

# Nutritional benefits from domatia inhabitants in an ant–plant interaction: interlopers do pay the rent

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## Summary

**1.** How a symbiosis originates and is maintained are important evolutionary questions. Symbioses in myrmecophytes (plants providing nesting for ants) are believed to be maintained by protection and nutrients provided by specialist plant-ants in exchange for nesting spaces (called domatia) and nourishment offered by ant-plants. However, besides the benefits accrued from housing protective ants, the mechanisms contributing to the fitness advantages of bearing domatia have rarely been examined, especially because the domatia trait is usually constitutively expressed, and many myrmecophytes have obligate mutualisms with single ant species resulting in invariant conditions.

**2.** In the unspecialized ant-plant *Humboldtia brunonis* (Fabaceae) that offers extrafloral nectar to ants, only some plants produce domatia in the form of hollow internodes. These domatia have a self-opening slit making them more prone to interlopers and are occupied mostly by non-protective ants and other invertebrates, especially arboreal earthworms. The protection mutualism with ants is restricted in geographical extent, occurring only at a few sites in the southernmost part of this plant's range in the Western Ghats of India.

**3.** We examined nutrient flux from domatia residents to the plant using stable isotopes. We found that between 9% (earthworms) and 17% (protective or non-protective ants) of nitrogen of plant tissues nearest the domatium came from domatia inhabitants. Therefore, interlopers such as earthworms and non-protective ants contributed positively to the nitrogen budget of localized plant modules of this understorey tree. <sup>15</sup>N-enriched feeding experiments with protective ants demonstrated that nutrients flowed from domatia inhabitants to nearby plant modules. Fruit set did not differ between paired hand-pollinated inflorescences on domatia and non-domatia bearing branches. This was possibly due to the nutrient flux from domatia to adjacent branches without domatia within localized modules.

**4.** This study has demonstrated the nutritive role of non-protective ants and non-ant invertebrates, hitherto referred to as interlopers, in an unspecialized myrmecophyte. Our study suggests that even before the establishment of a specialized ant–plant protection mutualism, nutritional benefits conferred by domatia inhabitants can explain the fitness benefits of bearing domatia, and thus the maintenance of a trait that facilitates the establishment of a specialized ant–plant symbiosis.

**Key-words:** arboreal earthworm, myrmecophyte, myrmecotrophy, nutritional ecology, stable isotopes, trophic interaction

## Introduction

Beneficial symbioses can be mediated by the emergence of novel traits. For instance, experimental affixing of artificial dwellings for arachnids, that is, arachnodomatia, on leaves

improved the fitness of the experimental plants, bearing these domatia by providing shelters for predatory spider mites (Agrawal & Karban 1997). Myrmecophytes, a dominant plant form in many tropical ecosystems, are plants that provide dwellings, that is, domatia, for ants in the form of swollen hollow internodes, thorns, stipules, leaf pouches or chambers within epiphytic tubers (Davidson &

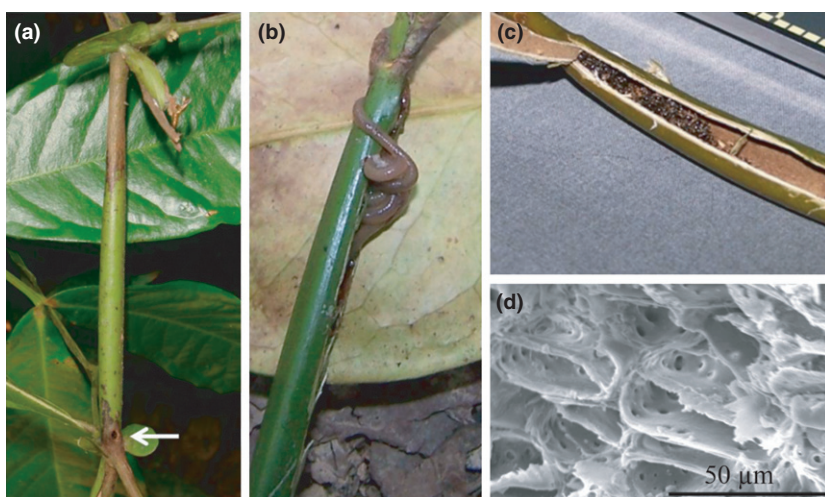
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McKey 1993). The fitness benefits of the domatia trait in a myrmecophyte have rarely been directly investigated (Gaume *et al.* 2005b). This is because, in addition to the difficulty of measuring lifetime fitness, domatia are usually constitutively expressed and it is difficult therefore to examine the fitness of plants with and without domatia. Domatia are, however, believed to contribute to the fitness of myrmecophytes by housing ants that protect plants from herbivory (Heil & McKey 2003; Rico-Gray & Oliveira 2007; Rosumek *et al.* 2009; Trager *et al.* 2010) and that also contribute to the nitrogen budget of the plant either directly through absorption by the plant of nitrogen from ant wastes (Gay 1993; Treseder, Davidson & Ehleringer 1995; Sagers, Ginger & Evans 2000; Fischer *et al.* 2003; Solano & Dejean 2004; Defosse *et al.* 2011; Bazile *et al.* 2012; Dejean *et al.* 2012, 2013; Gegenbauer *et al.* 2012) or via fungi cultivated by the ants within the domatia (Leroy *et al.* 2011). Those protective ant–plant mutualisms, in which nutrient flux also occurs between ants and plants, are usually obligate and specialized interactions in which the domatia of individual plants are usually occupied by a single ant colony (Sagers, Ginger & Evans 2000; Fischer *et al.* 2003; Defosse *et al.* 2011). In such mutualisms, the beneficial ant partner and the domatia trait co-occur. Such specialized interactions provide limited insight into the origin and maintenance of the domatia trait since all plants bear domatia, and most plants and domatia are occupied by beneficial ants. Our understanding of the evolution of domatia and the symbioses they facilitate in myrmecophytes would therefore be greatly enhanced by investigation of non-specialized ant-plants which may harbour non-protective domatia residents, or of those that do not always produce domatia.

Fortunately, domatia in some myrmecophytes house non-protective ants as well as non-ant invertebrates (Dejean & Djieto-Lordon 1996; Krombein *et al.* 1999; Rickson *et al.* 2003; Gaume *et al.* 2006; Moog *et al.* 2008). Such domatia residents are usually referred to as ‘interlopers’ and are considered either opportunistic commensals or

parasites of the ant–plant mutualism (Gaume & McKey 1998). Whether these domatia residents contribute nutrients to their hosts is not known. Only two studies have reported cases where galls or domatia-like structures are induced by ants; in such cases, ants stimulate the production of their own dwellings (Blüthgen & Wesenberg 2001; Edwards *et al.* 2009). The unspecialized ant-plant *Humboldtia brunonis* Wallich (Fabaceae) is therefore unique since within the same population some individuals bear caulinary domatia (Fig. 1) while others do not (Gaume *et al.* 2005b), although all individuals bear extrafloral nectaries on the leaves and bracts of floral buds. This system provides the opportunity for natural experiments on the fitness benefits of bearing domatia (Gaume *et al.* 2005b).

The domatia of *H. brunonis* are occupied by 16 species of ants across its distribution range, of which only one ant, *Technomyrmex albipes* (Dolichoderinae), provides significant protection against herbivory and that too at only restricted locations (Shenoy & Borges 2010). Besides ants, *H. brunonis* domatia are occupied by myriad non-ant invertebrates, of which an arboreal earthworm *Perionyx pullus* (Gaume *et al.* 2006) (Fig. 1b) is the most abundant, occupying up to 40% domatia in certain sites (Shenoy & Borges 2010), followed in abundance by *Crematogaster dohrni*, an ant that castrates flowers at the bud stage by damaging reproductive organs (Gaume, Zacharias & Borges 2005a). These domatia residents, with the exception of a resident pollinating bee, *Braunsapis puangensis* (Shenoy & Borges 2008), do not offer any obvious benefit to the host plant. However, trees bearing domatia had greater fruit set than those without domatia (Gaume *et al.* 2005b). The fitness benefit was even greater when the domatia were occupied by the protective ant (Gaume *et al.* 2005b). This suggests that other benefits accrue to plants that bear domatia, besides the protection gained by harbouring protective ants. Such benefits that are unrelated to biotic defence could contribute to explaining why domatia are maintained in populations from which the mutualistic ant is absent.



**Fig. 1.** Domatium of *Humboldtia brunonis* (a) caulinary domatium with arrow pointing to self-opening slit. (b) Earthworms emerging from an experimentally opened domatium. (c) The inside of an ant-occupied domatium. (d) Scanning electron microscope image of the cells of the inner wall of a domatium, showing pitted cell walls.

We therefore investigated whether the increased fitness of domatia-bearing trees of *H. brunonis* could be due to nutritional benefits acquired by such trees from their domatia inhabitants. Because *H. brunonis* is an understory woody tree, and may have localized, modular circulation of nutrients as occurs in many woody plants (Sprugel, Hinckley & Schaap 1991; Orians, Ardón & Mohammad 2002; Zanne *et al.* 2006), we specifically examined whether within a domatia-bearing tree, domatia-related trophic benefits are restricted to the domatia-bearing modules. We used stable isotopes of carbon and nitrogen to measure nutrient flow.

In order to understand nutrient flux between domatia residents and the ant-plant, and possible fitness benefits of such flux to the plant, we asked the following questions:

1. Is host extrafloral nectar (EFN) an important carbon source for ants residing in the domatia?
2. Does the host plant acquire nitrogen derived from the domatia inhabitants? If so, what fractions of localized plant tissue nitrogen are contributed separately by protective ants, non-protective ants and arboreal earthworms?
3. Do domatia-bearing modules have greater fruit set than branches without domatia on the same tree?

The present study thus investigated trophic interaction between *H. brunonis* and its domatia residents and tests nutritional or trophic mutualism as a basis for the maintenance of the domatia trait in unspecialized ant-plants, in addition to, or in the absence of a protection mutualism with ants.

## Materials and methods

### STUDY SPECIES AND SITE

*Humboldtia brunonis* (Fabaceae) is a dominant understory tree, 6–10 m tall, endemic to the low elevation, tropical, wet evergreen forests of the Western Ghats of India (Ramesh & Pascal 1997) and found in a narrow mountainous strip between 11°10'N and 13°45' N. The plant is clonal with a maximum distance of 8.75 m between any two neighbouring non-clones (Dev, Shenoy & Borges 2010). It occurs in dense patches of about 0.25 km<sup>2</sup> (J. Chanam, pers. comm.), and each such patch may consist of different clones. The flowering season is from December to April (Shenoy & Borges 2008) during which the plant also produces young leaves. The bracts of the flower buds, young leaves and stipules bear active extrafloral nectaries at which ant activity is high. In a population, only some of the trees bear caulinary domatia (Gaume *et al.* 2005b); each domatium is formed by a single internode, which is swollen and hollow towards the distal end, and with a tapering non-hollow base at the proximal region (Fig. 1a). A self-opening slit is present at the distal end of each domatium (Fig. 1a). In a domatia-bearing tree, the domatia are formed only at some internodes, of some branches. In a single branch, there may be several (up to five) domatia, arranged either in a continuous series or interspersed with normal non-swollen internodes (J. Chanam & R.M. Borges, unpublished data). Adjacent domatia are not connected internally.

Extrafloral nectar contribution to ant diets was investigated at Agumbe Reserve Forest (13°31'N, 75°5'E; 633 m a.s.l.), whereas all other experiments were conducted in Sampaje Reserve Forest (12°29'N, 75°35'E; 665 m a.s.l.) within Karnataka state.

### STABLE ISOTOPE ANALYSIS

In nature, the heavier stable isotopes of several dietary elements get enriched from one trophic level to the next, and this trophic enrichment is used to study the flux of these elements in a food chain (Peterson & Fry 1987; Fry 2006). We used the stable isotopes of carbon and nitrogen to investigate nutrient flux in our system. The composition of stable isotopes in a sample is expressed as a  $\delta$  (delta) value relative to a standard and is measured in ‰ (permil). The  $\delta$  value for nitrogen, which has two stable isotopes <sup>14</sup>N and <sup>15</sup>N, is expressed as follows:  $\delta^{15}\text{N}\text{‰} = [(R_{\text{sample}} - R_{\text{std}})/R_{\text{std}}] \times 1000$  where  $R_{\text{sample}} = ^{15}\text{N}/^{14}\text{N}$  of sample and  $R_{\text{std}} = ^{15}\text{N}/^{14}\text{N}$  of standard, that is, atmospheric nitrogen. The carbon isotope composition ( $\delta^{13}\text{C}$ ) is similarly calculated where the  $R_{\text{std}}$  for carbon is the <sup>13</sup>C/<sup>12</sup>C of the PeeDee Belemnite standard (Peterson & Fry 1987).

All samples were analysed using an elemental analyser connected with an isotope ratio mass spectrometer (IRMS, Delta<sup>PLUS</sup>, Thermo Fisher, Germany) at the National Facility for Stable Isotope Studies in Biological Sciences, University for Agricultural Sciences, Bangalore. To ensure accuracy in measurements, laboratory standards were analysed after every eight to ten samples, to confirm that the standard deviation of their  $\delta$  values was always less than 0.1‰ for carbon and 0.15‰ for nitrogen with the analytical uncertainty of the instrument never more than 0.06‰. We used bovine serum albumin (BSA, analytical grade from Sigma, product number: A2153) as a laboratory standard for nitrogen and potato starch (Qualigens, product number: 20725) for carbon. Both laboratory standards were pre-calibrated against international standards. While the potato starch was calibrated against ANU sucrose (with a Craig corrected  $\delta^{13}\text{C}$  of -10.4‰), the BSA was calibrated against USGS32 and IAEA-N-2 ammonium sulphate.

We measured natural  $\delta^{13}\text{C}$  values of EFN, and that of ants dwelling in the domatia, as well as those present on non-*H. brunonis* plants to determine the utilization of EFN as food by domatia-dwelling ants. We also measured natural  $\delta^{15}\text{N}$  values of various plant tissues, as well as of ant and non-ant invertebrates, to determine the nitrogen flow from the domatia dwellers to the host plant (details of samples provided below). We conducted experiments using <sup>15</sup>N-enriched glycine (98 atom% <sup>15</sup>N, molecular weight 76.06, Sigma-Aldrich) as tracers to detect nutrient flow. <sup>15</sup>N is an extremely rare stable isotope of nitrogen in nature (Fry 2006), and therefore, experimental enrichment of individual organisms with this isotope is a reliable way to track nitrogen flux in trophic interactions. The incorporation of nutrients from domatia inhabitants to the host plant system was calculated using atom per cent excess of <sup>15</sup>N (Fischer *et al.* 2003; Romero *et al.* 2006; Hood-Nowotny & Knols 2007).

### THE CONTRIBUTION OF HOST EFN TO THE DIET OF ANTS RESIDENT WITHIN DOMATIA

Samples of *Crematogaster dohrni*, a non-protective ant species residing within domatia of *H. brunonis* plants ( $n = 9$  domatia) as well as those present on non-*H. brunonis* plants ( $n = 8$ ), were collected in clean sterile vials, desiccated using silica gel in the field, and later in a hot air oven at 60°C. EFN samples ( $n = 9$ ) were also collected using micropipettes with sterile tips from the trees from which the domatia ants were collected, placed in sterile GC-MS injection vials and immediately stored in liquid nitrogen in the field. In the laboratory, EFN samples were absorbed onto small wedges of Whatman glass-fibre filter papers. These filter papers had been pre-heated to 450°C overnight in a muffle furnace to remove all traces of organic matter prior to EFN application. The glass-fibre filter papers with absorbed EFN were then evaporated in a laminar hood to prevent microbial growth during the



evaporation. These samples were then placed in silver capsules for IRMS analysis. For this question, we analysed only  $\delta^{13}\text{C}$  values of the samples since EFN is mostly sugar- and thereby, carbon-rich and has relatively low contents of nitrogen.

#### ABSORPTION OF NUTRIENTS BY THE HOST PLANT FROM DOMATIA

1. We performed a pulse-chase experiment by injecting 100  $\mu\text{L}$  of a solution containing 12.5 mM of  $^{15}\text{N}$  glycine in HPLC-grade water (after Fischer *et al.* 2003) into the domatia (via the self-opening slit) of *H. brunonis* plants in the following sample groups: young unoccupied domatia, domatia occupied by the protective ant *T. albipes*, domatia occupied by the 'parasitic' ant *C. dohrni* and domatia occupied by earthworms (two domatia in each group; totally eight domatia were treated and their values were pooled for statistical analyses). Prior to the experiment, the ant-occupied domatia were first emptied of the occupants by tapping the domatia. The earthworm-domatia could not be emptied, and the earthworms remained inside the domatia during the course of the experiment. Also, prior to the experiment, a control leaf from a node adjacent to each domatium was collected. 100  $\mu\text{L}$  of a solution containing 12.5 mM of  $^{15}\text{N}$  glycine in HPLC-grade water was injected into each domatium via the self-opening slit. The experimental branches with the domatia and the adjacent internodes were harvested after 24 h. A distant non-domatia bearing branch with leaves was also collected for each experimental tree to determine whether the label travelled to distant parts away from the experimental domatium. All plant samples were thoroughly rinsed in distilled water. Each experimental branch was separated into the following parts: domatia wall (including only the woody layer), domatia inner lining, domatia base, proximal and distal leaves (with respect to domatia), domatia-bearing branch and leaf on non-domatia bearing branch. Samples were desiccated in silica gel till they were brought back to the laboratory, where they were dried in an oven at 60°C, ground into fine powder in a motorized pulverizer, homogenized and stored at -20°C until IRMS analysis.

2. To determine whether the host plant acquired nutrients derived from ants residing in the domatia, we performed a pulse experiment by feeding sucrose solution labelled with  $^{15}\text{N}$  glycine to domatia-inhabiting ants for 2 weeks (after Fischer *et al.* 2003). We chose six plants with domatia that were exclusively occupied by one of the following ants: the protective ant *T. albipes* ( $n = 2$  plants) as well as the non-protective *C. dohrni* ( $n = 2$  plants) and *Cataulacus taprobanae* ( $n = 2$  plants). One ant-occupied domatium was selected from each of these plants for the feeding experiment. These ant species were the most common ant residents in the domatia at this site. Values were pooled across ant species for this question ( $n = 6$  domatia). On the first day, a leaf from the node just adjacent to each experimental domatium was collected as a control leaf. About 5–7 worker ants that were moving in and out of the domatium were also collected in clean vials as control ants.  $^{15}\text{N}$  glycine was dissolved in 30% sucrose solution and fed to the ants on alternate days for 2 weeks, such that a total of 10.04 mg  $^{15}\text{N}$  glycine was provided to each ant nest by the end of the feeding experiment. We made sure that all the droplets of the solution were consumed by the ants in each feeding episode. For every feeding, a young leaf obtained from a non-experimental *H. brunonis* plant was attached to the internode adjacent to the domatia, and droplets of the feeding solution were applied on it. This was done to avoid direct contact between the labelled solution and any other part of the plant outside the domatia. A roof of plastic sheets was erected over each experimental domatium so that the ant-occupied domatia remained undisturbed and feeding could continue even during the monsoon rain. After 2 weeks of feeding, followed by 1 week of no intervention, the domatia-bearing

branch was harvested, along with a distant non-domatia bearing branch with leaves from each experimental tree. The domatia containing the ant nests were separated and placed in glass tubes containing silica gel. The plant material was separated into proximal and distal (with respect to domatium) leaves, domatia-bearing branch and leaf on domatia-bearing branch, non-domatia bearing branch and leaf on non-domatia bearing branch. The plant tissues were cleaned, dried, pulverized and stored as before. The domatia containing the ants were opened, the ants removed and dried in the oven. The domatia were separated into inner lining, wall and base (the domatium base being the solid woody region in the proximal part of the domatium internode where the swollen cavity ends).

3. We determined the fraction of host tissue nitrogen contributed by domatia inhabitants such as protective ants, non-protective ants and arboreal earthworms in the following way. Since *H. brunonis* is a tree, it was not feasible to measure the per cent nitrogen incorporation for the whole plant. We therefore determined the per cent nitrogen incorporation only for the branch that bore the domatium, and not for the whole host tree. In such localized host plant tissues, we investigated the fraction of plant nitrogen that was contributed by the various inhabitants using (i) the enriched samples of ant occupants in which ants were fed on  $^{15}\text{N}$ -enriched solution in the previous experiment and (ii) unenriched samples. For the unenriched ant samples, we collected domatia occupied by protective domatia-dwelling ants (*T. albipes*,  $n = 5$  domatia) and non-protective domatia-dwelling ants (*C. dohrni*,  $n = 3$  domatia), one domatium per tree, from trees on which no feeding with labelled solutions had occurred. We also collected domatia occupied by the arboreal earthworm (*P. pullus*,  $n = 4$  domatia). The natural (unenriched) samples were especially important for estimation of nitrogen contribution to the plant by earthworms since feeding experiments were not feasible for them. Since there was not enough solid debris in the domatia to collect separately, we scraped and collected the inner lining of the domatia which could have absorbed excreta, frass and dead tissues of the occupants as nutrient sources. The control plant tissues were branches that had unoccupied domatia and the inner linings of these domatia, collected from trees other than those from which enriched and unenriched samples were obtained. The per cent nitrogen of host plant tissue contributed by domatia occupants was calculated using atom per cent excess (APE) of nitrogen (Fischer *et al.* 2003; Romero *et al.* 2006).

$$\%N_{\text{received}} = (\text{APE}_{\text{dom.branch}}/\text{APE}_{\text{dom.inner lining}}) \times 100$$

where APE =  $^{15}\text{N}$  atom per cent of sample -  $^{15}\text{N}$  atom per cent of control; dom.branch = domatia-bearing branch; dom.inner lining = inner lining of domatia.

#### FRUIT SET OF DOMATIA-BEARING AND NON-DOMATIA MODULES

In order to investigate whether nutrition from domatia inhabitants may contribute to greater fruit set of domatia-bearing branches, we compared fruit set between 20 pairs of inflorescence buds, borne on domatia-bearing and non-domatia bearing branches, every pair on a different tree ( $n = 20$  trees). Inflorescence buds were bagged to exclude natural pollination. The opened flowers were subsequently hand-pollinated with equal amounts of pollen obtained from the same sources, on an equal number of florets. Since *H. brunonis* flowers are not self-pollinated (Shenoy & Borges 2008) and *H. brunonis* tree clumps are also clonal (Dev, Shenoy & Borges 2010), pollen donors were distant conspecific plants whose flowers had also been previously bagged. After pollination, the flowers were rebagged to avoid deposition of any more pollen from natural pollinators and also to protect against herbivores. The fruit set in these two groups was compared.

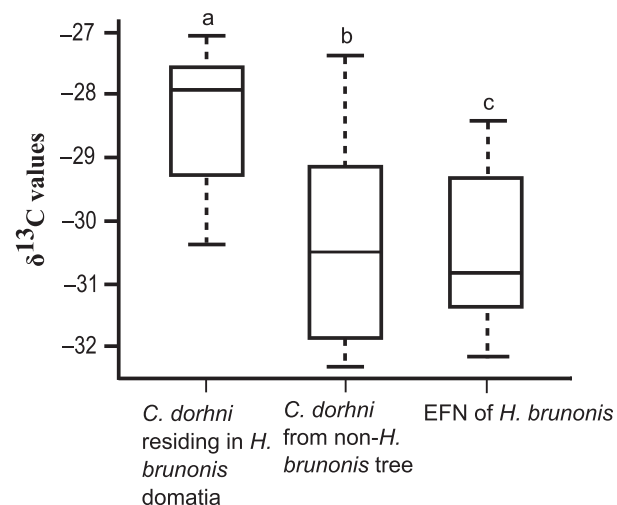
## STATISTICAL ANALYSES

All statistical analyses were performed using R software (version 2.14.1). Since the data were not normally distributed, the variation in  $\delta$  isotope values within and across groups was compared using Kruskal-Wallis ANOVAs, followed by *post hoc* Wilcoxon rank-sum tests for unpaired samples after Bonferroni correction. The paired fruit set values were compared using a Wilcoxon matched-pairs signed-rank test.

## Results

## CONTRIBUTION OF HOST EFN TO DOMATIA-RESIDENT ANTS

Since heavier isotopes in the diet are enriched in the body of the consumer, an increase in 2–3 ‰ in  $\delta^{13}\text{C}$  value was expected with trophic level. There was significant variation between the  $\delta^{13}\text{C}$  values of *C. dohrni* ants residing in the domatia, *C. dohrni* collected from non-*H. brunonis* trees and EFN (Kruskal-Wallis ANOVA:  $\chi^2 = 7.78$ , d.f. = 2,  $P = 0.02$ ) (Fig. 2). The average  $\delta^{13}\text{C}$  of *C. dohrni* ants residing in the domatia was  $-28.42\text{‰}$  and was greater than that of the EFN of the host tree which was  $-30.47\text{‰}$  (Wilcoxon rank-sum test,  $W = 71$ ,  $P = 0.006$ ). The average  $\delta^{13}\text{C}$  value of the *C. dohrni* collected from non-*H. brunonis* trees was  $-30.35\text{‰}$ ; this value was lower than that of conspecifics that reside in the domatia of *H. brunonis* (Wilcoxon rank-sum test,  $W = 57$ ,  $P = 0.046$ ) and similar to that of EFN of *H. brunonis* (Wilcoxon rank-sum test,  $W = 37$ ,  $P = 0.96$ ) (Fig. 2). The  $\delta^{13}\text{C}$  enrichment in domatia-dwelling *C. dohrni* compared to EFN and compared to non-



**Fig. 2.** Carbon isotope  $\delta^{13}\text{C}$  values of *Crematogaster dohrni* ants dwelling in the domatia of *Humboldtia brunonis* ( $n = 9$  domatia), *C. dohrni* from non-*H. brunonis* trees ( $n = 8$ ) and EFN of *H. brunonis* ( $n = 9$ ). The lower and upper boundaries of the box plots indicate the lower and upper quartile values, respectively, while the bar in the box indicates the median. The error bars indicate the minimum and the maximum values. Different letters above the boxes indicate significant differences between the groups (Wilcoxon rank-sum tests after Bonferroni correction).

domatia-dwelling conspecific ants suggests that EFN is an important carbon source for domatia-dwelling ants.

## FLUX OF DOMATIA NUTRIENTS INTO THE HOST PLANT

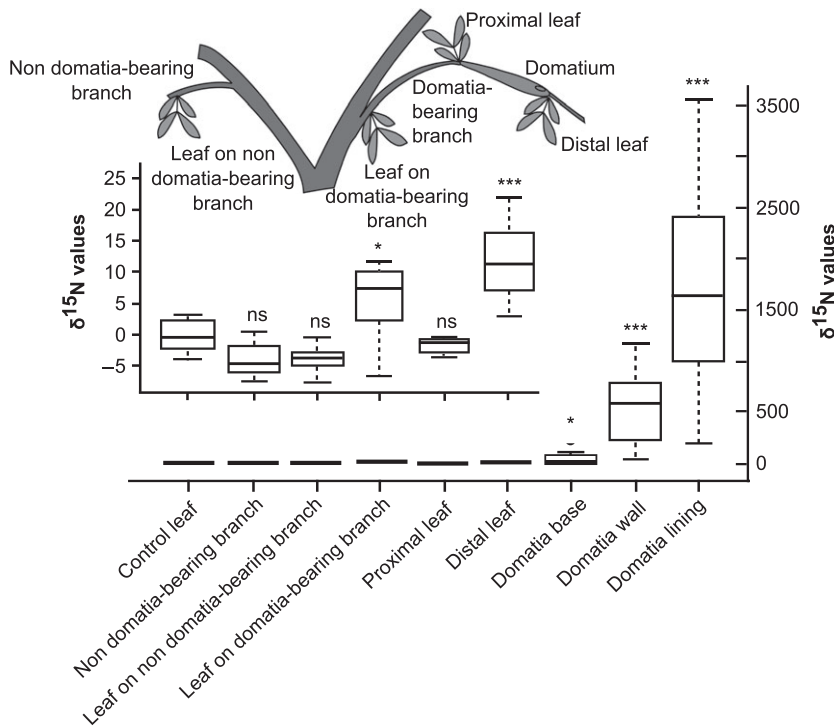
**1.** We found that the plant absorbed nutrients that were directly injected into the domatia. The injected labelled nitrogen solution was transferred by 24 h not only into the domatia wall, and domatia base, but was also detected in the leaves borne on the distal node of the domatia internodes, as well as the leaves borne on the branch bearing the domatia (Fig. 3). The  $\delta^{15}\text{N}$  values of these samples were significantly higher compared to the controls, though tissues closer to the injection site were far more enriched than those further away (Fig. 3). However, at the end of 24 h, there was no significant increase either in  $\delta^{15}\text{N}$  values of the leaf and branch samples of the non-domatia bearing branches, or of the leaves on the domatia-bearing branch (Fig. 3).

**2.** In the ant-feeding experiment, we found that ant-derived nutrients were absorbed from the domatia into the host plant. There was non-uniform movement into the plant tissue of the labelled solution consumed by ants from the experimental domatia to other tissues, with the highest  $\delta^{15}\text{N}$  values occurring in the ant workers and the inner wall of the domatia (Fig. 4). The  $\delta^{15}\text{N}$  value of the inner domatium wall was the highest of all the plant samples as it was in direct contact with the ants within the domatia. Nevertheless, even the average  $\delta^{15}\text{N}$  value of a distant non-domatia bearing branch was significantly greater than that of the control leaf, showing that the labelled solution processed by the ants reached far beyond the domatia-bearing module into the other non-domatia bearing modules within 3 weeks of the start of the experiment. Most of the samples had  $\delta^{15}\text{N}$  values higher than the  $\delta^{15}\text{N}$  value of the control sample except for the leaf on the domatia-bearing branch ( $W = 49$ ,  $P = 0.083$ ), and the leaf borne on a distant non-domatia bearing branch ( $W = 23$ ,  $P = 0.38$ ) (Fig. 4). Comparisons with control values could not be made for the ant eggs and larvae owing to sample size limitations.

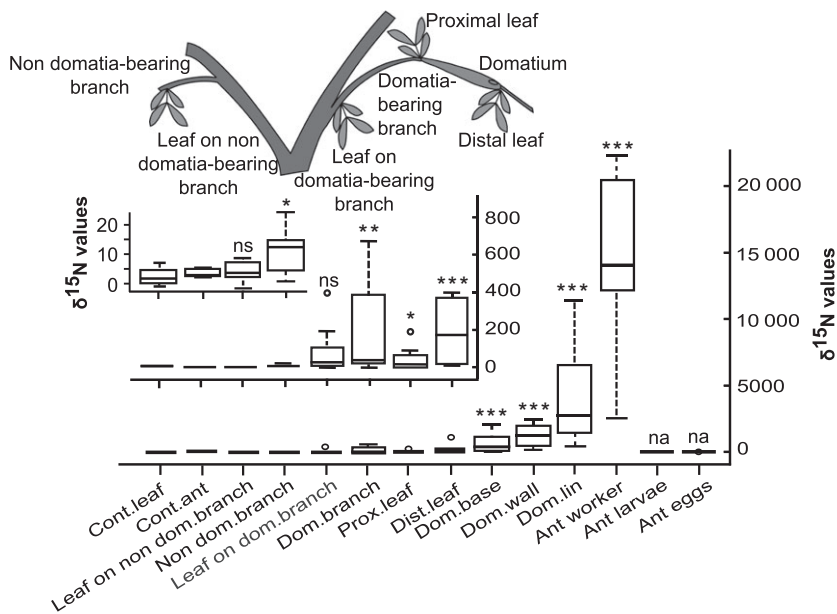
**3.** Domatia inhabitants contributed to a considerable fraction of host tissue nitrogen (Table 1). For the enriched samples, the per cent nitrogen incorporated into the host tissue (domatia-bearing branch) from domatia-dwelling ants (all ant species pooled together) was 5.65%. For unenriched samples, the per cent nitrogen incorporated into the host tissue for different ant species, viz. *C. dohrni* (castration parasitic ant) and *T. albipes* (the protective ant), was 17.12% and 16.77%, respectively, and for the earthworm was 8.73%. These results suggest a significant nutrient flux to the plant from protective ants as well as interloping domatia inhabitants, such as castrating ants and earthworms.

## FRUIT SET OF DOMATIA-BEARING MODULES

Fruit set was not significantly different between the domatia-bearing and non-domatia bearing branches (Wilcoxon



**Fig. 3.** Flow of <sup>15</sup>N-labelled glycine injected into domatia (*n* = 8 domatia) to proximate and distal plant parts. Samples were collected 24 h after injection. The lower and upper boundaries of the box plots indicate the lower and upper quartile values, respectively, while the bar in the box indicates the median. The error bars indicate minimum and maximum values, excluding outliers. Outlier values in a sample class, if any, are indicated by open circles. Values of each sample class in the experimental samples are compared to those of the control leaf using Wilcoxon rank-sum tests with Bonferroni corrections. ns = non-significant, \* = *P* < 0.05, \*\* = *P* < 0.01, \*\*\* = *P* < 0.001.



**Fig. 4.** Flow of <sup>15</sup>N-labelled glycine fed to domatia-dwelling ants (*n* = 8 domatia). Samples were collected 3 weeks after the first feeding. The abbreviations represent the following samples: cont.leaf = control leaf; cont.ant = control ant; leaf on non-dom. branch = leaf on non-domatia-bearing branch; non-dom. branch = non-domatia-bearing branch; leaf on dom. branch = leaf on domatia-bearing branch; dom. branch = domatia-bearing branch; prox. leaf = proximal leaf; dist. leaf = distal leaf; dom. base = domatium base; dom. wall = domatium wall; dom. lining = domatium lining. Box plot depictions as in legend for Fig. 3. Each sample class is compared to the control (plant tissues with control leaf, and ant worker values with control ant) using Wilcoxon rank-sum tests with Bonferroni corrections. ns = non-significant, \* = *P* < 0.05, \*\* = *P* < 0.01, \*\*\* = *P* < 0.001, na = not available.

matched-pairs signed-rank test, *V* = 118, *P* = 0.37). The fruit set itself was very low with only 25 out of 385 florets in domatia-bearing, and 29 out of 484 florets in non-domatia-bearing branches bearing fruit even after hand pollination.

**Discussion**

The <sup>15</sup>N-enriched feeding experiments with protective and non-protective ants demonstrated that nutrients flowed from domatia inhabitants to nearby plant modules from domatia-bearing branches. Non-protective ants residing

within the domatia were important consumers of EFN and thus contributed to nutrient efflux as well as influx in this ant–plant system.

A considerable per cent of nitrogen in the domatia-bearing branch of the host tree was contributed by protective and non-protective ants, as well as the dominant resident earthworm. Our study has clearly demonstrated the nutritive role of non-protective ants and non-ant invertebrates, hitherto referred to as interlopers, in an unspecialized myrmecophyte, suggesting that trophic benefits conferred by domatia inhabitants can contribute to explaining the fitness benefits of bearing domatia, even before the

**Table 1.** Percentage of nitrogen of host plant tissue contributed by the domatia occupant (%N<sub>received</sub>), calculated using atom per cent (AP) values. The host plant tissue considered for the calculations is the domatia-bearing branch (dom.branch), while the nutrient source is the inner lining of the domatia (dom.inner lining). Control values are obtained from unoccupied domatia and the corresponding branches bearing them. Sample sizes indicate numbers of domatia

	AP <sub>dom.branch</sub> (Mean ± SD)	AP <sub>dom.inner lining</sub> (Mean ± SD)			
(i) Control					
Unoccupied domatia (n = 4)	0.3659245 ± 0.00103	0.363809 ± 0.00431			
	AP <sub>dom.branch</sub> (Mean ± SD)	AP <sub>dom.inner lining</sub> (Mean ± SD)	APE <sub>dom.branch</sub> (AP <sub>sample</sub> - AP <sub>control</sub> )	APE <sub>dom.inner lining</sub> (AP <sub>sample</sub> - AP <sub>control</sub> )	% N <sub>received</sub>
(ii) Unenriched					
<i>Crematogaster dohrni</i> (n = 3)	0.366223 ± 0.00076	0.365550 ± 0.00061	0.000298	0.0017411	17.125
<i>Technomyrmex albipes</i> (n = 5)	0.3664124 ± 0.0004	0.366718 ± 0.00189	0.000488	0.002909	16.771
<i>Perionyx pullus</i> (n = 5)	0.3660746 ± 0.0005	0.365529 ± 0.00023	0.000150	0.0017198	8.728
(iii) Enriched					
Ants (all species pooled, n = 8)	0.442454 (±0.10282)	1.718022 (±1.4514)	0.076529	1.354212	5.651

establishment of a specialized ant–plant protection mutualism. A nutritional mutualism can promote the maintenance of a trait such as a caulinary domatium that facilitates the symbiosis between ants and plants.

The genus *Crematogaster* is believed to be largely an exudate feeder, consuming honeydew or EFN (Davidson 1997), although it may also engage in carnivory (Davidson *et al.* 2003). The  $\delta^{13}\text{C}$  value of the domatia-dwelling *Crematogaster dohrni* ants was about 2‰ higher than that of EFN. Since ants residing in *H. brunonis* feed on the EFN of the host, this  $\delta^{13}\text{C}$  value of these domatia-dwelling *C. dohrni* ants may be a consequence of trophic enrichment of the food resource (here, EFN) in the ants. The 2‰ trophic enrichment for carbon is within the range reported for other studies (Post 2002; Blüthgen, Gebauer & Fiedler 2003; Spence & Rosenheim 2005; Caut, Angulo & Courchamp 2009). The conspecific *C. dohrni* samples collected from a nest on a non-*H. brunonis* tree, unlike those residing in the *H. brunonis* domatia, had an average  $\delta^{13}\text{C}$  value that did not show any enrichment compared to EFN. This suggests that these non-domatia-dwelling ants do not consume *H. brunonis* EFN. Our results therefore suggest that EFN is an important component of the diet of the domatia-dwelling castration parasite *C. dohrni*. Such domatia-dwelling ants, although failing to provide protection services to their hosts, could gain carbon from the plant while contributing up to 17% of nitrogen to the domatium-bearing branch they occupy.

We found that domatia allow absorption and flow of nutrients into the host plant. The  $^{15}\text{N}$ -labelled solution injected directly into domatia was not only absorbed through the domatia wall, but was also detected in the leaf distal to the domatia internode and the leaf borne on the domatia-bearing branch. However, there was no significant

increase in  $\delta^{15}\text{N}$  of tissues further downstream compared to the control leaves. This could be because the labelled nitrogen progressed only up to that point within the 24-h duration of the experiment. In the ant-feeding experiments, there was significant increase in  $\delta^{15}\text{N}$  values of not only the inner wall of the domatia, but also of branch tissues of the domatia-bearing and distant non-domatia bearing branches. In other studies also (Fischer *et al.* 2003; Defosse *et al.* 2011), tissues near the experimental domatia had higher  $\delta^{15}\text{N}$  values compared to distant tissues when sampled after 2 weeks or less. The highest  $\delta^{15}\text{N}$  value was present in the worker ants that consumed the labelled solution and may have transferred it to nest mates via trophallaxis. During the experiment, there was no increase in  $\delta^{15}\text{N}$  values in ant larvae and eggs. The much greater  $\delta^{15}\text{N}$  values of the tissues nearer the experimental domatia compared to relatively distant tissues suggests time-dependent flow of the labelled solution in the plant even to modules far away. In a pulse-chase experiment on a distantly related myrmecophyte *Leonardoxa africana* within the same caesalpinoid legume tribe as *Humboldtia*, the labels were highest in the tissues near the application site at the time of the experiment, but after 2 years were homogeneously distributed throughout the plant (Defosse *et al.* 2011).

Although the feeding experiment was performed only with ants as these were the only suitable domatia residents for such an experiment, our results can be extrapolated to other non-ant domatia invertebrates. The nutrients derived from the defecation or collected debris from these other invertebrate occupants could be similarly absorbed by the host plant. This finding is of considerable importance especially for the arboreal earthworm *Perionyx pullus*, which occupies many of the domatia in this system. Up to 40%



of domatia are occupied by earthworms at some sites (Shenoy & Borges 2010). Till date, earthworms were considered interlopers with no contribution to the host plant since they have antagonistic interactions with ants (Gaume *et al.* 2006) and could be efficiently driving out ants from the domatia, thus contributing to their dominance in the domatia especially at the driest site (Shenoy & Borges 2010). Since the earthworms complete their entire development within the domatia during the dry season (Gaume *et al.* 2006), they probably feed on loose dead pith tissue in the domatia cavity, and their excreta could be delivered in a form that is easily absorbed and metabolized by the host plant. These earthworms have been observed out of the domatia, crawling on the leaves and stems of *H. brunonis* only during the rainy season, when they probably mate (Gaume *et al.* 2006). Prior to the monsoon, only immature stages of earthworms are found within the domatia (R. M. Borges, pers. comm.). It is plausible that the earthworms feed on leaf epiphylls and debris on the stem while they are out of the domatia, which later contributes to plant nutrition after they move back into the domatia where egg cocoons are deposited. However, this is only speculation at this time and requires further investigation. Earthworms and ants sometimes co-occupy a single domatium in which they are separated by a carton partition constructed by the ants within the domatium (Gaume *et al.* 2006; J. Chanam, pers. comm.). In such cases, earthworms may also acquire nutrients from the ants or from previously evicted residents. The nutrient flux from non-ant invertebrates to plants in myrmecophytes is comparable to the absorption of invertebrate-derived nutrients in tank-bromeliads in which the leaf bases impound rainwater, forming phytotelmata that harbour myriad invertebrate communities (Romero *et al.* 2006; Céréghino *et al.* 2011). In these bromeliads, there is nutrient flux from the nutrient-rich water in the phytotelmata into the plant via special absorptive trichomes present in the leaf base (Benzing 1970; Benzing *et al.* 1976). In the epiphytic myrmecophyte *Dischidia major*, arboreal roots enter the leaf pouch domatia to absorb nutrients (Treseder, Davidson & Ehleringer 1995). In the caulinary domatia of myrmecophytes, however, special absorptive organs have not been observed; instead, the inner wall of the domatia is usually lined with cells having pitted cell walls, bearing numerous plasmodesmata, that could facilitate the flow of the nutrients from the domatia into the plant (Defosse *et al.* 2011; Gegenbauer *et al.* 2012). We observed such pitted cell walls in *H. brunonis* also (Fig. 1d) suggesting a conduit of nutrient flow from the domatia to the plant.

For nitrogen incorporation from domatia inhabitant to the plant, our study reports results of the atom per cent excess method for both enriched and unenriched samples of ants (*T. albipes* or *C. dohrni*), and both methods show considerable contribution of nitrogen to nearby tissues by protective ants as well as non-protective domatia residents. The values of per cent incorporation for domatia occupied by either *T. albipes* or *C. dohrni* are higher for the natural

samples than the enriched ones where the ants were experimentally fed with <sup>15</sup>N-labelled sugar solution. This is likely to be because the enriched samples measure absorption within the limited period of the feeding experiment, whereas the natural samples measure absorption over a much longer period, and are therefore more indicative of the real nitrogen flux between domatia inhabitants and plant tissues. Our results therefore have a major implication for the way relationships between myrmecophytes and non-protective 'interlopers' should be viewed. Furthermore, the domatia of *H. brunonis* have a self-opening slit, which may enable interlopers such as earthworms to occupy domatia and invade the system. Such invasion by interlopers, such as generalist ants, also occurs in the open leaf pouch domatia of some myrmecophytes (Moog *et al.* 2008). The contribution of earthworms to the nitrogen budget of the plant may therefore facilitate the maintenance of domatia even in those seasonally dry northern sites where earthworms dominate over ants in domatia occupancy and where the protection mutualism between ants and plants is absent (Shenoy & Borges 2010; J. Chanam & R.M. Borges, unpublished data).

Very few studies have demonstrated that ants contribute directly to the reproductive success of ant-plants (Vesprini, Galetto & Bernardello 2003; Gaume, Zacharias & Borges 2005a; Trager *et al.* 2010; Leroy *et al.* 2012). In *H. brunonis*, the nutrients absorbed from domatia did not significantly increase the fruit set on domatia-bearing branches compared to adjacent non-domatia bearing modules. This non-significant difference may be due to the fact that the nutrients absorbed from the domatia were not restricted to the domatia-bearing module, as shown by our ant-feeding experiment results. The hundreds of domatia on larger trees therefore serve as nitrogen sources and adjacent modules as nitrogen sinks. Previous studies (Shenoy & Borges 2008) on *H. brunonis* have reported very low natural fruit set (<3.5%). The invertebrate-derived nutrients from the domatia may therefore be crucial in ensuring greater fruit set of domatia-bearing trees and could contribute to explaining the greater fitness of trees with the domatia trait independent of the presence of the protective ant *T. albipes* as a resident within the tree (Gaume, Zacharias & Borges 2005a).

Nitrogen is a limiting nutrient for plants in most ecosystems (Vitousek & Howarth 1991). Plants growing in nitrogen-limiting situations often adopt the strategy of carnivory in which they directly trap and digest prey, extracting nitrogen and other nutrients from their carcasses (Ellison & Gotelli 2001; Millett *et al.* 2012). Other plants in such environments enter into symbioses with nitrogen-fixing bacteria and house these mutualists in root nodules (Desbrosses & Stougaard 2011). Although *H. brunonis* is a leguminous tree, it belongs to the legume tribe Detarieae in the subfamily Caesalpinioideae (Bruneau *et al.* 2001). Nodulation is believed to be rare in Caesalpinioideae, largely restricted to the tribe Caesalpinieae, and considered absent in *Humboldtia* (Faria *et al.* 1989). It is



therefore possible that the nitrogen obtained from domatia inhabitants is important in the nutritional ecology of *H. brunonis* and may contribute to its dominance in the understory. However, nitrogen may not be the only nutrient gained from invertebrate domatia residents. Phosphorus is a more limiting nutrient in tropical forests (Vitousek 1984; Hedin *et al.* 2009), and perhaps what the plant gains from its domatia residents is phosphorus or other minerals and organic nutrients. However, phosphorus has only one stable isotope, which makes its flux from domatia resident to plant much more difficult to investigate. Supplementing non-domatia bearing plants with phosphorus and comparing their resultant fitness to that of domatia-bearing plants may provide insight into the role of phosphorus in this system. This awaits future research.

To conclude, it is therefore possible that by providing nesting spaces for invertebrates, plant domatia mediated the origin of a generalized trophic-cum-protection mutualism with its motley residents, which was later refined to a more specialized trophic-cum-protection mutualism with specialist ants in many ant-plant species.

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