predators contribute to the nutrition of bromeliads, as already reported by Romero *et al.* (2006, 2010) and Gonçalves *et al.* (2011, 2014, 2016). Since many species of bromeliads live in oligotrophic environments such as rocky outcrops, sandy soils and canopy forests, these plants need to obtain nutrients from sources other than soil. Here, we showed that species of distinct subfamilies and types possess contrasting resource-use strategies, as they showed distinct ecophysiological plasticity. So far, CAM metabolism and the trichomes have dominated discussions about the evolution of Bromeliaceae, in which CAM allowed the evolution of epiphytism, for example, and epiphytism allowed speciation (Givnish *et al.*, 2014; Donoghue and Sanderson, 2015). In turn, this study highlights the exceptional influence of storage of amino acids for bromeliad occurrence in nutrient-poor environments, which may have favoured its radiation into a wide range of environments. The metabolic pathways associated with amino acid production and transamination, and the presence of a tank may be the important directions for future studies on Bromeliaceae evolution.

SUPPLEMENTARY DATA

Supplementary data are available online at ww.aob.oxfordjournals.org and consist of the following. Table S1: ANOVA of the percentage of nitrogen in bromeliad leaves derived from

Dendropsophus nanus faeces, and nested ANOVA (bromeliad species nested within subfamilies) summarizing the effects of treatments (faeces and control) on the total nitrogen concentration, total nitrogen incorporated, soluble protein, total amino acids and asparagine concentrations, relative growth rate of leaves and leaf mass per area of *Ananas bracteatus*, *Quesnelia arvensis*, *Aechmea blanchetiana*, *Neoregelia cruenta* (Bromelioideae) and *Vriesea gigantea*, *Vriesea bituminosa*, *Tillandsia cyanea* (Tillandsioideae). Table S2: ANOVA of the percentage of nitrogen in bromeliad leaves derived from *Dendropsophus nanus* faeces, and nested ANOVA (bromeliad species nested within ecophysiological types) summarizing the effects of treatments (faeces and control) on the total nitrogen concentration, total nitrogen incorporated, soluble protein, total amino acids and asparagine concentrations, relative growth rate of leaves and leaf mass per area of *Ananas bracteatus* (ecological type II), *Quesnelia arvensis* (type III), *Aechmea blanchetiana* (type III), *Neoregelia cruenta* (type III), *Vriesea gigantea* (type IV), *Vriesea bituminosa* (type IV) and *Tillandsia cyanea* (type V).

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