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ANA ZANGIROLAME GONÇALVES

CONTRIBUIÇÃO DE ANIMAIS PARA A ECOLOGIA NUTRICIONAL DE BROMÉLIAS: TESTES COM ISÓTOPOS ESTÁVEIS DE ^{15}N E RESPOSTAS FISIOLÓGICAS

Dissertação apresentada ao Instituto de Biologia para obtenção do Título de Mestre em Ecologia.

Orientador: Prof. Dr. Gustavo Quevedo Romero

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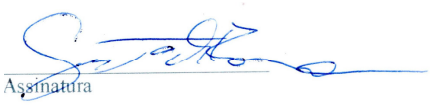

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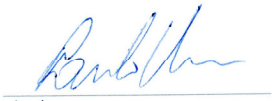

Assinatura

Prof. Dr. Luciano Freschi

Assinatura

Prof. Dr. João Vasconcellos-Neto

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SUMÁRIO

RESUMO

Inúmeros organismos vivem associados a plantas da família Bromeliaceae e rejeitos derivados desses organismos (e.g., fezes e detritos vegetais) podem contribuir para a nutrição das bromélias. A aranha *Psecas chapoda* (Salticidae) habita *Bromelia balansae*, *Ananas comosus* e *Aechmea distichantha* (Bromelioideae) em uma grande extensão geográfica. Dependendo da estrutura da roseta e dos tricomas absorventes, estas bromélias podem absorver mais ou menos nitrogênio derivado da aranha. A obtenção de nitrogênio pode variar de acordo com o período do ano (e.g., seco vs. chuvoso) e também devido à presença de microorganismos associados às folhas das bromélias. No presente estudo utilizamos métodos isotópicos e fisiológicos para responder as seguintes questões: (1) a aranha contribui para a nutrição e crescimento de *B. balansae*, *An. comosus* e *Ae. distichantha*? (2) quais respostas fisiológicas (i.e., concentrações de clorofilas, carotenóides e proteínas solúveis) as plantas apresentam por ganharem nitrogênio derivado da aranha? (3) existe variação sazonal na absorção de nitrogênio proveniente das aranhas? (4) as bactérias associadas à filosfera de *B. balansae* facilitam a absorção de nutrientes por estas plantas? Nossos resultados mostraram que *P. chapoda* favorece nutricionalmente suas três bromélias hospedeiras. Entretanto, nossos resultados indicam que o mutualismo entre aranhas e bromélias é sazonalmente restrito gerando resultados condicionais. A variação interespecífica na obtenção de nitrogênio ocorreu provavelmente devido às diferentes performances e rotas fotossintéticas de cada espécie. Enquanto *B. balansae* parece utilizar nitrogênio para crescimento, *Ae. distichantha* aparentemente acumula nitrogênio para condições de estresse nutricional. Adicionalmente, mostramos que plantas com densidade natural de bactérias acumularam 57% mais proteínas solúveis e cresceram 13% mais do que as bromélias que tiveram a abundância de bactérias

reduzidas com antibióticos. Estes resultados sugerem pela primeira vez que bactérias aceleram a ciclagem de nutrientes na filosfera e podem favorecer nutricionalmente estas plantas.

ABSTRACT

Many organisms live associated with Bromeliaceae plants and materials derived from these organisms (e.g., faeces and plant debris) may contribute to bromeliad nutrition. The spider *Psecas chapoda* (Salticidae) lives in *Bromelia balansae*, *Ananas comosus* and *Aechmea distichantha* (Bromelioideae) in a large geographic extent. Depending on the structure of the rosette and trichomes, these bromeliads may absorb more or less nitrogen derived from spiders. The acquisition of nitrogen may vary according to the seasons (e.g., dry vs. wet) and also due to the presence of microorganisms associated with bromeliad leaves. In this study we used physiological and isotopic methods to answer the following questions: (1) Do spiders contribute to the nutrition and growth of *B. balansae*, *An. comosus* and *Ae. distichantha*? (2) Which physiological responses (i.e., chlorophylls, carotenoids and soluble protein concentrations) the plants have by receiving nitrogen from spiders? (3) Is there seasonal variation in the absorption of nitrogen from spiders? (4) Do bacteria associated with *B. balansae* phyllosphere facilitate nutrient absorption by these plants? Our results showed that *P. chapoda* nutritionally improve their three host plants. However, our results indicate that this mutualism is seasonally restricted generating conditional outcomes. The interespecific variation in nitrogen acquisition occurred probably due to different performances and photosynthetic routes of each plant species. While *B. balansae* appear to use nitrogen for growth, *Ae. distichantha* apparently accumulate nitrogen for nutritional stress conditions. Additionally, we showed that plants with natural density of bacteria accumulated 57% more soluble proteins and grew 13% more than bromeliads that had their abundance of bacteria reduced with antibiotics. These results suggest for the first time that bacteria accelerate nutrient cycling in the phyllosphere and may nutritionally favor these plants.

1. INTRODUÇÃO GERAL

Bromeliaceae: características morfo-fisiológicas e evolução

As impressionantes formas funcionais e ecológicas das mais de 2800 espécies de Bromeliaceae são favorecidas pelas suas folhas justapostas em forma de roseta e pela presença de tricomas foliares (Benzing, 2000). As rosetas podem ou não acumular nutrientes e água das chuvas, no primeiro caso formando o fitotelmata (i.e., tanque), e os tricomas podem ter diferentes graus de desenvolvimento (Sakai & Sanford, 1980; Benzing, 1986, 2000). Devido a estas peculiaridades morfológicas, as bromélias apresentam ampla distribuição geográfica, desde florestas tropicais a savanas secas, campos rupestres e regiões semi-áridas (Crayn *et al.*, 2004; Gitaí *et al.*, 2005), podendo ocorrer desde o nível do mar até áreas montanhosas, com modos de vida terrestre, epífita e até formas mais extremas como as epífitas atmosféricas, as quais são capazes de absorver nutrientes da atmosfera por deposição seca, junto ao vapor d'água e à chuva (Benzing, 1986, 2000; Crayn *et al.*, 2004).

As bromélias têm ocorrência praticamente Neotropical, com apenas a espécie *Pitcairnia feliciana* presente no oeste do continente africano (Benzing, 1986, 2000). Esta família de plantas provavelmente se diversificou no início do período Terciário, entre 40 e 65 milhões de anos atrás e seu registro fóssil compreende poucos fragmentos de folhas preservadas, flores e pólens (Gómez, 1972). Características como a evolução dos tricomas foliares e aumento de sua capacidade absortiva, redução do sistema radicular, redução do número de folhas, presença de fitotelmata e diversificação no metabolismo do carbono [i.e., metabolismo ácido das crassuláceas (CAM)] permitiram a ocorrência dessas plantas em diversos habitats (Benzing & Burt, 1970; Benzing, 2000). A família Bromeliaceae é monofilética e dividida nas subfamílias Pitcairnioideae, Bromelioideae e Tillandsioideae (Terry *et al.*, 1997; Crayn *et al.*, 2004).

A subfamília Pitcairnioideae é considerada parafilética e grupo irmão de Bromelioideae devido ao compartilhamento de ancestral comum entre *Puya* e Bromelioideae, demonstrada a partir da análise de nucleotídeos e de DNA do cloroplasto (Ranker *et al.,* 1990; Terry *et al.*, 1997). Os organismos da subfamília Pitcairnioideae mantêm as características plesiomórficas da família, i.e., terrestrialidade, tricomas pouco desenvolvidos e metabolismo C3 na maioria das espécies (Benzing *et al.*, 1985). Esta subfamília é predominantemente terrestre e depende das raízes na aquisição de nutrientes, uma vez que seus tricomas são estruturalmente os mais simples de Bromeliaceae, são encontrados em pouca quantidade e não desempenham substancialmente a função absortiva (Benzing, 2000). Entretanto, espécies de *Brocchinia* possuem tricomas desenvolvidos e fitotelmata, podem ser epífitas, mirmecófitas, carnívoras e hospedeiras de cianobactérias fixadoras de N_2 (Benzing *et al.*, 1985; Benzing, 2000). A espécie *Brocchinia reducta*, por exemplo, apresenta características de planta carnívora, i.e., mecanismos de atração, fixação, digestão e absorção de presas (Givnish *et al.*, 1994). Devido a estas características, considera-se que *Brocchinia* é o primeiro gênero que divergiu em Pitcairnioideae, sendo grupo irmão de Bromeliaceae e apresentando relação filogenética próxima à Tillandsioideae (Benzing *et al.*, 1985; Crayn *et al.*, 2004).

A subfamília Bromelioideae é monofilética e a maioria das espécies possui metabolismo CAM (Benzing, 2000). Alguns gêneros (e.g., *Ananas*, *Bromelia*) são terrestres, não formam fitotelmata, apresentam tricomas não especializados e raízes bem desenvolvidas responsáveis pela absorção de nutrientes e água do solo (Benzing & Burt, 1970; Benzing, 2000; Endres & Mercier, 2003). Entretanto, outros gêneros desta subfamília (e.g., *Aechmea*, *Neoregelia*, *Quesnelia*) são epífitas, formam fitotelmata, possuem tricomas especializados em absorver

compostos nitrogenados (e.g., aminoácidos) e suas raízes fixam o vegetal ao substrato (Benzing & Burt, 1970; Benzing, 2000).

A subfamília Tillandsioideae é monofilética e possui as características morfo-fisiológicas mais derivadas da família (Benzing *et al.*, 1985; Benzing, 2000). A maioria das espécies de Tillandsioideae é epífita, algumas apresentam fitotelmata (e.g., *Vriesea*) enquanto outras são conhecidas como epífitas atmosféricas (e.g., algumas *Tillandsia*) (Benzing, 1986, 2000; Martin, 1994). As espécies desta subfamília possuem os tricomas foliares mais desenvolvidos entre Bromeliaceae, capazes de absorver água e compostos nitrogenados, enquanto suas raízes são finas e basicamente responsáveis pela fixação ao substrato (Sakai & Sanford, 1980; Benzing, 1986, 2000). Alguns gêneros possuem metabolismo de carbono C3, enquanto as espécies que ocupam os habitats com menor disponibilidade de água possuem metabolismo CAM (Benzing, 1986, 2000).

Interações entre animais e Bromeliaceae

A configuração tridimensional das bromélias, bem como suas folhas organizadas em rosetas e o fitotelmata, formam um habitat adequado para a ocorrência de inúmeros animais e até mesmo de algumas plantas (Benzing, 2000). Muitas espécies de epífitas são importantes para macacos e sagüis, que desfolham a vegetação do dossel em busca de comida. Por exemplo, *Leontopithecus rosalia*, o mico-leão dourado da Mata Atlântica brasileira, e *Callithrix geoffroyi*, o sagüi-de-cara-branca, forrageiam entre as bromélias epífitas para garantir sua dieta onívora (Leme & Marigo, 1993). Por outro lado, algumas espécies de macacos podem se alimentar das inflorescências dessas plantas, destruindo-as (Freeze &

Oppenheimer, 1981). As aves são muito importantes por polinizar e dispersar as sementes das bromélias, utilizando-as como sítio de forrageio e na construção de seus ninhos (Benzing, 2000). Por exemplo, *Pseudocolaptes lawrecii* passa 74% da sua vida no fitotelmata das bromélias, movendo-se de planta para planta em busca de invertebrados (Sillett, 1994). As espécies *Cacicus haemorrhous* e *Platypsaris rufus* dependem de *Tillandsia usneoides* para a construção de seus ninhos (Pizo, 1994), assim como algumas espécies de pássaros constroem seus ninhos em *Puya raimondii* (Pitcairnioideae), além de polinizá-la (Rees & Roe, 1980).

As bromélias fitotelmatas formam um microhabitat propício à ocorrência de anuros, especialmente para completarem seu ciclo de vida. Em apenas uma estação, Richardson *et al.* (2000) coletaram 37 anuros em 20 bromélias, mostrando a grande abundância desses organismos nestas plantas. Muitos anuros permanecem durante toda sua vida em Bromeliaceae e alguns podem ocupar uma única espécie de planta (Abendroth, 1971; Benzing, 2000), como *Eleuthrodactylus jasperi* que depende das bromélias para completarem seu ciclo de vida (Benzing, 2000). O caranguejo *Metopaulias depressus* também depende do reservatório de água das bromélias como berçário e permanece em bromélias epífitas durante todo seu ciclo de vida (Diesel & Schuh, 1993; Diesel, 1992, 1997). As fêmeas executam o cuidado parental aos jovens, alterando as concentrações de cálcio, oxigênio e o pH da água disponível a partir da coleta constante de conchas ricas em $CaCO_3$ que são depositadas no fitotelmata (Diesel & Schuh, 1993; Diesel, 1992, 1997). O fitotelmata de *Aechmea nudicaulis* diminui a dependência do opilião *Bourguyia albiornata* em relação às chuvas, que utiliza estas bromélias na deposição de seus ovos e cuidado com as ninfas (Machado & Oliveira, 2002). As bromélias também são muito visitadas por formigas devido ao microhabitat favorável à sua sobrevivência, por formarem jardins de formigas, por serem sítios de

nidificação casuais ou mesmo por serem plantas mirmecófitas (Blüthgen *et al.,* 2000; Benzing, 2000; Cogni & Oliveira, 2004). Por exemplo, *Tillandsia bulbosa* apresenta modificações morfológicas para abrigar formigas (i.e., mirmecófitas), estabelecendo relações espécie-específicas (Huxley, 1980).

Associações entre aranhas e Bromeliaceae são comuns (Barth *et al.,* 1988; Dias & Brescovit, 2004; Romero & Vasconcellos-Neto, 2004a,b, 2005a,b,c; Romero, 2005, 2006). Por exemplo, espécies do gênero *Cupiennius* (Ctenidae) se associam especialmente com Bromeliaceae e Musaceae, onde se escondem durante o dia enquanto permanecem ativos durante a noite, utilizando estas plantas para caçar e encontrar parceiros (Barth *et al.*, 1988). Recentemente, Romero (2006) mostrou que nove espécies de aranhas Salticidae vivem associadas à Bromeliaceae em diversos tipos de vegetação em várias regiões do Brasil, Bolívia, Argentina e Paraguai. *Psecas chapoda* (Salticidae), uma das aranhas bromelícolas neotropicais mais bem conhecidas, utiliza a planta *Bromelia balansae* (Bromelioideae) como sítio de forrageamento, acasalamento, postura de ootecas e berçário, bem como abrigo contra predadores e fogo (Rossa-Feres *et al.,* 2000; Romero & Vasconcellos-Neto, 2004b, 2005a,b; Romero, 2006; Omena & Romero, 2008). Em contraste com outras espécies de salticídeos bromelícolas (e.g., *Psecas sp.*, *Eustiromastix nativo* e *Coryphasia sp.*), que podem habitar até oito espécies de bromélias em regiões litorâneas, *P. chapoda* habita somente *B. balansae*, *Ananas comosus* e *Aechmea distichantha* em regiões de cerrado, campo rupestre e margens de florestas semidecíduas (Romero, 2006).

Sistema de estudo

A aranha *Psecas chapoda* (Peckham & Peckham, 1984) (Salticidae) vive quase exclusivamente em *Bromelia balansae* (Bromeliaceae) (Figura 1A e B) em grande extensão geográfica, abrangendo Paraguai, Bolívia e quatro estados brasileiros, Mato Grosso (MT), Mato Grosso do Sul (MS), São Paulo (SP) e Rio Grande do Sul (RS), mas também pode ser encontrada em outras duas espécies de Bromeliaceae, *Ananas comosus* em agroecossistemas e *Aechmea distichantha* em ambientes naturais (Romero, 2006). Esta espécie de aranha promove a nutrição de *B. balansae* enquanto a utiliza como refúgio, sítio de forrageamento, acasalamento e berçário para os imaturos (Figura 1C e D) (Rossa-Feres *et al.*, 2000; Romero & Vasconcellos-Neto, 2004b, 2005a,b; Romero, 2006; Omena & Romero, 2008).

Apesar da diversidade de organismos encontrados na roseta de Bromeliaceae, poucos estudos demonstraram o fluxo de nutrientes de animais para bromélias (Romero *et al.*, 2006, 2008, 2010). Utilizando métodos isotópicos de ^{15}N, Romero *et al.* (2006) mostraram que *P. chapoda* contribuiu com 18% do nitrogênio total de *B. balansae* e que bromélias com aranhas cresceram 15% mais do que aquelas sem aranhas nas rosetas. Estes autores discutem que, na natureza, esta contribuição deve ser ainda maior, pois a aranha produz inúmeros detritos ricos em nitrogênio (e.g., exúvias, teia, ovos mortos) que podem se acumular nas folhas destas plantas. Adicionalmente, Romero *et al.* (2008) mostraram que a contribuição de *P. chapoda* para *B. balansae* pode variar dependendo da densidade de aranhas entre diferentes áreas. Bromélias de áreas abertas mostraram valores de $\delta^{15}N$ mais altos em relação às bromélias de florestas e, portanto, este sistema mutualístico tem resultados condicionais espacialmente dependentes (Romero *et al.*, 2008). Como esta aranha ocorre em três espécies de bromélias com diferenças morfo-fisiológicas e hábitos distintos (terrestre e/ou epifítico) (Benzing *et al.*, 1976; Benzing, 1986, 2000), a aranha pode favorecer suas plantas hospedeiras em proporções

diferentes. Enquanto a bromélia terrestre (*B. balansae*) é mais dependente do solo na aquisição de nitrogênio, a bromélia-tanque (*Ae. distichantha*) pode ser mais dependente de detritos orgânicos presentes em suas folhas e, assim, *Ae. distichantha* pode se beneficiar mais nutricionalmente que *B. balansae* pela interação com a aranha.

Objetivos gerais

Esta dissertação teve como objetivo geral mostrar que enquanto inúmeros animais habitam bromélias, eles podem contribuir para a nutrição destas plantas por meio do fornecimento de compostos nitrogenados. Os objetivos principais deste trabalho foram: (1) determinar se a aranha *P. chapoda* contribui para a nutrição e crescimento de *B. balansae*, *An. comosus* e *Ae. distichantha* de forma semelhante ou distinta; (2) determinar quais respostas fisiológicas (i.e., concentrações de clorofilas, carotenóides e proteínas solúveis) as plantas apresentam por ganharem nitrogênio derivado da aranha; (3) determinar se existe variação sazonal na absorção de nitrogênio proveniente das aranhas e conseqüente diferença no crescimento das três espécies de bromélias; e (4) determinar se as bactérias associadas à filosfera de *B. balansae* facilitam a absorção de nutrientes por estas plantas.

O primeiro capítulo deste trabalho relata o mutualismo digestivo entre a aranha *Psecas chapoda* (Salticidae) e suas três espécies de bromélias hospedeiras, *Bromelia balansae*, *Ananas comosus* e *Aechmea distichantha*. O segundo capítulo mostra que microorganismos associados à filosfera (i.e., folhas) de bromélias podem acelerar a ciclagem de nutrientes neste compartimento das plantas, favorecendo-as nutricionalmente. O terceiro capítulo mostra o fluxo de nutrientes de animais para espécies de Bromelioideae e Tillandsioideae

(Bromeliaceae). Uma vez que espécies destas diferentes subfamílias apresentam atributos morfo-fisiológicos distintos, estas plantas podem se beneficiar nutricionalmente em proporções distintas quando interagem com animais.

Figura 1. Roseta de *Bromelia balansae* (A), fêmea adulta de *Psecas chapoda* (B), fêmea adulta em sua ooteca e (C) ovos da aranha.

LITERATURA CITADA

Abendroth A. 1971. Bromeliads and frogs. *Journal Bromeliad Society* **21**: 83-84.

Barth FG, Seyfarth EA, Bleckmann H, Schüch W. 1988. Spiders of the genus *Cupiennius* Simon 1891 (Araneae, Ctenidae). I. Range distribution, dwelling plants, and climatic characteristics of the habitats. *Oecologia* **77**: 187-193.

Benzing DH. 1986. Foliar specializations for animal-assisted nutrition in Bromeliaceae. In: *Insects and the plant surface*. Juniper B, Southwood R. (eds). Edward Arnold.

Benzing DH. 2000. *Bromeliaceae: profile of an adaptive radiation*. Cambridge University Press.

Benzing DH, Burt KM. 1970. Foliar permeability among twenty species of the Bromeliaceae. *Bulletin of the Torrey Botanical Club* **5**: 269-279.

Benzing DH, Henderson K, Kessel B, Sulak J. 1976. The absortive capacities of bromeliad trichomes. *American Journal of Botany* **63**: 1009-1014.

Benzing DH, Givinish TJ, Bermudes D. 1985. Absorptive trichomes in *Brocchinia reducta* (Bromeliaceae) and their evolutionary and systematic significance. *Systematic Botany* **10**: 81-91.

Blüthgen N, Vehaagh M, Goitía W, Blüthgen N. 2000. Ant nests in tank bromeliads – an example of non-specific interaction. *Insectes Sociaux* **47**: 313-316.

Cogni R, Oliveira PS. 2004. Patterns in foraging and nesting ecology in the neotropical ant, *Gnamptogenys moelleri* (Formicidae, Ponerinae). *Insectes Sociaux* **51**: 123-130.

Crayn DM, Klaus W, Smith JAC. 2004. Multiple origins of crassulacean acid metabolism and the epiphytic habit in the neotropical family Bromeliaceae. *Proceedings of the National Academy of Sciences* **101**: 3703-3708.

Dias SC, Brescovit AD. 2004. Microhabitat selection and co-occurrence of *Pachistopelma rufonigrum* Pocock (Aranae, Theraphosidae) and *Nothroctenus fuxico* sp. nov. (Aranae, Ctenidae) in tank bromeliads from Serra de Itabaiana, Sergipe, Brazil. *Revista Brasileira de Zoologia* **21**: 789-796.

Diesel R. 1992. Managing the offspring environment – brood care in the bromeliad crab, *Metopaulias depressus*. *Behavioral Ecology and Sociobiology* **30**: 125-134.

Diesel R. 1997. Maternal control of calcium concentration in the larval nursery of the bromeliad crab, *Metopaulias depressus* (Grapsidae). *Proceedings of the Royal Society of London Series B-Biological Sciences* **264**: 1403-1406.

Diesel R, Schuh M. 1993. Maternal care in the bromeliad crab *Metopaulias depressus* (Decapoda) – maintaining oxygen, pH and calcium levels optimal for the larvae. *Behavioral Ecology and Sociobiology* **32**: 11-15.

Endres L, Mercier H. 2003. Amino acid uptake and profile in bromeliads with different habitats cultivated in vitro. *Plant Physiology and Biochemistry* **41**: 181-187.

Freeze CH, Oppenheimer JR. 1981. The capuchin monkey, genus *Cebus*. In *Ecology and Behavior of Neotropical Primates*. Colmbra-Filho AF, Mittermeier RA. (eds). Vol 1. Academy of Brazilian Science.

Gitaí J, Horres R, Benko-Iseppon AM. 2005. Chromosomal features and evolution of Bromeliaceae. *Plant Systematics and Evolution* **253**: 65-80.

Givnish TJ, Burkhardt EL, Happel EE, Weintraub JD. 1994. Carnivory in the bromeliad *Brocchinia reducta*, with a cost/benefit model for the general restriction of carnivorous plants to sunny, moist, nutrient-poor habitats. *The American Naturalist* **124**: 479-497.

Gómez LD. 1972. *Karatophyllum bromelioides* L. D. Gomez (Bromeliaceae), nov. gen. et. sp., del Terciario Medio de Costa Rica. *Revista de Biologia Tropical* **20**: 221-229.

Huxley C. 1980. Symbiosis between ants and epiphytes. *Biological Reviews* **55**: 321-340.

Leme EMC, Marigo LC. 1993. *Bromélias na Natureza*. Marigo Comunicação Visual.

Machado G, Oliveira PS. 2002. Maternal care in the neotropical harvestman *Bourguyia albiornata* (Arachnida: Opiliones): oviposition site selection and egg protection. *Behaviour* **139**: 1509-1524.

Martin CE. 1994. Physiological ecology of the Bromeliaceae. *The Botanical Review* **1**: 1-82.

Omena PM, Romero GQ. 2008. Fine-scale microhabitat selection in a bromeliad-dwelling jumping spider (Salticidae). *Biological Journal of the Linnean Society* **94**: 653-662.

Pizo MA. 1994. Bromeliad use by Atlantic forest birds at Fazenda Intervales in southeastern Brazil. *Bromélia* **1**: 3-7.

Ranker TA, Soltis DE, Soltis PS, Gilmartin AJ. 1990. Subfamilial phylogenetic relationships of the Bromeliaceae: evidence from chloroplast DNA restriction site variation. *Systematic Botany* **15**: 425-434.

Rees WE, Roe NA. 1980. *Puya raimondii* (Pitcairnioideae, Bromeliaceae) and birds: an hypothesis on nutrient relationships. *Canadian Journal of Botany* **58**: 1262-1268.

Richardson BA, Richardson MJ, Scatema FN, McDowells WH. 2000. Effects of nutrient availability and other elevational changes on bromeliad populations and their invertebrate communities in a humid tropical forest in Puerto Rico. *Journal of Tropical Ecology* **16**: 167-188.

Romero GQ. 2005. *Associações entre aranhas Salticidae e Bromeliaceae: história natural, distribuição espacial e mutualismos.* Tese de doutorado, Universidade Estadual de Campinas, Campinas, São Paulo.

Romero GQ. 2006. Geographic range, habitats and host plants of bromeliad-living jumping spiders (Salticidae). *Biotropica* **38**: 522-530.

Romero GQ, Vasconcellos-Neto J. 2004a. Spatial distribution patterns of jumping spiders associated with terrestrial bromeliads. *Biotropica* **36**: 596-601.

Romero GQ, Vasconcellos-Neto J. 2004b. Beneficial effects of flower-dwelling predators on their host plant. *Ecology* **85**: 446-457.

Romero GQ, Vasconcellos-Neto J. 2005a. Population dynamics, age structure and sex ratio of the bromeliad-dwelling jumping spider, *Psecas chapoda* (Salticidae). *Journal of Natural History* **39**: 153-163.

Romero GQ, Vasconcellos-Neto J. 2005b. The effects of plant structure on the spatial and microspatial distribution of a bromeliad-living jumping spider (Salticidae). *Journal of Animal Ecology* **74**: 12-21.

Romero GQ, Vasconcellos-Neto J. 2005c. Spatial distribution and microhabitat preference of *Psecas chapoda* (Peckham & Peckham) (Araneae, Salticidae). *The Journal of Arachnology* **33**: 124-134.

Romero GQ, Mazzafera P, Vasconcellos-Neto J, Trivelin PCO. 2006. Bromeliad-living spiders improve host plant nutrition and growth. *Ecology* **87**: 803-808.

Romero GQ, Vasconcellos-Neto J, Trivelin PCO. 2008. Spatial variation in the strength of mutualism between a jumping spider and a terrestrial bromeliad: evidence from the stable isotope ^{15}N. *Acta Oecologica* **33**: 380-386.

Romero GQ, Nomura F, Gonçalves AZ, Dias NYN, Mercier H, Conforto EC, Rossa-Feres DC. 2010. Nitrogen fluxes from treefrogs to tank epiphytic bromeliads: na isotopic and physiological approach. *Oecologia* **162**: 941-949.

Rossa-Feres DC, Romero GQ, Gonçalves-De-Freitas E, Feres RJF. 2000. Reproductive behavior and seasonal occurrence of *Psecas viridipurpureus* (Salticidae, Araneae). *Revista Brasileira de Biologia* **60**: 221-228.

Sakai WS, Sanford WG. 1980. Ultrastructure of the water-absorbing trichomes of pineapple (*Ananas comosus*, Bromeliaceae). *Annals of Botany* **46**: 7-11.

Sillett TS. 1994. Foraging ecology of epiphyte-searching insectivorous birds in Costa Rica. *Condor* **96**: 863-877.

Terry RG, Brown GH, Olmstead RG. 1997. Examination of subfamilial phylogeny in Bromeliaceae using comparative sequence of the plastid locus *ndhF1*. *American Journal of Botany* **84**: 664-670.

2. CAPÍTULO I

Gonçalves AZ, Mercier H, Mazzafera P, Romero GQ. 2011

Spider-fed bromeliads: seasonal and interspecific variation in plant performance

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Spider-fed bromeliads: seasonal and interspecific variation in plant performance

Ana Zangirólame Gonçalves[1], Helenice Mercier[2], Paulo Mazzafera[3] and Gustavo Quevedo Romero[1]*

[1]*Departamento de Biologia Animal, , Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), CP 6109, CEP 13083-970, Campinas, São Paulo, Brazil*

[2] *Departamento de Botânica, Instituto de Biociências, Universidade de São Paulo (USP), Rua do Matão, 277, CEP 05508-900, São Paulo, Brazil.*

[3] *Departamento de Biologia Vegetal, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), CP 6109, CEP 13083-970, Campinas, São Paulo, Brazil*

* Corresponding author: gq_romero@yahoo.com.br

Running title: spider-fed bromeliads

- *Background and Aims* Several animals that live on bromeliads can contribute to plant nutrition through nitrogen provisioning (digestive mutualism). The bromeliad-living spider *Psecas chapoda* (Salticidae) inhabits and breeds on *Bromelia balansae* in regions of South America, but in specific regions can also appear on *Ananas comosus* plantations and *Aechmea distichantha*.
- *Methods* Using isotopic and physiological methods in greenhouse experiments, we evaluated the role of labeled (^{15}N) spider feces and *Drosophila melanogaster* flies in the nutrition and growth of each host plant, as well as seasonal variation in the importance of this digestive mutualism.
- *Key Results* Spiders contributed 0.6 ± 0.2% (mean ± SE; dry season) to 2.7 ± 1% (wet season) to the total nitrogen in *B. balansae*, 2.4 ± 0.4% (dry) to 4.1 ± 0.3% (wet) in *An. comosus* and 3.8 ± 0.4% (dry) to 5 ± 1% (wet) in *Ae. distichantha*. In contrast, flies did not contribute to the nutrition of these bromeliads. Chlorophylls and carotenoid concentrations did not differ among treatments. Plants that received feces had higher soluble protein concentrations and leaf growth (RGR) only during the wet season.
- *Conclusions* These results indicate that the mutualism between spiders and bromeliads is seasonally restricted generating a conditional outcome. There was interspecific variation in nutrient uptake, probably related to each species' performance and photosynthetic pathways. Whereas *B. balansae* seems to use nitrogen for growth, *Ae. distichantha* apparently stores nitrogen for stressful nutritional conditions. Bromeliads absorbed more nitrogen coming from spider feces than from flies, reinforcing the beneficial role played by predators in these digestive mutualisms.

Key words: Bromelioideae, *Bromelia balansae*, *Ananas comosus*, *Aechmea distichantha*, chlorophylls and carotenoid, soluble protein, spider-bromeliad interactions, stable isotope ^{15}N, nitrogen flux.

INTRODUCTION

Plant performance can be strongly influenced by predators (Herrera and Pellmyr, 2002; Knight *et al.*, 2006). For example, it is well known that predators can decrease plant fitness by capturing or chasing away pollinators (Knight *et al.*, 2006; Gonçalves-Souza *et al.*, 2008). In contrast, plant performance can be improved if predators decrease the damage to floral tissues caused by phytophages (Rico-Gray and Oliveira, 2007; Romero *et al.*, 2008a). However, there is a less known phenomenon by which predators can improve plant performance by contributing to plant nutrition (Romero *et al.*, 2006). To date, the most common examples of this type of nutritional interaction are from ant–plant systems (Treseder *et al.*, 1995; Sagers *et al.*, 2000; Fischer *et al.*, 2003; Solano and Dejean, 2004), from digestive mutualism involving *Pameridea* bugs (Miridae) and their carnivorous host plant *Roridula* (Ellis and Midgley, 1996; Anderson and Midgley, 2002, 2003) and from amphibians and spiders that inhabit bromeliads (Romero *et al.*, 2006; Inselsbacher *et al.*, 2007; Romero *et al.*, 2010). In this kind of mutualism animals contribute to plant nutrition and performance, and receive variable benefits from the plants.

Plants of the large Neotropical family Bromeliaceae can shelter numerous organisms, including bacteria, algae, fungi, invertebrates, vertebrates and even vascular plants (Gutiérrez

et al., 1993; Benzing, 2000; Machado and Oliveira, 2002; Romero, 2006). Associations between spiders and Bromeliaceae are widespread in South America (Romero and Vasconcellos-Neto, 2005a; Romero, 2006). For instance, Romero (2006) reported that nine species of the spider family Salticidae live in association with Bromeliaceae in diverse types of vegetation in various regions ranging from Brazil, Bolivia, Argentina and Paraguay. Some bromeliads are terrestrial with well developed root systems and their leaves forming rosettes that contribute little to nutrient uptake (Benzing, 1986, 2000). Many bromeliad species have adaptations that allow them to occupy xeric, nutrient-poor environments (Benzing, 2000). Some of them are tank based, being dependent of animals for nutrition. Others are myrmecophytes with ant-houses or ant-nest gardens and, finally, some of them are atmospherics with a dense indumentum of absorbing hairs where the substrates serve primarily for anchorage (Benzing, 1986, 2000). Bromeliad leaves are organized in rosettes that sometimes accumulate rain water (phytotelmata), and have trichomes on the foliage surface which are specialized for absorbing water and nutrients. Additionally, the organisms associated with Bromeliaceae may contribute to plant nutrition and performance. However, despite a growing knowledge of the number of associations between predators and Bromeliaceae, so far only a few studies have evaluated their role as digestive mutualists of Bromeliaceae (Romero *et al.*, 2006, 2008b, 2010).

Digestive mutualism involving animals and Bromeliaceae was first suggested by Benzing (1986, 2000) and empirically sustained by Romero *et al.* (2006, 2008b) which showed that the Neotropical jumping spider *Psecas chapoda* (Salticidae) provides the terrestrial bromeliad *Bromelia balansae* with nitrogen derived from its debris (e.g., feces). *Psecas chapoda* inhabits and breeds almost exclusively on this bromeliad species in several

regions of South America, including Brazil, Bolivia and Paraguay (Romero, 2006). In this by-product mutualism (see Romero *et al.*, 2008b), the bromeliad architecture can benefit spiders by providing foraging, mating and egg laying sites, shelter against predators and fire, and nurseries for spiderlings (Romero and Vasconcellos-Neto, 2005a,b,c; Omena and Romero, 2008). In turn, bromeliads can absorb nutrients from spider debris (e.g., feces, spider silk, prey carcass and exuviae) through specialized leaf trichomes or roots (Romero *et al.*, 2006, 2008b).

In addition to *B. balansae*, *P. chapoda* also inhabit two other Bromelioideae species: the commercial *Ananas comosus* (pineapple) and *Aechmea distichantha*. Since these three Bromelioideae species have variable life styles and modes of nutrient uptake, these spider-plant systems are suitable for testing animal contributions to plant nutrition and performance in the Bromelioideae subfamily.

In this study, we used isotopic (^{15}N) and physiological methods to evaluate the contribution of *P. chapoda* feces as a nitrogen source in sustaining the nutrition and growth of *B. balansae*, *An. comosus* and *Ae. distichantha*, and the seasonal variation in these spider-bromeliad relationships. In addition, we used ^{15}N labeled *Drosophila melanogaster* flies to test whether insects that eventually fall into the bromeliad rosette and phytotelmata are also a source of nutrients for these plants. Specifically, our study addressed the following questions: (1) which bromeliad species derives the most nitrogen from spider feces? (2) Does seasonal variation affect the absorption of the nitrogen originating from *P. chapoda*? (3) Do concentrations of chlorophylls, carotenoid and soluble protein change in response to the nitrogen obtained from *P. chapoda*? (4) Does the nitrogen from *P. chapoda* affect the growth of the three bromeliad species over dry and wet seasons?

MATERIAL AND METHODS

Organisms and experiment design

Pineapple is typically found in large plantations, but also grows naturally in cerrado vegetation and semi-deciduous forests in South America (Romero, 2006). Like *B. balansae*, this species has well-developed roots for acquiring nutrients (Benzing, 1986, 2000). *Bromelia balansae* and *An. comosus* do not have phytotelmata, but they are able to accumulate a few millimeters of rain water at the base of their rosettes (Romero *et al.*, 2006). *Aechmea distichantha* is terrestrial, epiphytic or lithophilic, has phytotelmata (Borgo and Silva, 2003; Romero *et al.*, 2007) and poorly developed roots, which have the nearly exclusive function of attaching the rosette to the substrate (Benzing, 1986, 2000). Of these three bromeliads, *Ae. distichantha* has more developed and numerous epidermic trichomes, which are specialized in acquiring complex nitrogen molecules (e.g., amino acids) (Martin, 1994; Benzing, 2000). In contrast, *B. balansae* and *An. comosus* have trichome foliage that apparently does not absorb complex organic nutrients (Benzing, 2000). Whereas *B. balansae* is a C3 plant, the photosynthetic pathways of *An. comosus* and *Ae. distichantha* are based on crassulacean acid metabolism (CAM) (Martin, 1994). Since the water conservative CAM mode of photosynthesis often occurs in epiphytic species, especially in those lacking the phytotelmata and in terrestrial species that occupy arid sites (Martin, 1994), CAM species are expected to depend more on foliar uptake of spider feces.

To test the seasonal variation on the contribution of *P. chapoda* to the nutrition and growth of *B. balansae*, *An. comosus* and *Ae. distichantha*, experiments were conducted in the

dry (from May 9 to July 5, 2006) and wet (from March 4 to April 30, 2007) seasons. The average monthly rainfall in the dry season (May to July) varied from 25 to 38 mm and the minimum and maximum temperatures were 13°C and 28°C, respectively. In the wet season (March and April) the rainfall reached 413 mm, with a minimum temperature of 20°C and a maximum temperature of 32°C (INPE/CPTEC, 2008).

In this experiment small young bromeliads with similar biomass and size (foliar length varying from 20 to 25 cm) were used to minimize the effect of nitrogen dilution. Moreover, all plants had an equivalent size to those plants that support up to two *P. chapoda* individuals (6^{th} to 8^{th} instars) (Romero *et al.*, 2006). *Bromelia balansae* plants were obtained from seeds from the same cohort. *Ananas comosus* plants were obtained from pineapple crowns and *Ae. distichantha* plants were collected from mountain top rocky outcrops in Monte Verde, Minas Gerais State, in the southeastern Brazil region. All bromeliads were planted in pots (14.5 cm in diameter, 14.5 cm high). These bromeliads remained for six months and one year in a greenhouse before the start of experiments of the dry and wet seasons, respectively. This procedure was important to avoid any contact of the bromeliads with spiders or other organisms and to allow acclimation of plants. We raised each bromeliad species according to its type of substrata: during the two experiments (dry and wet seasons), *B. balansae* was kept in a nutrient poor sandy soil (the same type of soil used by Romero *et al.*, 2006) and *Ae. distichantha* was kept in triturated *Pinus* sp. rind when this became an epiphytic bromeliad. In the first experiment (dry season) *An. comosus* plants were kept in a reddish-yellow clay soil with medium sandy texture, while in the second experiment (wet season) they were kept in the same sandy soil used for *B. balansae*. Any variation in ^{15}N values among soils from pots was not relevant here once the bromeliads received enriched debris. All plants were kept in a

net greenhouse (mesh diameter: 1 mm) exposed to seasonal conditions (dry and wet). The plants were watered with limited amounts of water just to avoid excessive desiccation; for this, we used an automatic irrigation system using three fine spraying sprinklers, each with a capacity of 6L h^{-1}, which worked for 15 min every 6 h.

Nitrogen flux from spiders to bromeliads

To quantify the nitrogen flux from spiders to bromeliads, spiders were fed ^{15}N-labeled *Drosophila melanogaster* flies and their feces were stored for later application to the bromeliads. The flies were cultured from eggs in a medium of ^{15}N-labeled yeast. The labeled yeast was obtained by raising commercial yeast on a Difco-Bacto carbon-based medium with ammonium sulfate [$(^{15}NH_4)_2SO_4$, 10% excess atoms, from Cambridge Isotope Laboratories, MA]. Details on the laboratory procedure for yeast and fly enrichment can be found in Romero *et al.* (2006).

Fifty *P. chapoda* females (between 6th and 8th instars) were collected in the field (São José do Rio Preto municipality, São Paulo State) from *B. balansae* and kept in glass flasks of approximately 7 cm diameter and 10 cm in height. Each spider was fed every three days with 15 enriched flies, an interval sufficient for the spider to capture all the flies and produce feces (see Romero *et al.*, 2006). At three-day intervals the feces in each flask was diluted in 500 µL distilled water and then frozen and stored in polypropylene tubes. Additionally, at three-day intervals, 15 flies were frozen (-18°C) for use in the experiments.

In the dry season, the experiment had two treatments: every three days *B. balansae*, *An. comosus* and *Ae. distichantha* specimens either (1) received feces produced by two

spiders (n = 5 plants) or (2) did not receive feces (n = 5 plants). In the wet season the experiment had three treatments: every three days *B. balansae*, *An. comosus* and *Ae. distichantha* specimens either (1) received feces produced by two spiders (n = 5 plants), (2) received one enriched fly (n = 5 plants) or (3) did not receive feces or flies (n = 5 plants). Thawed flies and feces were applied to the central part of the rosette and the tank of the bromeliads at the base of the leaves. The biomass of spider feces and flies used in the different treatments was similar, i.e., two spiders produced 0.24 ± 0.04 mg feces (n = 4) every three days, whereas one fly weighed 0.23 ± 0.02 mg (n = 6). These biomass values did not differ statistically (t-test: P = 0.636). The water in *Ae. distichantha* tank came from the irrigation system (subterraneous water) and rainwater. The original water found in this species in the field was not maintained in the experiment since macro and microorganisms could affect the results of isotopic analyses. Two new leaves of each plant were randomly collected in July 8, 2006 (dry season) and in May 3, 2007 (wet season). Only parts of leaves that had no contact to labeled debris and flies were analyzed. The leaves were homogenized together and were dried at 60°C, crushed to obtain a fine powder and this material was stored dry in polypropylene tubes until isotopic analyses.

Isotopic analyses

The ^{15}N atoms percent values and total nitrogen concentration (total N μg mg^{-1} dry leaf tissue) of the bromeliad leaves, the enriched feces from spiders fed with enriched flies and enriched flies were determined with an isotope ratio mass spectrometer (20-20 mass spectrometer, PDZ Europa, Sandbach, England) after sample combustion to N_2 at 1000°C by

an on-line elemental analyzer (PDZ Europa ANCA-GSL) in the Stable Isotope Facility at the University of California at Davis. To calculate the nitrogen fraction in the plants receiving feces of *P. chapoda* and fly *D. melanogaster* (f_A), we used the two-source (i.e., soil and feces/flies) mixing model equations with a single isotopic signature (e.g., $\delta^{15}N$) described by Phillips and Gregg (2001). Additionally, we considered the ^{15}N fractioning during its assimilation and the metabolic process of the plants, according to the following equation (McCutchan *et al.*, 2003):

$$f_A = \frac{\delta_M - \delta_B - \Delta\delta^{15}N}{\delta_A - \delta_B}$$

where f_A is the proportionate contribution of labeled *D. melanogaster* or feces of *P. chapoda* (%), δ_M is the isotope ratio of the plants that received feces or flies, δ_A and δ_B are the isotope ratios of potential nitrogen sources (feces/flies and soil, respectively) and $\Delta\delta^{15}N$ is the trophic shift for nitrogen between diet (e.g., feces, flies or soil) and consumer (e.g., bromeliads). The values of $\Delta\delta^{15}N$ used were +3.3 ± 0.26‰ (mean ± standard error) for the plants that received feces and +1.4 ± 0.2‰ for the plants that received flies (McCutchan *et al.,* 2003).

The nitrogen acquisition values were compared among bromeliad species and treatments (feces and flies), and between seasons using ANOVA; Fisher Least Square Difference (LSD) post hoc tests were used for pair-wise comparisons. For nitrogen acquisition comparisons between the two seasons, the data from the plants that received enriched flies were removed from the analysis because this treatment was only carried out during the wet season.

Analyses of plant pigments and protein

To determine if the nitrogen derived from *P. chapoda* had some influence on the physiology of host plants, bromeliads from the wet season experiment were analyzed for concentrations of chlorophyll, carotenoid and soluble proteins. The leaves used in these analyses were different from those used in isotopic analysis. The procedures to obtain chlorophyll *a*, *b*, *a*+*b* and carotenoid contents were those of Lichtenthaler (1987). Six fresh leaves from the intermediate part of the rosettes were randomly chosen and cut in small pieces; 1 g was frozen in liquid nitrogen and homogenized with 7 mL of 80% acetone. The extract was filtered using filter paper, which had been previously dampened with 2 mL of the same solvent, and the residue retained in the filter paper was washed three times with 4 mL of solvent. The combined filtered extracts were volume adjusted to 20 mL and the absorbance was measured with a spectrophotometer at 470 nm, 647 nm and 663 nm.

The soluble proteins were extracted from 1 g of leaf first cut into small pieces, frozen in liquid nitrogen and homogenized with 3 mL of ultra-pure Milli-Q water. The homogenate was centrifuged at 12,000 rpm (g) for 10 minutes and the supernatant (15µL) 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.

Data on the concentrations (µg g^{-1} fresh leaf mass) of chlorophyll *a*, chlorophyll *b*, chlorophyll *a*+*b*, carotenoids and soluble protein were $\log_{10}$ transformed and then compared among treatments (feces, flies and controls) and bromeliad species using two-way ANOVA.

Bromeliad growth

To test if the nitrogen derived from spiders affected plant growth and whether growth varied seasonally, two new leaves from each experimental plant were randomly chosen and their lengths were measured at the beginning and at the end of the experiments. A previous study showed that leaf length is the best growth measure for bromeliads having hard and narrow leaves (see Romero *et al.*, 2006). Since leaf removal would affect plant growth, we did not take other measurements, such as leaf biomass. Actually, significant linear regressions have been detected between leaf length and its dry biomass before this study (*B. balansae*: $r^2 = 0.84$, $P < 0.001$; *An. comosus*: $r^2 = 0.88$, $P < 0.001$; *Ae. distichantha*: $r^2 = 0.80$, $P < 0.001$). The relative growth rate (RGR) was calculated from these data in both experimental seasons using the following equation:

$$RGR = \frac{Ln_{l\,final} - Ln_{l\,initial}}{t_2 - t_1}$$

in which $Ln_{l\,final}$ and $Ln_{l\,initial}$ 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 time in days between the initial and final measurements. The obtained RGR values were compared among treatments, bromeliad species and between seasons using a two or three-way ANOVA; Fisher Least Square Difference (LSD) post hoc tests were used for pair-wise comparisons. For RGR comparisons between the two experimental seasons, the data from the plants that received enriched flies were removed from the analysis.

RESULTS

Nitrogen flux from spiders to bromeliads

The $\delta^{15}N$ values of enriched flies, of *P. chapoda* spiders that fed on the enriched flies and of spider feces indicate that these materials were enriched in the experiments during the dry and wet seasons (Table 1). *Psecas chapoda* contributed nutritionally to the host plants *B. balansae*, *An. comosus* and *Ae. distichantha* (Fig. 1). Nevertheless, the nitrogen derived from *P. chapoda* feces was absorbed in different proportions among the three bromeliads (Table 2); *B. balansae* and *Ae. distichantha* derived less and higher nitrogen from *P. chapoda*, respectively (Fig. 1) and its absorption was greater in the wet than in the dry season (Table 2, Fig. 1). Whereas *P. chapoda* contributed from 0.6 ± 0.2% (mean ± SE; dry season) to 2.7 ± 1% (wet season) of the total nitrogen contributed to *B. balansae*, it contributed from 2.4 ± 0.4% (dry) to 4.1 ± 0.3% (wet) of the total nitrogen of *An. comosus* and from 3.8 ± 0.4% (dry) to 5 ± 1% (wet) of the total nitrogen contributed to *Ae. distichantha* (Fig. 1A, B). The nitrogen derived from *D. melanogaster* flies was lower than those derived from spider feces, and did not differ among the bromeliad species (Table 2, Fig. 1B).

Plant pigments and protein

Chlorophyll and carotenoid concentrations in *B. balansae*, *An. comosus* and *Ae. distichantha* did not differ among treatments (Table 3, Fig. 2). Chlorophylls and carotenoids differed among bromeliad species (Table 3); whereas *An. comosus* had the highest concentrations of chlorophylls and carotenoids, *B. balansae* and *Ae. distichantha* shared similar concentrations (Fig. 2). In contrast to pigments, significant differences were found for soluble proteins among treatments, bromeliad species and their interactions (Table 3). *Ananas*

comosus and *Ae. distichantha* showed increased protein concentrations when they received spider feces with the highest concentrations in *An. comosus* (Fig. 2).

Bromeliad growth

Whereas the addition of feces in the dry season only marginally affected the growth (RGR) of the bromeliads (P = 0.052), the addition of feces or flies during the wet season improved plant growth (P = 0.046; Table 4, Fig. 3A, B). In this season the bromeliads receiving feces grew more, however this difference was mainly observed because of the response of *B. balansae* to the different treatments (Fig. 3B). While *B. balansae* grew more when receiving feces, the RGR of the other species were not affected by feces addition (Fig. 3B). Moreover, feces contributed more than flies to *B. balansae* growth (Fig. 3B).

In the dry season, the RGR differed among the three bromeliad species (P = 0.028; Table 4) and it was higher in *An. comosus*, followed by *Ae. distichantha* and *B. balansae* (Fig. 3A; Fisher LSD, *An. comosus* vs. *B. balansae*: P = 0.010; *An. comosus* vs. *Ae. distichantha*: P = 0.036; *B. balansae* vs. *Ae. distichantha*: P = 0.546). In the wet season, the RGR also differed significantly among the three bromeliad species (P < 0.001; Table 4). Nevertheless, *An. comosus* had greater growth, followed by *B. balansae* and *Ae. distichantha* (Fig. 3B; Fisher LSD, *An. comosus* vs. *B. balansae*: P < 0.001; *An. comosus* vs. *Ae. distichantha*: P < 0.001; *B. balansae* vs. *Ae. distichantha*: P < 0.001). All bromeliads grew more during the wet than in the dry season (Table 4, Fig. 3).

DISCUSSION

Our results indicate that *P. chapoda* spiders contribute to the nutrition of their three bromeliad hosts, *B. balansae*, *An. comosus* and *Ae. distichantha*, by nitrogen provisioning through their feces. Therefore, the by-product mutualism between the spider *P. chapoda* and the plant *B. balansae* described recently by Romero *et al.* (2006, 2008b) can be extended to other host bromeliad species, such as *Ae. distichantha* and *An. comosus*. The three host plants of *P. chapoda* typically grow in poor soils due to high rates of weathering and leaching, or even in the outcrop of granitic rocks and in plant trunks (Romero, 2006; Romero *et al.*, 2007), where the availability of nutrients is very low (Benzing, 2000; Press *et al.*, 2006). Thus, the nitrogen derived from spiders seems to be of great benefit for these bromeliads. Since diverse spider species live associated with several other Bromelioideae species in various geographical regions (see Romero, 2006), we suggest that this type of digestive by-product mutualism extends to other spider-bromeliad systems.

The two CAM plants (*Ae. distichantha* and *An. comosus*) absorbed more nitrogen than the C3 bromeliad (*B. balansae*) in both the dry and wet seasons. Crassulacean acid metabolism can confer a higher efficiency of water use to these CAM plants even under severe hydric conditions (Martin, 1994). However, even the CAM plants showed a reduction in foliar nitrogen absorption and growth in the dry season compared to the wet season. Therefore, although CAM plants may be able to better use the available water supply, environmental harsh conditions (e.g., reduced humidity and temperature) may limit their performance. Griffiths *et al.* (1989) showed that the photosynthetic parameters of the CAM bromeliad *Tillandsia flexuosa* were substantially reduced during the dry season. In the wet season, greater water availability and higher temperatures also favor CAM plant growth and nitrogen absorption (Griffiths, 1988). In addition, greater environmental humidity can

increase the contact of nutrients with trichome surfaces, improving trichome nutrient absorption (Benzing, 2000).

Aechmea distichantha was the species that absorbed the greatest amount of nitrogen derived from spider feces, likely because it has poorly developed roots (Benzing, 1986) and larger, higher developed epidermic trichomes than *B. balansae* and *An. comosus* (Martin, 1994). Since *Ae. distichantha* inhabits rocky inselbergs (Romero, 2006), this species might depend more on animal detritus for its nutrition, absorbing nutrients via its leaves. Many authors (Benzing, 1986, 2000; Romero *et al.*, 2006, 2008b, 2010) suggested that epiphytic tank-bromeliads can benefit more from animal association than can terrestrial bromeliads. Terrestrial bromeliads appear to have plesiomorphic traits (i.e., C3 photosynthetic pathway, developed roots and fewer epidermic trichomes), while epiphytic and atmospheric bromeliads appear to have apomorphic traits (i.e., phytotelmata, developed trichomes) that allowed their adaptive distribution through oligotrophic environments (Medina, 1974; Benzing, 1986, 2000; Crayn *et al.*, 2004). Biotic and abiotic factors, such as oligotrophy and leaf traits, affect a plant's nutritional options regarding the use of fauna for trophic advantage (Benzing, 1986, 2000; Armbruster *et al.*, 2002; Leroy *et al.*, 2009). Since *Ae. distichantha* has apomorphic leaf traits and occurs in oligotrophic environments, this species uses spiders for trophic advantage.

The C3 plant *B. balansae* and the epiphytic CAM plant *Ae. distichantha* differed only marginally in their growth in the dry season. In contrast, in the wet season *B. balansae* grew better but did not accumulate protein, while *Ae. distichantha* grew less and had increased soluble protein content. Soluble proteins are one of the main forms of store nitrogen in plants (Frommer *et al.,* 1994) and their increase can reflect a plant's good nutritional status (Barneix

and Causin, 1996). Whereas *B. balansae* may be allocating nitrogen derived from spiders to vegetative growth and clonal reproduction, the CAM plant may be allocating nitrogen to other ends (i.e., storage, reproduction and metabolism). In a greenhouse experiment, Benzing (1983) reported that, even with added fertilizer, epiphytic bromeliads did not significantly increase in size, suggesting that these plants have a slow growth rate associated with an adaptive response to survive in extremely oligotrophic environments. Nutrients absorbed by *Ae. distichantha* are apparently stored for nutritional stress conditions. The terrestrial bromeliad *B. balansae*, on the other hand, may be adapted to a less limited environment which could explain the lower accumulation of soluble proteins compared to *Ae. distichantha*. Although *B. balansae* has more absorbing trichomes than the CAM plant *An. comosus* (Benzing and Burt, 1970), the latter absorbed more nitrogen and also had the highest concentration of chlorophylls, carotenoids and soluble proteins. This likely occurred because *An. comosus* is a fast growing species and has a high demand for nitrogen (Endres and Mercier, 2001). Although few studies have investigated the efficiency of nitrogen uptake in CAM bromeliads, they are possibly more capable of using available nitrogen from a variety of sources in the environment than C3 plants (Oaks, 1994; Kerbauy, 2004).

Both C3 and CAM plants were affected by the dry season conditions indicating that under field conditions the benefits of this spider-plant mutualism may be limited to certain periods of the year, i.e., it is seasonally restricted. When mutualism is considered from a cost and benefit perspective, it becomes clear that outcomes must in fact be extremely dynamic in space and time, along a continuum of possible outcomes (Bronstein, 1994). A great number of factors have been shown to influence these outcomes, especially the biotic and abiotic factors at the site in which the interaction takes place (Thompson, 1988; Bronstein, 1994). For

example, in the interaction between *Roridula* plants and their mutualistic hemipteran *Pameridea*, Anderson and Midgley (2007) showed that plants had negative growth rates with no hemipterans, positive growth rates with intermediate hemipteran densities and negative growth rate with very high hemipteran densities. This research shows that mutualisms are a dynamic process which has variable outcomes. In the case of bromeliads and *P. chapoda*, bromeliads probably have benefit mainly in the rainy period of the year, even if they are inhabited by spiders during all seasonal periods (see Romero and Vasconcellos-Neto, 2005c). These results could be associated with abiotic factors like temperature and quantity of rainwater, since in the rainy season these factors do not limit plant growth. Recently, Romero *et al.* (2008b) showed that this system is also spatially restricted, i.e., bromeliads in areas where spiders are found in greater abundance derive more ^{15}N than in areas where spiders are found in low density. Although few studies have investigated conditionality in spider-plant mutualistic systems, a knowledge of spatial and temporal conditional outcomes may be relevant for a better understanding of the evolution of these types of interactions.

In contrast, the three studied bromeliads absorbed very little nitrogen from *D. melanogaster* flies (simulating insects that eventually fall in the rosettes) compared to *P. chapoda* feces (i.e., guanine, see Romero *et al.*, 2006). A similar event was described by Ellis and Midgley (1996) and Anderson and Midgley (2002) for the digestive mutualism between the *Pameridea roridulae* hemiptera and its *Roridula gorgonias* host plant. Feeding on insects, *P. chapoda* spiders channel organic matter into the bromeliad rosettes and even excrete simple compounds (e.g., guanin) that can be directly absorbed by the plant's trichomes. On the other hand, insect chitin needs to be mineralized by bacteria and/or other microorganisms before it becomes available. Even *Ae. distichantha*, which has a tank and, therefore, would be

expected to derive more nitrogen from insect carcasses (see Romero *et al.*, 2006), had similar nitrogen obtention to the other species that were studied. These results reinforce the beneficial role of predators for epyphitic tank plants.

In conclusion, *P. chapoda* improved the performance and/or growth of their three host bromeliads. However, their effects varied temporally generating a conditional outcome in this digestive mutualistic event. There was a strong interspecific variation in nutrient uptake, which is probably related to each species' performance and photosynthetic pathways, i.e., CAM plants absorbed more nitrogen than the C3 bromeliad in both dry and wet seasons, but even the CAM plants reduced nitrogen uptake and growth in the dry season.Whereas *B. balansae* seems to use nitrogen for growth, *Ae. distichantha* apparently stores nitrogen for stressful nutritional conditions. Additionally, bromeliads absorbed more nitrogen coming from spider feces than from flies, reinforcing the beneficial role played by predators in these digestive mutualisms.

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LITERATURE CITED

Anderson B, Midgley JJ. 2002. It takes two to tango but three is a tangle: mutualists and cheaters on the carnivorous plant *Roridula. Oecologia* **132**: 369-373.

Anderson B, Midgley JJ. 2003. Digestive mutualism, an alternate pathway in plant carnivory. *Oikos* **102**: 221-224.

Anderson B, Midgley JJ. 2007. Density-dependent outcomes in a digestive mutualism between carnivorous *Roridula* plants and their associated hemipterans. *Oecologia* **152**: 115-120.

Armbruster P, Hutchinson RA, Cotgreave P. 2002. Factors influencing community structure in a South American tank bromeliad fauna. *Oikos* **96**: 225-234.

Barneix AJ, Causin HF. 1996. The central role of amino acids on nitrogen utilization snd plant growth. *Journal of Plant Physiology* **149**: 358-362.

Benzing DH. 1983. Vascular epiphytes: a survey with special reference to their interactions with other organisms. In: Sutton SL, Whitmor TC, Chadwick AC, eds. *Tropical rain forest: ecology and management*. Oxford: Blackwell Scientific Publication, 11-24.

Benzing DH. 1986. Foliar specializations for animal-assisted nutrition in Bromeliaceae. In: Juniper B, Southwood R, eds. *Insects and the plant surface*. London: Edward Arnold, 235-256.

Benzing DH. 2000. *Bromeliaceae: profile of an adaptive radiation*. New York: Cambridge University Press.

Benzing DH, Burt KM. 1970. Foliar permeability among twenty species of the Bromeliaceae. *Bulletin of the Torrey Botanical Club* **5**: 269-279.

Borgo M, Silva SM. 2003. Epífitos vasculares em fragmentos de Floresta Ombrófila Mista, Curitiba, Paraná, Brasil. *Revista Brasileira de Botânica* **3**: 391-401.

Bradford MM. 1976. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *Analytical Biochemistry* **72**: 248-254.

Bronstein JL. 1994. Conditional outcomes in mutualistic interactions. *Trends in Ecology and Evolution* **9**: 214-217.

Crayn DM, Winter K, Smith JAC. 2004. Multiple origins of crassulacean acid metabolism and the epiphytic habit in the Neotropical family Bromeliaceae. *Proceedings of the National Academy of Science* **101**: 3703-3708.

Ellis AG, Midgley JJ. 1996. A new plant-animal mutualism involving a plant with sticky leaves and a resident hemipteran insect. *Oecologia* **106**: 478-481.

Endres L, Mercier H. 2001. Influence of nitrogen forms of the growth and nitrogen metabolism of bromeliads. *Journal of Plant Nutrition* **24**: 29-42.

Fischer RC, Wanek W, Richter A, Mayer V. 2003. Do ants feed plants? A ^{15}N labeling study of nitrogen fluxes from ants to plants in the mutualism of *Pheidole* and *Piper*. *Journal of Ecology* **91**: 126-134.

Frommer WB, Kwart M, Hirner B, Fischer WN, Hummel S, Ninnemann O. 1994. Transporters for nitrogenous compounds in plants. *Plant Molecular Biology* **26**: 1651-1670.

Gonçalves-Souza T, Omena PM, Souza JC, Romero GQ. 2008. Trait-mediated effects on flowers: artificial spiders deceive pollinators and decrease plant fitness. *Ecology* **89**: 2407-2413.

Griffiths H. 1988. Carbon balance during CAM: an assessment of respiratory CO_2 recycling in the epiphytic bromeliads *Aechmea nudicaulis* and *Aechmea fendleri. Plant, Cell and Environment* **11**: 603-611.

Griffiths H, Lüttge U, Popp M et al. 1989. Ecophysiology of xerophytic and halophytic vegetation of a coastal alluvial plain in northern Venezuela. IV. *Tillandsia flexuosa* Sw. and *Schomburgkia humboldtiana* Reichb., epiphytic CAM plants. *New Phytologist* **111**: 273-282.

Gutiérrez MO, Lavin MC, Ayala FC, Perez AJ. 1993. Arthropods associated with *Bromelia hemisphaerica* (Bromeliales: Bromeliaceae) in Morelos, Mexico. *Florida Entomologist* **76**: 616-621.

Herrera CM, Pellmyr O. 2002. *Plant–animal interactions: an evolutionary approach*. Massachusetts: Blackwell Science.

INPE/CPTEC. 2008. Centro de previsão de tempo e estudos climáticos, available at http://www.cptec.inpe.br.

Inselsbacher E, Cambui CA, Richter A, Stange CF, Mercier H. 2007. Microbial activities and foliar uptake of nitrogen in the epiphytic bromeliad *Vriesea gigantea*. *New Phytologist* **175:** 311-320.

Kerbauy GB. 2004. *Fisiologia vegetal*. Rio de Janeiro: Editora Guanabara Koogan S.A.

Knight TM, Chase JM, Hillebrand H, Holt RD. 2006. Predation on mutualists can reduce the strength of trophic cascades. *Ecology Letters* **9**: 1173-1178.

Leroy C, Corbara B, Dejean A, Céréghino R. 2009. Ants mediate foliar structure and nitrogen acquisition in a tank-bromeliad. *New Phytologist* **183**: 1124-1133.

Lichtenthaler HK. 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. *Methods in Enzymology* **148**: 350-382.

Machado G, Oliveira PS. 2002. Maternal care in the neotropical harvestman *Bourguyia albiornata* (Arachnida: Opiliones): oviposition site selection and egg protection. *Behaviour* **139**: 1509-1524.

Martin CE. 1994. Physiological ecology of the Bromeliaceae. *The Botanical Review* **1**: 1-82.

McCutchan JHJ, Lewis WMJ, Kendall C, McGrath CC. 2003. Variation in trophic shift for stable isotope ratios of carbon, nitrogen and sulfur. *Oikos* **102**: 378-390.

Medina E. 1974. Dark CO_2 fixation, habitat preference and evolution within the Bromeliaceae. *Evolution* **28**: 677-686.

Oaks A. 1994. Efficiency of nitrogen utilization in C3 and C4 cereals. *Plant Physiology* **106**: 407-414.

Omena PM, Romero GQ. 2008. Fine-scale microhabitat selection in a bromeliad-dwelling jumping spider (Salticidae). *Biological Journal of the Linnean Society* **94**: 653-662.

Phillips DL, Gregg JW. 2001. Uncertainty in source partitioning using stable isotopes. *Oecologia* **127**: 171-179.

Press F, Siever R, Grotzinger J, Jordan TH. 2006. *Para entender a terra*. Porto Alegre: Bookman Press.

Rico-Gray V, Oliveira PS. 2007. *The ecology and evolution of ant-plant interactions*. Chicago: The University of Chicago Press.

Romero GQ. 2006. Geographic range, habitats and host plants of bromeliad-living jumping spiders (Salticidae). *Biotropica* **38**: 522-530.

Romero GQ, Vasconcellos-Neto J. 2005a. The effects of plant structure on the spatial and microspatial distribution of a bromeliad-living jumping spider (Salticidae). *Journal of Animal Ecology* **74**: 12-21.

Romero GQ, Vasconcellos-Neto J. 2005b. Spatial distribution and microhabitat preference of *Psecas chapoda* (Peckham & Peckham) (Araneae, Salticidae). *The Journal of Arachnology* **33**: 124-134.

Romero GQ, Vasconcellos-Neto J. 2005c. Population dynamics, age structure and sex ratio of the bromeliad-dwelling jumping spider, *Psecas chapoda* (Salticidae). *Journal of Natural History* **39**: 153-163.

Romero GQ, Mazzafera P, Vasconcellos-Neto J, Trivelin PCO. 2006. Bromeliad-living spiders improve host plant nutrition and growth. *Ecology* **87**: 803-808.

Romero GQ, Santos AJ, Wienskoski EH, Vasconcellos-Neto J. 2007. Association of two new *Coryphasia* species (Araneae, Salticidae) with tank-bromeliads in southeastern Brazil: habitats and patterns of host plant use. *The Journal of Arachnology* **35**: 181-192.

Romero GQ, Souza JC, Vasconcellos-Neto J. 2008a. Anti-herbivore protection by mutualistic spiders and the role of plant glandular trichomes. *Ecology* **89**: 3105-3115.

Romero GQ, Vasconcellos-Neto J, Trivelin PCO. 2008b. Spatial variation in the strength of mutualism between a jumping spider and a terrestrial bromeliad: evidence from the stable isotope ^{15}N. *Acta Oecologica* **33**: 380-386.

Romero GQ, Nomura F, Gonçalves AZ et al. 2010. Nitrogen fluxes from treefrogs to tank epiphytic bromeliads: an isotopic and physiological approach. *Oecologia* **162**: 941-949.

Sagers CL, Ginger SM, Evans RD. 2000. Carbon and nitrogen isotopes trace nutrient exchange in an ant–plant mutualism. *Oecologia* **123**: 582-586.

Solano PJ, Dejean A. 2004. Ant-fed plants: comparison between three geophytic myrmecophytes. *Biological Journal of the Linnean Society* **83**: 433-439.

Thompson JN. 1988. Variation in interspecific interactions. *Annual Review of Ecology and Systematics* **19**: 65-87.

Treseder KK, Davidson DW, Ehleringer JR. 1995. Absorption of ant-provided carbon and nitrogen by a tropical epiphyte. *Nature* **357**: 137-139.

Table 1. *Average $\delta^{15}N$ values of (A) natural abundance and enriched spiders, spider faeces and Drosophila flies in the dry and wet seasons, and of (B)* Bromelia balansae*,* Aechmea distichantha *and* Ananas comosus *leaves which received enriched faeces and Drosophila flies, and control, during the dry (May to July 2006) and wet seasons (March to April 2007)*

Treatment	$\delta^{15}N$ values (se)	*N*
A)		
Dry season		
Faeces		
Natural abundance	12.10 (2.71)	3
Enriched	3054.41 (151.55)	4
Drosophila melanogaster		
Natural abundance	3.07 (0.19)	2
Enriched	3027.61 (173.52)	5
Spider (adult female)		
Natural abundance	17.08	1
Enriched	2108.11 (384.48)	4
Wet season		
Faeces		
Natural abundance	12.1 (2.71)	3
Enriched	1797.21 (63.09)	5
Drosophila melanogaster		
Natural abundance	3.07 (0.19)	2
Enriched	2366.79 (40.16)	5
Spider (adult female)		
Natural abundance	17.08	1
Enriched	1215.99 (253.11)	5
B)		
Dry season		
B. balansae		
Faeces	23.28 (7.27)	5
Control	1.84 (1.21)	5
An. comosus		
Faeces	78.56 (11.64)	5
Control	1.71 (1.71)	4
Ae. distichantha		

Faeces	117.91 (12.43)	5
Control	- 1.03 (1.47)	5
Wet season		
B. balansae		
Faeces	59.62 (18.28)	5
Flies	8.84 (2.84)	5
Control	6.66 (1.46)	5
An. comosus		
Faeces	82.90 (4.72)	5
Flies	13.69 (2.58)	5
Control	5.19 (2.17)	5
Ae. distichantha		
Faeces	108.71 (17.53)	5
Flies	17.86 (2.85)	5
Control	14.57 (1.47)	5

The standard errors of means are in parentheses.

n, Number of replicates

Table 2. *Analyses of variance (ANOVA) comparing the amount of nitrogen of the bromeliad species* Bromelia balansae*,* Ananas comosus *and* Aechmea distichantha *that was derived from* Psecas chapoda *faeces and* Drosophila melanogaster *flies (treatments) in different seasons (dry: May to July 2006; wet: March to April 2007). Significance of* $P < 0.05$ *is highlighted in bold*

Source of variation	d.f.	MS	*F*	*P*
Comparing treatments (only for wet season)				
Treatments	1	100.8	58.90	**<0.001**
Bromeliads	2	3.929	2.089	**<0.001**
Treatments x Bromeliads	2	3.422	1.820	0.1838
Error	24	1.881		
Comparing seasons (only for treatment "faeces")				
Seasons	1	20.83	16.78	**<0.001**
Bromeliads	1	19.08	15.36	**<0.001**
Seasons x Bromeliads	2	0.5083	0.4094	0.6686
Error	24	1.242		

Table 3. *Analyses of variance (ANOVA) summarizing the effects of different treatments (*Psecas chapoda *faeces,* Drosophila melanogaster *flies and control) on chlorophylls* a, b, a+b, *carotenoids and soluble proteins concentrations in* Bromelia banlansae, Ananas comosus *and* Aechmea distichantha, *in the wet season experiment. Significance of* $P < 0.05$ *is highlighted in bold*

Source of variation	d.f.	MS	*F*	*P*
Chlorophyll *a*				
Treatments	2	0.0070	0.37	0.69
Bromeliads	2	0.8377	44.82	**<0.001**
Treatments x Bromeliads	4	0.0214	1.14	0.351
Error	36	0.0187		
Chlorophyll *b*				
Treatments	2	0.0003	0.016	0.984
Bromeliads	2	0.8091	48.28	**<0.001**
Treatments x Bromeliads	4	0.0314	1.87	0.136
Error	36	0.0168		
Chlorophyll *a+b*				
Treatments	2	0.0044	0.25	0.779
Bromeliads	2	0.8282	47.18	**<0.001**
Treatments x Bromeliads	4	0.0219	1.25	0.308
Error	36	0.0176		
Carotenoids				
Treatments	2	0.0023	0.16	0.852
Bromeliads	2	0.7997	55.78	**<0.001**
Treatments x Bromeliads	4	0.0271	1.89	0.133
Error	36	0.0143		
Soluble proteins				
Treatments	2	1.6728	70.38	**<0.001**
Bromeliads	2	2.6749	112.54	**<0.001**
Treatments x Bromeliads	4	0.7921	33.32	**<0.001**
Error	36	0.0238		

Table 4. *Analyses of variance (ANOVA) summarizing the effects of different treatments (*Psecas chapoda *faeces,* Drosophila melanogaster *flies and control) on the foliar growth rate (RGR) of* Bromelia banlansae*,* Ananas comosus *and* Aechmea distichantha*, during the dry (May to July, 2006) and wet seasons (March to April, 2007). Significance of* $P < 0.05$ *is highlighted in bold*

Source of variation	d.f.	MS	*F*	*P*
Dry season				
Treatments	1	0.00044	4.12	0.052
Bromeliads	2	0.00044	4.15	**0.028**
Treatments x Bromeliads	2	0.00003	0.31	0.738
Error	23			
Wet season				
Treatments	2	0.00008	3.34	**0.046**
Bromeliads	2	0.00113	46.98	**<0.001**
Treatments x Bromeliads	4	0.000004	0.15	0.960
Error	36			
Comparing periods (only for treatment "faeces")				
Periods	1	0.0074	120.02	**<0.001**
Treatments (faeces vs. control)	1	0.0005	8.80	**0.005**
Bromeliads	2	0.0010	16.40	**<0.001**
Periods x Treatments	1	0.00004	0.72	0.398
Periods x Bromeliads	2	0.0001	3.08	0.055
Treatments x Bromeliads	2	0.00002	0.478	0.627
Periods x Treatments x Bromeliads	2	0.000008	0.130	0.878
Error	47	0.00006		

Figure captions

Fig.1. The percentage of nitrogen in *Bromelia balansae*, *Ananas comosus* and *Aechmea distichantha* derived from *Psecas chapoda* faeces and *Drosophila melanogaster* flies during the (A) dry- (May to July, 2006) and (B) wet- (March to April, 2007) season experiments. In the dry-season experiment no flies were used. Values were obtained from the two-source mixing models equations (see Materials and Methods for details). Bars indicate the s.e. and letters indicate *post-hoc* comparisons by Fisher's LSD ($\alpha < 0.05$).

Fig.2. Chlorophylls *a*, *b*, *a*+*b*, carotenoids and soluble protein concentrations for *Bromelia balansae*, *Ananas comosus* and *Aechmea distichantha* during treatments with *Psecas chapoda* faeces, *Drosophila melanogaster* flies and control, as indicated, in the wet season experiment. Bars indicate the s.e. Letters indicate *post-hoc* comparisons by Fisher's LSD ($\alpha < 0.05$) and their absence indicates no statistical differences among treatments. Species were analysed separately.

Fig.3. Relative growth rate (RGR) of the leaves of *Bromelia balansae*, *Ananas comosus* and *Aechmea distichantha* from different treatments (*Psecas chapoda* faeces, *Drosophila melanogaster* flies and control) during the (A) dry- (May to July, 2006) and (B) wet- (March to April, 2007) season experiments. Bars indicate the s.e. Letters indicate *post-hoc* comparisons by Fisher's LSD ($\alpha < 0.05$) and their absence indicate no statistical differences among treatments. Species were analysed separately.

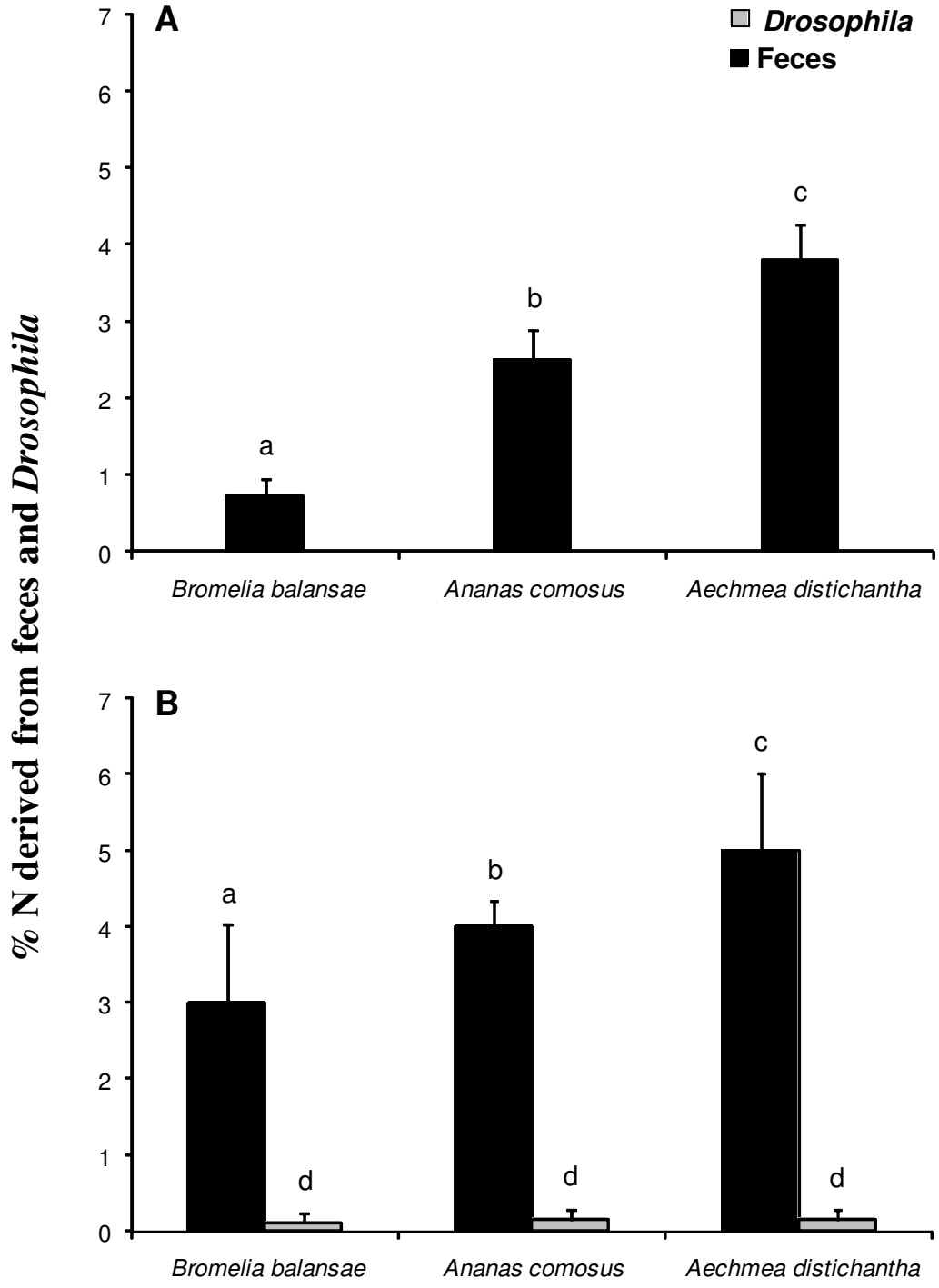
% N derived from feces and Drosophila
A
Drosophila
Feces
7
6
5
4
3
2
1
0
a
b
c
Bromelia balansae
Ananas comosus
Aechmea distichantha
B
a
b
c
d
d
d
Bromelia balansae
Ananas comosus
Aechmea distichantha

Fig. 1

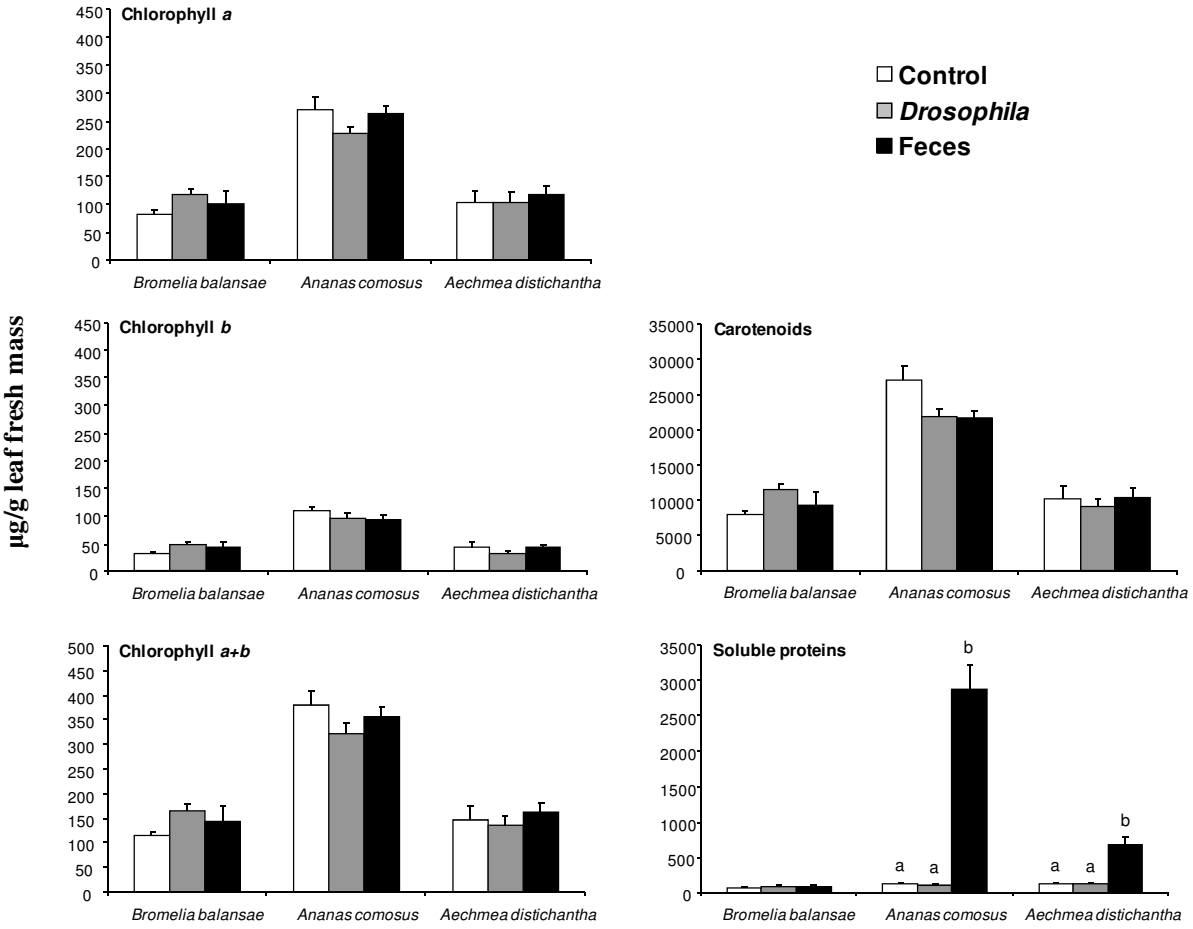
Chlorophyll a
Chlorophyll b
Carotenoids
Chlorophyll a+b
Soluble proteins
µg/g leaf fresh mass
Control
Drosophila
Feces
Bromelia balansae
Ananas comosus
Aechmea distichantha
a
b

Fig. 2

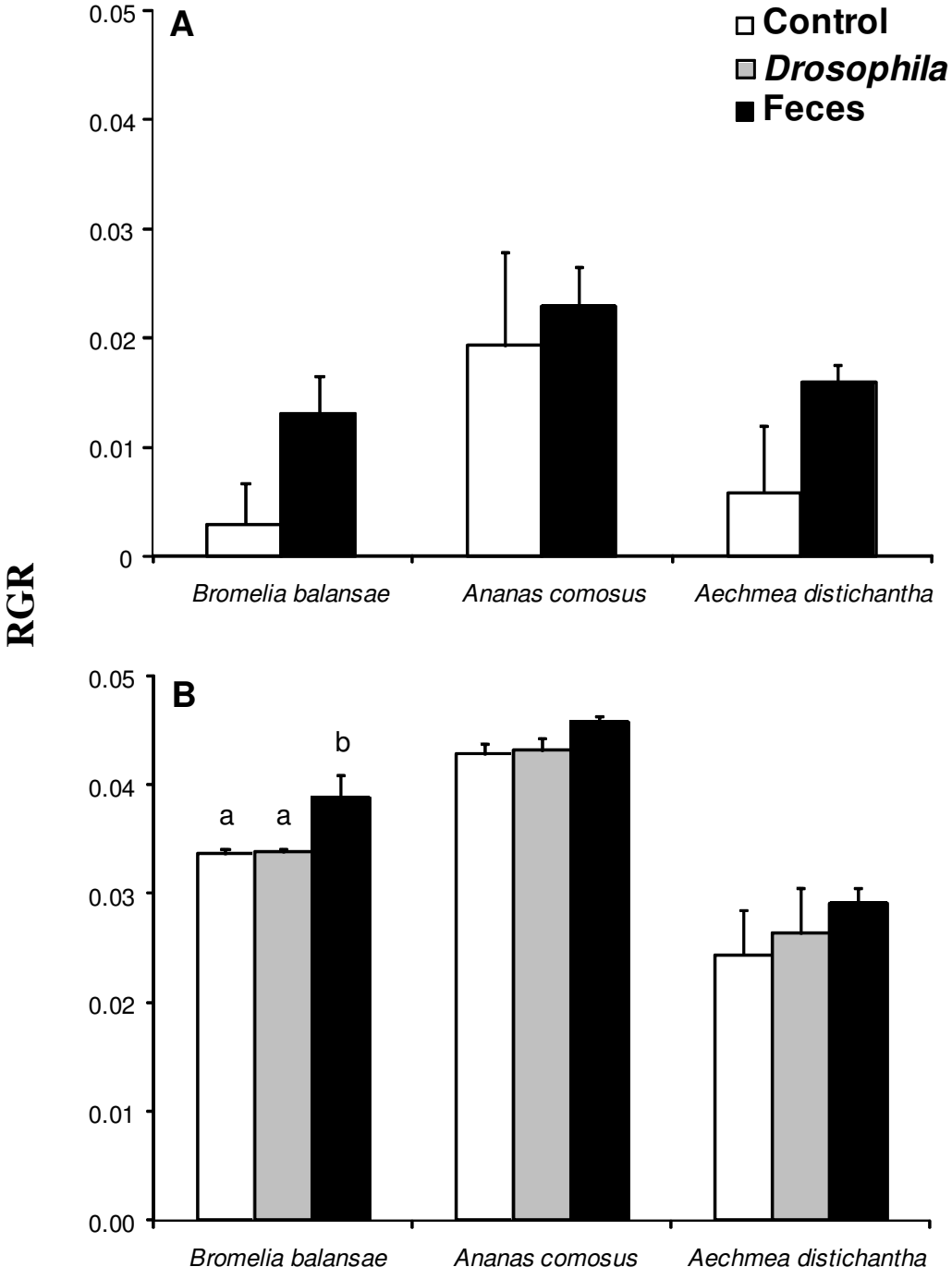
A
Control
Drosophila
Feces
RGR
0.05
0.04
0.03
0.02
0.01
0
Bromelia balansae
Ananas comosus
Aechmea distichantha
B
a
a
b
0.05
0.04
0.03
0.02
0.01
0.00
Bromelia balansae
Ananas comosus
Aechmea distichantha

Fig. 3

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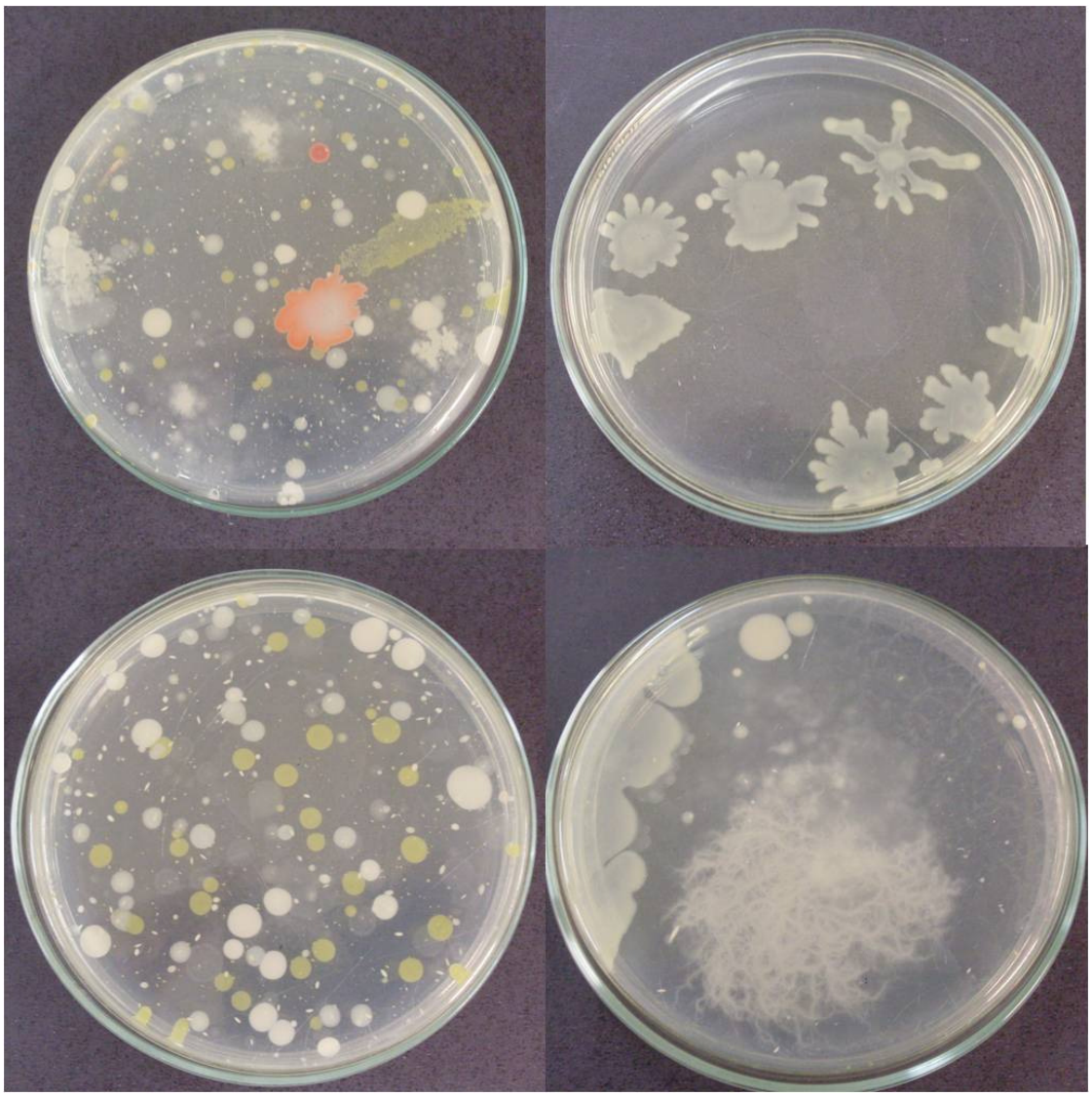
Ciclagem de nutrientes na filosfera de bromélias

(Manuscrito não submetido)



Ciclagem de nutrientes na filosfera de bromélias

Ana Zangirólame Gonçalves*, Fernando Leite Hoffmann†, Helenice Mercier‡, Paulo Mazzafera§ and Gustavo Quevedo Romero*

** Departamento de Biologia Animal, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), CP 6109, CEP 13083-970, Campinas, São Paulo, Brazil*

† Departamento de Engenharia e Tecnologia de Alimentos, IBILCE, Universidade Estadual Paulista (UNESP), Rua Cristóvão Colombo, 2265, CEP 15054-000, São José do Rio Preto, São Paulo, Brazil

‡ Departamento de Botânica, Instituto de Biociências, Universidade de São Paulo (USP), Rua do Matão, 277, CEP 05508-900, São Paulo, Brazil

§ Departamento de Biologia Vegetal, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), CP 6109, CEP 13083-970, Campinas, São Paulo, Brazil

A superfície das folhas ou filosfera é habitada por uma alta diversidade de microorganismos (*1*), muitos dos quais podem desempenhar papel essencial na ciclagem de nutrientes (*2*). Bactérias que mineralizam compostos orgânicos na filosfera podem disponibilizar nutrientes para plantas. Neste trabalho manipulamos a abundância de bactérias sobre a filosfera da bromélia *Bromelia balansae* (Bromeliaceae) e mostramos que plantas com densidade natural de bactérias acumularam 57% mais proteínas solúveis e cresceram 13% mais do que as bromélias que tiveram a abundância de bactérias reduzidas com antibióticos. Estes resultados sugerem pela primeira vez que bactérias aceleram a ciclagem de nutrientes na filosfera e podem favorecer nutricionalmente as plantas.

A superfície das folhas ou filosfera é um habitat propício à ocorrência de microorganismos (*1*) e sua diversidade é incalculável (*3-5*). Entretanto, a maioria dos trabalhos descreve a fixação de N por bactérias (*6*) enquanto pouco se sabe sobre a ciclagem de nutrientes no dossel de florestas tropicais. Detritos de origem animal e vegetal podem acumular na filosfera de plantas (*7-8*) e algumas delas, como bromélias, possuem estruturas epidérmicas (i.e., tricomas) especializadas em absorver nutrientes e água que estejam em contato com a filosfera (*7*). Portanto, microorganismos que ocorrem na filosfera de bromélias podem mineralizar compostos orgânicos complexos (e.g., folhas, carcaças de animais), disponibilizando nutrientes simples (e.g., compostos nitrogenados simples) que podem ser absorvidos diretamente pelos tricomas epidérmicos. Neste estudo, utilizamos o mutualismo digestivo entre a aranha *Psecas chapoda* (Salticidae), sua bromélia hospedeira *Bromelia balansae* (Bromeliaceae) e as bactérias que ocorrem em sua filosfera (*8*) para testar se estes

microorganismos aceleram a ciclagem de nutrientes que estão em contato com a filosfera e favorecem a nutrição destas plantas.

Para testar esta hipótese, cinco bromélias mantidas em casa de vegetação foram submetidas aos seguintes tratamentos a cada três dias: (*Antibiótico*) fezes das aranhas, bactérias e antibióticos, (*Sem antibiótico*) fezes das aranhas, bactérias e água destilada estéril e (*Controle*) nada receberam. Enriquecemos as aranhas e conseqüentemente suas fezes com ^{15}N a partir de sulfato de amônio enriquecido [$(^{15}NH_4)_2SO_4$, 10% de excesso de átomos, da Cambridge Isotope Laboratories, MA], a fim de quantificar o N das plantas proveniente das aranhas em diferentes abundâncias de bactérias. Coletamos bactérias em campo (município de São José do Rio Preto, São Paulo) sobre *B. balansae* e as aplicamos nas bromélias experimentais, bem como aplicamos os antibióticos clorofenicol e amoxicilina no tratamento *Antibiótico*. Ao final do experimento, o $\delta^{15}N$ das aranhas, fezes e bromélias foi obtido no laboratório Stable Isotope Facility (Universidade da Califórnia), contamos a quantidade de unidades formadoras de colônias de bactérias (UFCs) sobre as bromélias de campo e naquelas utilizadas no experimento (utilizando meio Plate Count Agar e meio mínimo contendo apenas guanina como fonte de N e carbono), quantificamos a concentração de proteínas solúveis nas bromélias (*9*) e sua taxa de crescimento relativo (ver Material de Suporte On-line).

As aranhas contribuíram com 10,7 ± 1,9% (média ± erro padrão) do N presente nas bromélias que receberam antibióticos (*Antibiótico*) e 27,1 ± 4,4% do N das bromélias que não receberam antibióticos (*Sem antibiótico*). As bromélias que obtiveram a maior quantidade de N a partir da aranha também apresentaram a maior quantidade de UFCs ($P < 0,001$), produziram 57% mais proteínas solúveis ($P < 0,001$; Fig 1A) e cresceram 13% mais ($P < 0,001$; Fig 1B) que as bromélias com menor abundância de bactérias.

Nossos resultados mostram pela primeira vez a importância de microorganismos da filosfera na ciclagem de nutrientes e disponibilização dos mesmos para plantas tropicais. Estes microorganismos não somente favoreceram nutricionalmente as plantas, como também seu desenvolvimento, por meio do aumento na produção de proteínas solúveis e do crescimento. Em um bioma como a Mata Atlântica, no qual se estima existir até 13 milhões de espécies de bactérias na filosfera (*5*), estes microorganismos podem desempenhar inúmeras funções na ciclagem de nutrientes, não somente na fixação de N como têm sido relatado até então (*6*). Sendo responsáveis pela mineralização de matéria orgânica e disponibilização de íons simples para a absorção das plantas (*10*), os microorganismos podem, portanto, desempenhar importante papel na dinâmica de nutrientes no dossel de florestas tropicais.

Referências

1. Ruinen, J. *Nature* **177**, 220-221 (1956).

2. McArthur, J. V. *Microbial Ecology, an evolutionary approach* (Academic Press, California, 2006).

3. Beattie, G. A. & Lindow, S. E. *Annu. Rev. Phytopathol* **33**, 145-172 (1995).

4. Andrews, J. H. & Harris, R. F. *Annu. Rev. Phytopathol* **38**, 145-180 (2000).

5. Lambais, M. R., Crowley, D. E., Cury, J. C., Büll, R. C. & Rodrigues, R. R. *Science* **312**, 1917 (2006).

6. Lindow, S. E. & Brandl, M. T. *Appl. Environ. Microbiol.* **69**, 1875-1883 (2003).

7. Benzing, D. H. *Bromeliaceae: profile of an adaptive radiation* (Cambridge University Press, Cambridge, 2000).

8. Romero, G. Q., Mazzafera, P., Vasconcellos-Neto, J. & Trivelin, P. C. O. *Ecology* **87**, 803-808 (2006).

9. Bradford, M. M. *Anal. Biochem.* **72**, 248-254 (1976).

10. Hodge, A., Robinson, D. & Fitter, A. *Trends Plant Sci.* **5**, 304-308 (2000).

Legenda da figura.

Fig. 1. (A) Concentração de proteínas solúveis e **(B)** taxa de crescimento relativo (TCR) em *Bromelia balansae* quando submetida aos seguintes tratamentos: (*Controle*) nada recebeu; (*Antibiótico*) fezes produzidas pela aranha *Psecas chapoda*, bactérias coletadas em campo sobre *Bromelia balansae* e antibióticos clorofenicol e amoxicilina; e (*Sem antibiótico*) fezes da aranha, bactérias e água destilada estéril. Barras indicam erro padrão e comparações entre tratamentos são significativas a $P < 0,05$.

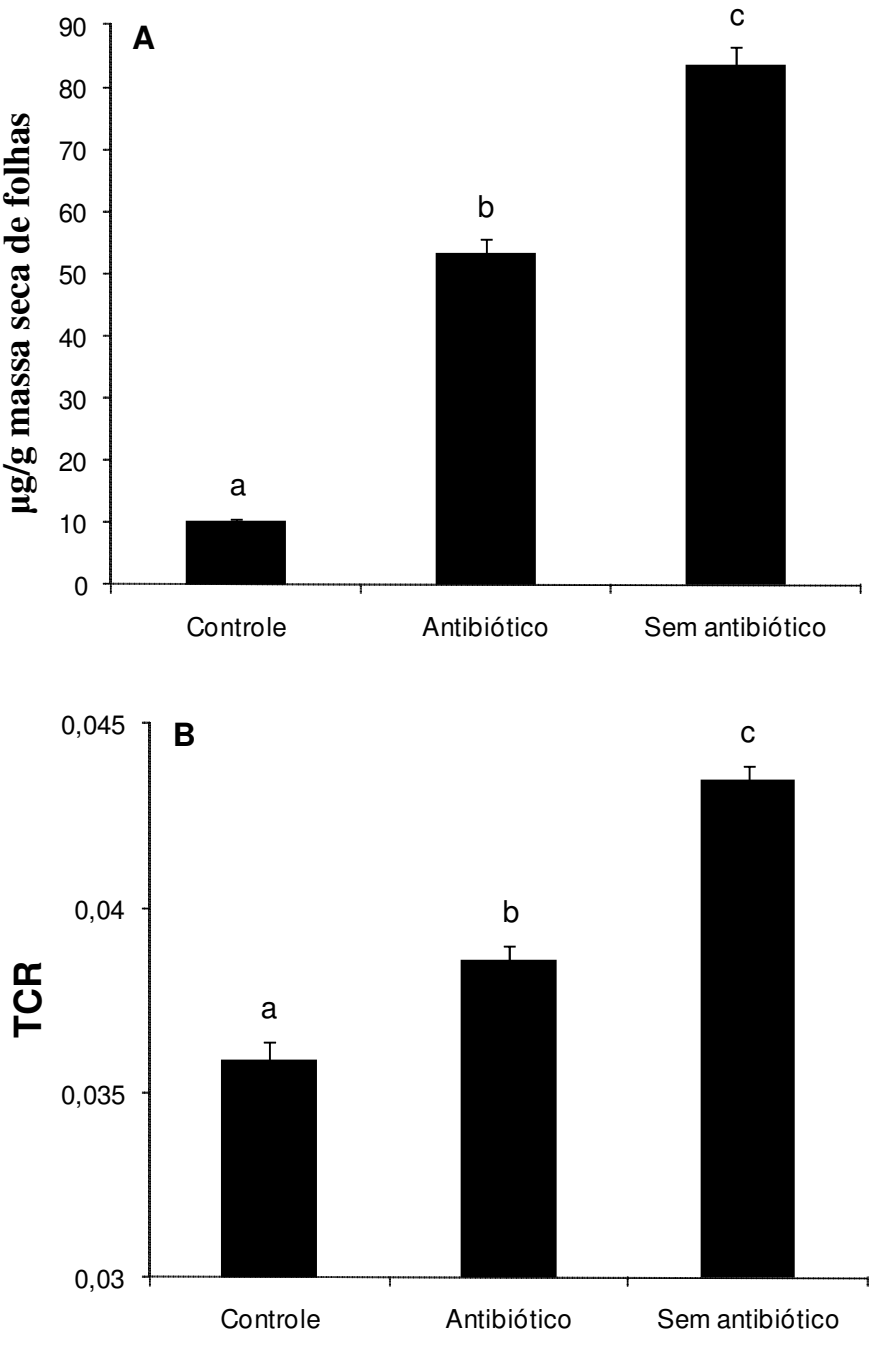

A
µg/g massa seca de folhas
90
80
70
60
50
40
30
20
10
0
a
b
c
Controle
Antibiótico
Sem antibiótico
B
TCR
0,045
0,04
0,035
0,03
a
b
c
Controle
Antibiótico
Sem antibiótico

Fig. 1

Material de Suporte On-line

Material e métodos

Bactérias na filosfera. Para confirmar a presença e a abundância de bactérias nas folhas de *Bromelia balansae* foi feita a contagem de unidades formadoras de colônias (UFCs) presentes em cinco bromélias coletadas em campo (município de São José do Rio Preto, Estado de São Paulo) e em cinco bromélias mantidas em casa de vegetação por aproximadamente 7,5 anos, diferente daquelas utilizadas no experimento. A quantidade de UFCs foi comparada entre as bromélias destes dois locais, pois as plantas de casa de vegetação poderiam apresentar menor abundância de bactérias por estarem afastadas do habitat natural. A base da roseta de cada bromélia (10 cm acima do solo) foi cortada em pedaços de aproximadamente 1 cm^2, misturados de forma homogênea e 10 g desse material foi adicionado a 90 mL de água destilada estéril. Foram feitas contagens das UFCs diluídas em 10 mL até 10^{10} mL de água destilada estéril. Alíquotas de 1 mL de cada diluição foram adicionadas em meio de cultura sólido Plate Count Agar (PCA; Neogen® Corporation, Michigan) e em meio mínimo sólido com 5 g de guanina como única fonte de carbono e nitrogênio (Referência S1). Foi utilizado meio contendo guanina, pois é a principal fonte de N presente nas fezes da aranha *Psecas chapoda* (Referência S2). Os meios foram mantidos em 48h a 35°C para posterior contagem das UFCs.

Ciclagem de nutrientes. Para testar se microorganismos presentes na filosfera favorecem a ciclagem de nutrientes disponibilizando-os às plantas, foi feito experimento em casa de vegetação com sistema de irrigação automática com liberação de 6L/h, ativado por 15 min a

cada 6 h. O experimento foi desenvolvido de 9 de março a 9 de maio de 2009, em São José do Rio Preto (estado de São Paulo). Cada cinco bromélias foram submetidas aos seguintes tratamentos a cada três dias: (*Antibiótico*) fezes produzidas pela aranha *Psecas chapoda*, bactérias e antibióticos; (*Sem antibióticos*) fezes das aranhas, bactérias e água destilada estéril; e (*Controle*) nada receberam. As bromélias utilizadas no experimento eram provenientes de mesma coorte, com porte semelhante (e.g., comprimento foliar entre 20 e 25 cm) para minimizar os efeitos da diluição do nitrogênio e tinham porte equivalente às bromélias de campo que suportam duas aranhas de 6^{th} a 8^{th} instar (Referência S2). As bromélias foram mantidas em vasos de 14,5 cm de diâmetro e 14,5 cm de altura, em solo arenoso e pobre em nutrientes (Referência S2), durante 7,5 anos sem contato com aranhas.

As aranhas foram alimentadas com moscas *Drosophila melanogaster* enriquecidas com ^{15}N e suas fezes foram estocadas para posterior aplicação nas bromélias. As moscas foram cultivadas em meio de cultura com levedura marcada com ^{15}N, obtida a partir de levedura comercial cultivada em meio Difco-Bacto à base de carbono, com sulfato de amônio [$(^{15}NH_4)_2SO_4$, 10% de excesso de átomos, do Cambridge Isotope Laboratories, MA]. Para maiores detalhes do enriquecimento de levedura e moscas ver ref. S2. Vinte aranhas (6^{th} a 8^{th} instar) foram coletadas em campo sobre *B. balansae* e mantidas em laboratório. Cada aranha recebeu 15 moscas enriquecidas a cada três dias. Neste mesmo intervalo, as fezes de cada aranha foram coletadas e diluídas em 500 µL de água destilada estéril para posterior aplicação nas bromélias.

As soluções com bactérias foram preparadas a cada 10 dias, mesmo intervalo de aplicação desta solução nas bromélias. Para cada solução, cinco bromélias foram coletadas em campo e a base de suas rosetas (10 cm acima do solo) foram misturadas a 2 L de água

destilada estéril. Cada bromélia dos tratamentos *Antibiótico* e *Sem antibiótico* receberam 200 mL desta solução, que foi borrifada manualmente em toda a roseta. Esta aplicação foi feita seis vezes até o final do experimento.

As bromélias do tratamento *Antibiótico* receberam 100 mL de solução com antibióticos, enquanto que as bromélias do tratamento *Sem antibiótico* receberam 100 mL de água destilada estéril em intervalos de cinco dias, totalizando 12 aplicações até o final do experimento. Ambas as soluções foram borrifadas manualmente em toda a roseta. A solução com antibióticos foi preparada a partir de 10 mg de clorofenicol e 10 mg de amoxicilina diluídos em 5 mL de metanol 1% e posteriormente diluídos em 495 mL de água destilada estéril. Os antibióticos, bem como suas quantidades aplicadas não prejudicaram o metabolismo das plantas, visto que clorofenicol e amoxicilina são antibióticos pouco tóxicos para as plantas e as concentrações aplicadas foram baixas (i.e., 0,02 mg/mL de cada antibiótico por planta a cada cinco dias). Alguns trabalhos mostraram que o uso contínuo de antibióticos pode causar lesões e alterações foliares nas plantas (Referências S3, S4), mas estas alterações não foram observadas durante este experimento. Ao final do experimento, foi feita a contagem de UFCs para todas as bromélias, em PCA e meio mínimo, como descrito anteriormente.

Análises isotópicas. Os valores de $\delta^{15}N$ das moscas, das fezes e das bromélias foram determinados por espectrômetro de massa (20-20 espectrômetro de massa, PDZ Europa, Sandbach, England) após a combustão da amostra para N_2 a 1000°C com analisador elementar on-line (PDZ Europa ANCA-GSL), no laboratório Stable Isotope Facility, University of California em Davis. A fração de nitrogênio absorvido pelas bromélias (f_A) foi

calculada a partir das equações do modelo de mistura com duas fontes de nitrogênio (i.e., solo e fezes) e um elemento isotópico (e.g., $\delta^{15}N$; Referência S5). O fracionamento do ^{15}N durante a assimilação de nitrogênio e os processos metabólicos das plantas foi considerado nas equações, segundo a equação (Referência S6):

$$f_A = \frac{\delta_M - \delta_B - \Delta\delta^{15}N}{\delta_A - \delta_B}$$

na qual f_A é a fração de nitrogênio absorvido pelas bromélias (%), δ_M é a razão isotópica das bromélias que receberam fezes, δ_A e δ_B são as razões isotópicas das potenciais fontes de nitrogênio (fezes e solo, respectivamente) e $\Delta\delta^{15}N$ é a diferença de ^{15}N de acordo com a mudança trófica entre dieta (e.g., fezes ou solo) e consumidor (e.g., bromélias). Os valores de $\Delta\delta^{15}N$ utilizados foram + 3,3 ± 0,26‰ [(média ± erro padrão); Referência S6].

Análise de proteínas solúveis. Para determinar se a presença de bactérias e o nitrogênio proveniente das aranhas influenciam a concentração de proteínas solúveis das plantas, as folhas das bromélias foram cortadas em pedaços (1 cm^2) e 1 g dessas folhas foram congelados em nitrogênio líquido e homogeneizados em 3 mL de água ultra pura Milli-Q. Essa mistura foi centrifugada a 12.000 rpm por 10 min e o sobrenadante (15 µL) foi utilizado para medir a concentração de proteínas solúveis (Referência S7). A absorbância foi medida usando espectrofotômetro a 595 nm e a curva padrão foi obtida com albumina bovina. Os resultados da concentração de proteínas solúveis (µg/g de massa fresca das folhas) foram transformados em $\log_{10}$ e posteriormente comparados entre os tratamentos usando one-way ANOVA.

Crescimento das plantas. Para testar se a presença de bactérias favorece o crescimento das plantas, duas folhas novas (i.e., da primeira ou segunda camada da roseta) de cada bromélia foram escolhidas aleatoriamente e tiveram seus comprimentos medidos no início e no final do experimento. O comprimento foliar está diretamente relacionado com a biomassa seca das folhas (regressão linear simples: $r^2 = 0,84$, $P < 0,001$) e é descrito como a melhor medida de crescimento para bromélias com folhas duras e estreitas (Referência S2). As folhas das bromélias mostraram crescimento contínuo durante o experimento e sua taxa de crescimento relativo (TCR) foi calculada a partir da seguinte equação:

$$TCR = \frac{Ln_{l\,final} - Ln_{l\,initial}}{t_2 - t_1}$$

na qual $Ln_{l\,final}$ e $Ln_{l\,inicial}$ são, respectivamente, o logaritmo natural do comprimento foliar ao final do experimento e o comprimento foliar inicial, sendo que $t_2 - t_1$ é o tempo em dias entre as medições inicial e final. A TCR foi comparada entre os tratamentos usando one-way ANOVA.

Texto suporte

As bromélias coletadas em campo apresentaram $2,96\times10^7 \pm 1,5\times10^7$ UFCs (média ± erro padrão) em meio de cultura PCA e $2,71\times10^6 \pm 1,64\times10^6$ UFCs em meio mínimo. As bromélias mantidas em casa de vegetação (que não participaram do experimento) apresentaram $3,03\times10^7 \pm 2,99\times10^7$ UFCs em meio PCA e nove morfotipos de colônias. Destes nove morfotipos, apenas seis cresceram em meio mínimo contendo guanina.

As bromélias do experimento que receberam antibióticos apresentaram $2,9\times10^3 \pm 4,9\times10^2$ UFCs, ou seja, menor abundância de bactérias ($P < 0,001$) em relação às bromélias

que não receberam antibióticos e as bromélias controle, que apresentaram $7{,}18\times10^6 \pm 1{,}52\times10^6$ UFCs e $3{,}44\times10^6 \pm 1{,}09\times10^6$ UFCs, respectivamente.

Referências

S1. J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular cloning: a laboratory manual* (Cold Spring Harbor Laboratory Press, New York, vol. 1, 1989).

S2. G. Q. Romero, P. Mazzafera, J. Vasconcellos-Neto, P. C. O. Trivelin, *Ecology* **87**, 803 (2006).

S3. L. G. Nickell, A. C. Finlay, *J. Agric. Food. Chem.***2**, 178 (1954).

S4. P. W. Brian, *Annu. Rev. Plant.Physiol.* **8**, 413 (1957).

S5. D. L. Phillips, J. W. Gregg, *Oecologia* **127**, 171 (2001).

S6. J. H. McCutchan, W. M. Lewis, C. Kendall, C. C. McGrath, *Oikos* **102**, 378 (2003).

S7. M. M. Bradford, *Anal. Biochem.* **72**, 248 (1976).

Tabela S1. Valores de $\delta^{15}N$ das moscas *Drosophila melanogaster*, das fêmeas adultas de *Psecas chapoda*, de suas fezes e das bromélias *Bromelia balansae* que receberam os seguintes tratamentos: (*Antibiótico*) fezes da aranha *P. chapoda*, solução com bactérias coletadas em campo sobre *B. balansae* e antibióticos clorofenicol e amoxicilina; (*Sem antibiótico*) fezes da aranha, solução com bactérias e água destilada estéril; e (*Controle*) que nada recebeu

Tratamento	$\delta^{15}N$ (EP)	*N*
Drosophila melanogaster		
Abundância natural	3,07 (0,19)	2
Enriquecido	1530,51 (76,95)	5
Aranha (fêmea adulta)		
Abundância natural	17,08	1
Enriquecido	1028,18 (68,93)	5
Fezes		
Abundância natural	12,1 (2,71)	3
Enriquecido	1234,07 (113,16)	4
Bromelia balansae		
Antibiótico	131,76 (19,34)	5
Sem antibiótico	258,90 (43,85)	5
Controle	8,17 (0,64)	5

Erro padrão encontra-se entre parênteses

n, Número de réplicas

Gonçalves AZ, Mercier H, Ladaslav S, Oliveira RS, Romero GQ.

Fluxo de nutrientes de animais para Bromelioideae e Tillandsioideae (Bromeliaceae)

(Manuscrito não submetido)

Fluxo de nutrientes de animais para Bromelioideae e Tillandsioideae (Bromeliaceae)

Ana Zangirólame Gonçalves[1], Helenice Mercier[2], Ladaslav Sodek[3], Rafael Silva Oliveira[3] and Gustavo Quevedo Romero[1]

[1]Departamento de Biologia Animal, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), CP 6109, CEP 13083-970, Campinas, São Paulo, Brazil

[2]Departamento de Botânica, Instituto de Biociências, Universidade de São Paulo (USP), Rua do Matão, 277, CEP 05508-900, São Paulo, Brazil

[3]Departamento de Biologia Vegetal, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), CP 6109, CEP 13083-970, Campinas, São Paulo, Brazil

Resumo

Uma vez que a roseta das bromélias pode abrigar inúmeros organismos, estes podem contribuir para a nutrição e o desempenho destas plantas. Entretanto, poucos estudos demonstraram fluxo de nitrogênio de animais e detritos vegetais para Bromeliaceae. As espécies de Bromelioideae e Tillandsioideae (Bromeliaceae) apresentam tricomas foliares com capacidades absortivas diferentes e, desta forma, estas plantas podem se beneficiar nutricionalmente em proporções distintas quando interagem com animais. Esta hipótese foi testada em condições controladas em casa de vegetação utilizando métodos isotópicos (^{15}N) e fisiológicos; fezes de um anuro (*Dendropsophus nanus*, usado como modelo de predador) foram aplicadas em espécies de Bromelioideae e Tillandsioideae. Fezes contribuíram em maior proporção para nutrição de *Tillandsia cyanea,* seguida de *Ananas bracteatus*, *Quesnelia arvensis*, *Aechmea blanchetiana*, *Neoregelia cruenta*, *Vriesea gigantea* e *Vriesea bituminosa*. Enquanto bromélias da subfamília Bromelioideae (*An. bracteatus*, *Q. arvensis* e *Ae. blanchetiana*) aumentaram a concentração de proteínas solúveis após aplicação de fezes e cresceram (e.g., *An. bracteatus*), as bromélias da subfamília Tillandsioideae não acumularam proteínas e não cresceram. Este estudo sugere que, enquanto espécies de Bromelioideae transformam nitrogênio em uma forma diretamente utilizável (i.e., proteínas solúveis) para crescimento imediato, as espécies de Tillandsioideae absorvem nitrogênio, porém, não o utilizam diretamente em seu crescimento, talvez o acumulando na forma de aminoácidos (e.g., asparagina).

Palavras-chave: Bromeliaceae, tricomas, fluxo de nitrogênio, *Ananas*, *Aechmea*, *Quesnelia*, *Neoregelia*, *Vriesea*, *Tillandsia*.

Introdução

Bromeliaceae compreende 56 gêneros e aproximadamente 2885 espécies de plantas neotropicais distribuídas em diversos habitats, desde florestas tropicais a savanas secas, campos rupestres e regiões semi-áridas (Crayn *et al.,* 2004; Gitaí *et al.,* 2005). As bromélias podem ocorrer desde o nível do mar até áreas montanhosas e possuem modos de vida terrestre, epífita e até formas mais extremas, como as epífitas atmosféricas (i.e., plantas capazes de absorver nutrientes e água diretamente da atmosfera) (Benzing, 1986, 2000; Crayn *et al.*, 2004). A distribuição desta família em diversos habitats está associada à evolução de seus tricomas foliares e aumento de sua capacidade absortiva, redução do sistema radicular, redução do número de folhas, presença de fitotelmata e diversificação no metabolismo do carbono (Benzing & Burt, 1970; Benzing, 2000). Esta família de plantas é monofilética e compreende as subfamílias Pitcairnioideae, Bromelioideae e Tillandsioideae (Terry *et al.*, 1997; Crayn *et al.*, 2004).

A subfamília Pitcairnioideae é considerada parafilética (Ranker *et al.*, 1990; Terry *et al.*, 1997) e mantém as características plesiomórficas da família, i.e., terrestrialidade, tricomas menos desenvolvidos e metabolismo C3 na grande maioria das espécies (Benzing *et al.*, 1985). Esta subfamília é predominantemente terrestre e depende das raízes na aquisição de nutrientes, uma vez que seus tricomas são estruturalmente os mais simples de Bromeliaceae e não desempenham substancialmente a função absortiva (Benzing, 2000). Entretanto, espécies

de *Brocchinia* possuem tricomas desenvolvidos e fitotelmata, podem ser epífitas, mirmecófitas, carnívoras e hospedeiras de cianobactérias fixadoras de N_2 (Benzing *et al.*, 1985; Benzing, 2000). A espécie *Brocchinia reducta*, por exemplo, apresenta características de planta carnívora, i.e., mecanismos de atração, fixação, digestão e absorção de presas (Givnish *et al.*, 1994). A subfamília Bromelioideae é monofilética e a maioria das espécies possui metabolismo CAM (Benzing, 2000). Alguns gêneros de Bromelioideae (e.g., *Ananas, Bromelia*) são terrestres, não formam fitotelmata, apresentam tricomas não especializados e raízes bem desenvolvidas responsáveis pela absorção de nutrientes e água do solo (Benzing & Burt, 1970; Benzing, 2000; Endres & Mercier, 2003). Entretanto, outros gêneros desta subfamília (e.g., *Aechmea*, *Neoregelia*, *Quesnelia*) são epífitas, formam fitotelmata, possuem tricomas especializados em absorver compostos nitrogenados (e.g., aminoácidos) e suas raízes apenas fixam o vegetal ao substrato (Benzing & Burt, 1970; Benzing, 2000). A subfamília Tillandsioideae é monofilética e possui as características morfo-fisiológicas mais derivadas da família (Benzing *et al.*, 1985; Benzing, 2000). A maioria das espécies de Tillandsioideae é epífita, algumas apresentam fitotelmata (e.g., *Vriesea*), enquanto outras são conhecidas como epífitas atmosféricas (e.g., algumas *Tillandsia*), pois são capazes de absorver nutrientes diretamente da atmosfera (Benzing, 1986, 2000; Martin, 1994). Os membros dessa subfamília possuem os tricomas foliares mais desenvolvidos entre Bromeliaceae, capazes de absorver água e compostos nitrogenados, enquanto suas raízes são finas e basicamente responsáveis pela fixação ao substrato (Sakai & Sanford, 1980; Benzing, 1986, 2000).

A roseta das bromélias é um microhabitat úmido que pode abrigar inúmeros organismos, como microorganismos, invertebrados, vertebrados e plantas vasculares (Gutiérrez *et al.*, 1993; Benzing, 2000; Machado & Oliveira, 2002; Cogni & Oliveira, 2004;

Dias & Brescovit, 2004; Romero & Vasconcellos-Neto, 2004, 2005; Romero, 2005, 2006). Fêmeas de opiliões (e.g., *Bourguyia albiornata*) e várias espécies de formigas utilizam Bromeliaceae como sítios de nidificação (Blüthgen *et al.,* 2000; Benzing, 2000; Machado & Oliveira, 2002; Cogni & Oliveira, 2004). Existem bromélias mirmecófitas que apresentam modificações morfológicas (e.g., *Tillandsia bulbosa*) para abrigar formigas, estabelecendo relações espécie-específicas (Huxley, 1980). O caranguejo *Metopaulias depressus* permanece em bromélias epífitas durante todo seu ciclo de vida e utiliza o reservatório de água das axilas destas bromélias como berçário (Diesel & Schuh, 1993; Diesel, 1992, 1997). Espécies de Anura, como *Eleuthrodactylus jasperi*, podem ser completamente dependentes das bromélias para completarem seu ciclo de vida (Benzing, 2000). Associações entre aranhas e bromélias são comuns (Barth *et al.,* 1988; Dias & Brescovit, 2004; Romero & Vasconcellos-Neto, 2004, 2005; Romero, 2005, 2006), podendo até ocorrer interações mutualísticas entre estes organismos (Romero *et al.*, 2006, 2008).

Apesar de inúmeros estudos terem descrito interações entre animais e Bromeliaceae, poucos demonstraram o fluxo de nitrogênio de animais para bromélias (Romero *et al.,* 2006, 2008, 2010). Enquanto espécies de Bromelioideae podem ser moderadamente beneficiadas pelas interações com animais, espécies de Tillandsioideae podem ser grandemente beneficiadas com estas interações por portarem tricomas epidérmicos mais desenvolvidos (Benzing, 1986, 2000). De fato, estudos independentes, usando métodos diferentes, mostraram que enquanto 18% do nitrogênio total de uma espécie de Bromelioideae (*Bromelia balansae*) derivou de animais (aranhas Salticidae; Romero *et al.*, 2006), animais contribuíram mais para a nutrição de uma espécie de Tillandsioideae (*Vriesea bituminosa*; 28% e 50% do nitrogênio derivado de anfíbios e cupins, respectivamente; Romero *et al.*, 2010). Neste estudo

utilizamos métodos isotópicos (^{15}N) e fisiológicos para avaliar a contribuição de animais para a nutrição de várias espécies de bromélias das subfamílias Bromelioideae e Tillandsioideae. Especificamente, este estudo abordou as seguintes questões: (1) Espécies que possuem características derivadas da família (i.e., epifitismo, fitotelmata, tricomas desenvolvidos) absorvem mais nitrogênio que espécies com características plesiomórficas (i.e., terrestrialidade, tricomas menos desenvolvidos)? (2) Espécies com características derivadas têm aumento na sua concentração de proteínas solúveis quando interagem com animais? (3) O nitrogênio absorvido favorece o crescimento diferenciado entre as espécies com características derivadas e plesiomórficas?

Material e métodos

Organismos

Representantes das subfamílias Bromelioideae (*Ananas bracteatus*, *Aechmea blanchetiana*, *Neoregelia cruenta* e *Quesnelia arvensis*) e Tillandsioideae (*Vriesea bituminosa*, *Vriesea gigantea* e *Tillandsia cyanea*) foram utilizadas para testar a contribuição de animais para a nutrição de bromélias com diferenças morfo-fisiológicas. Destas bromélias, não há descrições da ocorrência de animais em *An. bracteatus* (terrestre) e *T. cyaneae* (epífita).

Aechmea blanchetiana (Bromelioideae) possui fitotelmata, apresenta hábito terrestre, epifítico, ocupa solos arenosos em vegetação de mussununga, campos nativos em Linhares (ES) e restingas em Trancoso (BA) (Romero & Vasconcellos-Neto, 2004). Esta espécie de bromélia é ocupada pelas aranhas *Eustiromastix nativo* e *Psecas* sp. em Linhares e *E. nativo* e lagartos do gênero *Mabuya* em Trancoso (Romero & Vasconcellos-Neto, 2004; Romero,

2006). *Neoregelia cruenta* (Bromelioideae) tem hábito terrestre, possui fitotelmata e é encontrada em áreas de restinga e afloramentos rochosos no litoral brasileiro (Cogliatti-Carvalho *et al.,* 2001; Fernandes *et al.,* 2002; Romero, 2006). Esta espécie de bromélia é ocupada pelas aranhas *Psecas* sp. e *Coryphasia* sp. (Romero, 2006), pelo lagarto *Mabuya macrorhyncha* (Scincidae) na restinga da Barra de Maricá (RJ) (Filho *et al.,* 2001) e por diversas espécies de anuros, principalmente *Xenohyla truncata*, *Scinax alter* e *S. cuspidatus* (Silva *et al.*, 1989). *Quesnelia arvensis* (Bromelioideae) tem hábito terrestre e epifítico, possui fitotelmata e é ocupada pela aranha *Coryphasia* sp. (Romero, 2006), além de ser a bromélia freqüentemente utilizada como sítio de nidificação pela formiga *Gnamptogenys moelleri* (Ponerinae) (Cogni & Oliveira, 2004). *Vriesea bituminosa* (Tillandsioideae) tem hábito terrestre e epifítico, forma fitotelmata e é habitada pela aranha *Coryphasia* sp. em Monte Verde (MG) (Romero, 2006) e pelo anuro *Scinax hayii* (Hylidae) em áreas de Mata Atlântica, desde o estado do Espírito Santo até o estado de Santa Catarina (Romero *et al.*, 2010). *Vriesea gigantea* (Tillandsioideae) tem hábito epifítico, forma fitotelmata (Endres & Mercier, 2001a,b) e é ocupada pelas aranhas *Asaphobelis physonychus*, *Coryphasia* sp. (Romero, 2006) e por anuros (Eterovick, 1999).

O anuro *Dendropsophus nanus* (Hylidae) é um predador de pequeno porte e produz fezes em pequena quantidade, assim como outros anuros de pequeno porte que ocorrem em bromélias. Este animal não é predador bromelícola restrito, porém, foi utilizado neste estudo apenas como modelo generalizado de predador porque muitas espécies de anfíbios são bromelícolas e muito conspícuos sobre estas plantas em florestas tropicais (ver Romero *et al.*, 2010). Fezes de anfíbios são ricas em nitrogênio (i.e., uréia; Lehninger *et al.*, 1993; Duellman

& Trueb, 1994), assim como fezes de muitos outros predadores bromelícolas (e.g., Romero *et al.*, 2006).

Fluxo de nutrientes de dejetos de predadores para bromélias: comparando Bromelioideae e Tillandsioideae

Para testar se bromélias da subfamília Tillandsioideae são mais beneficiadas por associações com animais do que as da família Bromelioideae, desenvolvemos experimentos em casa de vegetação com sistema de irrigação automática com liberação de 8L/h, ativado por 15 min a cada 2 h. O experimento foi feito de 9 de fevereiro até 9 de abril de 2009, em São José do Rio Preto (estado de São Paulo) e teve os seguintes tratamentos: a cada dois dias, seis indivíduos de cada espécie de bromélia receberam: (1) fezes produzidas por um anuro e (2) controle que nada recebeu. Cinqüenta anuros (machos adultos) foram coletados em campo na região de Nova Itapirema (Estado de São Paulo) e mantidos em laboratório em frascos de vidro (7 cm de diâmetro e 10 cm de altura). Cada anuro recebeu 20 moscas *Drosophila melanogaster* enriquecidas a cada dois dias. As fezes produzidas por um anuro, a cada dois dias, foram coletadas com pinça e armazenadas individualmente para posterior aplicação na base da roseta das bromélias. As moscas foram enriquecidas a partir do seu crescimento em meio de cultura contendo levedura marcada com ^{15}N, obtida a partir de levedura comercial cultivada em meio Difco-Bacto à base de carbono, com sulfato de amônio [$(^{15}NH_4)_2SO_4$, 10% de excesso de átomos, do Cambridge Isotope Laboratories, MA]. Para maiores detalhes do enriquecimento de levedura e moscas ver Romero *et al.* (2006).

As bromélias utilizadas no experimento foram plantadas em vasos (14,5 cm de diâmetro, 14,5 cm de altura) contendo solo homogêneo a partir da mistura de casca de *Pinus*

sp., vermiculita e turfa; o substrato (solo) foi o mesmo para todas as espécies estudadas. As plantas tiveram biomassa e tamanho semelhantes (e.g., comprimento foliar variando de 10 a 15 cm).

Análises isotópicas

A concentração total de nitrogênio ($^{15}N + {}^{14}N$) nas folhas das bromélias e os valores de $\delta^{15}N$ das moscas, das fezes e das bromélias foram determinados por espectrômetro de massa (20-20 espectrômetro de massa, PDZ Europa, Sandbach, England) após a combustão da amostra para N_2 a 1000°C com analisador elementar on-line (PDZ Europa ANCA-GSL), no laboratório Stable Isotope Facility, University of California em Davis. A fração de nitrogênio proveniente das fezes absorvido pelas bromélias (f_A) foi calculada a partir das equações do modelo de mistura com duas fontes de nitrogênio (i.e., solo e fezes) e um elemento isotópico (e.g., $\delta^{15}N$; ver Phillips & Gregg, 2001). O fracionamento do ^{15}N durante a assimilação de nitrogênio e os processos metabólicos das plantas foram considerados nas equações, segundo a equação (McCutchan *et al.*, 2003):

$$f_A = \frac{\delta_M - \delta_B - \Delta\delta^{15}N}{\delta_A - \delta_B}$$

na qual f_A é a fração de nitrogênio proveniente das fezes absorvido pelas bromélias (%), δ_M é a razão isotópica das bromélias que receberam fezes, δ_A e δ_B são as razões isotópicas das potenciais fontes de nitrogênio (fezes e solo, respectivamente) e $\Delta\delta^{15}N$ é a diferença de ^{15}N de acordo com a mudança trófica entre dieta (e.g., fezes ou solo) e consumidor (e.g., bromélias). Os valores de $\Delta\delta^{15}N$ utilizados foram + 3,3 ± 0,26‰ [(média ± erro padrão); McCutchan *et al.*, 2003].

Análise da concentração de proteínas solúveis

Para determinar se o nitrogênio absorvido pelas bromélias influencia a concentração de proteínas solúveis, as folhas de cada espécie de bromélia foram cortadas em pedaços pequenos (1 cm^2) e 1 g dessas folhas foi congelado em nitrogênio líquido e homogeneizado em 3 mL de água ultra pura Milli-Q. Essa mistura foi centrifugada a 12.000 rpm por 10 min e o sobrenadante (15 µL) utilizado para medir a concentração de proteínas (Bradford, 1976). A absorbância foi medida usando espectrofotômetro a 595 nm e a curva padrão foi obtida com albumina bovina. Os resultados da concentração de proteínas solúveis (µg/g de massa fresca das folhas) foram transformados em $\log_{10}$ e posteriormente comparados entre os tratamentos usando ANOVA de dois fatores. O teste post hoc Fisher Least Square Difference (LSD) foi utilizado para comparações entre pares de resultados.

Crescimento das bromélias

Para testar se a absorção de nitrogênio favorece o crescimento das plantas, duas folhas novas de cada bromélia foram escolhidas aleatoriamente e tiveram seus comprimentos medidos no início e no final do experimento. O comprimento foliar está diretamente relacionado com a biomassa seca das folhas (regressão linear simples; *An. 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; *Ae. 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). As folhas das bromélias mostraram crescimento contínuo durante o experimento e sua taxa de crescimento relativo (TCR) foi calculada a partir da seguinte equação:

$$TCR = \frac{Ln_{lfinal} - Ln_{linitial}}{t_2 - t_1}$$

na qual $Ln_{l\ final}$ e $Ln_{l\ inicial}$ são, respectivamente, o logaritmo natural do comprimento foliar ao final do experimento e o comprimento foliar inicial, sendo que $t_2 - t_1$ é o tempo em dias entre as medições inicial e final. A TCR foi comparada entre os tratamentos usando two-way ANOVA. O teste post hoc Fisher Least Square Difference (LSD) foi utilizado para comparações entre pares de resultados.

Resultados

As moscas, os anuros e suas fezes foram enriquecidos (Tabela 1). A concentração total de nitrogênio ($^{15}N + {}^{14}N$) nas folhas das bromélias não diferiu entre os tratamentos (Fig. 1). A contribuição de dejetos de predadores variou entre as espécies estudadas (Fig. 2) e foi maior em *Tillandsia cyanea*, na qual 8,4 ± 1,3% (média ± erro padrão) do seu nitrogênio derivaram do anuro, seguida de *Ananas bracteatus* (6,8 ± 1,2%), *Quesnelia arvensis* (3,5 ± 0,5%), *Aechmea blanchetiana* (3,2 ± 0,5%), *Neoregelia cruenta* (2,6 ± 0,6%), *Vriesea gigantea* (2,5 ± 0,4%) e *Vriesea bituminosa* (2 ± 0,2%).

A concentração de proteínas solúveis (Fig. 3) diferiu entre os tratamentos ($F_{1,70}$ = 10,11; P = 0,002), entre as espécies de bromélias ($F_{6,70}$ = 85,56; P < 0,001) e o efeito do tratamento diferiu entre as espécies de bromélias ($F_{6,70}$ = 9,49; P < 0,001). As espécies de Bromelioideae, *An. bracteatus*, *Q. arvensis* e *Ae. blanchetiana* foram as únicas espécies que tiveram aumento significativo na concentração de proteínas solúveis após o recebimento de nitrogênio do predador (*An. bracteatus*: $F_{1,10}$ = 23,20; P < 0,001; *Q. arvensis*: $F_{1,10}$ = 34,50; P < 0,001; *Ae. blanchetiana*: $F_{1,10}$ = 15,02; P = 0,003). *Ananas bracteatus* e *V. bituminosa*

tiveram a maior concentração de proteínas solúveis em relação às outras espécies (Fisher LSD, *Ananas bracteatus* vs. *V. bituminosa*: P = 0,408; *Ananas bracteatus* ou *V. bituminosa* vs. as outras espécies: P < 0,001; Fig. 3), enquanto *T. cyanea* apresentou a menor concentração de proteínas solúveis (Fisher LSD, *T. cyanea* vs. as outras espécies: P < 0,001; Fig. 3).

O crescimento das bromélias diferiu entre os tratamentos ($F_{1,70}$ = 6,88; P = 0,011), entretanto esta diferença somente ocorreu devido à resposta de *An. bracteatus* ao recebimento de fezes do anuro ($F_{1,10}$ = 10,55; P = 0,008; Fig. 4). O crescimento das bromélias diferiu entre as espécies ($F_{6,70}$ = 26,72; P < 0,001), sendo o maior em *An. bracteatus* (Fisher LSD, *An. bracteatus* vs. as outras espécies: P < 0,001).

Discussão

Conforme esperávamos, *Tillandsia cyanea* (Tillandsioideae) foi a espécie que mais derivou nitrogênio de predadores. Possivelmente, isto é resultante de tricomas foliares desenvolvidos, característicos de bromélias da subfamília Tillandsioideae, com ultra-estrutura elaborada e inúmeras mitocôndrias que auxiliam na função absortiva (Sakai & Sanford, 1980; Benzing, 1986, 2000). A absorção rápida e em maior quantidade é importante para *Tillandsia*, pois essas plantas ocupam habitat com chuvas esparsas, onde a água e os minerais precisam ser absorvidos rapidamente quando disponíveis (Benzing *et al.*, 1976). No entanto, as outras espécies de Tillandsioideae (*V. gigantea* e *V. bituminosa*) absorveram quantidade semelhante de nitrogênio em relação às espécies de Bromelioideae estudadas. De fato, Sakai & Sanford (1980) sugeriram que a capacidade absortiva dos tricomas de Bromelioideae com fitotelmata

pode ser comparável à Tillandsioideae. Nossos resultados corroboram os pressupostos de Sakai & Sanford (1980). A quantidade, qualidade e disposição dos tricomas nas folhas de Bromelioideae com fitotelmata podem beneficiá-las de forma semelhante em relação às espécies de Tillandsioideae. Este pode ser um tema para futuras pesquisas.

As espécies de Bromelioideae (*An. bracteatus*, *Q. arvensis* e *Ae. blanchetiana*), com exceção de *N. cruenta*, foram as únicas espécies estudadas que tiveram aumento na concentração de proteínas solúveis após o recebimento de nitrogênio. Ao contrário do esperado, nenhuma espécie de Tillandsioideae acumulou proteínas solúveis em suas folhas após aplicação das fezes de predadores. Estes resultados sugerem que Bromelioideae e Tillandsioideae podem alocar o nitrogênio absorvido de animais para rotas fisiológicas distintas. Alguns trabalhos mostraram que *Ananas comosus* (Bromelioideae) apresenta menor concentração de asparagina nas folhas e crescem mais, enquanto *V. gigantea* (Tillandsioideae) acumula asparagina nas folhas e sua taxa de crescimento é lenta (Endres & Mercier, 2001a,b, 2003). Asparagina não é detectada nas análises de proteínas solúveis (Bradford, 1976) e parece ser o aminoácido mais produzido por bromélias quando recebem nitrogênio, por estar associada ao armazenamento e à reutilização deste nutriente quando o mesmo falta no ambiente (Endres & Mercier, 2001a,b, 2003). Adicionalmente, Benzing (1983) observou que apesar da aplicação de fertilizante em bromélias atmosféricas, estas pouco cresceram. Este autor concluiu que plantas epífitas têm capacidade limitada de crescimento mesmo na adição de nutrientes, podendo ser uma resposta adaptativa para sobreviver em ambientes oligotróficos. Portanto, enquanto espécies de Bromelioideae parecem absorver nitrogênio para transformá-lo em uma forma diretamente utilizável (i.e., proteínas solúveis), as espécies de

Tillandsioideae parecem absorver nitrogênio para acumulá-lo, talvez sob a forma de asparagina para reutilização do mesmo em condições de estresse nutricional.

Neste estudo mostramos que animais contribuem para a nutrição de bromélias, assim como demonstrado por Romero *et al.* (2006, 2008, 2010). Romero *et al.* (2006, 2008) mostraram a existência de mutualismo digestivo entre *Bromelia balansae* e a aranha *Psecas chapoda*, no qual 18% do nitrogênio desta espécie de bromélia derivou da atividade da aranha (i.e., fezes e carcaças de presas). Esta aranha também contribui nutricionalmente com as outras duas bromélias nas quais ocorre, *Ananas comosus* e *Aechmea distichantha* (Capítulo I). Em outro estudo, Romero *et al.* (2010) demonstraram que o anuro bromelícola *Scinax hayii* (Hylidae) contribui com aproximadamente 27,7% do nitrogênio de *Vriesea bituminosa*. Uma vez que muitas espécies de bromélias vivem em ambientes oligotróficos, como afloramentos rochosos, solos arenosos e no dossel de florestas, estas plantas necessitam obter nutrientes de outras fontes. Assim, a partir das folhas organizadas em rosetas que acumulam água e nutrientes e dos tricomas epidérmicos absorventes (Sakai & Sanford, 1980; Martin, 1994; Benzing, 1986, 2000), muitos animais que utilizam bromélias durante seu ciclo de vida podem se tornar uma importante fonte de nutrientes para estas plantas.

Predadores contribuíram em maior proporção para nutrição de *T. cyanea*. As bromélias da subfamília Tillandsioideae não acumularam proteínas e pouco cresceram, enquanto as bromélias da subfamília Bromelioideae (*An. bracteatus*, *Q. arvensis* e *Ae. blanchetiana*) aumentaram a concentração de proteínas solúveis após aplicação de fezes e cresceram (e.g., *An. bracteatus*). Aparentemente parece não existir uma relação direta entre absorção de nitrogênio, concentração de proteínas solúveis nas folhas e crescimento. Este trabalho mostra que as interpretações não são simples a cerca da complexidade dos tricomas, de seu poder de

absorção, associados com as diferentes subfamílias e sua evolução. Estudos detalhados que reúnam capacidade absortiva dos tricomas, sua ultra-estrutura, sua quantidade e distribuição nas folhas podem mostrar que, mesmo em subfamílias com características consideradas plesiomórficas, a absorção de nitrogênio proveniente de animais pode ser representativa. Adicionalmente, estudos fisiológicos detalhados são necessários para mostrar as diferentes rotas de utilização do nitrogênio entre as diferentes subfamílias de bromélias.

Literatura citada

Barth FG, Seyfarth EA, Bleckmann H, Schüch W. 1988. Spiders of the genus *Cupiennius* Simon 1891 (Araneae, Ctenidae). I. Range distribution, dwelling plants, and climatic characteristics of the habitats. *Oecologia* **77**: 187-193.

Benzing DH. 1983. Vascular epiphytes: a survey with special reference to their interactions with other organisms. In: *Tropical rain forest: ecology and management*. Sutton SL, Whitmor TC, Chadwick AC (eds). Blackwell Scientific Publication.

Benzing DH. 1986. Foliar specializations for animal-assisted nutrition in Bromeliaceae. In: *Insects and the plant surface*. Juniper B, Southwood R (eds). Edward Arnold.

Benzing DH. 2000. *Bromeliaceae: profile of an adaptive radiation*. Cambridge University Press.

Benzing DH, Burt KM. 1970. Foliar permeability among twenty species of the Bromeliaceae. *Bulletin of the Torrey Botanical Club* **5**: 269-279.

Benzing DH, Henderson K, Kessel B, Sulak J. 1976. The absortive capacities of bromeliad trichomes. *American Journal of Botany* **63**: 1009-1014.

Benzing DH, Givinish TJ, Bermudes D. 1985. Absorptive trichomes in *Brocchinia reducta* (Bromeliaceae) and their evolutionary and systematic significance. *Systematic Botany* **10**: 81-91.

Blüthgen N, Vehaagh M, Goitía W, Blüthgen N. 2000. Ant nests in tank bromeliads – an example of non-specific interaction. *Insectes Sociaux* **47**: 313-316.

Bradford MM. 1976. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *Analytical Biochemistry* **72**: 248-254.

Cogliatti-Carvalho L, Freitas AFN, Rocha CFD, Sluys MV. 2001. Variação na estrutura e na composição de Bromeliaceae em cinco zonas de restinga no Parque Nacional da Restinga de Jurubatiba, Macaé, RJ. *Revista Brasileira de Botânica* **24**: 1-9.

Cogni R, Oliveira PS. 2004. Patterns in foraging and nesting ecology in the neotropical ant, *Gnamptogenys moelleri* (Formicidae, Ponerinae). *Insectes Sociaux* **51**: 123-130.

Crayn DM, Klaus W, Smith JAC. 2004. Multiple origins of crassulacean acid metabolism and the epiphytic habit in the neotropical family Bromeliaceae. *Proceedings of the National Academy of Sciences* **101**: 3703-3708.

Dias SC, Brescovit AD. 2004. Microhabitat selection and co-occurrence of *Pachistopelma rufonigrum* Pocock (Aranae, Theraphosidae) and *Nothroctenus fuxico* sp. nov. (Aranae, Ctenidae) in tank bromeliads from Serra de Itabaiana, Sergipe, Brazil. *Revista Brasileira de Zoologia* **21**: 789-796.

Diesel R. 1992. Managing the offspring environment – brood care in the bromeliad crab, *Metopaulias depressus*. *Behavioral Ecology and Sociobiology* **30**: 125-134.

Diesel R. 1997. Maternal control of calcium concentration in the larval nursery of the bromeliad crab, *Metopaulias depressus* (Grapsidae). *Proceedings of the Royal Society of London Series B-Biological Sciences* **264**: 1403-1406.

Diesel R, Schuh M. 1993. Maternal care in the bromeliad crab *Metopaulias depressus* (Decapoda) – maintaining oxygen, pH and calcium levels optimal for the larvae. *Behavioral Ecology and Sociobiology* **32**: 11-15.

Duellman WE, Trueb L. 1994. *Biology of amphibians*. The Johns Hopkins University Press.

Endres L, Mercier H. 2001a. Influence of nitrogen forms of the growth and nitrogen metabolism of bromeliads. *Journal of Plant Nutrition* **24**: 29-42.

Endres L, Mercier H. 2001b. Ammonium and urea as nitrogen sources for bromeliads. *Journal of Plant Pysiology* **158**: 205-212.

Endres L, Mercier H. 2003. Amino acid uptake and profile in bromeliads with different habitats cultivated in vitro. *Plant Physiology and Biochemistry* **41**: 181-187.

Eterovick PC. 1999. Use and sharing of calling and retreat sites by *Phyllodytes luteolus* in a modified environment. *Journal Herpetol* **33**:17-22.

Fernandes J, Chaloub RM, Reinert F. 2002. Influence of nitrogen supply on the photoprotective response of *Neoregelia cruenta* under high and low light intensity. *Functional Plant Biology* **29**: 757-762.

Filho PT, Barbosa OR, Paes V, Ribas SC, Almeida JR. 2001. Structure of claws and toes of two tropidurid lizard species of Restinga from Southeastern Brazil: adaptations to the vertical use of the habitat. *Revista Chilena de Anatomia* **19**.

Gitaí J, Horres R, Benko-Iseppon AM. 2005. Chromosomal features and evolution of Bromeliaceae. *Plant Systematics and Evolution* **253**: 65-80.

Givnish TJ, Burkhardt EL, Happel EE, Weintraub JD. 1994. Carnivory in the bromeliad *Brocchinia reducta*, with a cost/benefit model for the general restriction of carnivorous plants to sunny, moist, nutrient-poor habitats. *The American Naturalist* **124**: 479-497.

Gutiérrez MO, Lavin MC, Ayala FC, Perez AJ. 1993. Arthropods associated with *Bromelia hemisphaerica* (Bromeliales: Bromeliaceae) in Morelos, Mexico. *Florida Entomologist* **76**: 616-621.

Huxley C. 1980. Symbiosis between ants and epiphytes. *Biological Reviews* **55**: 321-340.

Lehninger AL, Nelson DL, Cox MM. 1993. Amino acid oxidation and the production of urea. In: *Principles of biochemistry*. Lehninger AL, Nelson DL, Cox MM (eds). 2nd ed. Worth Publisher.

Machado G, Oliveira PS. 2002. Maternal care in the neotropical harvestman *Bourguyia albiornata* (Arachnida: Opiliones): oviposition site selection and egg protection. *Behaviour* **139**: 1509-1524.

Martin CE. 1994. Physiological Ecology of the Bromeliaceae. *The Botanical Review* **1**: 1-82.

McCutchan JHJ, Lewis WMJ, Kendall C, McGrath C. 2003. Variation in trophic shift for stable isotope ratios of carbon, nitrogen and sulfur. *Oikos* **102**: 378-390.

Phillips DL, Gregg JW. 2001. Uncertainty in source partitioning using stable isotopes. *Oecologia* **127**: 171-179.

Ranker TA, Soltis DE, Soltis PS, Gilmartin AJ. 1990. Subfamilial phylogenetic relationships of the Bromeliaceae: evidence from chloroplast DNA restriction site variation. *Systematic Botany* **15**: 425-434.

Romero GQ. 2005. *Associações entre aranhas Salticidae e Bromeliaceae: história natural, distribuição espacial e mutualismos.* Tese de doutorado, Universidade Estadual de Campinas, Campinas, São Paulo.

Romero GQ. 2006. Geographic range, habitats and host plants of bromeliad-living jumping spiders (Salticidae). *Biotropica* **38**: 522-530.

Romero GQ, Vasconcellos-Neto J. 2004. Spatial distribution patterns of jumping spiders associated with terrestrial bromeliads. *Biotropica* **36**: 596-601.

Romero GQ, Vasconcellos-Neto J. 2005. The effects of plant structure on the spatial and microspatial distribution of a bromeliad-living jumping spider (Salticidae). *Journal of Animal Ecology* **74**: 12-21.

Romero GQ, Mazzafera P, Vasconcellos-Neto J, Trivelin PCO. 2006. Bromeliad-living spiders improve host plant nutrition and growth. *Ecology* **87**: 803-808.

Romero GQ, Vasconcellos-Neto J, Trivelin PCO. 2008. Spatial variation in the strength of mutualism between a jumping spider and a terrestrial bromeliad: evidence from the stable isotope ^{15}N. *Acta Oecologica* **33**: 380-386.

Romero GQ, Nomura F, Gonçalves AZ, Dias NYN, Mercier H, Conforto EC, Rossa-Feres DC. 2010. Nitrogen fluxes from treefrogs to tank epiphytic bromeliads: an isotopic and physiological approach. *Oecologia* **162**: 941-949.

Sakai WS, Sanford WG. 1980. Ultrastructure of the water-absorbing trichomes of pineapple (*Ananas comosus*, Bromeliaceae). *Annals of Botany* **46**: 7-11.

Silva HR, Britto-Pereira MC, Caramaschi U. 1989. Frugivory and seed dispersal by *Hyla truncata*, a neotropical treefrog. *Copeia* **3**: 781-783.

Terry RG, Brown GH, Olmstead RG. 1997. Examination of subfamilial phylogeny in Bromeliaceae using comparative sequence of the plastid locus *ndhF*[1]. *American Journal of Botany* **84**: 664-670.

Tabela 1. Valores de $\delta^{15}N$ das moscas *Drosophila melanogaster*, do anuro *Dendropsophus nanus* e de suas fezes

Tratamento	$\delta^{15}N$ (EP)	n
Drosophila melanogaster		
Abundância natural	3,07 (0,19)	2
Enriquecido	1530,51 (76,95)	5
Anuro (macho adulto)		
Abundância natural	12,64 (0,37)	2
Enriquecido	542,04 (82,58)	5
Fezes		
Abundância natural	8,97 (0,21)	3
Enriquecido	1046,47 (50,10)	5

Erro padrão encontra-se entre parênteses

n, Número de réplicas

Tabela 2. Valores de $\delta^{15}N$ para as folhas de *Ananas bracteatus, Neoregelia cruenta, Quesnelia arvensis, Aechmea blanchetiana, Vriesea bituminosa, Vriesea gigantea* e *Tillandsia cyanea* submetidas aos seguintes tratamentos: fezes do anuro *Dendropsophus nanus* e controle

Tratamento	$\delta^{15}N$ (EP)	n
An. bracteatus		
Fezes	70,43 (11,79)	6
Controle	10,68 (0,31)	6
N. cruenta		
Fezes	46,59 (5,72)	6
Controle	9,21 (0,31)	6
Q. arvensis		
Fezes	52,64 (4,62)	6
Controle	8,33 (0,31)	6
Ae. blanchetiana		
Fezes	46,42 (5,08)	6
Controle	11,56 (0,29)	6
V. bituminosa		
Fezes	30,33 (1,39)	6
Controle	8,75 (0,38)	6
V. gigantea		
Fezes	38,86 (3,85)	6
Controle	9,38 (0,44)	6
T. cyanea		
Fezes	84,57 (11,98)	6
Controle	9,02 (0,34)	6

Erro padrão encontra-se entre parênteses

n, Número de réplicas

Legendas das figuras

Fig. 1. Média da concentração total de nitrogênio ($^{15}N + ^{14}N$) nas folhas de *Ananas bracteatus*, *Neoregelia cruenta*, *Quesnelia arvensis*, *Aechmea blanchetiana*, *Vriesea bituminosa*, *Vriesea gigantea* e *Tillandsia cyanea*. Barras indicam erro padrão.

Fig. 2. Porcentagem de nitrogênio de *Ananas bracteatus*, *Neoregelia cruenta*, *Quesnelia arvensis*, *Aechmea blanchetiana*, *Vriesea bituminosa*, *Vriesea gigantea* e *Tillandsia cyanea* derivado do anuro *Dendropsophus nanus*. Valores foram obtidos a partir das equações do modelo de mistura com duas fontes (solo e fezes). Barras indicam erro padrão.

Fig. 3. Concentração de proteínas solúveis em µg/g de massa seca de folhas em *Ananas bracteatus*, *Neoregelia cruenta*, *Quesnelia arvensis*, *Aechmea blanchetiana*, *Vriesea bituminosa*, *Vriesea gigantea* e *Tillandsia cyanea* submetidas aos seguintes tratamentos: fezes do anuro *Dendropsophus nanus* e controle. Barras indicam erro padrão. Comparações post-hoc entre tratamentos são significantes a $P < 0,05$.

Fig. 4. Taxa de crescimento relativo (TCR) de *Ananas bracteatus*, *Neoregelia cruenta*, *Quesnelia arvensis*, *Aechmea blanchetiana*, *Vriesea bituminosa*, *Vriesea gigantea* e *Tillandsia cyanea* submetidas aos seguintes tratamentos: fezes do anuro *Dendropsophus nanus* e controle. Barras indicam erro padrão. Comparações post-hoc entre tratamentos são significantes a $P < 0,05$.

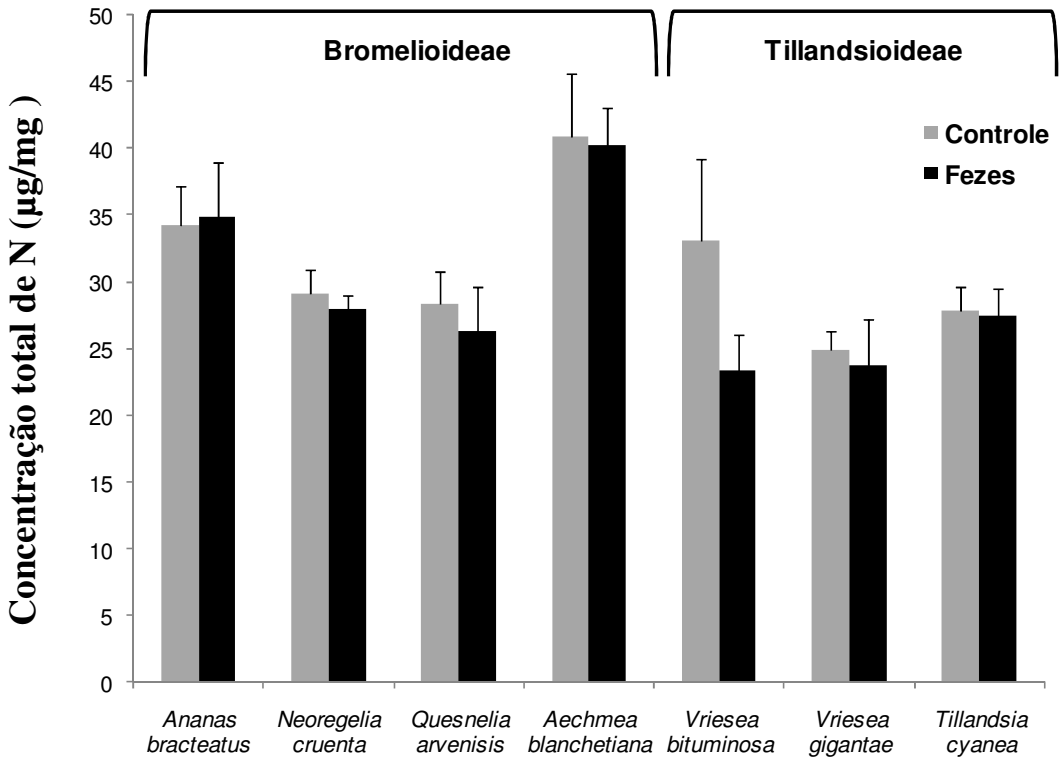

Concentração total de N (µg/mg)
50
45
40
35
30
25
20
15
10
5
0
Bromelioideae
Tillandsioideae
Controle
Fezes
Ananas bracteatus
Neoregelia cruenta
Quesnelia arvenisis
Aechmea blanchetiana
Vriesea bituminosa
Vriesea gigantae
Tillandsia cyanea

Fig. 1

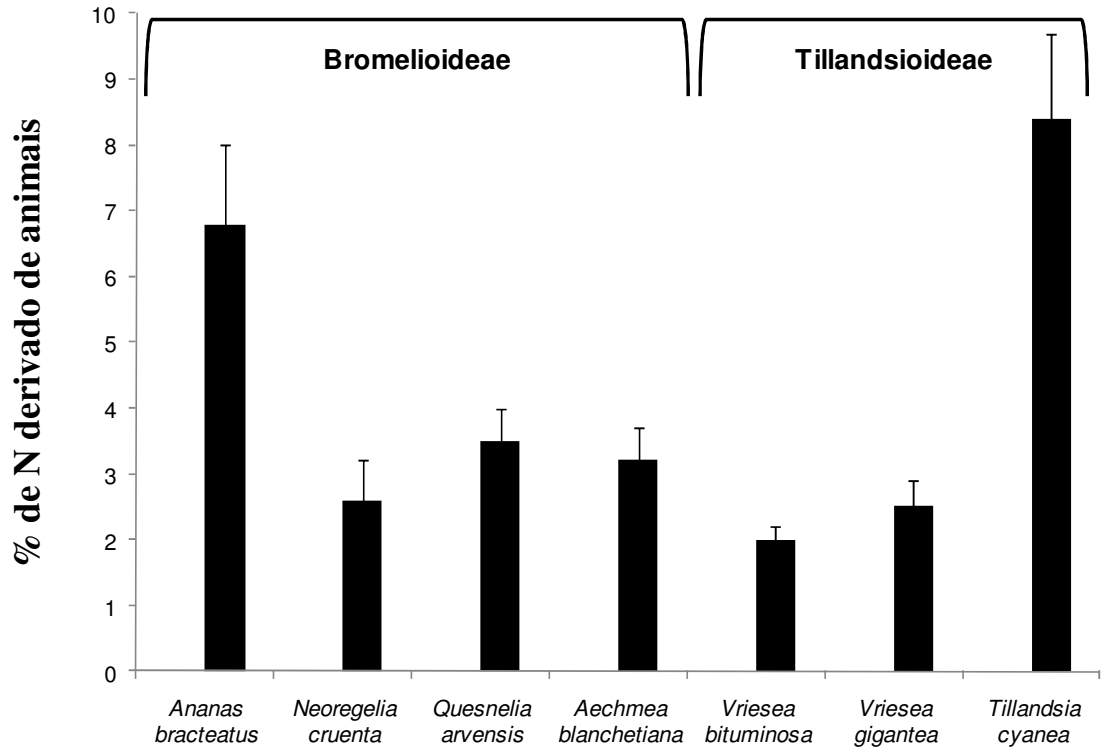

% de N derivado de animais
10
9
8
7
6
5
4
3
2
1
0
Bromelioideae
Tillandsioideae
Ananas bracteatus
Neoregelia cruenta
Quesnelia arvensis
Aechmea blanchetiana
Vriesea bituminosa
Vriesea gigantea
Tillandsia cyanea

Fig. 2

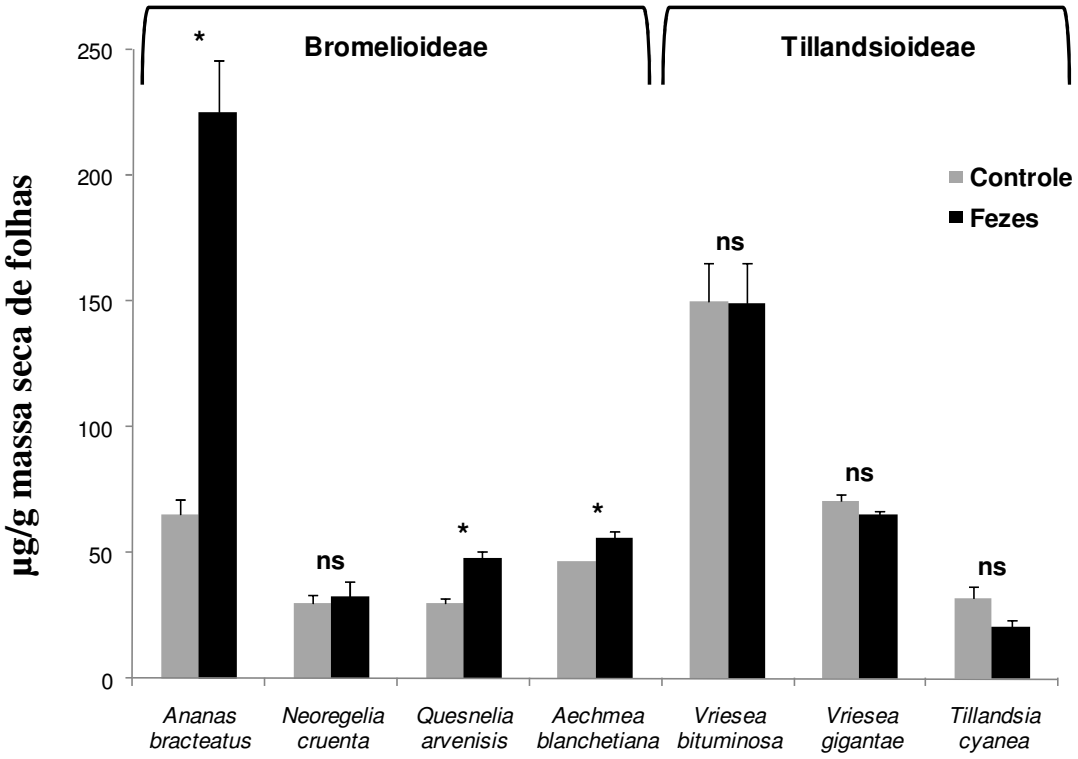

Bromelioideae
Tillandsioideae
µg/g massa seca de folhas
250
200
150
100
50
0
Controle
Fezes
*
ns
*
*
ns
ns
ns
Ananas bracteatus
Neoregelia cruenta
Quesnelia arvenisis
Aechmea blanchetiana
Vriesea bituminosa
Vriesea gigantae
Tillandsia cyanea

Fig. 3

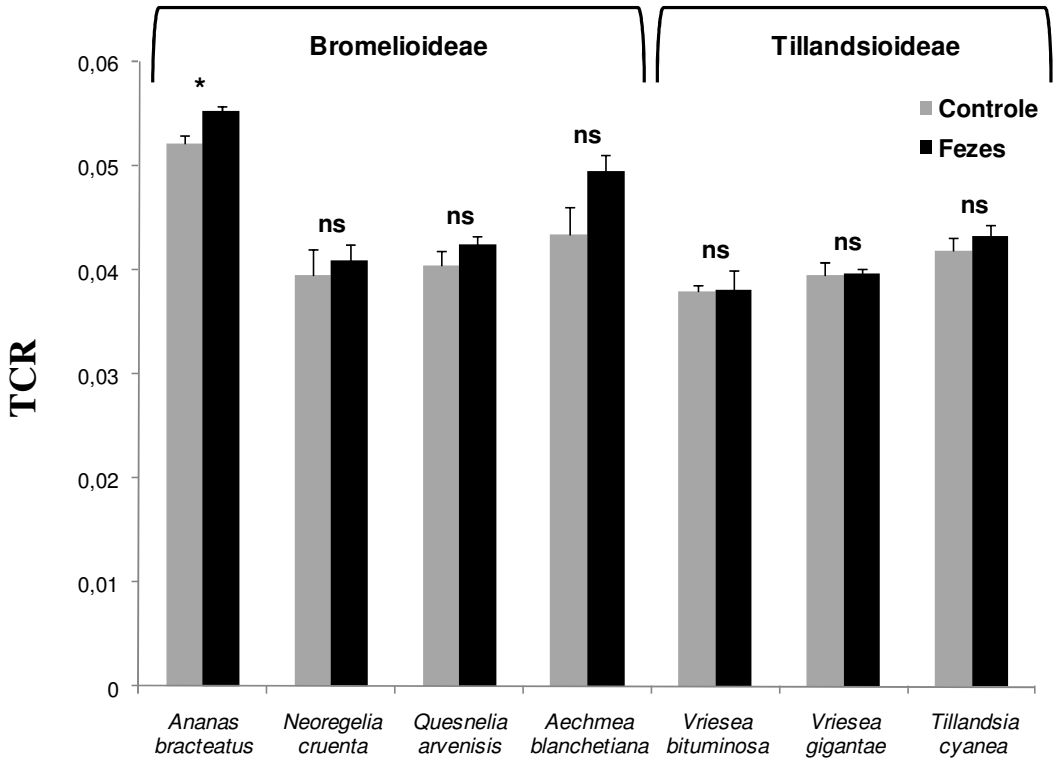

Bromelioideae
Tillandsioideae
Controle
Fezes
TCR
0,06
0,05
0,04
0,03
0,02
0,01
0
*
ns
ns
ns
ns
ns
ns
Ananas bracteatus
Neoregelia cruenta
Quesnelia arvenisis
Aechmea blanchetiana
Vriesea bituminosa
Vriesea gigantae
Tillandsia cyanea

Fig. 4

5. SÍNTESE

Neste trabalho realizamos experimentos em casa de vegetação para testar se a aranha *Psecas chapoda* contribui para a nutrição de suas três bromélias hospedeiras, *Bromelia balansae*, *Ananas comosus* e *Aechmea distichantha*, e se esta contribuição muda de acordo com a sazonalidade (e.g., seco vs. chuvoso). Esta aranha contribui, não somente nutricionalmente, mas também para a produção de proteínas solúveis e para o crescimento de suas três bromélias hospedeiras. Entretanto, esta contribuição ocorreu somente no período chuvoso, indicando que este mutualismo digestivo tem resultados condicionais sazonalmente dependentes. Adicionalmente, ocorreu variação interespecífica na absorção de nitrogênio entre estas espécies de plantas, provavelmente relacionada às diferentes performances e rotas fotossintéticas de cada espécie. Enquanto a bromélia terrestre *B. balansae* parece utilizar o nitrogênio absorvido para crescimento, a espécie epífita *Ae. distichantha* aparentemente acumula nitrogênio para condições de estresse nutricional. Os resultados do experimento descrito no terceiro capítulo reforçam esta hipótese: de modo geral, enquanto espécies de Bromelioideae transformam nitrogênio em uma forma diretamente utilizável (i.e., proteínas solúveis) para crescimento imediato, as espécies de Tillandsioideae (que possuem características derivadas da família Bromeliaceae, i.e., epifitismo, fitotelmata, tricomas desenvolvidos) absorvem nitrogênio, porém, não o utilizam diretamente em seu crescimento, talvez o acumulando na forma de aminoácidos (e.g., asparagina). Adicionalmente, mostramos neste trabalho que bactérias presentes na filosfera de bromélias podem auxiliá-las nutricionalmente, favorecendo sua produção de proteínas solúveis e seu crescimento. As bactérias da filosfera provavelmente disponibilizam compostos orgânicos simples (que podem ser absorvidos facilmente pelos tricomas foliares) por meio do processo de mineralização dos compostos orgânicos complexos retidos na roseta destas plantas.

6. ANEXO

Romero GQ, Nomura F, Gonçalves AZ, Dias NYN, Mercier H, Conforto EC, Rossa-Feres DC. 2010

Nitrogen fluxes from treefrogs to tank epiphytic bromeliads: an isotopic and physiological approach

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Nitrogen fluxes from treefrogs to tank epiphytic bromeliads: an isotopic and physiological approach

Gustavo Q. Romero[1]*, Fausto Nomura[2], Ana Z. Gonçalves[3], Natacha Y. N. Dias[4], Helenice Mercier[5], Elenice de C. Conforto[1], Denise de C. Rossa-Feres[1]

[1] Departamento de Zoologia e Botânica, IBILCE, Universidade Estadual Paulista (UNESP), Rua Cristóvão Colombo, 2265, CEP 15054-000, São José do Rio Preto, São Paulo, Brazil

[2] Departamento de Ecologia, ICB, Universidade Federal de Goiás (UFG), CP 131, CEP 7401-970, Goiânia, Goiás, Brazil

[3] Pós-graduação em Ecologia, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), CP 6109, CEP 13083-970, Campinas, São Paulo, Brazil

[4] Pós-graduação em Biologia Animal, Universidade Estadual Paulista (UNESP), Rua Cristóvão Colombo, 2265, CEP 15054-000, São José do Rio Preto, São Paulo, Brazil.

[5] *Departamento de Botânica, Instituto de Biociências, Universidade de São Paulo (USP), rua do Matão, 277, CEP 05508-900, São Paulo, Brazil.*

Abstract

Diverse invertebrate and vertebrate species live in association with plants of the large Neotropical family Bromeliaceae. Although previous studies have assumed that debris of associated organisms improves plant nutrition, so far little evidence supports this assumption. In this study we used isotopic (^{15}N) and physiological methods to investigate if the treefrog *Scinax hayii*, which uses the tank epiphytic bromeliad *Vriesea bituminosa* as a diurnal shelter, contributes to host plant nutrition. In the field, bromeliads with frogs had higher $\delta^{15}N$ values than those without frogs. Similar results were obtained from a controlled greenhouse experiment. Linear mixing models showed that frog feces and dead termites used to simulate insects that eventually fall inside the bromeliad tank contributed respectively 27.7% (± 0.07 SE) and 49.6% (± 0.50 SE) of the total nitrogen of *V. bituminosa*. Net photosynthetic rate was higher in plants that received feces and termites than in controls; however, this effect was only detected in the rainy, but not in the dry season. These results demonstrate for the first time that vertebrates contribute to bromeliad nutrition, and that this benefit is seasonally restricted. Since amphibian-bromeliad associations occur in diverse habitats in South and Central America, this mechanism for deriving nutrients may be important in bromeliad systems throughout the neotropics.

Key-words: bromeliad-frog interactions, digestive mutualism, nutrient provisioning, Tillandsioideae, *Scinax hayii*

Introduction

Nutrient fluxes across ecological compartments can exert profound direct and indirect effects on the recipient system (Purtauf and Scheu 2005). Although transfer of nutrients from primary producers to higher trophic levels is well established, inverse nutrient fluxes, i.e., from animals to plants, is a much less recognized process (e.g., Anderson and Polis 1999). Nutrient fluxes from animals to plants can occur at multiple scales. For example, on a broad scale, sea bird guano improves the net primary productivity of plants on oceanic islands (Anderson and Polis 1999; Wait et al. 2005), and resource pulses of cicadas (*Magicicada* spp.) in 17-year cycles improves growth and reproduction in temperate forest plants (Yang 2004). On a smaller scale, several studies have reported nutrient fluxes from ants to myrmecophilous plants (Rico-Gray et al. 1989; Treseder et al. 1995; Sagers et al. 2000; Fischer et al. 2003; Solano and Dejean 2004), from mutualistic *Pameridea* bugs (Miridae) to the carnivorous host plant *Roridula* (Anderson and Midgley 2002, 2003), and from the jumping spider *Psecas chapoda* to its Bromeliaceae host plant, *Bromelia balansae* (Romero et al. 2006, 2008). Although diverse invertebrates and vertebrates live associated with plants of the large Neotropical family Bromeliaceae, little is known about whether and how these animals contribute to their host plants performance.

Many species in the family Bromeliaceae typically live in low-nutrient environments (rock outcrops, sandy soils, tree canopies), but have their leaves organized in rosettes, an

arrangement that forms a tank or phytotelmata, which allows them to intercept and retain debris and water (Benzing 2000). Minerals and water accumulated in the rosette can be absorbed through specialized trichomes (Sakai and Sanford 1980; Benzing et al. 1985). Whereas plants of the subfamilies Pitcairnioideae and Bromelioideae seem to be unable to absorb large organic molecules through their trichomes, plants of the subfamily Tillandsioideae (e.g., *Vriesea*) can take complex organic compounds, such as amino acids (Endres and Mercier 2003; Cambuí and Mercier 2006). Therefore, bromeliads of this subfamily could derive nutrients from predator feces and arthropod carcasses in their tanks (Romero et al. 2006).

Many anuran species from South and Central America use bromeliads during their life cycles, although the degree of specialization in the use of this microhabitat varies among species (Peixoto 1995; Richardson 1999; Carvalho-e-Silva et al. 2000; Schineider and Teixeira 2001, Teixeira et al. 2002; Haddad and Prado 2005). For example, some species are specialized, spending their entire life cycle in association with bromeliads, reproducing and feeding among the plant axils, and producing tadpoles with morphological and behavioral adaptations (e.g., *Syncope antenori*, Krugel and Richter 1995; *Phyllodytes luteolus*, Giaretta 1996). Other species use bromeliads during the reproductive period, as calling or oviposition sites or as microhabitat for tadpole development (e.g., *Physalaemus spiniger,* Haddad and Pombal 1998; *Aplastodiscus sibilatus*, Cruz et al. 2003; *Dendrobates pumilio*, Young 1979). At the other extreme, some anurans use bromeliads only as diurnal shelters (e.g., *Eleutherodactylus johnstonei*, Ovaska 1991; *Aparasphenodon brunoi*, Teixeira et al. 2002; *Dendropsophus nahdereri* and *Scinax perereca*, Conte and Rossa-Feres 2006; and *Scinax hayii*, F. Nomura, pers. obs.). Even in those anuran species that only use bromeliad plants as

diurnal shelters, different degrees of specialization can be noted. For instance, *Aparasphenodon brunoi* have a hyperossified helmet-like cranium that protects the species against predators in the bromeliad's tanks (Teixeira et al. 2002), while *Scinax hayii*, also common in bromeliads, has no evident morphological modifications (Carvalho-e-Silva et al. 2000, F. Nomura pers. obs.).

In southeastern Brazil, the treefrog *Scinax hayii* (Hylidae) commonly uses the tank epiphytic bromeliad *Vriesea bituminosa* (Tillandsioideae) as a diurnal shelter, thus comprising a suitable system to test nitrogen fluxes and reciprocal benefits in frog-bromeliad systems. In addition, insects that fall into bromeliad tanks might also contribute to bromeliad nutrition. In this study we conducted field observations and surveys, and performed a controlled greenhouse experiment during two seasons (dry and wet seasons) to address the following questions: (1) Does *S. hayii* contribute to *V. bituminosa* nutrition through its debris (feces)? (2) Does the bromeliad absorb nitrogen derived from insects that die inside its tanks? (3) How much of the bromeliads nitrogen is derived from treefrog feces and from dead insects? (4) How does nitrogen uptake from treefrogs and dead insects influence the physiology of the bromeliad (e.g., gas exchanges, protein and chlorophyll concentrations)? (5) Do these responses vary in different seasons?

Material and methods

Study system

Vriesea bituminosa (Tillandsioideae) is a tank epiphytic bromeliad that commonly inhabits trees in Brazilian Rainforests, but can also occur in inselberg and cerrado vegetation (Romero

2006; Versieux and Wendt 2007, G. Q. Romero, pers. obs.). Its rosettes are large and colorful, and accumulate large amounts of rainwater (range for four small bromeliads: 240-260 ml). Because of these traits, it is preferred by landscape designers for use in private gardens, and is frequently extracted from the wild for this type of commercial use; thus it is an endangered species (Versieux and Wendt 2007). This plant's rosette can shelter several animal groups, including spiders, ants and frogs (Romero 2006, G.Q. Romero, personal observations, F. Nomura, unpubl. data).

The treefrog *Scinax hayii* (Hylidae) frequently uses *V. bituminosa* as diurnal shelter. This is a common, medium sized frog (about 43 mm total length) belonging to the *Scinax ruber* clade (Faivovich et al. 2005), which is endemic of the Atlantic Rainforest and occurs from Espírito Santo to Santa Catarina states, southeastern Brazil. This species lives in primary and secondary lowland and montane forests, along forest edges, in secondary vegetation, and even inside houses. Usually it occurs on low vegetation near streams and ponds (Carvalho-e-Silva and Carvalho-e-Silva 2004). It breeds in pools along streams and ponds between September and March, and often shelters in bromeliad rosettes during the day (F. Nomura, pers. obs.). It commonly returns to the same bromeliad after a night of reproduction (F. Nomura, unpublished data). Individuals are usually found alone in the bromeliads, but sometimes a single bromeliad can shelter up to three individuals of S. *hayii* (F. Nomura, pers. obs.).

Nutrient fluxes from frogs to plants: tests using isotopic methods

Field surveys

We first investigated the isotopic signature of ^{15}N (natural abundance) of field *V. bituminosa* bromeliads that were either inhabited by treefrogs or uninhabited. Bromeliads of similar size, at heights varying from 0.85 to 8.0 m, were randomly inspected and the presence/absence of frogs was recorded. These epiphytic bromeliads inhabited diverse host species and likely accumulated litter fallen from variable host tree types. Of the 31 bromeliads inspected, 10 had frogs. For each bromeliad inspected we collected one randomly selected leaf from the median layer (third-fourth node) for isotopic analysis. The survey was done in December 2006 near a natural lake at a humid mountain summit (1291 m asl) in the Atlantic Rainforest (i.e., ombrophitic dense high-montane forest) near Atibaia city (23º10'S; 46º31'W), São Paulo State, in southeastern Brazil. The local climate shows a distinct dry/cold (May–September) and a wet/warm (October–April) season. Mean annual rainfall is 128 mm and mean annual temperature is 20 °C (CIIAGRO 2008).

Experimental design

The nitrogen flux from frogs and insects to *V. bituminosa* was experimentally tested in a greenhouse at the Universidade Estadual Paulista, São José do Rio Preto city. The feces added to the plants were obtained from frogs of *S. hayii* fed with workers of the termite *Nasutitermes* sp. (Termitidae) collected in a field at the University. To obtain feces, 11 frogs (adults) were collected in the field (Atibaia city). They were kept in five 65 x 40 x 50 cm terraria in the laboratory and fed termites *ad libitum* for 64 days. The frog feces were collected daily, dried in a vacuum oven, weighed and then stored individually in polypropylene tubes. To avoid or minimize any effect of imprint of food the frogs ate prior to capture, the first feces produced

were discarded, and the reminder feces were randomly applied in the experimental bromeliads (treatment 1 below). Termites were also collected, dried, weighed and stored to be used in the experiment. The experiment had three treatments: (1) six bromeliads received frog feces inside the tank, (2) six bromeliads received termites, and (3) six control bromeliads received no organic matter. For the experimental treatments, either one feces acorn (mean ± 1 SD of the dry weight: 0.152 g ± 0.011; n = 50) or the same amount of dry weight of termites were deposited in the central tank of bromeliads at two-day intervals over 103 days, from January 25 to May 17, 2006.

Experimental bromeliads were cultured in the greenhouse, and thus had no previous contact with animals. They were of the same cohort, and were cultured in pots (20 cm width, 15 cm height) containing vegetal earth maintained in shade, under a 50% sunlight regime to simulate their natural environments. The size of the experimental bromeliads was similar (leaf length: 25cm) and corresponded to those in nature which support only one frog. Bromeliads were watered through an automatic irrigation system by fine spray using three sprinklers, each with capacity of 8L/ h, which worked for 15 min every 2 h.

At the end of the experiment (May 17), we collected two new leaves (first node) of each experimental bromeliad for isotopic determinations (^{15}N).

Isotopic analyses

The bromeliad leaves from both the field and the greenhouse experiments were washed for at least 3 min in a current of water and scrubbed by hand to eliminate contamination (organic particles or mites). Bromeliad leaves were oven-dried for 30 h at 65ºC, ground to a fine

powder in a ball mill, and transferred to airtight containers. Frog feces and muscle tissue, as well as samples of termites used in the experiment (n = 5) were frozen and dried for isotopic determinations. Stable isotope analyses were done by the Stable Isotope Facility at the University of California at Davis. Stable isotope ratios of N and C, as well as nitrogen concentration (μg of total N/mg of dried plant tissue), were determined by continuous flow isotope ratio mass spectrometer (IRMS) (20-20 mass spectrometer, PDZ Europa, Sandbach, England) after sample combustion to CO_2 and N_2 at 1000°C by an on-line elemental analyzer (PDZ Europa ANCA-GSL). Isotope ratios of C for leaves of field and greenhouse bromeliads were determined to characterize the photosynthetic pathway (i.e., C_3 or CAM) of *V. bituminosa*.

Natural ^{15}N and ^{13}C abundances were calculated using $\delta^{15}N = (R_{sample}/R_{standard} - 1)$ X 1000 (‰ versus At-air) and $\delta^{13}C = (R_{sample}/R_{standard} - 1)$ X 1000 (‰ versus V-PDB), where R is the ratio of mass 15/14 and 13/12 for nitrogen and carbon, respectively.

Physiological responses

Gas exchange analyses

Net photosynthetic rate per leaf unit area (A), transpiration rate (E), stomatal conductance (gs), intercellular CO_2 concentration (Ci), and vapor pressure difference between plant and environment (Δw) were measured on intact, attached leaves of all experimental bromeliads in the greenhouse using an ADC LCA-4 infra-red portable gas analyzer system and PLCB-4 leaf chamber (ADC, Hoddesdon, UK). The chamber was positioned at the middle, central region of mature leaves from the median rosette nodes (3^{th} to 4^{th} node). Gas exchange data were

recorded as soon as readings became stable, usually 60-120 s after leaf insertion into the chamber. Three to five replications were carried out for each experimental rosette, but only mean values were used in the statistical analyses. Measurements were done on clear days at two experimental periods, one at the end of rainy season (April 12, 2007) and one in the dry season (May 17, 2007), from 8:00 to 10:30 a.m. During the measurements air temperature and relative air humidity were 31.2±0.1°C and 48.3±0.4% (SE) in April 12, and 31.5±0.3°C and 38.9±0.7% in May 17. Mean photosynthetic photon flux density (PPFD) in April 12 and May 17 were 1548±17.7 and 1131±50.2 μmol m^{-2} s^{-1} (SE), respectively.

Chlorophyll and protein analyses

Immediately after the leaf gas exchange measurements on May 17, leaves were collected for biochemical analyses. Concentrations of chlorophyll a and b, carotenoid, and total soluble protein (TSP) were determined from the same bromeliad leaf. Total chlorophyll (a+b) was obtained from fresh leaves (1 g), by cutting in little pieces, freezing in liquid nitrogen and homogenizing at ca. 4°C in 7 ml of cold 80% acetone (v:v, acetone:water). The homogenized product was filtered in a paper funnel previously sprayed with 2 ml of cold 80% acetone, and the residue was washed three times with 4 ml of cold 80% acetone; the volume was topped up to 20 ml with acetone at 80%. The absorbance was measured in a spectrophotometer (Ultrospec 3000; Cambridge, England) at 470 nm, 647 nm and 663 nm and then calculated through equations developed by Lichtenthaler (1987).

Total soluble protein was determined according to Bradford (1976). Fresh leaves (1 g) were cut in little pieces, frozen in liquid nitrogen and homogenized in ca. 3 ml of ultra-pure

water. After centrifugation at 12,000 rpm for 10 min, the supernatant (15 µl) was mixed with 185 µl of the Comassie Brilliant Blue G-250 dye solution, obtained from 100 mg of the dye dissolved in 95% ethanol with 100 ml of 85% phosphoric acid. After five minutes, absorbance was read in a spectrophotometer at 595 nm. A calibration curve of protein concentration was made with bovine serum albumin (BSA).

Statistical analyses

$\delta^{15}N$ and nitrogen concentration values were compared between field bromeliads with and without *S. hayii* using ANCOVA, with presence/absence of frogs as a fixed factor (1 level) and distance of the bromeliads from the lake and height of the bromeliads on trees as the covariates; the dependent variable presented homogeneous variance (Cochran's test; P = 0.815). Data on $\delta^{15}N$ and nitrogen concentration values of experimental bromeliads were compared among treatments using ANOVA, with treatment as a fixed effect (2 levels). Data on $\delta^{15}N$ presented homogeneous variance (Cochran's test; P = 0.485); data on nitrogen concentration were log transformed to equalize the variances. Fisher Least Square Difference (LSD) *post hoc* test was used for pair-wise comparisons.

To determine the fraction of nitrogen (% $N_{df\ feces\ or\ termites}$) that experimental bromeliads derived from soil, termites and frog feces (mixture), we used linear mixing models developed by Phillips and Gregg (2001). Through sensitivity analysis, this model assesses the relative importance of the isotopic signature difference between two sources (e.g., soil and frog feces, or soil and termite carcasses), the standard deviation of isotopic signatures in the sources and mixed populations, sample size, analytical standard deviation, and evenness of the source

populations to determine the standard error (SE) of source proportion estimates (Phillips and Gregg 2001). Mean $\delta^{15}N$ values of leaves of *V. bituminosa* that received termites or feces from frogs that fed on termites were used as the signature for source mixture, mean $\delta^{15}N$ values of leaves of the control plants were used as the soil end-member, and mean $\delta^{15}N$ values for termites or feces from frogs that fed on termites were used as the animal end-member. Fractionation for plant absorption of animal debris is largely unknown. However, a previous unpublished study (A.Z. Gonçalves et al.) detected only an insignificant fractionation in nitrogen fluxes from *Drosophila* flies and spider feces (guanine) to the three bromeliad species *Bromelia balansae*, *Aechmea distichantha* and *Ananas comosus*; Thus, fractionation of N was not calculated here.

Data on net photosynthetic rate per leaf unit area (A), transpiration rate (E), stomatal conductance (gs), intercellular CO_2 concentration (Ci), and vapor pressure difference between plant and environment (Δw) were $\log_{10}$ or $\log_{10}$ (n+1) transformed for variance homogenizations and then compared using repeated measures ANOVA, with treatment as a fixed effect (2 levels) and sampling dates (April 12 and May 17, 2007) as the repetition factor. Separate analyses of variance were also performed to test the influence of feces and termites for specific sampling dates. Data on chlorophyll a, chlorophyll b, chlorophyll a+b, carotenoid and soluble protein concentrations (µg/g fresh leaf mass) were $\log_{10}$ transformed and compared among treatments using one way ANOVA. When necessary, Fisher Least Square Difference (LSD) *post hoc* test was used for paired comparisons.

All analyses of variance were run using Type III sums of squares (SS). All statistical analyses were performed using GLM. The mean values (±1 SE) presented in the figures, tables, and text, were computed directly from untransformed data.

Results

In the field, bromeliads with frogs had higher $\delta^{15}N$ values than those without frogs (Fig. 1a; ANCOVA: $F_{1, 25} = 5.31$, $P = 0.029$). In contrast, the nitrogen concentration in the leaf tissues did not differ between bromeliads with and without frogs (Fig. 1b; ANCOVA; $F_{1, 23} = 0.46$, $P = 0.50$). The covariates "distance from the margin" and "height of the bromeliad", as well as the interaction terms between these covariates and treatment (presence/absence of frogs) did not affect the results of $\delta^{15}N$ ($P \geq 0.36$) or nitrogen concentration ($P \geq 0.65$). In the greenhouse experiment, $\delta^{15}N$ values of bromeliad leaves differed among treatments ($F_{2, 15} = 10.5$, $P = 0.001$). $\delta^{15}N$ values were higher after treatment with frog feces and termites compared to control plants (ANOVA/Least Significant Difference; feces vs. control: $P < 0.001$; termites vs. control: $P = 0.004$; Fig. 1c), and there was no statistical difference between treatments with feces and termites (ANOVA/LSD; $P = 0.339$; Fig. 1c). Similar results were also obtained for nitrogen concentration (Fig. 1d; $F_{2, 15} = 3.95$, $P = 0.042$; pair-wise comparisons: feces vs. control: $P = 0.032$; termites vs. control: $P = 0.025$, feces vs. termites: $P = 0.897$).

The $\delta^{15}N$ values of frog feces, termites and frog tissue were 6.15±1.1 (SE), 3.22±0.12 and 0.88±0.65 (n = 5), respectively. Using the linear mixing model we estimated that frog feces and dead termites contributed 27.7% (± 0.07 SE) and 49.6% (± 0.50 SE) respectively, of the total N of *V. bituminosa*. The feces and termite bodies, including soft and hard (chitin) parts, were decomposed after 4-6 days of application inside the bromeliad tanks.

Vriesea bituminosa had $\delta^{13}C$ values varying from -26.01 to -30.29 ‰ (n = 31), corresponding to the range of the C_3 bromeliads. Net photosynthetic rate per leaf unit area, transpiration rate, stomatal conductance, intercellular CO_2 concentration, and vapor pressure difference between plant and environment did not differ among the treatments in our analyses of variance considering the repeated measures analyses of variance (Table 1). However, separate one way analyses of variance for season showed that net photosynthetic rate (A), transpiration rate (E), and vapor pressure difference between plant and environment (Δw) differed among treatments (Fig. 2) during the rainy season (A: $F_{2, 15} = 4.13$, $P = 0.037$; E: $F_{2, 15} = 4.26$, P 0.034; Δw: $F_{2, 15} = 3.74$, $P = 0.048$), but not at the beginning of the dry season (Fig. 2). Net photosynthetic rate and transpiration rate were higher in plants that received feces and termites than the control plants during the rainy season (Fig. 2). Though transpiration rate (E), stomatal conductance (gs), and intercellular CO_2 concentration increased from the wet (April 12) to the dry season (May 17), the net photosynthetic rate of bromeliads that received feces and termites decreased to levels of control plants in this period (Fig. 2, Table 1).

Concentrations of chlorophyll a, chlorophyll b, chlorophyll a+b, and carotenoids tended to be higher in the frog feces treatment, but the values did not differ significantly among the treatments (Table 2). In contrast, total soluble protein concentration differed among the treatments ($F_{2, 15} = 14.39$, $P < 0.001$), and was higher for bromeliads that received termites and control than those that received feces (Table 2).

Discussion

Our results clearly demonstrated a nitrogen flux from the associated treefrog *S. hayii* to the tank epiphytic bromeliad *V. bituminosa*. Since in our experiment we did not use frog urine, which is a nitrogen-rich compound (urea; Lehninger et al. 1993; Duellman and Trueb 1994), we suggest that the contribution of frogs to bromeliad nutrition might be even higher in the field. This anuran species primarily uses bromeliads as a diurnal shelter, and should excrete an amount of their feces and urine in reproductive and foraging sites when outside bromeliads (i.e., lakes, ponds, streams). In contrast, several other amphibian species are more specialized in bromeliads and use these microhabitats throughout their life cycle as reproductive, foraging, calling, and egg laying sites, and as microhabitat for tadpole development (Young 1979; Krugel and Richter 1995; Giaretta 1996; Haddad and Pombal 1998; Cruz et al. 2003). In these frog-bromeliad systems, bromeliads likely derive more nutrients from frogs. This should be a suitable theme for future research.

Whereas frog feces contributed 27.7% of the total nitrogen of the Tillandsioideae species *V. bituminosa*, dead termites used to simulate insects that fall in bromeliad tanks contributed 49.6% of the bromeliad nitrogen. Contrasting results were obtained by Romero et al. (2006), which verified that a terrestrial bromeliad *Bromelia balansae* (Bromelioideae) derived only 3% of nitrogen from insect carcass, whereas it derived 15% from spider feces. It is well established that terrestrial bromeliads without extensive phytotelmata from the subfamily Bromelioideae (e.g., *Bromelia*, *Ananas*) bear less specialized absorptive leaf trichomes and are assumed to depend mostly on their roots for soil inorganic nutrient acquisition (e.g., Endres and Mercier 2003). In contrast, tank-bromeliads with epiphytic habits from the subfamily Tillandsioideae (e.g., *Vriesea*) have specialized foliar trichomes, thus are better adapted to use larger organic molecules containing N (Owen and Thomson 1988;

Endres and Mercier 2001). Our findings support the hypothesis of Romero et al. (2006), which suggested that tank-bromeliads may benefit even more from animal nutrient input than do terrestrial bromeliads.

The contrasting difference between feces and termite contribution to bromeliad nutrition (27.7% vs. 49.6%) was unexpected and can be partially explained by the fact that urine was not used in the experiments, as mentioned above. In the digestion process, an amount of termite nitrogen could have been sequestered in frog urine, or assimilated by the predator. Even so, we believe that field bromeliads should not take up large quantity of nitrogen derived from insect carcass. Only small numbers of insects that fall inside the bromeliad tank die; many survive by climbing out of the tank on the rosette leaves (G. Q. Romero, unpubl. data). In contrast to carnivorous bromeliads, which attract insects by releasing nectar-like odors from extra-floral nectaries in the rosettes (e.g., *Brocchinia reducta*, Pticairnioidea; Givnish et al. 1984), *V. bituminosa* has no apparent mechanism of insect attraction (G. Q. Romero, pers. obs.). However, it has a tank filled by rain water that can be used as diurnal shelter against predators and/or desiccation by associated insectivorous predators, like amphibians. By foraging outside the bromeliads, frogs can concentrate a considerable amount of nitrogen-rich compounds (feces and urine) inside the tank bromeliad when returning to their diurnal shelter, in an analogous way to carnivorous plants. The water from the tank may thus help the plant digest and use larger organic compounds not completely digested by the frogs. Yet, mineralization of nitrogen compounds could be further accelerated by microorganisms living in the tanks of *Vriesea* bromeliads (see Inselsbacher et al. 2007).

Bromeliads from the field that sheltered frogs had higher $\delta^{15}N$ values than those without frogs. However, while field bromeliads in the absence of frogs had similar total

nitrogen concentration compared to those with frogs, control plants from the experiment had smaller nitrogen concentration than those that received termites or feces. These results indicate that field bromeliads take up certain amount of nitrogen from frogs (data on $\delta^{15}N$), but in the absence of these vertebrates they do not become nutritionally depleted. In the field, these bromeliads likely take up organic compounds or minerals derived from litter, which accumulate in large amounts in their tank and is quickly decomposed by detritivores (see Ngai and Srivastava 2006). Similarly, Romero et al. (2008) showed that *Bromelia balansae* from open grasslands were associated with the spider *Psecas chapoda* and had higher $\delta^{15}N$ values compared to forest bromeliads; however, forest bromeliads had higher N concentrations. Therefore, bromeliads in the field seem to use different sources of nitrogen. The influence of variable N sources (i.e., animal or vegetal) on bromeliad nutrition and growth deserves more attention, and is a suitable theme for future research.

Studies have independently reported that several amphibian species live associated to bromeliads (see introduction), and that urea supply, a soluble compound rich in amphibian excretes (Lehninger et al. 1993; Duellman and Trueb 1994), can increase free-$NH4^+$ and total amino acids in tissues of *Vriesea gigantea* (Endres and Mercier 2001). In this study we show that nitrogen derived from feces of bromeliad-dwelling frogs in fact improve net photosynthetic rate of *V. bituminosa*. Intriguingly, bromeliads that received frog feces had soluble proteins decreased by a half. This result suggests that *V. bituminosa* is able to store differently the nitrogen depending on the source accumulated in the tank. Bromeliads in which the only source was soil or soil plus termite may have stored nitrogen as soluble proteins, while bromeliads that received feces may accumulate nitrogen as amino-acids (e.g., asparagine), which is not detected by the Bradford method (see methods). Endres and Mercier

(2003) showed that *Vriesea gigantea* grown with ammonium, glutamine or glycine accumulated large amounts of asparagine (Asn). Accumulation of nitrogen sources as Asn may be a suitable way to improve growth in epiphytic bromeliads, once transamination reactions easily transform Asn to other amino-acids (Endres and Mercier 2003).

Several bromeliad species are tank epiphytes, therefore have no contact with the pedosphere and thus need to obtain N from other sources. Many epiphytic bromeliads live in association to amphibians, like *V. bituminosa*, thus frog-supplied N could provide a major benefit to these plants. However, the benefit provided by frogs observed here was temporally restricted, i.e., photosynthetic rate was high in the wet season, but decreased to control levels in the dry season. Decreased photosynthetic rate under drought conditions has been reported for other bromeliad species (reviewed in Martin 1994). It is well established that, for vascular epiphytes (including *Vriesea sanguinolenta*), the most relevant abiotic constraint for growth and vegetative functions is water shortage, while other factors, such as nutrient availability, are generally of less importance (Laube and Zotz 2003). Our results suggest that even in the presence of nitrogen-rich compounds derived from amphibian debris, the plants did not benefit during moisture-limited periods.

As previously thought, frogs associated with epiphytic bromeliads can contribute to host plant nutrition and performance (Endres and Mercier 2001). In addition, our results suggest that tank epiphytic bromeliads may derive more nitrogen from associated animals than terrestrial bromeliads, which lack tanks (see Romero et al. 2006). Although nitrogen derived from frog feces improved net photosynthetic rate of *V. bituminosa*, this type of benefit provided by frogs was temporally restricted and seems to be related to water stress during the dry season. This is the first study to show nutrient provisioning in a frog-bromeliad system.

Because several amphibians live associated with Bromeliaceae in South and Central America, this nutrient transfer phenomenon may be common throughout the Neotropics.

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References

Anderson B, Midgley JJ (2002) It takes two to tango but three is a tangle: mutualists and cheaters on the carnivorous plant *Roridula.* Oecologia 132: 369-373.

Anderson B, Midgley JJ (2003) Digestive mutualism, an alternate pathway in plant carnivory. Oikos 102:221-224.

Anderson WB, Polis GA (1999) Nutrient fluxes from water to land: seabirds affect plant nutrient status on Gulf of California islands. Oecologia 118:324-332.

Benzing DH (2000) Bromeliaceae: profile of an adaptative radiation. Cambridge University Press, Cambridge, UK.

Benzing DH, Givnish TJ, Bermudes D (1985) Absorptive trichomes in *Brocchinia reducta* (Bromeliaceae) and their evolutionary and systematic significance. Systematic Botany 10:81–91.

Bradford MM (1976) Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Analytical Biochemistry 72:248-254.

Cambuí CA, Mercier H (2006) Organic nitrogen nutrition in plants. Current Topics in Plant Biology 7:13-17.

Carvalho-e-Silva SP, Izecksohn E, Carvalho-e-Silva AMPT (2000) Diversidade e ecologia de anfíbios em restingas do sudeste brasileiro. Pp. 89–97. In Esteves FA, Lacerda LD (eds), Ecologia de restingas e lagoas costeiras. NUPEM/UFRJ, Macaé, Rio de Janeiro, Brazil.

Carvalho-e-Silva SP, Carvalho-e-Silva AMT (2004) *Scinax hayii*. In: IUCN 2008. 2008 IUCN Red List of Threatened Species. <www.iucnredlist.org>. Downloaded on 22 December 2008

CIIAGRO 2008. http://www.ciiagro.sp.gov.br

Conte CE, Rossa-Feres DC (2006) Diversidade e ocorrência temporal da anurofauna (Amphibia, Anura) em São José dos Pinhais, Paraná, Brasil. Revista Brasileira de Zoologia 23:162-175.

Cruz CAG, Pimenta BVS, Silvano DL (2003) Duas novas espécies pertencentes ao complexo de *Hyla albosignata* Lutz & Lutz, 1938, do leste do Brasil (Amphibia, Anura, Hylidae).

Boletim do Museu Nacional, Zoologia 503:1-13.

Duellman WE, Trueb L (1994) Biology of amphibians. The Johns Hopkins University Press, Baltimore. 696 p.

Endres L, Mercier H (2001) Ammonium and urea as nitrogen sources for bromeliads. Journal of Plant Physiology 158:205–212.

Endres L, Mercier H (2003) Amino acid uptake and profile in bromeliads with different habitats cultivated in vitro. Plant Physiology and Biochemistry 41:181–187.

Ehleringer JR, Osmond CB (2000) Stable Isotopes. In Pearcy RW, Ehleringer JR, Mooney HA, Rundel PW (eds), Plant Physiological Ecology: Field Methods and Instrumentation. Kluwer Academic Publishers, Dordrecht. pp. 281-300.

Faivovich J, Haddad CFB, Garcia PCA, Frost DR, Campbell JA, Wheeler WC (2005) Systematic review of the frog family Hylidae, with special reference to Hylinae: phylogenetic analysis and taxonomic revision. Bulletin of the American Museum of Natural History 294:1-240.

Fischer RC, Wanek W, Richter A, Mayer V (2003) Do ants feed plants? A ^{15}N labeling study of nitrogen fluxes from ants to plants in the mutualism of *Pheidole* and *Pipier*. Journal of Ecology 91:126-134.

Giaretta AA (1996) Reproductive specializations of the bromeliad hylid frog *Phyllodytes luteolus*. Journal of Herpetology 30:96-97.

Givnish TJ, Burkhardt EL, Happel RE, Weintraub JD (1984) Carnivory in the bromeliad *Brocchinia reducta*, with a cost/benefit model for the general restriction of carnivorous plants to sunny, moist, nutrient-poor habitats. American Naturalist 124:479–497.

Haddad CFB, Pombal JP (1998) Redescription of *Physalaemus spiniger* (Anura: Leptodactylidae) and description of two new reproductive modes. Journal of Herpetology 32:557-565.

Haddad CFB, Prado CPA (2005) Reproductive modes in frogs and their unexpected diversity in the Atlantic Forest of Brazil. BioScience 55:207-217.

Inselsbacher E, Cambui CA, Richter A, Stange CF, Mercier H, Wanek W (2007) Microbial activities and foliar uptake of nitrogen in the epiphytic bromeliad *Vriesea gigantea.* New Phytologist 175:311–320.

Krungel P, Richter S (1995) *Syncope antenori* - a bromeliad breeding frog with free-swimming, non-feeding tadpoles (Anura: Microhylidae). Copeia 1995:955–963.

Laube S, Zotz G (2003) Which abiotic factors limit vegetative growth in a vascular epiphyte? Functional Ecology 17:598-604.

Lehninger AL, Nelson DL, Cox MM (1993) Amino acid oxidation and the production of urea. In: Lehninger AL, Nelson DL, Cox MM (eds). Principles of biochemistry, 2nd ed. Worth Publisher, New York, pp 503–541.

Lichtenthaler HK (1987) Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods in Enzymology 148:350-382.

Martin GE (1994) Physiological ecology of the Bromeliaceae. The Botanical Review 60:1-82.

Ngai JT, Srivastava DS (2006) Predators accelerate nutrient cycling in a bromeliad ecosystem. Science 314:963.

Ovaska K (1991) Reproductive phenology, population structure, and habitat use of the frog *Eleutherodactylus johnstonei* in Barbados, West Indies. Journal of Herpetology 25:424-430.

Owen TP Jr., Thomson WW (1988) Sites of leucine, arginine, and glycine accumulation in the absorptive trichomes of a carnivorous bromeliad. Journal of Ultrastructure and Molecular Structure Research 101:215–223.

Peixoto OL (1995) Associação de anuros a bromeliáceas na Mata Atlântica. Revista da Universidade Rural, Série Ciência da Vida 17:75–83.

Phillips DL, Gregg JW (2001) Uncertainty in source partitioning using stable isotopes. Oecologia 127:171–179.

Purtauf T, Scheu S (2005) Nutrient and resource dynamics and food webs: Understanding the mutual relationships between the dynamics of food webs, resources, and nutrients. In: Ruiter P, Wolters V, Moore JC (eds). Dynamic food webs: multispecies assemblages, ecosystem development, and environmental change. Academic Press, MA, USA. 590 pp.

Richardson BA (1999) The bromeliad microcosm and the assessment of faunal diversity in a Neotropical Forest. Biotropica 31:321–336.

Rico-Gray V, Barber JT, Thien LB, Ellgaard EG, Toney JJ (1989) An unusual animal-plant interection: feeding of *Schomburgkia tibicinis* (Orchidaceae) by ants. American Journal of Botany 76:603-608.

Romero GQ (2006) Geographic range, habitats, and host plants of bromeliad-living jumping spiders (Salticidae). Biotropica 38:522–530.

Romero GQ, Mazzafera P, Vasconcellos-Neto J, Trivelin PCO (2006) Bromeliad-living spiders improve host plant nutrition and growth. Ecology 87:803-808.

Romero GQ, Vasconcellos-Neto J, Trivelin PCO (2008) Spatial variation in the strength of mutualism between a jumping spider and a terrestrial bromeliad: evidence from the stable isotope ^{15}N. Acta Oecologica 33:380-386.

Sagers CL, Ginger SM, Evans RD (2000) Carbon and nitrogen isotopes trace nutrient exchange in an ant-plant mutualism. Oecologia 123:582-586.

Sakai WS, Sanford WG (1980) Ultrastructure of the water-absorbing trichomes of pineapple (*Ananas comosus*, Bromeliaceae). Annals of Botany 46:7–11.

Schineider JAP, Teixeira RL (2001) Relacionamento entre anfíbios anuros e bromélias da restinga de Regência, Linhares, Espírito Santo, Brasil. Iheringia 91:41–48.

Solano PJ, Dejean A (2004) Ant-fed plants: comparison between three geophytic myrmecophytes. Biological Journal of the Linnean Society 83:433-439.

Teixeira RL, Schineider JAP, Almeida GI (2002) The occurrence of amphibians in bromeliads from a southeastern Brazilian Restinga habitat, with special reference to *Aparasphenodon brunoi* (Anura, Hylidae). Brazilian Journal of Biology 62:263–268.

Treseder KK, Davidson DW, Ehleringer JR (1995) Absorpition of ant-provided carbon and nitrogen by a tropical epiphyte. Nature 357:137-139.

Versieux LM, Wendt T (2007) Bromeliaceae diversity and conservation in Minas Gerais state, Brazil. Biodiversity and Conservation 16:2989–3009.

Wait DA, Aubrey DP, Anderson WB (2005) Seabird guano influences on desert islands: soil chemistry and herbaceous species richness and productivity. Journal of Arid Environments 60:681–695.

Yang LH (2004) Periodical cicadas as resource pulses in North American forests. Science 306: 1565-1567.

Young AM (1979) Arboreal movement and tadpole-carrying behavior of *Dendrobates pumilio* Schmidt (Dendrobatidae) in Northeastern Costa Rica. Biotropica 11:238-239.

Table 1. Repeated measures analyses of variance (ANOVAs) examining the effect of termite and frog feces on bromeliad physiological variables: net photosynthetic rate per leaf area unit (A), transpiration rate (E), stomatal conductance (gs), intercellular CO_2 concentration (Ci), and vapor pressure difference between plant and environment (Δw). The sample treatments were performed on two sampling dates (April 12 and May 17, 2007).

Source of variation	df	*MS*	*F*	*P*
Net photosynthetic rate (A)				
Treatment	2	0.03163	1.88	0.187
Error	15	0.01685		
Time	1	0.04629	7.61	0.015
Time X Treatment	2	0.02038	3.35	0.062
Error	15	0.00608		
Transpiration rate (E)				
Treatment	2	0.061346	3.06	0.077
Error	15	0.020074		
Time	1	0.100103	7.80	0.014
Time X Treatment	2	0.008458	0.69	0.532
Error	15	0.012839		
Stomatal conductance (gs)				
Treatment	2	0.000039	1.51	0.252
Error	15	0.000025		
Time	1	0.000495	27.48	<0.001
Time X Treatment	2	0.000007	0.39	0.685
Error	15	0.000018		
Intercellular CO_2 concentration (Ci)				
Treatment	2	0.0018	0.39	0.685
Error	15	0.0045		
Time	1	0.2384	91.48	<0.001
Time X Treatment	2	0.0022	0.86	0.443
Error	15	0.0026		
Vapor pressure difference (Δw)				
Treatment	2	0.04204	3.33	0.064
Error	15	0.01264		
Time	1	0.08800	8.91	0.009

Time X Treatment	2	0.00999	1.01	0.387
Error	15	0.00988		

Table 2. Chlorophyll a, chlorophyll b, chlorophyll a+b, carotenoid and total soluble protein concentrations (µg/g fresh leaf mass) of bromeliads grown under three treatments (control, termites added and feces added). Each value represents the mean of six replicates, and standard errors are indicated between parentheses. Different letters indicate significant differences ($P<0.05$; ANOVA/Fisher LSD *post hot* test; $\alpha = 0.05$).

Parameters	Treatments		
	Control	Termites added	Feces added
Chlorophyll a	337.0 (13.9) a	352.7 (29.9) a	397.2 (46.7) a
Chlorophyll b	159.3 (7.7) a	153.6 (18.1) a	193.4 (30.1) a
Chlorophyll a + b	496.4 (20.5) a	506.3 (35.5) a	590.6 (76.3) a
Carotenoids	35651.5 (2368.7) a	37585.4 (3243.4) a	41460.8 (4442.8) a
Total soluble protein	479.0 (41.1) a	442.3 (42.8) a	240.8 (29.5) b

Figure captions

Fig.1. Mean (a) $\delta^{15}N$ and (b) total nitrogen concentration ($^{15}N + {}^{14}N$) values in leaf tissues from field-grown bromeliads with or without frogs, and mean (c) $\delta^{15}N$ and (d) total nitrogen concentration in leaf tissues from greenhouse-grown bromeliads that received either no supplementation (control), termites, or the feces of frogs that were fed termites. Different letters indicate significant differences ($P<0.05$; ANOVA/Fisher LSD *post hot* test; $\alpha = 0.05$). Error bars indicate ± 1 SE.

Fig.2. Values of net photosynthetic rate per leaf area unit (a), transpiration rate (b), stomatal conductance (c), intercellular CO_2 concentration (d), and vapor pressure difference between plant and environment (e) of *Vriesea bituminosa* rosettes that received termites, frog feces and control, measured on two sampling dates (April 12 and May 17, 2007). Different letters indicate significant differences ($P<0.05$; ANOVA/Fisher LSD post hot test; $\alpha = 0.05$). Error bars indicate ± 1 SE.

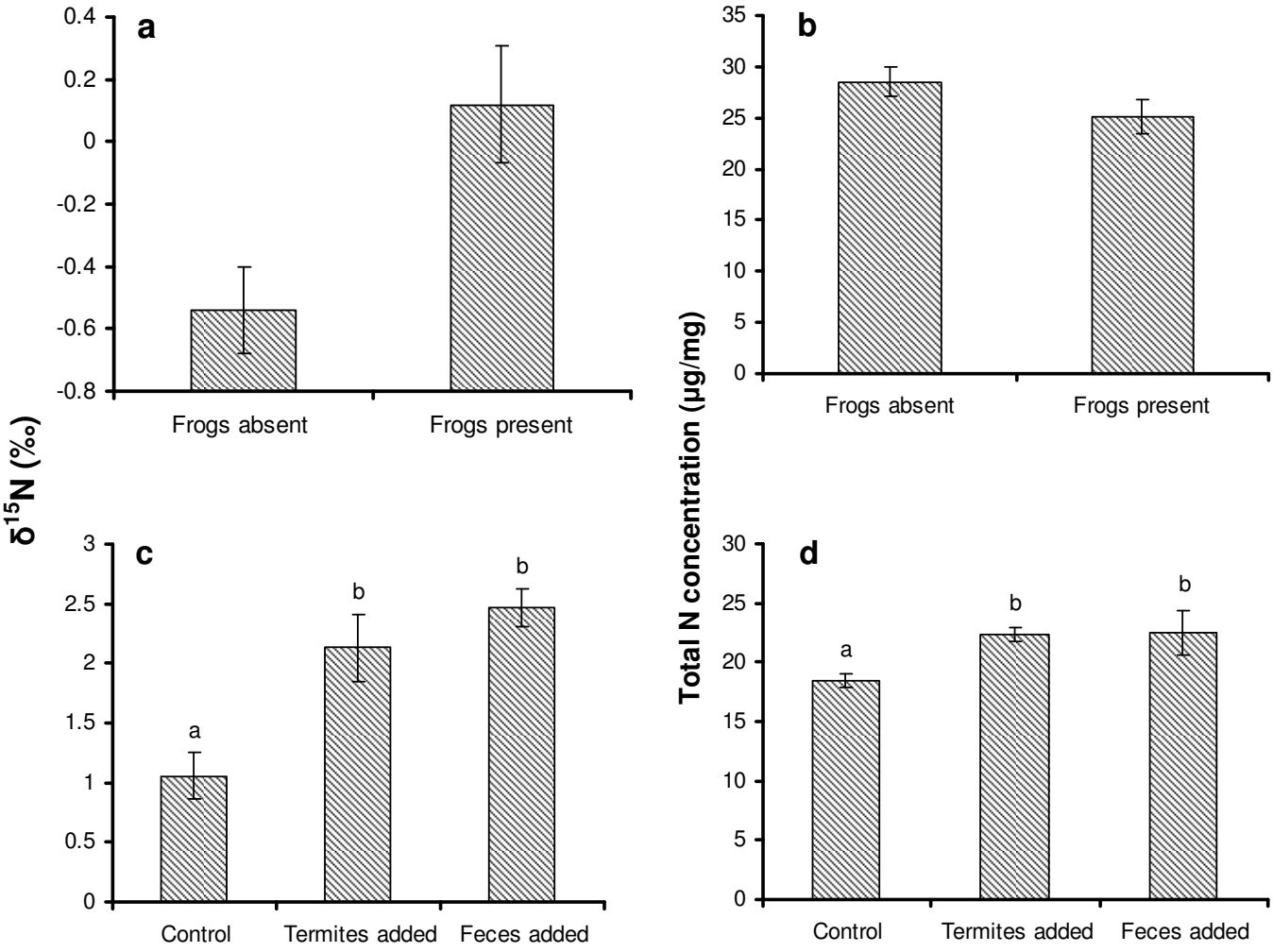
a
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
Frogs absent
Frogs present
δ15N (‰)
b
35
30
25
20
15
10
5
0
Frogs absent
Frogs present
Total N concentration (µg/mg)
c
3
2.5
2
1.5
1
0.5
0
a
b
b
Control
Termites added
Feces added
d
30
25
20
15
10
5
0
a
b
b
Control
Termites added
Feces added

Fig. 1

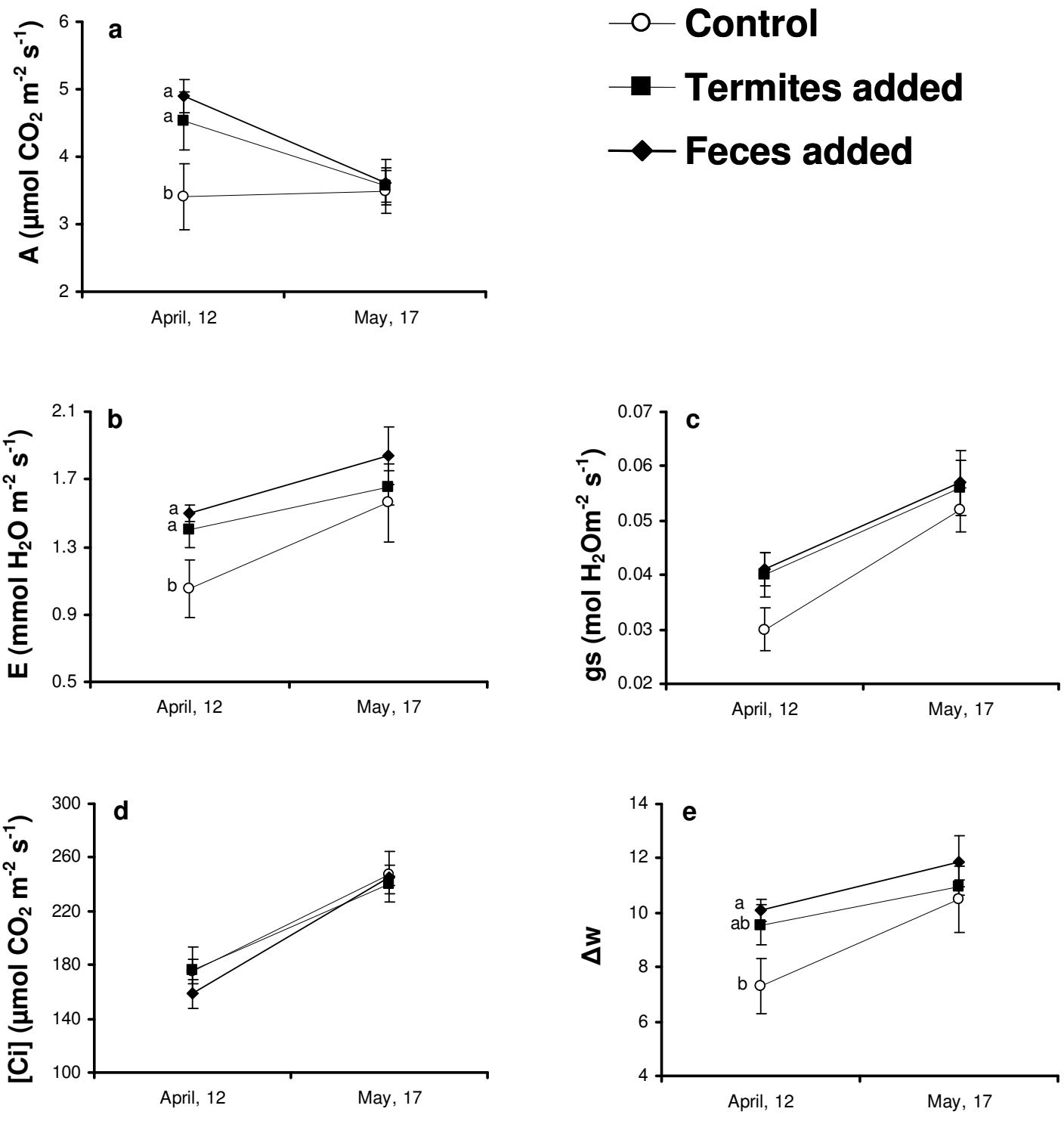
Control
Termites added
Feces added
a
A (µmol CO2 m-2 s-1)
6
5
4
3
2
a
a
b
April, 12
May, 17
b
E (mmol H2O m-2 s-1)
2.1
1.7
1.3
0.9
0.5
a
a
b
April, 12
May, 17
c
gs (mol H2Om-2 s-1)
0.07
0.06
0.05
0.04
0.03
0.02
April, 12
May, 17
d
[Ci] (µmol CO2 m-2 s-1)
300
260
220
180
140
100
April, 12
May, 17
e
Δw
14
12
10
8
6
4
a
ab
b
April, 12
May, 17

Fig. 2