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„Not just ant-plants: Fungal patches and diazotrophic prokaryotes in the *Cecropia/Azteca* ant-plant symbiosis"

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1 Abstract

Recent insights in the complex tripartite interaction of ants, plants and fungi in ant-plants revealed fungal patches as an integral part of ant-plant symbioses, with nutrient fluxes to both ants and plants. Fungal patches are found in phylogenetically distinct ant-plant associations within the hollow cavities provided by the host plants. Fungi of the order Chaetothyriales regularly inhabit these patches and ant larvae were found to feed on its hyphae. Colonization with microorganisms and fungi of the patch substrate made out of plant tissue with high C/N ratios could be promoted by nitrogen-fixing bacteria (diazotrophs) providing nitrogen.

This study investigated nitrogen fixation activity inside the hollow internodes of *Cecropia* plants inhabited by *Azteca* ants. After incubation in an artificial $^{15}N_2$ enriched atmosphere (75% $^{15}N_2$, 25% O_2), highest fixation rates were found in ant-made patches (0.019 $^{15}N_2$ µmol g^{-1} d^{-1}). In ant-made carton structures nitrogen fixation took place (0.007 $^{15}N_2$ µmol g^{-1} d^{-1}) in a significantly lower rate than in patches. Diazotrophic community analysis by means of *nifH* genes revealed the presence of Beta- and Alphaproteobacteria in both carton and patch. Interestingly, ant workers, queens and Coccoidea herded by the ants also displayed significantly elevated ^{15}N content after incubation, indicating a possible presence of endosymbionts.
Larvae accumulated a significant amount of nitrogen isotope from ^{15}N labelled patches, probably caused by feeding on fungal hyphae. Indications for ant mediated cultivation of Chaetothyriales were found as chaetothyrialean OTUs were host ant species specific.
In summary, this study showed for the first time that a diazotrophic community is present on the fungal patches and that members of this community actively fixate atmospheric nitrogen. Fungal patches in the *Cecropia/Azteca* association therefore constitute an as yet unknown nitrogen source. It becomes evident that ant-plant-fungi symbioses are not tripartite but rather multipartite symbiotic systems requiring further research to eventually understand their functioning.

1.1 German abstract

Neue Einsichten in die komplexe Dreifachinteraktion von Ameisen, Pflanzen und Pilzen in Ameisenpflanzen offenbarten Pilzpatches als wichtigen Bestandteil von Ameisen-Pflanzen Symbiosen. Pilzflächen (Patches) findet man in phylogenetisch verschiedenen Ameisen-Pflanzen Symbiosen innerhalb der von der Wirtspflanze bereitgestellten Wohnräume (Domatia). Pilze der Ordnung Chaetothyriales wachsen in diesen Patches und Ameisenlarven fressen deren Hyphen. Die Besiedelung mit Mikroorganismen und Pilzen des Patchmaterials, das aus Pflanzenmaterial mit hohem C/N Verhältnis besteht, könnte durch stickstofffixierende Bakterien gefördert werden.

Diese Studie untersuchte die Stickstofffixierungsrate innerhalb der von *Azteca* Ameisen besiedelten Internodien von *Cecropia* Pflanzen. Nach der Inkubation in $^{15}N_2$ angereicherter Atmosphäre (75% $^{15}N_2$, 25% O_2) wurden die höchsten Fixierungsraten in Patches gefunden (0.019 $^{15}N_2$ µmol g^{-1} d^{-1}). In von Ameisen erzeugten Kartonstrukturen wurde ebenfalls Stickstoff fixiert (0.007 $^{15}N_2$ µmol g^{-1} d^{-1}), aber signifikant weniger als in Patches. Analyse der *nifH* Gensequenz von diazotrophen Bakterien offenbarte die Präsenz von Beta-, und Alphaproteobacteria. Interessanterweise zeigten auch Ameisenlarven, Königinnen und Coccoidea signifikant höheren ^{15}N Anteil nach der Inkubation, was auf mögliche Endosymbionten hinweist.
Ameisenlarven akkumulierten signifikante Mengen an ^{15}N von markierten Patches, was auf Fressen von Pilzhyphen hindeutet. Anzeichen für Kultivierung von Chaetothyriales durch die Ameisen wurden gefunden, da Chaetothyriales OTUs Ameisenart-spezifisch waren.
Diese Studie zeigte zum ersten Mal, dass diazotrophe Bakterien in Pilzpatches vorkommen. Pilzpatches in der *Cecropia/Azteca* Symbiose bilden daher eine bisher unbekannte Stickstoffquelle.

2 Introduction

Myrmecophytes (ant-plants) are only found in the tropics and known to form mutualistic relationships with ants. Thereby, the plants offer hollow chambers as nesting space (domatia) and may provide nutrition rich food bodies as food for the inhabiting ant colony. The ants, in return, provide protection against overgrowth from surrounding vegetation, pathogens and herbivores (Rico-Gray & Oliveira, 2007). In this study, the myrmecophyte *Cecropia* spp. (Urticaceae) and its obligate ant inhabitant *Azteca* spp. (Dolichoderinae) are investigated, both of which are native to the neotropics.

Cecropia is a fast growing pioneer plant (Figure 1a) with hollow internodes internally separated from each other by hard sclerenchyma, so-called septa (Bailey, 1922). *Cecropia* trees are common in disturbed landscapes along roadsides, pastures and forest gaps. They are often inhabited by ants of which the obligate *Azteca* species display a competitive superiority to other ant genera (Longino, 1989). *Cecropia* plants show certain adaptations to the ant association such as hollow internodes (Longino, 1991) and a grooved area lacking latex ducts (prostoma) on each internode which is used by *Azteca* queens and workers to bite entrance holes (Bailey, 1922). Furthermore, two types of food bodies are provided: Pearl bodies are produced on the abaxial surface of young leaves and are thought to be rich on lipids (Sagers et al., 2000). Müllerian bodies are produced in trichome pads (trichilia) located on the abaxial petiole base (Figure 1b) and are rich on glycogen, lipids and amino acids (Marshall & Rickson, 1973). Trichilia and Müllerian bodies are unique to myrmecophytic *Cecropia* (Janzen, 1973; Rickson, 1976).

In Costa Rica, three out of four *Cecropia* species found are known to shelter ants and can be distinguished by their leaf shape and reproductive structures (Longino, 1991). *C. insignis* is found in forests, whereas *C. peltata* is most common in lowlands. *C. obtusifolia* is encountered in wet areas and high elevations on the pacific side of Costa Rica. According to Sagers, Ginger, and Evans (2000), *Cecropia peltata* plants obtain up to 93 % of their nitrogen from their ant inhabitants, which could be a major advantage for a pioneer plant.

At least four *Azteca* species (Figure 1j) are obligate *Cecropia* inhabitants in Central America, with *A. alfari* being the most abundant species complex (Longino 1989). *A. alfari* and *A. constructor* are found throughout Costa Rica, whereas *A. xanthochroa* is found everywhere except for dry Pacific lowlands, where *A. coeruleipennis* is abundant.

No host-plant specificity was found between *Azteca* and *Cecropia* species in Costa Rica, which indicated that pairings are most probably caused by habitat overlaps (Longino, 1989).

As *Azteca* ants do not leave the host plant, their major nutrient sources are Müllerian food bodies of their *Cecropia* host plant, honeydew and occasional prey (Longino, 1989; Rickson, 1976). Honeydew, which is readily used by the ants as carbohydrate source (Heil & McKey, 2003), is provided by mealy bugs (Pseudococcidae) and soft scales (Coccidae). Both are commonly found in *Azteca* inhabited *Cecropia* plants (Longino, 2007a). Coccidae of the genus *Cryptostigma* are known to be associated with *Azteca* ants (Kondo, 2010). On disturbance, ant workers are often observed carrying the Coccoidea to a safe place, confirming their importance for the colony (Bailey, 1922; pers. obs.).

After mating, *Azteca* queens enter the hollow stem of *Cecropia* saplings by biting an entrance hole through the prostoma without rupturing the phloem (Rico-Gray & Oliveira, 2007). Often, multiple queens have to compete for a limited amount of plants and are victims of parasitic wasps (Bailey, 1922; Longino 1991) making this the most susceptible stage of colony development. Once inside, the queen scrapes tissue from the inner domatium walls and closes the entry hole (Figure 1c) which is later occluded by a callus formed by the host plant (Bailey, 1922). Besides laying eggs the queen also creates a pellet from scraped off parenchyma, a so-called patch (Figure 1d, e, f). Multiple foundress queens can be found in separate or even the same internodes within one plant (Longino, 1989) Colony founding in *Cecropia* colonizing *Azteca* is claustral, the entrance is reopened only by the first ant workers (Longino, 1989). Young colonies consist of just the queen with brood and a patch (Figure 1d) and may be restricted to one or two internodes whereas established colonies inhabit almost the whole stem of large trees. The internodes are connected either through holes in the septa and/or through prostomata (Longino, 1991). In established colonies, multiple patches as well as prominent ant-made carton structures filled with brood can be found (Longino, 1991). Carton structures (Figure 1i) are widespread in the genus *Azteca*, many of which are arboreal and construct carton nests or runway galleries (Longino, 1991; Mayer & Voglmayr, 2009).

Usually, the patches commonly found in colonies at founding stage are rather small and brown, surrounded by white parenchyma of the plant walls (Figure 1d, e). In established colonies, however, patches become dark brown to black and are often twice the size of

the queen in diameter (pers. obs.). The function of these patches is not yet known, but their common occurrence indicates an importance for the colony.

Only recently, the importance of microorganisms among obligate ant-plant symbioses has been recognized (Mayer et al., 2014). Especially Chaetothyriales have been investigated because ants living in association with myrmecophytes regularly cultivate these ascomycete fungi (Voglmayr et al., 2011). In the African ant-plant *Leornardoxa africana* (Fabaceae) the mutualistic ant *Petalomyrmex phylax* was observed defecating on the fungi (Defossez et al, 2009). Furthermore, Blatrix et al. (2012) found in three phylogenetically independent ant-plant symbioses that the inhabiting ants harvest and feed the hyphae predominantly to their larvae.

Multiple species of slow-growing Chaetothyriales are especially adapted to oligotrophic habitats (Voglmayr et al., 2011), enabling them to colonize ant-made patches in domatia. The patches made by *Azteca* ants are commonly inhabited with black yeasts (Nepel et al., unpubl.).

The high Carbon/Nitrogen ratio of the patches made from plant parenchyma may be unfavorable for fungal growth. Thus, in order for the fungi to grow more efficiently, additional nitrogen sources could be beneficial, like it is the case in soil environments (Rousk & Bååth, 2007). This could be especially important during the colony founding stage, as the first small patch pellet is made only from parenchyma of the domatia walls but chaetothyrialean fungi are already present (Mayer et al., in prep.).

Nitrogen enrichment could be achieved through defecation of the queen on the patch. However, as the colony founding is claustral and the queen most probably lives from her muscle resources and fat bodies, it is questionable whether she is really defecating. Another possible nitrogen source may be atmospheric dinitrogen which can be reduced to ammonium via a nitrogenase enzyme found in various diazotrophic (nitrogen-fixing) prokaryotes (Fürnkranz et al., 2008). Nitrogen fixation accomplished by diazotrophs is considered to be a very important source of nitrogen in ecosystems such as tropical rainforests (Vitousek et al., 2002) and was already demonstrated in the phyllosphere of rainforest plants (Fürnkranz et al., 2008). Pinto-Tomás et al. (2009) showed in fungus gardens of leaf-cutter ant colonies that N_2 fixing bacteria constitute a source of nitrogen. A similar atmospheric N_2 retrieval system may have evolved in ant-made patches in domatia of *Cecropia* to enhance nitrogen availability.

This study thus investigated the fixation rates of patches found in *Cecropia* domatia inhabited by founding stage and established *Azteca* colonies. Nitrogen fixation should be especially important in young patches as old patches are often filled with ant debris (Figure 1h) which could increase nitrogen availability. Additionally, carton structures, ants, Coccoidea and epiphytes samples were investigated to offer a preliminary overview of nitrogen fixation activity in *Cecropia*. To accurately assess fixation rates, all samples were incubated in $^{15}N_2$ rich atmosphere. A complementary analysis of the dinitrogenase-reductase gene (*nifH*) was conducted to identify the involved diazotrophic microorganisms.

Furthermore, it was suggested that ants are harvesting and defecating on the fungal patches, as is the case in other ant-plant symbioses (Blatrix et al., 2012; Defossez et al., 2009). *In situ* observations with an endoscope and labelling of fungal patches with a ^{15}N-rich amino acid mixture were used to unveil possible interactions and nitrogen transfer from the patch to the ant inhabitants. Finally, ^{15}N labelled Müllerian bodies were presented to the ants to show destinations of harvested food bodies in the colony.

The *Cecropia/Azteca* symbiosis is a well-suited model system for the investigation of fungi and diazotrophs in myrmecophytes because (i) it is fast growing and found in high numbers in disturbed habitats (e.g. roadsides, plantations, pastures), (ii) high nitrogen gain from ant provided sources has already been shown (Sagers et al., 2000) and (iii) fungal patches are very common in *Azteca* inhabited *Cecropia* and the taxonomy of the fungi is already clear (Nepel et al., unpubl.).

3 Materials and Methods

3.1 Field site

Observations, sample collecting and experiments were conducted at the Tropical Research Station of the University of Vienna in La Gamba near the Golfo Dulce on the Southern Pacific Coast in Costa Rica (8°42'04"N 83°12'06"W, 70m above sea level) from August 5th to September 9th 2014. The average annual precipitation at the research station is approximately 5,800mm with an average temperature of around 28°C (Weissenhofer & Huber, 2008). All *Cecropia* plants were collected from surrounding areas of the station and the experiments were started at the collection day.

3.2 $^{15}N_2$ Nitrogen fixation activity in and on Cecropia

3.2.1 Collected samples

In order to assess the nitrogen fixation activity in the *Cecropia/Azteca* symbiosis, multiple sample types were obtained: *Azteca* queens and founding stage patches (referred to as Fpatch) were collected from *Cecropia* saplings. Patches and carton from established *Azteca* colonies inhabiting larger (> 2m) *Cecropia* trees were also collected. Additionally, larvae, pupae, worker and Coccoidea from the respective colonies were collected. Epiphytes from *Cecropia* stems and epiphylls were also taken from larger trees.

3.2.2 Incubation procedure

Samples were incubated in artificial $^{15}N_2$ rich atmosphere (75% $^{15}N_2$, 25% O_2) using 2mL gas chromatography (GC) glass vials with a cap with septum. The $^{15}N_2$ gas was stored in a gas bottle (98at% $^{15}N_2$, Campro Scientific, Netherlands), O_2 in a gas bag. Instead of using artificial atmosphere composition of 80% $^{15}N_2$, 20% O_2 which resembles natural atmosphere conditions, a slightly higher amount of O_2 was used to overcome possible problems with O_2 deficiency during incubation.

After placing the sample into the vial it was closed airtight. The septum was pierced with a 20mL syringe and the natural air was removed creating vacuum (Figure 2a). Then the septum was quickly pierced with another syringe with a valve, containing the artificial atmosphere (Figure 2b). The suction of the vacuum within the vial emptied the syringe immediately after opening the valve, if less gas was sucked in than the vial volume it was pressed inside manually. All these steps were conducted fast, so the samples remained in a vacuum only for short times (< 20 seconds). All incubation experiments took place in the laboratory of the research station. The samples were incubated in darkness at 27°C for either 48, 72 or 96 hours. Control samples were stored in Eppendorf tubes. All samples were dried in cryoboxes filled with silica gel. To prevent sample loss, the open vials were plugged with cotton wool. Ants and Coccoidea were killed by putting them in the freezer for half a day before drying. If necessary, silica gel was exchanged, so the samples were kept in constantly dry environment.

3.2.3 Founding stage colonies

Saplings with a clogged entry hole at the prostoma, indicating an *Azteca* queen inside (Figure 1c), were collected and opened at the research station. Colonies inhabiting only one internode with entry holes still sealed were considered to be in the founding stage.

Usually, only few eggs, larvae and pupae existed apart from the queen, rarely also workers were present. Fpatches (n = 5) and queens (n = 5) were collected and incubated. As the patches at founding stage were usually very small and did not allow splitting the same material for control and experiment, additional Fpatches (n = 5) and queens (n = 5) were collected to serve as control.

In addition to the *in vitro* experiments in which the samples were removed from their natural environment and incubated in glass vials, some founding queens were incubated *in situ* in their domatium to keep disturbance at a minimum. Recently colonized domatia were cut out of the plant maintaining the septum on each side of the internode. Carefully drilled holes in the plant walls of the internode assured gas diffusion. Several domatia were placed in large vials (70mL or 90mL, Figure 2c). In order to save expensive $^{15}N_2$ gas in the large volumes, Argon (Ar) stored in a gas bag was added to the artificial atmosphere (40 % $^{15}N_2$, 35 % Ar, 25% O_2). Unfortunately, the humidity within the vial due to the respiration of the plant material favored the growth of mold despite added silica gel and compromised the experiment. After incubation, the internodes were opened, the Fpatch (n = 5) and queen (n = 3) removed and put into separate Eppendorf vials.

3.2.4 Established colonies

In order to (i) compare nitrogen fixation rate in patches of founding queens and in patches from established colonies and (ii) the influence of domatium age on nitrogen fixation activity, also larger plants (>2 m) were harvested. The plant stem was cut in 3 parts with an equal amount of internodes. As new internodes develop apically, stem part 1 represented the youngest internodes with the youngest patches and carton structures within the colony. Stem part 2 was located in the middle and stem part 3 consisted of the oldest internodes found at the bottom of the plant.

Before opening, each stem part was put in the fridge for about 10 min to tranquilize the ant workers. Afterwards, it was cut longitudinally and larvae (n = 5), pupae (n = 5) and workers (n = 6) as well as carton structures (n = 25) and patches (n = 35) were collected. Carton and patches were pooled for each stem part. To identify influence of incubation time on $^{15}N_2$ fixation, patch and carton samples were incubated for either 48, 72 or 96 hours. Additionally, Coccoidea herded by the ants in the domatia, as well as epiphytes on the stem and epiphylls of the host plant were collected. The collected material of each sample was divided in two parts: One half was incubated *in vitro* in 2mL GC vials as

already described, the other half was kept as control in Eppendorf vials. This resulted in one incubated and one control sample for each sample type.

3.3 Interaction of ants with patches and Müllerian bodies

3.3.1 Behavioral observations

Investigation of behavioral interactions of the ants with the fungal patch was conducted with a Canon EOS 5D MarkII camera equipped with a Special Vario Objectiv (Karl Storz GmbH, Germany) and an endoscope (Karl Storz, Hopkins II). For illumination, red light from a LED connected to the endoscope was used. Unfortunately, no observation in the field was possible, as it proved to be very hard to locate the position of patches from the outside and the endoscope had to be positioned on the opposite for good recordings. As a consequence, domatia at founding stage were harvested and the endoscope was inserted through the septum, allowing recording of a whole internode (Figure 2f). The live recordings could be followed on the screen of the camera.

3.3.2 ^{15}N labelled patch

To investigate possible nitrogen transfer to the ants, the patches of domatia of young colonies only inhabiting one internode were selected for labelling *in situ* with a 98at% Glycine ^{15}N amino acid mixture (Isotec Sigma-Aldrich, USA, referred to as AS-mix). A small window was cut into the cell wall of the domatia and a 4µl droplet of AS-mix was placed onto the patch using a 20µl syringe (GE healthcare, Belgium; Figure 2d). The droplet was quickly absorbed by the patch. If the queen or workers interacted with the patch or the droplet before complete absorption, the sample was excluded. After adding the AS-mix, the internode was closed again (Figure 2e) and put into a plastic bag to slow desiccation of the plant. The bags were stored outside but protected from rain. Each day, the domatia were checked for mold. After approximately 7 days of incubation time, which also depended on the condition of the plant, the queen, brood and, if present, workers were removed and stored in separate Eppendorf vials. The ants were killed by keeping them in the freezer for half a day. Additionally, the patch was collected as control for proper execution of the experiment because only successful labelled patches show elevated $^{15}N/^{14}N$ ratios. The storage and transport was identical to that of the $^{15}N_2$ incubated samples. Five colonies were selected, which contained at least 4 of the 5 sample types of interest (larvae: n= 4, pupae: n = 5, worker: n = 3, queen: n = 5 and patch: n = 5).

3.3.3 ^{15}N labelled Müllerian bodies

Another small experiment was conducted to investigate destinations of Müllerian bodies within *Azteca* colonies. Müllerian bodies collected from previously opened colonies were placed in 60µL ^{15}N AS-mix. The stems of 3 *Cecropia* plants inhabited by established colonies were cut to approximately 35cm in length from the top to reduce dilution of the label and were then strung to a terrace bar, hanging in midair. Fortunately, the ants remained on the plant and were fed thrice a day with labelled Müllerian bodies by placing them onto the branches near the trichilia. After three days, the colonies were opened and larvae (n = 2), pupae (n = 3), worker (n = 4) and queens (n = 2) were collected. All fungal patches found within one plant were pooled. Storage and transport was the same as in the $^{15}N_2$ experiments.

3.4 Isotope ratio mass spectroscopy

In Vienna, samples were selected for Isotope Ratio Mass Spectroscopy (IRMS). In order to keep information gain and analysis costs in a favorable ratio, a sample size of at approximately n = 5 per sample for each control and experiment type was intended, except from carton and patch samples from established colonies, of which 26 and 36 samples were analyzed. Different amounts were weighed in, depending on the sample type (Supplementary Table 1) and packed into tin capsules. Because of the excessive weight of the queen, the abdomen was dissected and only the gastrointestinal tract was analyzed. The legs from ant workers were removed.

The tin capsules were then combusted dryly at approximately 1000 °C to produce CO_2 and N_2 gases. An elemental analyzer connected to an isotope-ratio mass spectrometer (DeltaPLUS, Finnigan MAT, Bremen, Germany) was used for measuring N isotope ratios ($^{15}N/^{14}N$). Weighing and IRMS analysis were conducted at the Stable Isotope Laboratory at the University of Vienna for Environmental Research (SILVER).

For calculations of ^{15}N enrichment, the $^{15}N/^{14}N$ ratio is expressed either in δ (delta) values or atom percent (at%) which can be converted to each other. The atom percent value represents the amount of ^{15}N atoms per 100 N atoms and is the standard unit for atomic ratios. It is calculated using the following formula (Slater et al., 2001):

$$at\%\ ^{15}N = \frac{^{15}N}{^{14}N + {}^{15}N} * 100$$

Furthermore, the atom percent excess (APE) was calculated, which is the difference between at% of control samples (natural abundance) and at% after incubation ($^{15}N_2$-enrichment).

$$APE = at\%_{incubated} - at\%_{control}$$

For smaller differences between control and experiment $^{15}N/^{14}N$ ratios, the δ value can be used, which is a relative deviation from international standards (atmospheric dinitrogen) in per mill. The corresponding formula (Slater et al., 2001):

$$\delta\ ‰ = \left(\frac{R_{sample}}{R_{reference}} - 1\right) * 1000$$

R = atomic ratio ($^{15}N/^{14}N$)

δ excess values were calculated analogous to APE ($\delta_{inc} - \delta_{control}$). Furthermore, to obtain actual fixation activity over time, the accumulation rate of nitrogen per g dry weight per day was calculated using the following formula (Fürnkranz et al., 2008):

$$\mu mol^{15}N_2\ g^{-1}d^{-1} = \frac{C_N * APE * 1000}{t * Mr * 100}$$

C_N = mg N per g dry weight
APE = Atom percent excess
t = time in days
Mr = molecular weight of $^{15}N_2$ or ^{15}N in the labelling experiments

3.5 Statistical analysis

All statistical analyses were conducted using IBM SPSS Statistics Version 20 (IBM corp., USA).

3.5.1 ^{15}N at% before and after incubation

At first, all different sample types collected from both founding stage and established colonies (Larvae, pupae, worker, queens, Coccoidea, Fpatch, patch, carton, epiphytes and epiphylls) were tested for an increase in $^{15}N/^{14}N$ ratios after incubation. Sample sizes were low (n = 5) for most samples, not normally distributed and the variances were much higher after incubation. Therefore, ^{15}N at% before and after incubation were compared using multiple nonparametric Wilcoxon signed rank tests. For one patch sample (76_III_P), no control sample was collected and the mean of part 3 control patch samples was used. Similarly, for 2 control worker samples the mean was used. Only Fpatch and queen samples were compared using Mann-Whitney U tests as the control values were the means of Fpatches and queens collected from other *Azteca* colonies. To avoid alpha error inflation, the Holm-Bonferroni procedure was used to adjust the critical alpha level (Holm, 1979).

3.5.2 $^{15}N_2$ fixation rates of sample groups

Sample groups were formed to compare fixation activity between animal samples (larvae, pupae, worker, queen Coccoidea), ant-made structures (Fpatch, patch, carton) and host plant surface epiphytes (epiphylls, stem epiphytes). To control for variation in incubation times and weighed in mass, the $^{15}N_2$ fixation in µmol $^{15}N_2$ per gram dry weight per day was used and log transformed to achieve both normal distribution and homoscedasticity. A mixed general linear model was calculated, with the fixation rate as dependent variable, sample group as fixed factor and the collection plant as random factor. Least significant difference (LSD) post hoc tests were calculated to locate differences in fixation rates between the groups.

3.5.3 $^{15}N_2$ fixation rates of patches and carton in established colonies

Patches and carton samples collected from established colonies were further analyzed. A mixed general linear model was calculated with the log transformed fixation rate as dependent variable. Sample type (patch, carton) and stem part (1, 2, 3) were entered as fixed factors, the collection plant as random factor.

3.5.4 ^{15}N amino acid labelling experiments

In both AS-mix labelling experiments, at% values after incubation of larvae, pupae and worker were compared with control at% values obtained from ant samples of established colonies, whereas queens and Fpatches were compared to control at% values of samples of other founding stage colonies. As experiment and control samples thus came from different plants, Mann-Whitney U tests were conducted.

3.6 DNA extraction and sequence analysis

DNA was extracted of all patch and carton samples chosen for IRMS analysis following a slightly modified Quick-start Protocol of the *DNeasy Plant* mini kit (Qiagen, Germany). Furthermore, the DNA of workers of established colonies from which the queen was not collected was extracted for species determination. A negative control (only chemicals, no sample material) was also included to control for possible contamination.

3.6.1 nifH PCR runs and gel electrophoresis

The *nifH* gene encodes the dinitrogenase-reductase subunit and is necessary for diazotrophic activity. It has been established as a biomarker for the study of nitrogen-fixing bacteria (Gaby & Buckley, 2012). After multiple test runs, a decent PCR protocol was established (Supplementary Table 2, 3) yielding strong bands of the *nifH* gene sequence using the Ueda19F and R6 primer pair (Table 1). For amplification and cycle sequencing of the target gene sequence a thermocycler (Eppendorf Mastercycler Model MC5331 and MC5333) was used.

Table 1.
Primer pairs and their sequences used for *nifH* amplification. Direction refers to either forward or reverse primer read orientation.

Primer	Direction	Sequence (5'-3')	Reference
Ueda19F	Forward	GCIWTYTAYGGIAARGGIGG	Ueda et al. (1995)
R6	Reverse	TCIGGIGARATGATGGC	Marusina et al. (2001)

After the PCR run, a gel electrophoresis (0.75g Agar, 50mL Tris-acetate-EDTA (TAE) buffer, 0.5µl GelRed (Biotium Inc., USA) was used to test for successful *nifH* sequence amplification and product quality. Negative controls were also made (PCR grade water instead of DNA) to control for contamination. Successfully amplified samples were then prepared for Sanger sequencing (Sanger et al., 1977).

3.6.2 Cycle sequencing

In order to eliminate the primers and any protein impurities for cycle sequencing, the PCR products were purified with 1µl FastAP Thermosensitive Alkaline Phosphatase and Exonuclease I (Fermentas, Germany; Werle et al., 1994). The samples were kept for 30 minutes at 37 °C, and 15 minutes at 80 °C. For cycle-sequencing an ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit v.3.1 (Applied Biosystems, UK), and the PCR primers were used (Supplementary Table 2). After cycle sequencing (Supplementary Table 3), the products were analyzed using an automated DNA sequencer (ABI 3730xl Genetic Analyzer, Applied Biosystems).

3.6.3 Analysis of sequences

For the manual post-processing (removing ambiguities and noisy parts) of obtained sequences SeqMan Pro version 7.1.0 (DNASTAR, Lasergene, USA) was used. The corrected sequences were then imported and checked using MEGA 6.06 (Tamura et al., 2013). Similar sequences were obtained using a nucleotide Megablast search on the GenBank website (URL: http://blast.ncbi.nlm.nih.gov/). Sequences of the *nifH* region of deltaproteobacteria found on the GenBank were also included for rooting of the tree. Alignments were assembled in MEGA using the built-in Muscle program (Edgar, 2004). Only sequences with more than 200bp length were included. Sequences were imported to T-Rex (Boc et al., 2012) and a maximum likelihood phylogenetic tree was created using the RAxML method (Stamatakis, 2006) with 1000 iterations. The tree was further edited using the program Dendroscope 3 (Huson & Scornavacca, 2012) and Photoshop CS6 (Adobe Systems, USA).

3.6.4 Identifying ant species

The ant species of most colonies was identified morphologically using the extensive identification keys for *Azteca* queens provided by Longino (2007b).

In case that the queen, which is necessary for determination of *Azteca* species, was not collected, the DNA of workers of the respective colonies was analyzed using the nuclear gene of Long-Wave Rhodopsin (LWRh) and comparing it to already morphologically identified *Azteca* queens according to Pringle et al. (2012). For amplification of the target region the primer pair LR143F and LR639ER (Ward & Downie, 2005), was used (Table 2). PCR protocol and cycle sequencing preparation as well as sequence analysis were the same as in the *nifH* analysis (Supplementary Table 2, 3). The aligned and corrected sequences were compared to 16 reference LWRh sequences from already identified

Azteca ants (2 *A. coeruleipennis*, 4 *A. alfari*, 6 *A. xanthochroa*, 4 *A. constructor*, Nepel et al., unpubl.). A sequence identity matrix was created in MEGA showing the number of base substitutions per site between sequences. Analyses were conducted using the Maximum Composite Likelihood model (Tamura et al., 2004). The analysis involved 18 nucleotide sequences. The resulting similarity matrix was analyzed using the PROXSCAL algorithm of SPSS 20 for multidimensional scaling (MDS) of proximity data.

3.6.5 Chaetothyriales OTUs

Twenty patch samples were also analyzed using the internal transcribed spacer (ITS) region of ribosomal DNA which is commonly used for identification of fungi (Schoch et al., 2012). V9G (de Hoog & Gerrits van den Ende, 1998), chaeD-R and V9G, chaeDITS-R primer pairs (Table 2) were used. The chaeD-R and chaeDITS-R reverse primer have been designed to identify Chaetothyriales associated with domatia of ant-plants (Voglmayr et al., 2011; Nepel et al, unpubl.). The procedure was the same as in the previous DNA amplifications, only a different general PCR program was used (Supplementary Table 4). For some samples the V9G and chaeD-R primer pair yielded no bonds after PCR amplification. In these cases the V9G and chaeDITS-R primer pair was used. All 20 samples were successfully amplified and all but one sample were also successfully Sanger sequenced. Sequences were corrected and aligned using the same procedure and programs as in the *nifH* analysis with Chathothyriales OTUs provided by Nepel et al. (unpubl.) as reference sequences. Again, a maximum likelihood phylogenetic tree was created using the RAxML method with 1000 iterations and modified in Dendroscope 3 and Photoshop CS6.

Table 2.
Primer pairs used for *Azteca* worker and chaethothyrialean fungi identification. Direction (Dir) refers to either forward (Fwd) or reverse (Rev) primer read orientation.

Primer	Dir	Sequence (5'-3')	Reference
V9G	Fwd	TTACGTCCCTGCCCTTTGTA	de Hoog & Gerrits van den Ende (1998)
chaeD-R	Rev	GCCCTACCGCAGTTCCA	Nepel et al. (unpubl.)
chaeDITS-R	Rev	AGTACGTGCTACAAGAGC	Nepel et al. (unpubl.)
LR143F	Fwd	GACAAAGTKCCACCRGARATGCT	Ward, P. S., Downie D. A. (2005)
LR639ER	Rev	YTTACCGRTTCCATCCRAACA	Ward, P. S., Downie D. A. (2005)

Figure 1. Ants and plants.
(a) *Cecropia* sp. sapling. **(b)** A trichilium on the abaxial petiole base offering glycogen rich Müllerian bodies for their *Azteca* inhabitants. **(c)** Prostoma recently entered by an *Azteca* queen. It was closed again by the queen using parenchyma. **(d)** *Azteca constructor* queen standing on a founding stage patch (Fpatch) next to the clogged entrance hole. **(e)** Large founding stage patch inside a young internode. Abundant white parenchyma is still present on the plant wall. **(f)** Internode of an established colony, with brood, workers and a prominent patch area. A carton structure with Coccoidea is seen on the right. Almost all white parenchyma is scraped off the plant wall. **(g)** In established colonies, Müllerian bodies are sometimes deposited and incorporated into the patch. **(h)** Magnification of patch material reveals presence of dead ant body parts. Bar represents 7mm. **(i)** Internode of an established colony with both patch and carton and already brownish plant wall. **(j)** The three *Azteca* species found within *Cecropia* in the course of the study: *A. alfari*, which was inhabiting most of the collected *Cecropia* plants is shown in the first row. In the second row *A. constructor*. In the third row *A. xanthochroa*, the largest queen with characteristic orange color. The bars represent 0.7mm.

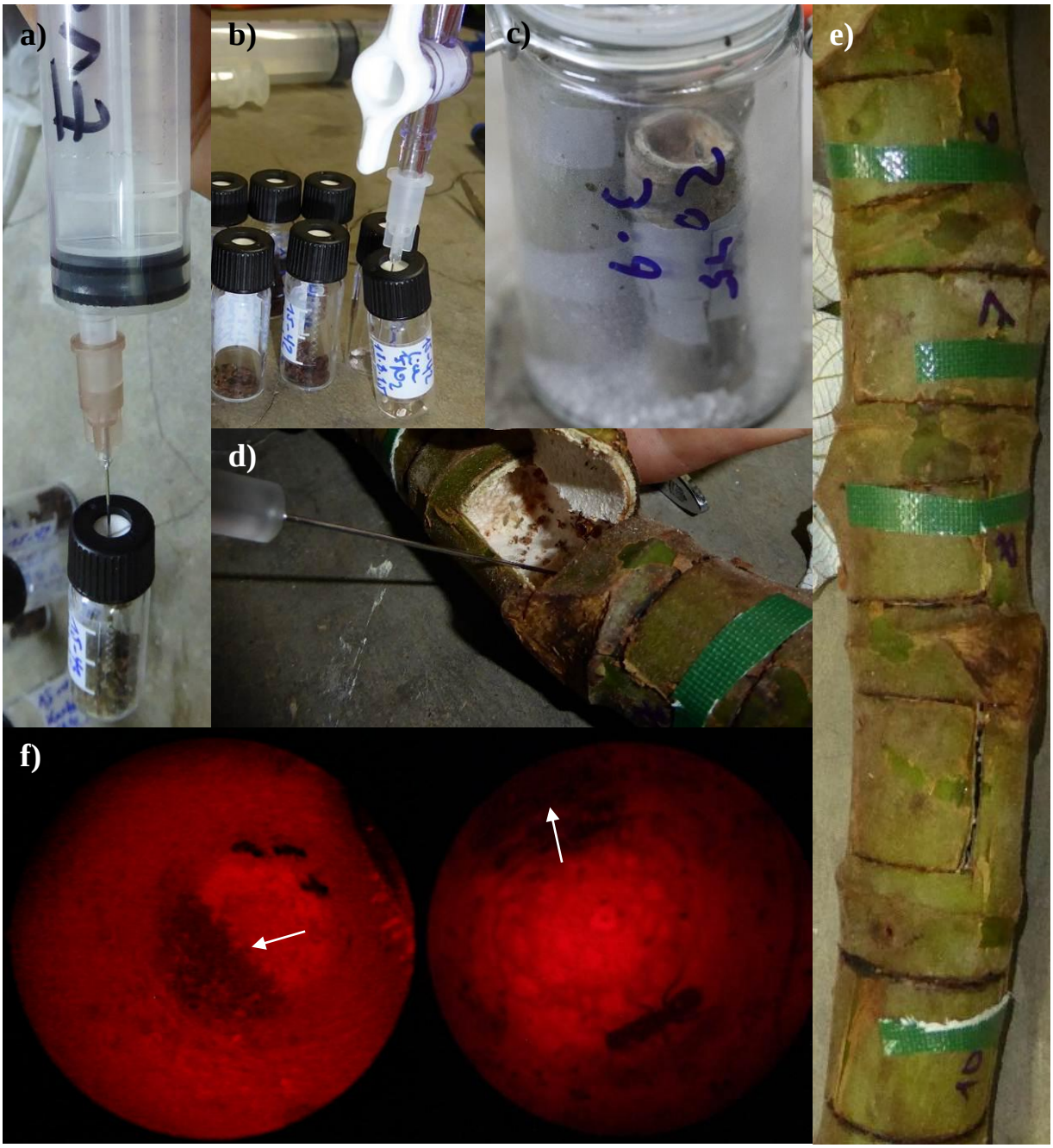

Figure 2. Incubation and labelling experiments.
(a) 2mL GC vials were evacuated using a syringe, creating vacuum. (**b)** A syringe containing the artificial atmosphere (75% $^{15}N_2$, 25% O_2) was quickly inserted into the vial containing vacuum. **(c)** Some patches and queens were incubated *in situ* within the internodes in large vials. Mold constituted a serious problem, however, caused by high humidity as seen on the picture. **(d)** In the patch labelling experiment, a window was cut into the internode and a 4µl ^{15}N AS-mix droplet was directly put onto the patch using a syringe. **(e)** Founding queens were found in many neighboring internodes. Once the patch was labelled, the internodes were closed again using tape. **(f)** Pictures taken while recording the inside of inhabited internodes of two different colonies with an endoscope inserted through a septum. White arrows point towards the patch area. On the left picture, workers are present while the queen is next to the endoscope and not seen. On the right picture, both queen and patch are seen, with ant workers not yet present. The wall on the back is a septum, separating one internode from another.

4 Results

4.1 $^{15}N_2$ fixation activity in and on Cecropia

An overview of all different sample types analyzed and their respective values can be seen in Table 3. The samples were collected from both founding stage (queens and Fpatches) and established colonies. The samples which underwent *in situ* incubation showed almost no increase in ^{15}N content (Table 3). Fpatch and queens were compared to the means of respective control samples collected separately, the other samples to control samples from the same colony. While patches and carton from established colonies were only used from *A. alfari* colonies, different ant species were analyzed in queen (2 *A. alfari*, 2 *A. constructor*, 1 *A. xanthochroa*) and Fpatch (3 *A. alfari*, 1 *A.* constructor, 1 *A. xanthochroa*) samples. In larvae, pupae and worker, one sample was from *A. constructor* while all others were from *A. alfari.*

Table 3.
Sample types of the nitrogen incubation experiments analyzed. ^{15}N fixation is given in atom percent excess (APE = incubated – control) and µmol $^{15}N_2$ per g dry weight per day. FPatch = Founding stage patch. Queen *in situ* and FPatch *in situ* were incubated in their domatia. All other samples were incubated *in vitro.* Values show means ± standard error of means.

Sample type	N	Incubation time [h]	^{15}N at%	Atom percent excess (APE)	µmol $^{15}N_2$ g^{-1} d^{-1}
FPatch	5	58.01 ± 5.71	0.72 ± 0.23	0.21 ± 0.10	0.022 ± 0.011
Patch	25	82.42 ± 2.68	0.51 ± 0.04	0.19 ± 0.05	0.019 ± 0.005
Carton	18	84.68 ± 2.99	0.44 ± 0.03	0.1 ± 0.04	0.007 ± 0.002
Larvae	5	77.62 ± 8.74	0.38 ± 0.01	0.01 ± 0.01	0.003 ± 0.001
Pupae	5	82.32 ± 9.34	0.38 ± 0.01	0.01 ± 0.01	0.002 ± 0.001
Worker	6	80.33 ± 5.1	0.39 ± 0.01	0.03 ± 0.01	0.004 ± 0.001
Queen	5	62.54 ± 5.8	0.4 ± 0.01	0.03 ± 0.01	0.008 ± 0.004
Coccoidea	5	68.46 ± 7.28	0.4 ± 0.02	0.03 ± 0.02	0.005 ± 0.003
Stem epiphytes	5	72.30 ± 0.05	0.48 ± 0.07	0.11 ± 0.07	0.024 ± 0.015
Epiphylls	5	72.70 ± 0.18	0.43 ± 0.02	0.07 ± 0.03	0.024 ± 0.01
Queen *in situ*	3	78.67 ± 15.33	0.37 ± >0.01	>0.001 ± >0.01	>0.001 ± >0.001
FPatch *in situ*	5	75.95 ± 11.41	0.37 ± >0.01	-0.28 ± 0.17	-0.056 ± 0.043

4.1.1 Ant species and incubation time

The small sample size of Fpatches did not allow statistical comparisons of fixation rates between ant species. However, the Fpatch fixation rate of the *A. xanthochroa* sample (0.06 $^{15}N_2$ µmol g^{-1} d^{-1}) was much higher than of *A. constructor* and *A. alfari* (both 0.01 $^{15}N_2$ µmol g^{-1} d^{-1}, see Supplementary Table 6).

From established colonies more patch and carton samples were initially analyzed than shown in Table 3. An investigation of sample sizes regarding the factors (incubation time, stem part, sample type and ant species revealed great sample imbalances concerning ant species and incubation time, with all samples either from *A. alfari* or *A. constructor*. The mean fixation rate of patches of *A. alfari* colonies (n = 25, 0.019 $^{15}N_2$ µmol g^{-1} d^{-1}) was 2.7 times as high as fixation activity in patches of *A. constructor* colonies (n = 8, 0.007 $^{15}N_2$ µmol g^{-1} d^{-1}). However, comparing the days of incubation revealed significantly lower $^{15}N_2$ fixation rates after 2 days than after 3 or 4 days (Kruskal-Wallis test, df = 2, n = 61, $p < .001$, see Supplementary Figure 1). Within 2 days of incubation time, almost all samples incubated were from *A. constructor*, whilst *A. alfari* was found most frequently in the 3 and 4 days incubation groups (see Supplementary Table 7). This is problematic, as the difference in fixation could be either caused by the shorter incubation time or the ant species.

Thus, as the main interest of investigation was the difference in fixation rate between patch and carton and between stem parts, the patch and carton samples which only were incubated for 2 days as well as the *A. constructor* samples were removed from analysis to allow an unbiased statistical analysis. No significant difference of fixation rates between 3 and 4 days of incubation time was found (Mann-Whitney test, n = 43, p = .662) and incubation times were pooled. It has to be noted, that all assumptions made regarding established colonies are therefore based on *A. alfari* and 2 or 3 days of incubation time.

4.1.2 ^{15}N at% before and after incubation

Comparison of ^{15}N at% values before and after incubation in $^{15}N_2$ enriched atmosphere revealed high N fixation in most samples (Table 3, Figure 3). Multiple Wilcoxon Signed rank tests were conducted, comparing control and incubated at% values. Both queens and Fpatches *in situ* did not differ from the control (Mann-Whitney tests, queen: n = 3, p = .7, Fpatch: n = 5, p = .548) which was most probably caused by methodological problems (mould) and were not further analyzed.

Larvae and pupae samples had no significant increase in ^{15}N at% during incubation time (Wilcoxon signed rank test, larvae: n = 5, p = .225, pupae: n = 5, p = .138). Carton and patch samples of established colonies differed highly significant from control samples (Wilcoxon signed rank test, patch: n = 25, p < .001; carton: n = 18, p < .001). Interestingly, worker samples also displayed significant $^{15}N_2$ accumulation (Wilcoxon signed rank test, n = 6, p = .028) and both epiphylls and stem epiphytes incorporated $^{15}N_2$ (Wilcoxon signed rank test, n = 5, p = .043 for both). In Fpatch and queens samples, Mann-Whitney tests were conducted, as the control samples were collected from other plants, revealing a significant increase in $^{15}N_2$ ratio (Mann-Whitney test, Fpatch: n = 5, p = 0.09; queens: n = 5, p = .009). Controlling for multiple testing, the Holm–Bonferroni method was used (Holm, 1979). As a result, only patch, carton, Fpatch and queen samples can be considered significantly different. Nevertheless, all samples with p < .05 are regarded to be nitrogen-fixing in this case, as multiple pair-wise testing is unavoidable in this type of study (e.g. Pinto-Tomás et al., 2009), pair testing was planned and the results show a clear increase in $^{15}N_2$ content, indicating a general effect (Moran, 2003).

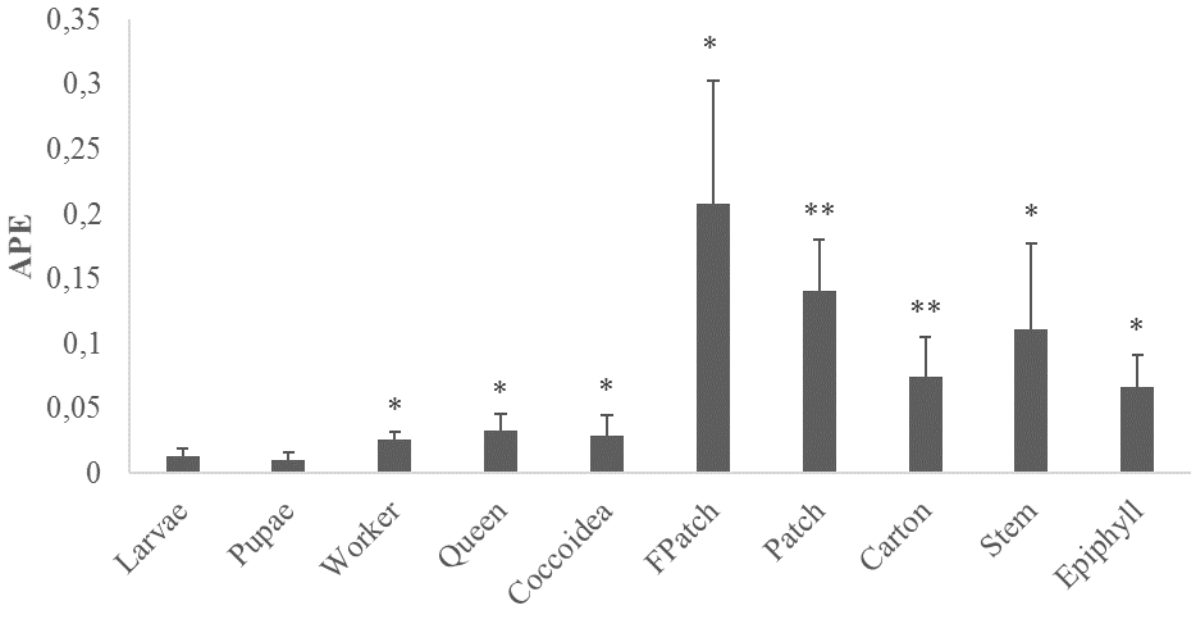

Figure 3.
Mean ^{15}N atom percent excess (APE) of all collected samples. Error bars show standard error of means. Stem = stem epiphyte. * = p < .05; ** = p < .001.

4.1.3 $^{15}N_2$ fixation rates of sample groups

Sample groups were formed to compare fixation activity between animal samples (larvae, pupae, workers, queens, Coccoidea: n = 25, one pupa sample was excluded due to negative fixation rate), ant-made structures (Fpatch, patch, carton: n = 48) and host plant surface epiphytes (epiphylls, stem epiphytes: n = 10). $^{15}N_2$ fixation in µmol $^{15}N_2$ per gram dry weight per day was used and log transformed to achieve both normal distribution and homoscedasticity. A mixed general linear model was conducted with sample groups as fixed factor and the plants from which the samples were collected as random factor. $^{15}N_2$ fixation rate differed significantly between the 3 sample groups (mixed general linear model, n = 83, F = 8.832, p = .005). LSD post hoc tests revealed that animal samples fixated significantly less than ant made structures (p = .002) and epiphytes (p = .006). No significant difference in $^{15}N_2$ fixation rate was found between epiphytes and ant made structures (p = .144; see Figure 4).

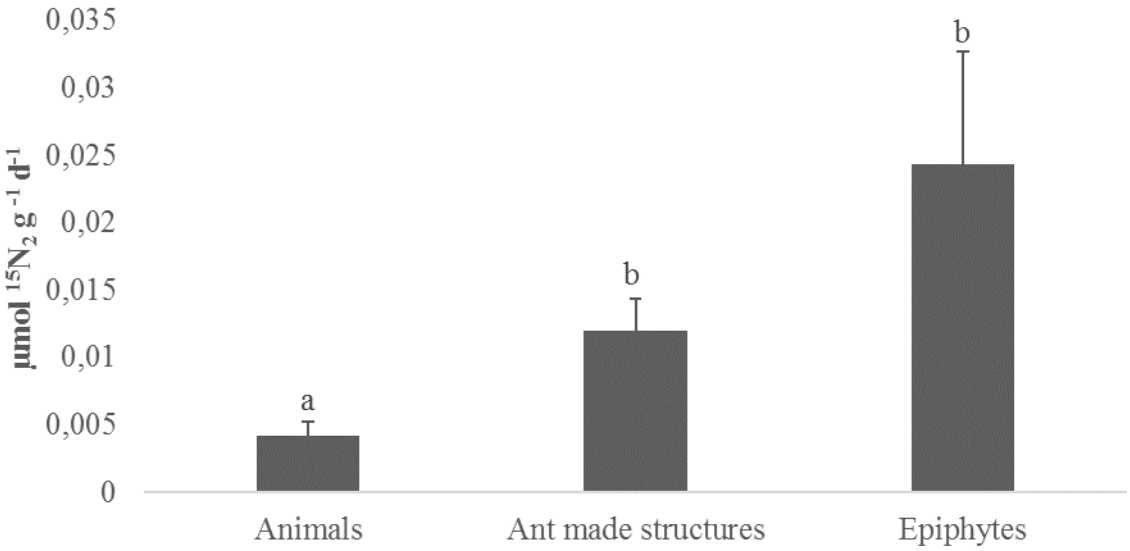

Figure 4.
Mean fixation rate of sample groups. Comparison using a mixed general linear mixed and LSD post hoc tests revealed significant (p < .01) lower fixation rates in animal samples. Letters correspond to significantly different fixation rates. Error bars show standard error of means.

4.1.4 $^{15}N_2$ fixation rates of patches and carton in established colonies

Patches and carton samples collected from established colonies were analyzed separately. Again $^{15}N_2$ fixation in µmol $^{15}N_2$ per gram dry weight per day was used and log transformed to achieve both normal distribution and homoscedasticity. A mixed general linear model was calculated comparing the fixation rate (µmol$^{15}N_2$ per gram dry weight

per day) between carton, patch samples (n = 18 and n = 25, respectively) and the stem parts (part 1: n = 14, part 2: n = 16, part 3: n = 13) with the plant from which they were collected included as random factor. A highly significant difference between carton and patch samples (mixed general linear model, n = 43, F = 15.51, p < .0001) was revealed, with a 2.7 times higher mean $^{15}N_2$ fixation rate found in patch samples than in carton (Figure 5). The $^{15}N_2$ fixation rate in the 3 stem parts was not significantly different (mixed general linear model, n = 43, F = .053, p = .948, see Supplementary Figure 2), although the mean fixation rate in patches from part 3 (0.026 $^{15}N_2$ µmol g^{-1} d^{-1}) was more than twice as high than in patches from part 1 (0.011 $^{15}N_2$ µmol g^{-1} d^{-1}, see Supplementary Table 8). An additional Kruskal-Wallis test only including patch samples (part 1: n = 9, part 2: n = 9, part 3: n = 7) also found no effect of patch age on the fixation rate (n = 25, df = 2, Chi – Square = 2.37, p = .467).

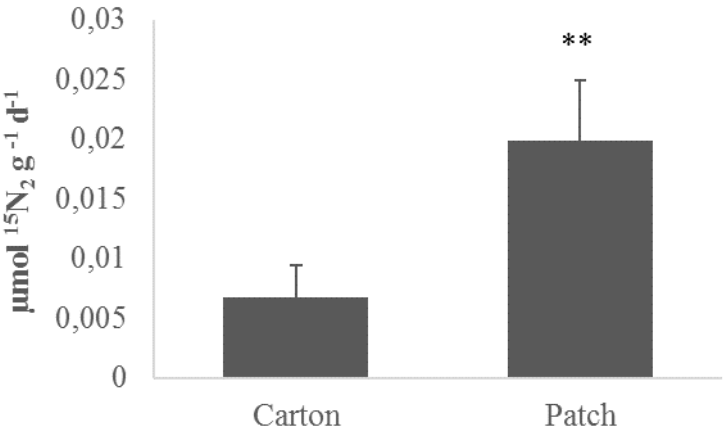

Figure 5.
Mean $^{15}N_2$ fixation of patch and carton samples after 3 and 4 days incubation time. The fixation rate in µmol $^{15}N_2$ per g dry weight per day is significantly higher in patch than in carton samples. Error bars show standard error of means. ** = p < .0001

4.1.5 Noticeable patch observations

During the experiments, dead ant bodies were often found in the patches of established colonies. Even dead queens had been integrated. On desiccation, hundreds of nematodes came to the patch surface. Also, in rare occasions, Müllerian bodies were found integrated in the patches (Figure 1g).

4.2 Interaction of ants with patches and Müllerian bodies

4.2.1 Behavioral observations

Despite relatively good recording quality and resolution (see Figure 2f), the observations failed to deliver evidence for harvesting of hyphae or defecation caused by several problems:

(i) Most of the recording time, queen or worker ants attacked the endoscope and covering it with parenchyma taken from the patch. Only after a certain amount of time, habituation occurred and the ants showed normal activities such as tending the brood and queen.

(ii) The battery of the camera only allowed observations of approximately 20 minutes and each battery change moved the camera, resulting again in a disturbance of the ants.

(iii) During prolonged observation, the plant began to desiccate, most probably inducing stress to the inhabiting ants.

4.2.2 ^{15}N amino acid labelled patch

The foundress patches of 5 colonies were infiltrated with a 98at% ^{15}N glycine amino acid mixture (AS-mix) and incubated between 6 to 8 days (mean = 7 days, see Table 4) to investigate the translocation of label. In 2 colonies, no worker were present. Also, in one of the plants investigated, no larvae were found. One of the 5 colonies was inhabited by *A. xanthochroa*, the remaining 4 by *A. alfari*.

Table 4.

Overview of all samples collected within internodes with ^{15}N glycine amino acid mix labelled patches. Larvae and worker samples are below n = 5. Columns show the means of incubation time, ^{15}N δ and µmol ^{15}N accumulation per g dry weight per day ± standard error of means. For ^{15}N δ excess (label – control) no standard error is given as the means of control samples of different plants were used.

Sample type	N	Incubation time [hr]	^{15}N δ	^{15}N δ excess	µmol ^{15}N per g d.w. per day
Larvae	4	164.66 ± 10.11	3.97 ± 2.27	7.9	$21.7*10^{-5}$ ± $12*10^{-5}$
Pupae	5	169.13 ± 9.01	1.84 ± 0.89	2.31	$4.58*10^{-5}$ ± $1.9*10^{-5}$
Worker	3	180.22 ± 6.49	0.83 ± 0.79	4.13	$10.8*10^{-5}$ ± $1.6*10^{-5}$
Queen	5	169.13 ± 9.01	0.46 ± 0.25	-0.014	$0.04*10^{-5}$ ± $3.9*10^{-5}$
Patch	5	169.13 ± 9.01	1062.8 ± 475.01	2105.1	$4500*10^{-5}$ ± $1600*10^{-5}$

Delta (δ) values were used for better visualization in this experiment as the accumulation rates of ^{15}N were very small. The elevated $^{15}N/^{14}N$ ratios of patches (Table 4) proved that the patches were successfully soaked with the ^{15}N amino acid solution (Mann-Whitney

test, n = 10, p = .008). The queens did not accumulate ^{15}N, as becomes evident from the negative mean δ excess value (n = 10, p = .31). Mann-Whitney tests comparing ^{15}N δ of larvae, pupae and worker samples respective control samples collected from established colonies revealed a significant accumulation of ^{15}N in larvae (n = 9, p = .032) whereas pupae and worker only showed a trend towards elevated ^{15}N ratios (pupae: n = 10, p = .31; worker: n = 9, p = .24; see also Figure 6)

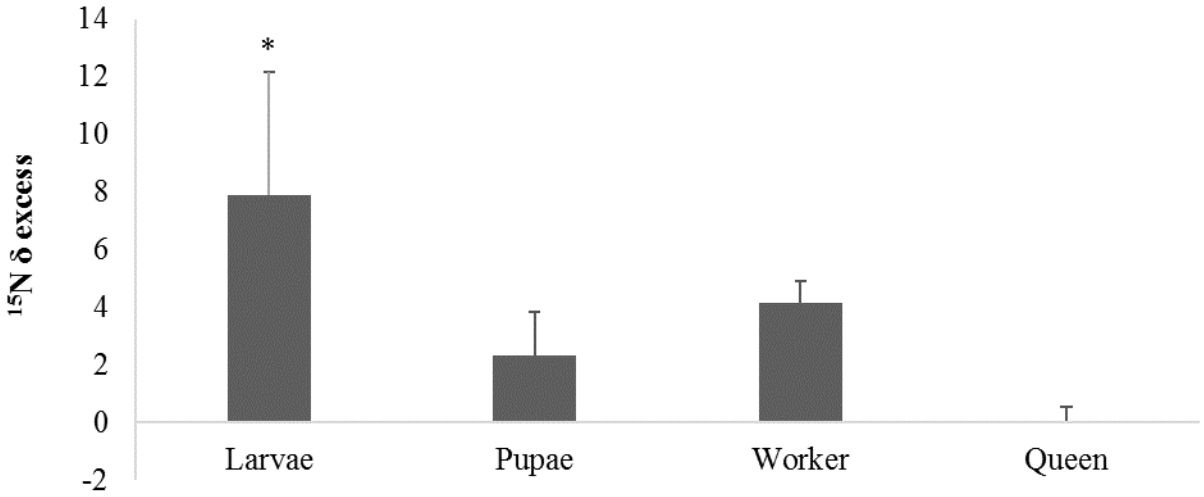

Figure 6.
Mean nitrogen δ excess values (labelled – control) of larvae (n = 4), pupae (n = 5), worker (n = 3) and queen (n = 5). Fpatch values are not depicted as they were manually labelled leading to very high δ excess values. Error bars show standard error of means. * p < .05.

4.2.3 ^{15}N amino acid labelled Müllerian bodies

Unfortunately, only very small sample sizes were obtained in the experiment (Table 5), as only three plants were investigated. Still, one plant was inhabited by two small colonies with workers (1 *A. alfari*, 1 *A. constructor*) which only became evident after opening. As a result, 4 worker and 4 patch samples were obtained. The other two plants were inhabited by *A. alfari* colonies each.

Table 5.
Overview of all samples of the Müllerian bodies experiment. Columns show incubation time and means of ^{15}N atom percent, atom percent excess (APE) and µmol ^{15}N accumulation per g dry weight per day ± standard error of means. In APE (label – control) no standard error is given as the means of control samples of different plants were used.

Sample type	N	Incubation time [hr]	^{15}N at%	APE	µmol ^{15}N per g d. w. per day
Larvae	2		0.591 ± 0.084	0.224	$90*10^{-3}$ ± $40*10^{-3}$
Pupae	3		0.367 ± 0.0001	0.007	$0.2*10^{-3}$ ± $0.04*10^{-3}$
Worker	4	72	0.495 ± 0.055	0.128	$39*10^{-3}$ ± $19*10^{-3}$
Queen	2		0.374 ± 0.0001	0.007	$5.7*10^{-3}$ ± $1.3*10^{-3}$
Patch	4		0.482 ± 0.094	0.112	$38*10^{-3}$ ± $21*10^{-3}$

Again, the ^{15}N at% of control ant samples from established colonies were used and compared to ^{15}N at% after incubation. Mann-Whitney tests were conducted for each of the sample types, revealing a significant increase in ^{15}N only in workers (n = 10, p = .029). The larvae, pupae and patch samples only indicate a trend towards accumulation (larvae: n = 7, p = .333; pupae: n = 8, p = .1; patch: n = 9, p = .343), with the larvae displaying the highest difference between experiment and control (Figure 7). The queen samples displayed no increase in ^{15}N at% (n = 7, p = .333).

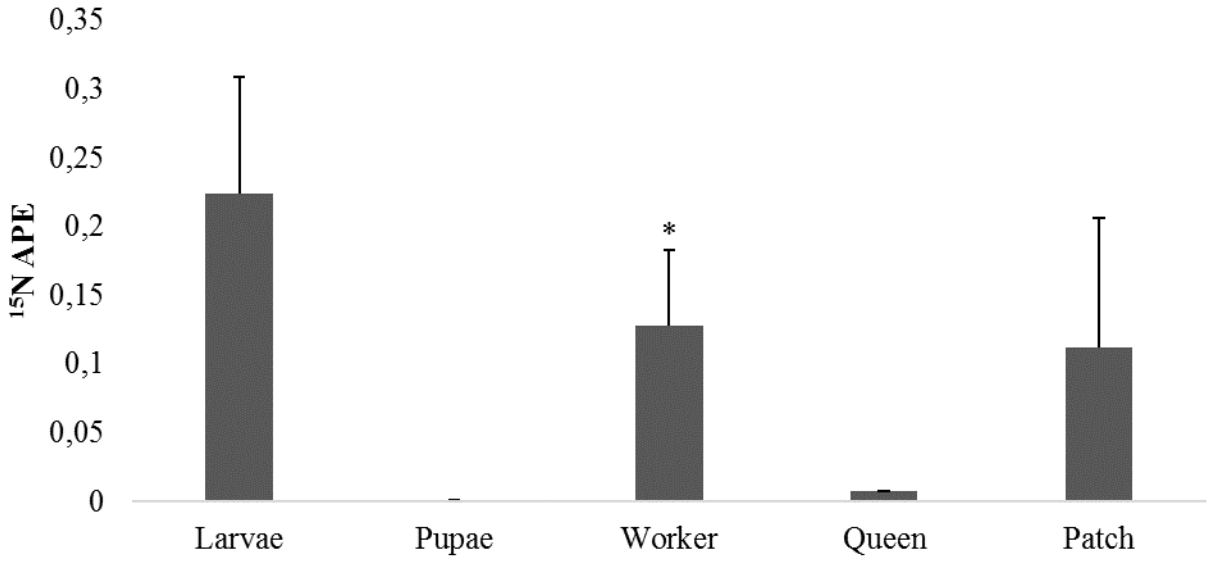

Figure 7.
Mean ^{15}N atom percent excess (APE; labelled – control) of ant and patch samples. Error bars show standard error of means. * $p < 0.05$.

4.3 DNA extraction and sequence analysis

4.3.1.1 nifH

PCR amplification of the DNA from patch (*n* = 16), carton (*n* = 25) as well as epiphyll (n = 1) and stem epiphyte (n = 3) samples using the Ueda19F and R6 primer pair (Marusina et al., 2001; Ueda et al., 1995) yielded PCR products in most samples indicating the presence of *nifH* dinitrogenase-reductase gene sequence products. The quantity of PCR product, as indicated by stronger bonds, was usually higher in patch than in carton samples (Figure 8).

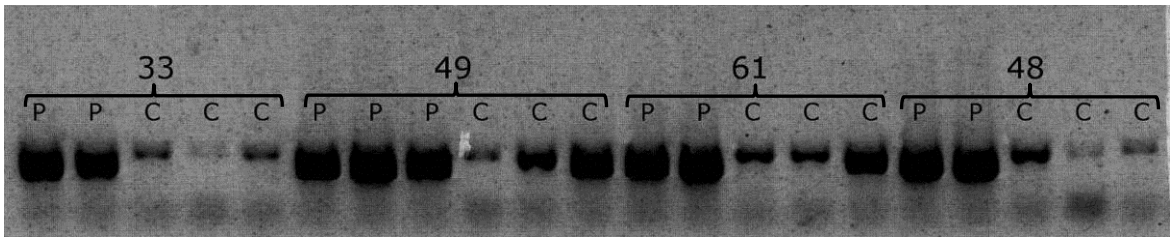

Figure 8.
Gel electrophoresis of 21 out of 45 samples after *nifH* sequence amplification using Ueda19F and R6 (Marusina et al., 2001; Ueda et al., 1995). The number corresponds to the colony number, P for patch and C for carton samples. Patches usually show strong bonds, whereas carton samples tend to have smaller quantities of target sequences after amplification. Some of the samples could not be amplified.

The DNA was not cloned prior to amplification because a limited diversity of diazotrophic bacteria was expected in this ant-shaped environment. However, sequences obtained from Sanger sequencing contained many wobbles due to simultaneous read of two different bases. This indicates multiple *nifH* genotypes within the samples. The patch samples were noisier than the carton samples. In total, 31 sequences out of 45 samples could be analyzed. None of the stem epiphytes samples yielded usable sequences. Only sequences with more than 200bp length were used for further phylogenetic analysis.

The phylogram (Figure 9) shows that neither patch (n = 15) and carton (n = 16), nor *A. alfari* (n = 25) and *A. constructor* (n = 6) samples form separate clades. In comparison with reference sequences, 79% of the sequences showed highest similarity with bacteria from the order of Rhizobiales (Alphaproteobacteria). The *nifH* sequence of the species *Mesorhizobium loti* (Rhizobiales) had the highest sequence similarity with 39% of the samples. All of the samples could be assigned to the classes of Alpha, - and Betaproteobacteria.

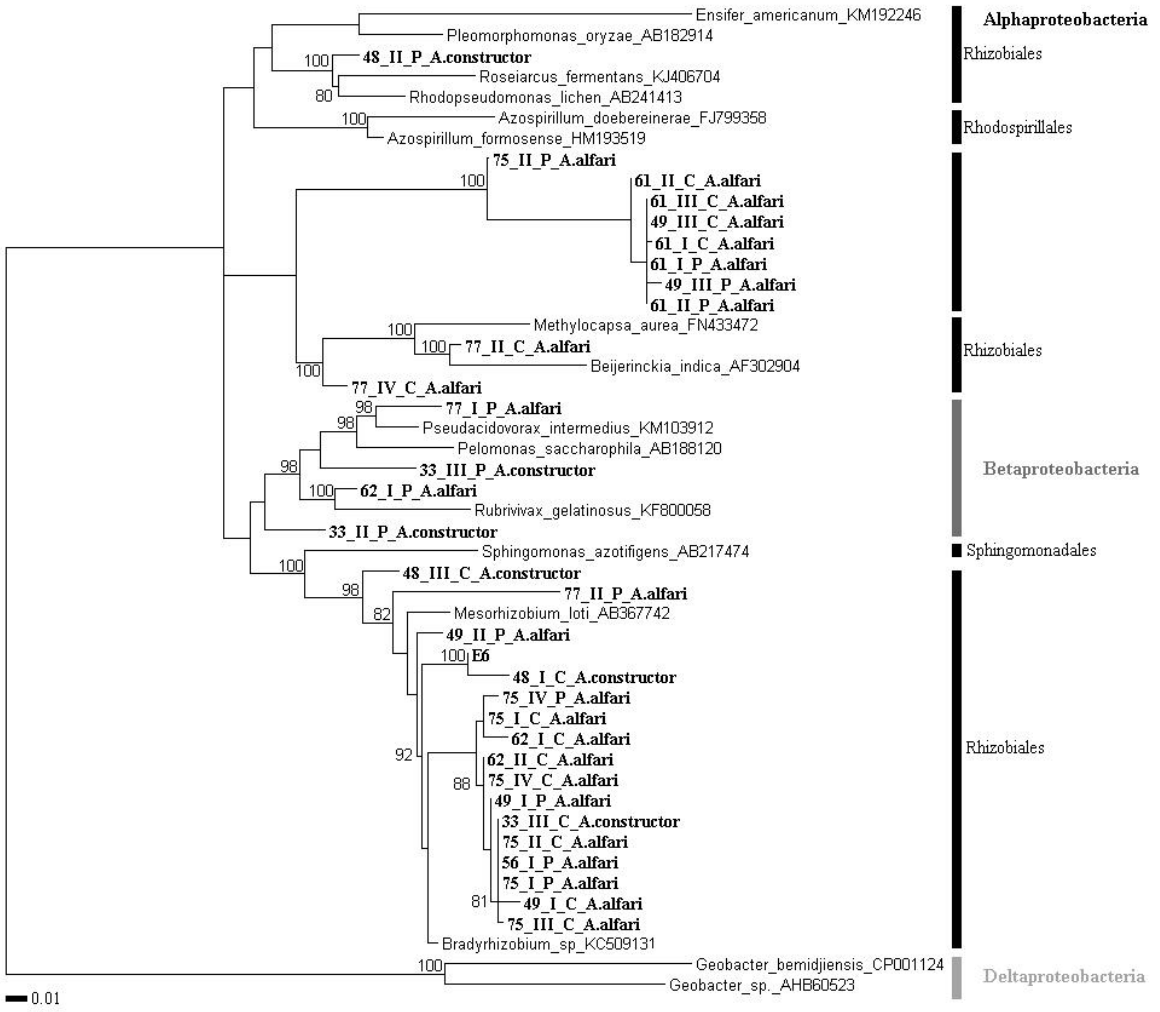

Figure 9.

A maximum likelihood phylogenetic tree with 32 *nifH* sequences from samples shown in bold and reference sequences. Only sequences with ≥ 200bp were analyzed. The number responds to the ant colony number. Roman numerals identify the stem part the sample was taken. P is a patch sample, C carton. All but colonies 33 and 48 were *A. alfari*. Numbers on the nodes represent bootstrap values (percentage of 1000 iterations that came to same result), only values > 70 are shown. All sample sequences are similar to Beta- and Alphaproteobacteria. Highest similarity was found with Rhizobiales.

4.3.1.2 Workers

All of the 14 established ant colonies could not be taxonomically assigned because the queen was missing. Except for 2 colonies PCR of LR143F and LR639ER (Ward & Downie, 2005) primer as barcode marker for *Azteca* gave good results (Figure 10). The workers of those two samples were identified morphologically (Longino, 2007b).

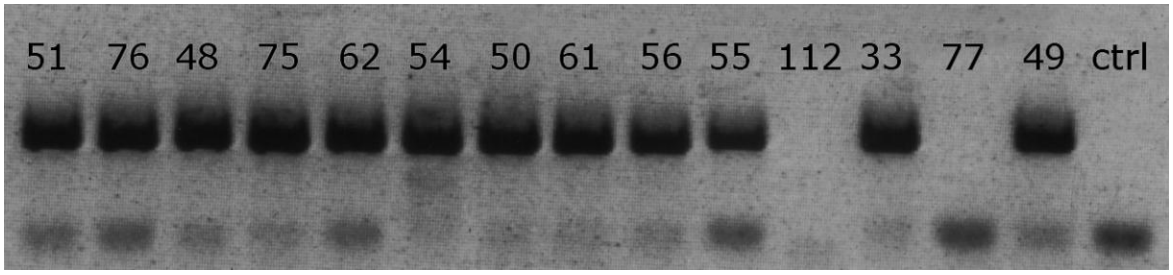

Figure 10.
Long-Wave Rhodopsin (LWRh) gel electrophoresis revealed strong bonds indicating successful amplification. Samples of colonies 122 and 77 did not work. On the right is the negative control (ctrl) which was used to check for contamination which did not occur.

The comparison of the sequences gained in this study and the Long-Wave Rhodopsin (LWRh) sequences of *Cecropia* inhabiting *Azteca* species from Nepel et al. (unpubl.) allowed a clear identification of the ant species as all samples clustered either with the sequence of *A. constructor* (33%) or *A.alfari* (67%). A 2-dimensional representation of the sequence identity matrix obtained by multidimensional scaling can be seen in Figure 11.

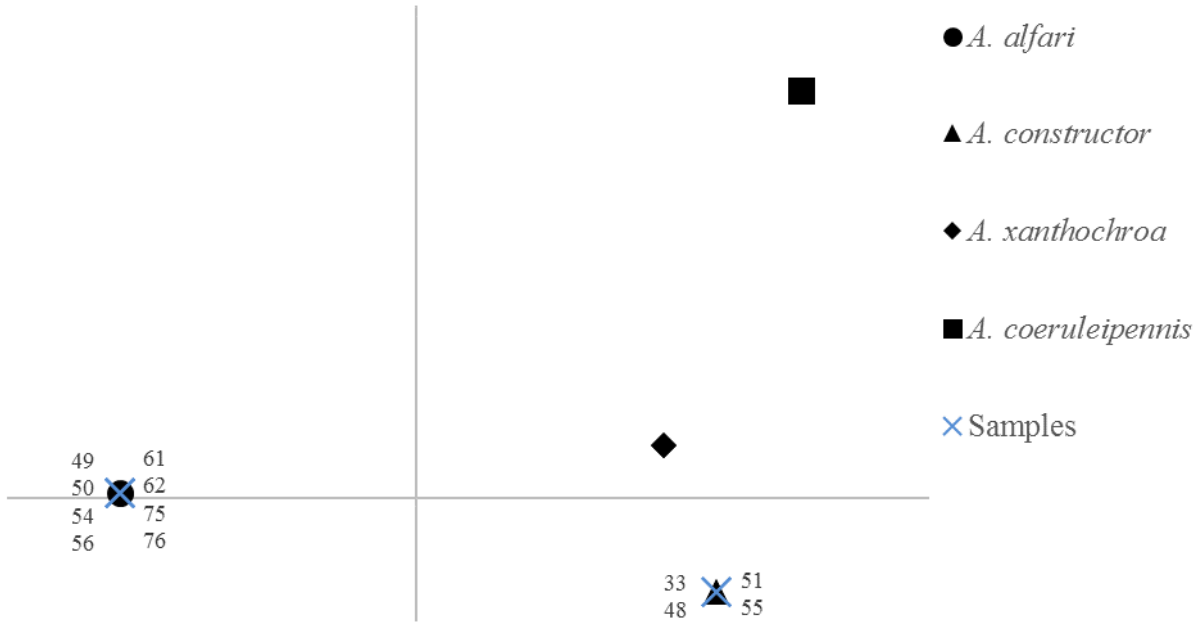

Figure 11.
Proximities of worker samples to specific ant species. Only *A. alfari* and *A. constructor* show clustering of worker samples. Surrounding numbers represent the identification number of different worker samples. The multidimensional scaling analysis was conducted with a normalized raw stress of 0.001.

4.3.1.3 Chaetothyriales OTUs

In most samples the V9G (De Hoog & Gerrits van den Ende 1998) and chaeD-R primer pair (Nepel et al. unpubl.) resulted in sufficient PCR product for Sanger sequencing. Samples with missing or weak bonds (Figure 12) were amplified using primers V9G and chaeDITS-R (Nepel et al. unpubl.). For 19 samples (Patch n = 12; Fpatch n = 4; carton n = 3, see Supplementary Table 5) the complete ITS or the partial long subunit (LSU) were obtained and affiliated to the OTUs established by Nepel et al (unpubl.).

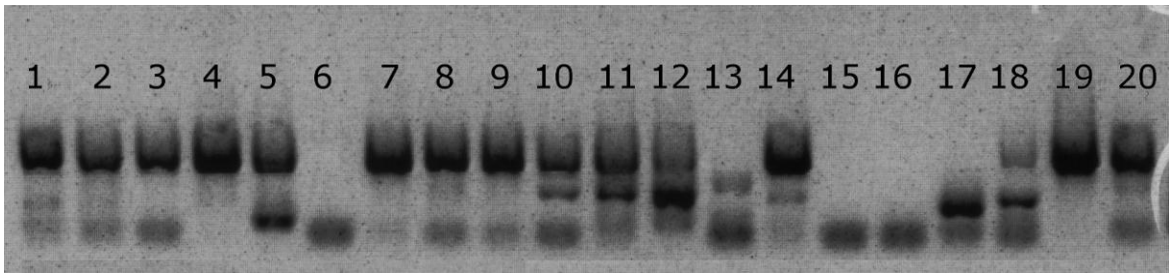

Figure 12.
Gel electrophoresis reveals that some chaethothyrialean target gene sequence amplifications did not work. This picture displays only V9G, chaeD-R primer pair results. All samples with missing bonds were then successfully amplified with a repeated PCR using the V9G and chaeDITS-R primer. The samples corresponding to the numbers can be seen in Supplementary Table 5.

A maximum likelihood phylogram of the sequence identity is shown in Figure 13. All but one of the *A. alfari* samples clustered in OTU2, while the *A. constructor* and *A. xanthochroa* sample clustered exclusively in OTU3. Thus, a high concordance between chaethothyrialean OTUs and ant species was found. Carton samples were only analyzed in *A. alfari* and clustered in the same clade. However, all foundress patches (n = 4) were found in the OTU3 clade.

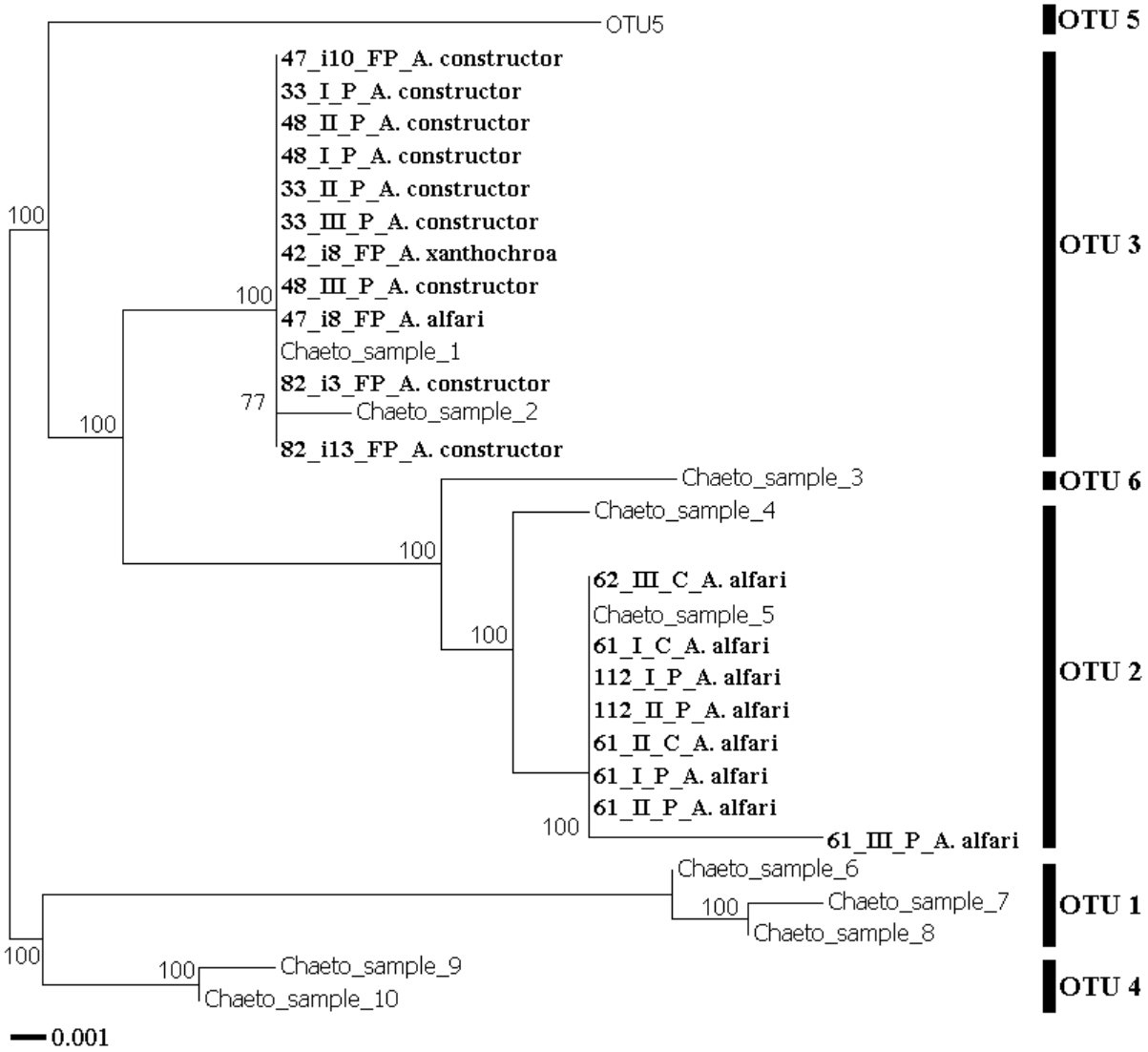

Figure 13.

Maximum likelihood phylogram of 19 analyzed samples and OTU reference sequences. All but one analyzed *A. alfari* sample cluster in the OTU2 clade, the *A. constructor* samples and one *A. xanthochroa* sample in the OTU3 clade. All Fpatches are found in the OTU3 clade, all carton samples in the OTU2 clade

5 Discussion

5.1 $^{15}N_2$ fixation in patches and carton

This present study clearly demonstrated for the first time that nitrogen fixation occurs in the domatia of *Cecropia* plants colonized by *Azteca* ants. Fungal patches which are inhabited by chaetothyrialean fungi (Ascomyceta) and found regularly in ant-inhabited domatia were of particular interest because of their involvement in nutrient cycles (Blatrix et al., 2012, Defossez et al., 2010, Voglmayr et al., 2011). $^{15}N_2$ fixation took place in ant-made patches of both established colonies and colonies at founding stage. This indicates colonization by diazotrophic prokaryotes already during colony founding. Interestingly, the mean $^{15}N_2$ fixation rate (measured in $^{15}N_2$ µmol g^{-1} d^{-1}) in patches of founding queens was similar to that of patches collected from established colonies. In established colonies, fixation rates between patches of different domatia age were not significantly different, although the fixation rate in patches of older, already abandoned domatia (0.026 $^{15}N_2$ µmol g^{-1} d^{-1}) which were used as waste deposit (Figure 1h) was more than twice as high than in patches of younger domatia (0.011 $^{15}N_2$ µmol g^{-1} d^{-1}) used for rearing larvae. Thus, nitrogen fixation does not only occur in patches at colony founding stage, but interestingly also in older patches despite detritus deposition which is probably an additional nitrogen source. Similar nitrogen fixation rates as in fungal patches in *Cecropia* were found in photosynthetic microbial mats present in intertidal zones, sustaining mat growth in this extreme habitat (Woebken et al., 2015). Likewise, nitrogen fixation in founding stage patches could facilitate colonization by other organisms. A reason for continuous nitrogen fixation in older patches could be steady nitrogen uptake by Chaetothyriales. Nothing is yet known about the interactions of chaetothyrialean fungi and diazotrophs but it could be cooperative, like it is the case in soil systems (Kaiser et al., 2014). Moreover, the mouth parts of *Sclerorhabditis* nematodes indicate feeding on bacteria (Huertas, 2014), which could also account for the constant nitrogen demand in older patches in which they are found in high abundances.

It can be concluded that patch colonization with diazotrophic bacteria occurs soon after ant colonization and patch formation. $^{15}N_2$ fixation tends to be higher with increasing patch age despite of additional detritus input.

Carton, which is commonly found in the domatia of many *Cecropia*-inhabiting *Azteca* colonies is a prominent ant-made structure and its main function seems to increase

available nesting space for the ant brood (Longino, 1991). Although nitrogen fixation took place also in carton (0.007 $^{15}N_2$ µmol g^{-1} d^{-1}), patch samples were found to fixate on average more than two times the amount of nitrogen per day (0.019 $^{15}N_2$ µmol g^{-1} d^{-1}). This could be due to either the different substrate composition or differences in the diazotrophic community of patch and carton. Moreover, the ant species could also have an impact on fixation rate, as both carton and patch structures are ant-made.

5.1.1 Substrate

Carton, made from parenchyma of the domatium wall is very high in carbon and most probably serves structural needs of the ant colony. Patches consist predominantly of Chaetothyriales (Ascomycota) which are known to be saprotrophic (Voglmayr et al., 2011). One purpose of patches thus seems the recycling of organic matter (Blatrix et al., 2012; Defossez et al., 2011; Voglmayr et al., 2011). This is further affirmed by the presence of deposited dead ant bodies. Furthermore, nematodes emerging on the surface of drying patch samples in high numbers (Esquivel, et al., 2012; pers. obs.) indicate nutrient availability. Therefore, the high fixation rates of the patch substrate are in accordance with its involvement in nutrient cycles.

5.1.2 Ant and plant species

Patches and carton structures are made by the inhabiting *Azteca* species suggesting a potential influence of ant species on fixation rate. Although founding stage patches were collected from *Azteca alfari*, *A. constructor* and *A. xanthochra*, the small sample sizes did not allow statistical comparisons between the species. In established colonies, a high abundance of *A. alfari* and only a small number of *A. constructor* colonies were found in the collection area whereas no established colonies of *A. xanthochroa* were present. The mean fixation rate of patches from *A. alfari* colonies (0.0199 $^{15}N_2$ µmol g^{-1} d^{-1}) was almost thrice as high as in patches of *A. constructor* (0.0069 $^{15}N_2$ µmol g^{-1} d^{-1}). This indicates an effect of ant species on fixation rate in patches. However, most of the *A. constructor* patch samples were incubated for only 2 days while the *A. alfari* samples were incubated for 3 or 4 days. Therefore, the difference in fixation rates could be caused by (i) actual lower fixation rates in *A. constructor* colonies or (ii) the shorter duration of the incubation as longer incubation may have an effect on the substrate. Despite indications for an effect of ant species on fixation rate, more research is needed to conclude on this.

The influence of *Cecropia* species on the $^{15}N_2$ fixation rate was not investigated, because *Azteca* ants do not exhibit host plant specificity (Longino, 1989). Furthermore, patches are created by the *Azteca* ants themselves and myrmecophytic *Cecropia* plants can live without ants albeit with growth constraints (Schupp, 1986). Accordingly, no major influence of *Cecropia* species on fixation rate was expected. Moreover, determination of saplings and small plants is rather difficult: Leaf form and differential characters are variable in young plants and flowers as well as fruits are still missing at this stage.

5.1.3 Diazotrophic community

A preliminary analysis of the *nifH* gene sequence coding for a subunit of the dinitrogenase-reductase enzyme complex (Gaby & Buckley, 2012) in collected patch and carton samples revealed so far no major differences between the community of diazotrophs in patches and carton. The *nifH* gene sequence from the analyzed samples clustered in Beta- and Alphaproteobacteria. More than 70% of the samples sequences were most similar to Rhizobia comprising the genera of *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, and *Sinorhizobium*, which are rhizosphere bacteria associated with root nodules of plants (Kaneko et al., 2000). It has to be noted, however, that many obtained sequences contained ambiguous bases, most probably caused by the presence of multiple *nifH* genotypes. This unexpected high bacterial diversity prevented more accurate community analyses. Therefore, next generation sequencing will be necessary to further determine diversity and distribution of diazotrophic bacteria in the *Azteca*-*Cecropia* ant-plant symbiosis.

Fürnkranz et al. (2008) has shown nitrogen fixation activity of epiphytes on a range of rainforest plants. This is also the case in *Cecropia*, as both epiphylls and stem epiphytes depict similar mean nitrogen fixation rates per day and gram as patch and carton samples. Unfortunately, only from one epiphyll sample the *nifH* sequence was successfully amplified and the differences of the diazotrophic community could not be investigated. This only sample clusters with patch and carton samples. It is, however, still too early for arguments on the origin of the community and more samples have to be investigated to prove this.

5.2 $^{15}N_2$ fixation in animals

Another interesting fact are the fixation rates found in *Azteca* queens (0.008 $^{15}N_2$ µmol g^{-1} d^{-1}) and workers (0.004 $^{15}N_2$ µmol g^{-1} d^{-1}) after incubation. Although animal samples had the lowest fixation rates of all samples analyzed, this is an interesting discovery and has

yet to be investigated in more detail. In *Azteca* worker ants, diazotrophs could be either endosymbiotic or located on the outer surface of the exoskeleton which was not removed for the analysis because of the small size of the ants. In the queen, however, the exoskeleton of the gaster was removed and only the digestive system was analyzed. Furthermore, fungal patches and ants were incubated separately to prevent feeding on fungal patch particles with labelled bacteria. Hence, the results suggest a possible presence of N_2 fixating endosymbionts in the gut of *Azteca* ants.

In leaf-cutter ants, endosymbionts capable of N_2 fixation, some of which belonging to *Rhizobiales*, were found in the genus *Acromyrmex* (Sapountzis et al., 2015). Furthermore, Pinto-Tomás (2009) also found N_2 fixation activity in ant brood of *Atta* leaf-cutting ants. Generally, insects with protein poor diets are thought to have nitrogen-fixing endosymbionts (Nardi et al. 2002). If this is also the case in *Azteca* workers remains questionable, as they feed on Müllerian bodies, honeydew and insect prey (Longino, 1989). For the queens which presumably live of fat reserves and flight muscles during their claustral colony founding, like it is the case in many other ant species (Hölldobler & Wilson, 1990), additional nitrogen sources obtained through endosymbionts could prove to be an advantage.

Cryptostigma sp., which are commonly found in internodes (Kondo, 2010), are providing *Azteca* ants with honeydew in exchange for protection (Bailey, 1922). Incubation experiments revealed fixation activity (0.005 µmol $^{15}N_2$ g^{-1} d^{-1}), which is in accordance with the assumption of Buchner (1965), that Hemiptera could obtain additional nitrogen from symbionts to sustain their fast growth. This assumption is, however, challenged by Smith (1948), which failed to find nitrogen fixation in aphids. Moreover, whether $^{5}N_2$ was fixed by endosymbionts rather than by diazotrophs located on the surface is questionable, as the Coccoidea were analyzed as a whole.

Thus, further investigation of probable N_2 fixation in *Azteca* ants and Coccoidea seems promising but is not the focus of this study.

5.3 Chaetothyriales

Patches are not only inhabited by diazotrophs and nematodes. Chaetothyriales are also commonly found in the *Cecropia*/*Azteca* symbiosis. Fungi of this order are often associated with ants and are obviously adapted to a life within ant domatia (Voglmayr et al., 2011). The analysis of the internal transcribed spacer (ITS) region of ribosomal DNA confirmed previous studies (Nepel et al., unpubl.). OTU2 was found in most samples of

A. alfari and OTU3 in all *A. constructor* and *A. xanthochroa* samples investigated. Therefore, an association of chaetothyrialean fungi species with a certain *Azteca* host ant species seems to be the case. This is well in accordance with Voglmayr et al. (2011) who assumed that the dominance of Chaetothyriales over competing fungi in ant domatia, to which it often succumbs in pure cultures, is probably established by the host ants. Altogether, the results indicate ant mediated cultivation of chaetothyrialen fungi also in the *Cecropia/Azteca* symbiosis. Since obligate *Azteca* ants do not leave their host plant (Longino, 1989), chaetothyrialean fungi could constitute an additional food source.

5.4 Interaction of ants with patches and Müllerian bodies

The interaction of *Azteca* ants and fungal patches is still poorly investigated. *In situ* endoscopic recordings of the inside of founding stage colonies failed to demonstrate hyphae harvesting by ants. Prolonged observations in the field with an improved procedure still has the potential to unveil defecation on and harvesting of fungal patches in the *Cecropia/Azteca* symbiosis, which would further improve our knowledge of interaction between ants and patches.

Instead, indications for an interaction between ants and the fungal hyphae of the patch were obtained by labelling foundress patches with a ^{15}N glycine amino acid-mixture. After 7 days, larvae, pupae and worker samples displayed elevated ^{15}N levels but only the larvae had a significant ^{15}N accumulation. Thus, larvae maybe indeed feed on hyphae from the fungi, as was observed by Blatrix et al. (2012) in other ant-plant-fungi symbioses. However, it is also possible that the larvae were deposited onto the patch by the worker and accumulation took place without actual feeding of the hyphae.

Müllerian bodies are sometimes deposited on patches as well (pers. obs.), which also becomes evident through elevated, though not significant, ^{15}N levels in patches in colonies after exposure to ^{15}N labelled Müllerian bodies. This indicates nitrogen transfer from Müllerian bodies to patch structures. This is in accordance with Defossez et al. (2009) who also found elevated ^{15}N levels in patches after offering labelled food and even recorded defecation of ants onto patches in the *Leonardoxa*/*Aphomomyrmex* ant-plant symbiosis. The analyzed workers displayed significantly elevated ^{15}N levels, which was expected, as they were harvesting the Müllerian bodies from the stem. However, since the label was not incorporated into the Müllerian bodies but applied on the outside, it is possible that the label remained on mandibles or legs of the workers during

transportation. This could have caused label distribution dissociated from the Müllerian bodies. More thoroughly experiments are needed to affirm this finding.

5.5 Conclusion

The most important finding of this study is that fixation of atmospheric dinitrogen occurs in fungal patches inside the internodes of *Cecropia* plants colonized with *Azteca* ants. This is completely new and for the first time demonstrated for an ant-plant association. Comparison of the carton and fungus patches showed a significantly higher fixation rate in patches than in carton indicating a function of the patches in nutrient cycling. The nitrogen fixation in older patches with N-rich organic detritus could be due to constant incorporation of nitrogen by growing fungi and nematodes which are thought to feed on bacteria (Huertas, 2014). Interactions of fungi and diazotrophs could be cooperative like in soil systems (Kaiser et al., 2014) and both ants and *Cecropia* plants seem to gain nutrients (Sagers et al., 2000) from this multipartite symbiotic system.

High species concordance of *Azteca* and Chaetothyriales indicate possible cultivation of fungi by *Azteca* ants. Feeding on the fungi, as already shown in other ant-plant symbioses (Blatrix et al., 2012; Defossez et al., 2011), could also be the case in the *Cecropia/Azteca* symbiosis.

Clearly, further research is needed to assess the presence and frequency of diazotrophic communities in *Cecropia/Azteca* and other ant-plant systems to evaluate their impact on the symbiosis and eventually understand nutrient fluxes within this complex entities.

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7 Zusammenfassung

Ameisenpflanzen sind in den Tropen häufig anzutreffen und bilden Lebensgemeinschaften mit Ameisen zum beidseitigen Vorteil. Die Pflanzen stellen oft Wohnraum und Nahrung für die Ameisen bereit, während letztere die Pflanzen vor Fraßschäden und Überwuchs schützen.

Neue Erkenntnisse bezüglich der Interaktion von Ameisen, Pflanzen und Pilzen in Ameisenpflanzen zeigten, dass sogenannte Pilzpatches eine wichtige Rolle für Nährstoffflüsse innerhalb der Symbiose einnehmen. Diese Flächen (Patches) aus Pflanzenmaterial und Detritus findet man in phylogenetisch verschiedenen Ameisen-Pflanzen Symbiosen innerhalb der Wohnräume (Domatien), die von den Pflanzen bereitgestellt werden. Pilze der Ordnung Chaetothyriales wachsen in diesen Patches, deren Pilzhyphen auch von Ameisen gefressen werden. Das Wachstum der Pilze auf diesen aus stickstoffarmen Pflanzenmaterial bestehenden Patches könnte durch stickstofffixierende Bakterien (Diazotrophen) gefördert werden.

Diese Studie untersuchte daher Pilzpatches in von *Azteca* Ameisen (Dolichoderinae) besiedelten *Cecropia* Pflanzen (Urticaceae) auf mögliche Stickstofffixierung. Zusätzlich wurde untersucht, ob auch *Azteca* Ameisen Pilzhyphen fressen, wie dies in anderen Ameisen-Pflanzen Interaktionen der Fall ist.

Cecropia Pflanzen findet man oft in gestörten, sonnenreichen Habitaten wie Straßenrändern oder Kahlschlägen. Ihre hohlen Stämme dienen als Nistplätze, glykogenreiche Müllersche Körperchen, die an sogenannten Trichilien an der Stängelbasis der Blätter dargeboten werden, als Nahrung für die *Azteca* Kolonien. Innerhalb der Domatien findet man häufig Pilzpatches sowie Kartonstrukturen, die in Brutkammern die nutzbare Oberfläche vergrößern.

Um Stickstofffixierung nachzuweisen, wurde das stabile Stickstoffisotop $^{15}N_2$ verwendet. Nach der Inkubation der Proben in $^{15}N_2$ angereicherter Atmosphäre (75% $^{15}N_2$, 25% O_2) zeigten sich die höchsten Fixierungsraten in den Pilzpatches (0,019 $^{15}N_2$ µmol pro Gramm pro Tag), die ähnlich hoch waren wie die von auf *Cecropia* wachsenden Epiphyten. In Kartonstrukturen wurde ebenfalls Stickstoff fixiert, aber mit 0,007 $^{15}N_2$ µmol/g/T nur circa halb so viel wie in Patches. Dies bestätigt die besondere Rolle, die Patches in Nährstoffkreisläufen innerhalb der Symbiose einnehmen.

Die Analyse von involvierten stickstofffixierenden Bakterien mithilfe der *nifH*

Gensequenz zeigte, dass diese zu den Ordnungen der Beta- und Alphaproteobacteria gehören und in Karton und Patches gleichermaßen vorkommen.
Zusätzliche Untersuchungen von *Azteca* Königinnen und Arbeitern zeigten auch bei diesen einen erhöhten Anteil an Stickstoffisotopen nach der Inkubation (0,008 bzw. 0,004 $^{15}N_2$ µmol/g/T). Dies deutet auf stickstofffixierende Endosymbionten hin, aber weitere Experimente sind nötig, um eindeutige Aussagen zu treffen.

Versuche, mit einem Endoskop direkte Nachweise von Ameisen beim Fressen von Pilzhyphen zu filmen, scheiterten. Ergänzende Experimente mit ^{15}N markierten Pilzpatches in *Azteca* Kolonien offenbarten jedoch, dass Ameisenlarven nach 7 Tagen einen signifikanten Anteil an Stickstoffisotopen akkumulierten, was auf das Fressen der markierten Pilzhyphen hindeutet.
Eine Analyse der charakteristischen ITS Gensequenz von Chaetothyriales Pilzen zeigte, dass in Patches und Karton von *Azteca alfari* und *A. constructor* Kolonien je eine andere Pilzspezies präsent ist. Das ist ein Hinweis auf eine mögliche Kultivierung der Chaetothyriales Pilze durch die Ameisen.

Diese Studie demonstrierte zum ersten Mal, dass stickstofffixierende Bakterien in Domatien von *Cecropia* existieren und aktiv Stickstoff fixieren. Besonders Pilzpatches bilden eine bisher unbekannte Stickstoffquelle in der *Cecropia*/*Azteca* Symbiose. Die Übereinstimmung von *Azteca* und Chaetothyriales Arten deutet darauf hin, dass die Pilze von den Ameisen kultiviert werden. Erste Hinweise für das Fressen von Pilzhyphen durch Ameisenlarven wurden ebenfalls erbracht.
Es wird offensichtlich, dass es sich bei Ameisen-Pflanzen Symbiosen um äußerst komplexe Systeme handelt. Weitere vielversprechende Untersuchungen sind notwendig, um die Vielfalt an stickstofffixierenden Bakterien und deren Nutzen zu erfassen sowie mögliches Vorkommen auch in anderen Ameisen-Pflanzen Symbiosen zu überprüfen.

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9 Appendix

9.1 IRMS

Supplementary Table 1.
Amounts weighed in for IRMS analysis for different sample types. Less amounts were taken for incubated samples. Ranges in mg are given for incubated and control samples.

	Patch	Carton	Animals	Epiphytes
$^{15}N_2$ incubated [mg]	0.8 – 1	2 – 2.5	0.1 – 0.3	1.5
control [mg]	0.8 – 1.5	2 – 2.7	0.3 – 0.6	2

9.2 PCR

Supplementary Table 2.
Mastermixes used for PCR and cycle sequencing.

General PCR		Cycle sequencing	
Used chemicals	Amount [µl]	Used chemicals	Amount [µl]
2x DreamTaq	7	Sequencing buffer [x5]	2
Ueda16 [10 µM]	1	Trehalose [1 M]	2
R6 [10 µM]	1	Primer [10 µM]	0.3
PCR grade H_2O	4.1	PCR grade H_2O	1.2
BSA [20 µg/µl]	0.3	BigDye v3.1 [2.5x]	0.5
DNA template	0.6	DNA template	4
Total	14		10

Supplementary Table 3.
PCR programs used for the two PCR runs

General PCR run				Cycle sequencing run			
Process	Time	Temp. [°C]	Number of cycles	Process	Time	Temp. [°C]	Number of cycles
Denature	4 min	94	1	Initial Denaturation	1 min	96	1
Annealing	45 sec	56	35	Denature	10 sec	96	40
Elongation	30 sec	72		Annealing	5 sec	50	
Denature	30 sec	94		Elongation	2 min	60	
Annealing	45 sec	52	1				
Final Elongation	10 min	72					

Supplementary Table 4.
General PCR run for the amplification of the chaetothyrialean fungi target sequence.

Process	Time	Temperature [°C]	Number of cycles
Initial denaturation	3 min	95	1
Denature	15 sec	95	
Annealing	20 sec	53	35
Elongation	1 min	72	
Final elongation	3 min	72	1

Supplementary Table 5.
Patch and carton samples analyzed for Chaetothyriales. Sample number (Samp no) is the assigned number during the DNA amplification and cycle sequencing. Sample type consists of the ant colony/plant number, type (P = patch, C = carton), stem part (I – III). Further the ant species is given. The OTU (operational taxonomic unit) column shows to which OTU the chaetothyrialean fungi of the samples are affiliated. For one sample, no sequence (seq.) was obtained.

Samp no	Sample type	Ant species	OTU	Samp no	Sample type	Ant species	OTU
1	33_I_P	*A. constructor*	3	11	61_II_P	*A. alfari*	2
2	33_II_P	*A. constructor*	3	12	61_III_P	*A. alfari*	2
3	33_III_P	*A. constructor*	3	13	42_i8_P	*A. xanthochroa*	3
4	48_I_P	*A. constructor*	3	14	47_i8_P	*A. alfari*	2
5	48_II_P	*A. constructor*	3	15	80_i6_P	*A. constructor*	no seq.
6	48_III_P	*A. constructor*	3	16	82_i3_P	*A. constructor*	3
7	61_I_C	*A. alfari*	2	17	82_i13_P	*A. constructor*	3
8	61_II_C	*A. alfari*	2	18	47_i10_P	*A. constructor*	3
9	62_III_C	*A. alfari*	2	19	112_I_P	*A. alfari*	2
10	61_I_P	*A. alfari*	2	20	112_II_P	*A. alfari*	2

9.3 Azteca species and incubation time

Supplementary Table 6.
Mean $^{15}N_2$ fixation rates of founding stage patches (Fptach), patches of established colonies, worker and queen samples from all 3 *Azteca* species encountered in this study. In *A. xanthochroa*, only samples from one founding stage colony were analyzed.

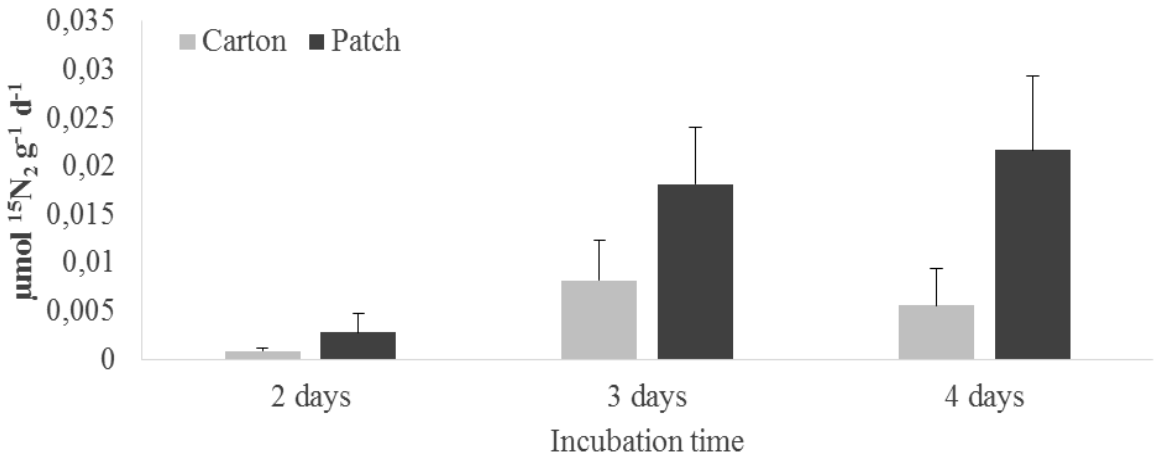
Sample type	*A. alfari*		*A. constructor*		*A. xanthochroa*	
	N	$^{15}N_2$ µmol g^{-1} d^{-1}	N	$^{15}N_2$ µmol g^{-1} d^{-1}	N	$^{15}N_2$ µmol g^{-1} d^{-1}
Fpatch	3	0.013 ± 0.006	1	0.01	1	0.06
Patch	25	0.019 ± 0.005	8	0.007 ± 0.003		
Worker	5	0.004 ± 0.001	1	0.003		
Queen	2	0.011 ± 0.01	2	0.009 ± 0.003	1	<0.001

Supplementary Table 7.
Frequencies of patch and carton samples and all factors of interest. *A. alfari* species are almost not present in the 2 days incubation group, whereas *A. constructor* is only found twice in the 3 and 4 days incubation groups. All *A. constructor* samples and 2 days of incubation were excluded from further analysis.

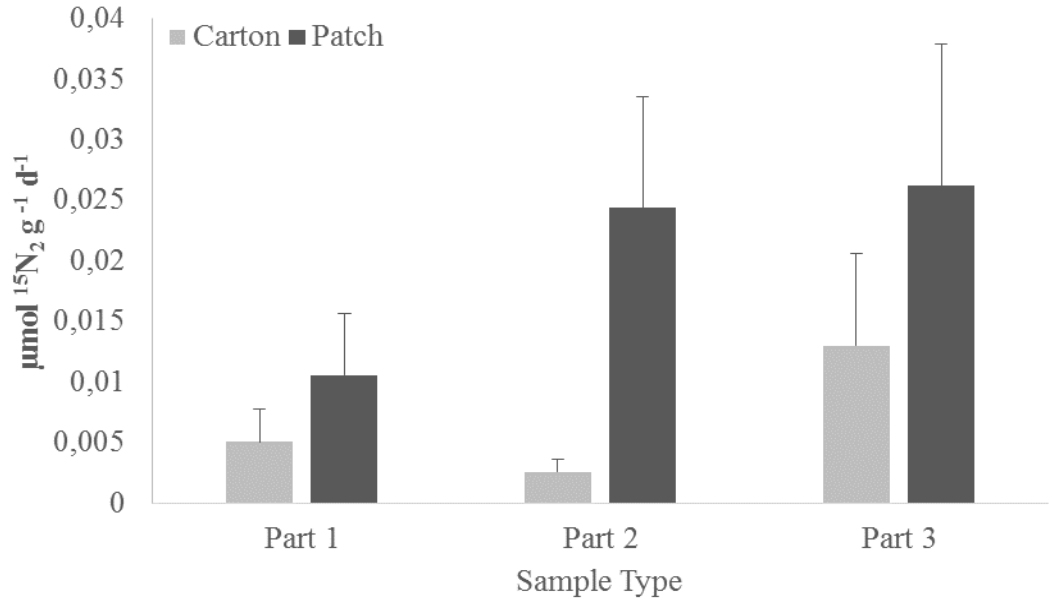
		2 days		3 days		4 days
Sample type	Stem part	*A. alfari*	*A. constructor*	*A. alfari*	*A. constructor*	*A. alfari*
Carton	Part 1	1	3	2		3
	Part 2		2	4		3
	Part 3		2	2		4
N carton		1	7	8		10
Patch	Part 1	1	2	5	1	4
	Part 2		2	5	1	4
	Part 3	1	2	3		4
N patch		2	6	13	2	12
Total		3	**13**	21	**2**	22

Supplementary Table 8.
Amount and mean fixation rates (µmol $^{15}N_2$ per g d.w. per day) of patch and carton samples of established *A. alfari* colonies for each steam part ± standard error of means.

Stem part	Patch		Carton	
	N	µmol $^{15}N_2$ per g d.w. per day	N	µmol $^{15}N_2$ per g d.w. per day
Part 1	9	0,011 ± 0,006	5	0,005 ± 0,002
Part 2	9	0,024 ± 0,003	7	0,003 ± 0,001
Part 3	7	0,026 ± 0,019	6	0,013 ± 0,008
Overall	25	0,019 ± 0.005	18	0,007 ± 0,003



Supplementary Figure 1.
Mean $^{15}N_2$ fixation rates of patch and carton samples after 2-4 days incubation time. The fixation rate in µmol $^{15}N_2$ per g dry weight per day of patch and carton samples of established colonies is higher the longer the samples were incubated (Kruskal-Wallis test, n = 61, df = 2, p < .001). Error bars show standard error of means.



Supplementary Figure 2.
Mean fixation rate of patch and carton samples of established *A. alfari* colonies. Part 1 resembles the youngest patch and carton structures, part 3 the oldest. No significant effect of age on fixation rate was found, although patches show a trend to elevated fixation rates with increasing age (mixed general linear model, n = 43, F = .053, p = .948).

9.4 Sample table

All samples from the different experiments which were selected for IRMS analysis. Coll. Date = collection date. In the Experiment column (Exp), $^{15}N_2$ refers to incubation in $^{15}N_2$ enriched atmosphere, AS to ^{15}N labelled founding stage patch (Fpatch) samples, M to ^{15}N labelled Müllerian bodies and C to the control condition. Colony stage (C. stge) refers to the developmental stage of the colony which is either established (est.) or at founding stage (fdg.). Each plant number (Pl no) corresponds to a harvested plant. Treatment (Tr) refers to the way the samples have been incubated, either within the internodes in large vials (*in situ* = S) or separately in vials (*in vitro* = V). The Sample (Samp) type column abbreviations are Work. = Worker, Coc. = Coccoidea, Stepi = Stemepiphyte, Epi = Epiphyll. The OTU column shows the assignment of analyzed chaetothyrialean target sequences to either OTU2 or OTU3. The *nifH* GenBank column displays the highest similarity with reference GenBank sequences in percent and the respective GenBank accession number separated by a slash.

Identifier	Coll. date	Exp	C. stge	Ant species	Pl no	Tr	Sampty pe	Inc time [hrs]	OTU	*nif*H GenBank
33_I_P_N	08.08.14	$^{15}N_2$	est.	*A. constructor*	33	V	Patch	31.63	OTU3	
33_II_P_N	08.08.14	$^{15}N_2$	est.	*A. constructor*	33	V	Patch	31.63	OTU3	89/AB217474
33_III_P_N	08.08.14	$^{15}N_2$	est.	*A. constructor*	33	V	Patch	31.63	OTU3	85/AB188120
33_I_C_N	08.08.14	$^{15}N_2$	est.	*A. constructor*	33	V	Carton	31.63		
33_II_C_N	08.08.14	$^{15}N_2$	est.	*A. constructor*	33	V	Carton	31.63		
33_III_C_N	08.08.14	$^{15}N_2$	est.	*A. constructor*	33	V	Carton	31.63		89/AB367742
48_I_P_N	12.08.14	$^{15}N_2$	est.	*A. constructor*	48	V	Patch	50	OTU3	93/KJ406704
48_II_P_N	12.08.14	$^{15}N_2$	est.	*A. constructor*	48	V	Patch	50	OTU3	
48_III_P_N	12.08.14	$^{15}N_2$	est.	*A. constructor*	48	V	Patch	50	OTU3	
48_I_C_N	12.08.14	$^{15}N_2$	est.	*A. constructor*	48	V	Carton	50		95/AB367742
48_II_C_N	12.08.14	$^{15}N_2$	est.	*A. constructor*	48	V	Carton	50		
48_III_C_N	12.08.14	$^{15}N_2$	est.	*A. constructor*	48	V	Carton	50		86/AB367742
49_I_P_N	13.08.14	$^{15}N_2$	est.	*A. alfari*	49	V	Patch	91.75		95/AB367742
49_II_P_N	13.08.14	$^{15}N_2$	est.	*A. alfari*	49	V	Patch	91.75		92/AB367742
49_III_P_N	13.08.14	$^{15}N_2$	est.	*A. alfari*	49	V	Patch	91.75		85/AB182914
49_I_C_N	13.08.14	$^{15}N_2$	est.	*A. alfari*	49	V	Carton	91.75		93/AB367742
49_II_C_N	13.08.14	$^{15}N_2$	est.	*A. alfari*	49	V	Carton	91.75		
49_III_C_N	13.08.14	$^{15}N_2$	est.	*A. alfari*	49	V	Carton	91.75		85/AB182914
50_I_P_N	18.08.14	$^{15}N_2$	est.	*A. alfari*	50	V	Patch	48		
50_III_P_N	18.08.14	$^{15}N_2$	est.	*A. alfari*	50	V	Patch	48		
50_I_C_N	18.08.14	$^{15}N_2$	est.	*A. alfari*	50	V	Carton	48		
51_I_C_N	19.08.14	$^{15}N_2$	est.	*A. constructor*	51	V	Carton	48		
54_I_P_N	23.08.14	$^{15}N_2$	est.	*A. alfari*	54	V	Patch	56.28		
54_II_P_N	23.08.14	$^{15}N_2$	est.	*A. alfari*	54	V	Patch	56.28		
54_II_C_N	23.08.14	$^{15}N_2$	est.	*A. alfari*	54	V	Carton	56.28		
55_I_P_N	24.08.14	$^{15}N_2$	est.	*A. constructor*	55	V	Patch	72		
55_II_P_N	24.08.14	$^{15}N_2$	est.	*A. constructor*	55	V	Patch	72		
56_I_P_N	25.08.14	$^{15}N_2$	est.	*A. alfari*	56	V	Patch	72.08		95/AB367742
56_II_P_N	25.08.14	$^{15}N_2$	est.	*A. alfari*	56	V	Patch	72.08		
56_III_P_N	25.08.14	$^{15}N_2$	est.	*A. alfari*	56	V	Patch	72.08		
56_II_C_N	25.08.14	$^{15}N_2$	est.	*A. alfari*	56	V	Carton	72.08		

61_I_P_N	27.08.14	$^{15}N_2$	est.	*A. alfari*	61	V	Patch	72.2	OTU2	85/FN433472
61_II_P_N	27.08.14	$^{15}N_2$	est.	*A. alfari*	61	V	Patch	72.2	OTU2	85/AB182914
61_III_P_N	27.08.14	$^{15}N_2$	est.	*A. alfari*	61	V	Patch	72.2	OTU2	
61_I_C_N	27.08.14	$^{15}N_2$	est.	*A. alfari*	61	V	Carton	72.42	OTU2	85/KM192246
61_II_C_N	27.08.14	$^{15}N_2$	est.	*A. alfari*	61	V	Carton	72.2	OTU2	83/AP007255
61_III_C_N	27.08.14	$^{15}N_2$	est.	*A. alfari*	61	V	Carton	72.42		86/AB182914
62_I_P_N	29.08.14	$^{15}N_2$	est.	*A. alfari*	62	V	Patch	76.33		92/AP012320
62_II_P_N	29.08.14	$^{15}N_2$	est.	*A. alfari*	62	V	Patch	76.33		
62_III_P_N	29.08.14	$^{15}N_2$	est.	*A. alfari*	62	V	Patch	76.33		
62_I_C_N	29.08.14	$^{15}N_2$	est.	*A. alfari*	62	V	Carton	76.33		86/AB367742
62_II_C_N	29.08.14	$^{15}N_2$	est.	*A. alfari*	62	V	Carton	76.33		95/AB367742
62_III_C_N	29.08.14	$^{15}N_2$	est.	*A. alfari*	62	V	Carton	76.33	OTU2	
75_I_P_N	01.09.14	$^{15}N_2$	est.	*A. alfari*	75	V	Patch	96.33		95/AB367742
75_II_P_N	01.09.14	$^{15}N_2$	est.	*A. alfari*	75	V	Patch	96.33		83/HM193519
75_III_P_N	01.09.14	$^{15}N_2$	est.	*A. alfari*	75	V	Patch	96.33		86/FJ799358
75_IV_P_N	01.09.14	$^{15}N_2$	est.	*A. alfari*	75	V	Patch	96.33		89/KC509131
75_I_C_N	01.09.14	$^{15}N_2$	est.	*A. alfari*	75	V	Carton	96.33		91/AB367742
75_II_C_N	01.09.14	$^{15}N_2$	est.	*A. alfari*	75	V	Carton	96.33		95/AB367742
75_III_C_N	01.09.14	$^{15}N_2$	est.	*A. alfari*	75	V	Carton	96.33		93/AB367742
75_IV_C_N	01.09.14	$^{15}N_2$	est.	*A. alfari*	75	V	Carton	96.33		94/AB367742
76_I_P_N	01.09.14	$^{15}N_2$	est.	*A. alfari*	76	V	Patch	96		
76_II_P_N	01.09.14	$^{15}N_2$	est.	*A. alfari*	76	V	Patch	96		
76_III_P_N	01.09.14	$^{15}N_2$	est.	*A. alfari*	76	V	Patch	96		
76_III_C_N	01.09.14	$^{15}N_2$	est.	*A. alfari*	76	V	Carton	96		
77_I_P_N	02.09.14	$^{15}N_2$	est.	*A. alfari*	77	V	Patch	96.58		88/KM103912
77_II_P_N	02.09.14	$^{15}N_2$	est.	*A. alfari*	77	V	Patch	96.58		90/KC509131
77_III_P_N	02.09.14	$^{15}N_2$	est.	*A. alfari*	77	V	Patch	96.58		
77_I_C_N	02.09.14	$^{15}N_2$	est.	*A. alfari*	77	V	Carton	96.58		
77_II_C_N	02.09.14	$^{15}N_2$	est.	*A. alfari*	77	V	Carton	96.58		
77_III_C_N	02.09.14	$^{15}N_2$	est.	*A. alfari*	77	V	Carton	96.58		
112_I_P_N	20.08.14	$^{15}N_2$	est.	*A. alfari*	112	V	Patch	72	OTU2	
112_II_P_N	20.08.14	$^{15}N_2$	est.	*A. alfari*	112	V	Patch	72	OTU2	
33_I_P_K	08.08.14	C	est.	*A. constructor*	33	V	Patch			
33_II_P_K	08.08.14	C	est.	*A. constructor*	33	V	Patch			
33_III_P_K	08.08.14	C	est.	*A. constructor*	33	V	Patch			
33_I_C_K	08.08.14	C	est.	*A. constructor*	33	V	Carton			
33_II_C_K	08.08.14	C	est.	*A. constructor*	33	V	Carton			
48_I_P_K	12.08.14	C	est.	*A. constructor*	48	V	Patch			
48_II_P_K	12.08.14	C	est.	*A. constructor*	48	V	Patch			93/AB241413
48_III_P_K	12.08.14	C	est.	*A. constructor*	48	V	Patch			
48_I_C_K	12.08.14	C	est.	*A. constructor*	48	V	Carton			
48_III_C_I	12.08.14	C	est.	*A. constructor*	48	V	Carton			
49_I_P_K	13.08.14	C	est.	*A. alfari*	49	V	Patch			

49_II_P_K	13.08.14	C	est.	*A. alfari*	49	V	Patch			
49_III_P_K	13.08.14	C	est.	*A. alfari*	49	V	Patch			
49_I_C_K	13.08.14	C	est.	*A. alfari*	49	V	Carton			
49_II_C_K	13.08.14	C	est.	*A. alfari*	49	V	Carton			
49_III_C_K	13.08.14	C	est.	*A. alfari*	49	V	Carton			
50_I_P_K	18.08.14	C	est.	*A. alfari*	50	V	Patch			
50_III_P_K	18.08.14	C	est.	*A. alfari*	50	V	Patch			
50_I_C_K	18.08.14	C	est.	*A. alfari*	50	V	Carton			
51_I_C_K	19.08.14	C	est.	*A. constructor*	51	V	Carton			
54_I_P_K	23.08.14	C	est.	*A. alfari*	54	V	Patch			
54_II_P_K	23.08.14	C	est.	*A. alfari*	54	V	Patch			
54_II_C_K	23.08.14	C	est.	*A. alfari*	54	V	Carton			
55_I_P_K	24.08.14	C	est.	*A. constructor*	55	V	Patch			
55_II_P_K	24.08.14	C	est.	*A. constructor*	55	V	Patch			
51_I_P_K	19.08.14	C	est.	*A. constructor*	51	V	Patch			
56_II_P_K	25.08.14	C	est.	*A. alfari*	56	V	Patch			
56_III_P_K	25.08.14	C	est.	*A. alfari*	56	V	Patch			
56_II_C_K	25.08.14	C	est.	*A. alfari*	56	V	Carton			
61_I_P_K	27.08.14	C	est.	*A. alfari*	61	V	Patch			
61_II_P_K	27.08.14	C	est.	*A. alfari*	61	V	Patch			
61_III_P_K	27.08.14	C	est.	*A. alfari*	61	V	Patch			
61_I_C_K	27.08.14	C	est.	*A. alfari*	61	V	Carton			
61_II_C_K	27.08.14	C	est.	*A. alfari*	61	V	Carton			
61_III_C_K	27.08.14	C	est.	*A. alfari*	61	V	Carton			
62_I_P_K	29.08.14	C	est.	*A. alfari*	62	V	Patch			
62_II_P_K	29.08.14	C	est.	*A. alfari*	62	V	Patch			
62_III_P_K	29.08.14	C	est.	*A. alfari*	62	V	Patch			
62_I_C_K	29.08.14	C	est.	*A. alfari*	62	V	Carton			
62_II_C_K	29.08.14	C	est.	*A. alfari*	62	V	Carton			
62_III_C_K	29.08.14	C	est.	*A. alfari*	62	V	Carton			
75_I_P_K	01.09.14	C	est.	*A. alfari*	75	V	Patch			
75_II_P_K	01.09.14	C	est.	*A. alfari*	75	V	Patch			
75_III_P_K	01.09.14	C	est.	*A. alfari*	75	V	Patch			
75_P_IV_K	01.09.14	C	est.	*A. alfari*	75	V	Patch			
75_I_C_K	01.09.14	C	est.	*A. alfari*	75	V	Carton			
75_II_C_K	01.09.14	C	est.	*A. alfari*	75	V	Carton			
75_III_C_K	01.09.14	C	est.	*A. alfari*	75	V	Carton			
76_I_P_K	01.09.14	C	est.	*A. alfari*	76	V	Patch			
76_II_P_K	01.09.14	C	est.	*A. alfari*	76	V	Patch			
76_III_K_K	01.09.14	C	est.	*A. alfari*	76	V	Carton			
77_I_P_K	02.09.14	C	est.	*A. alfari*	77	V	Patch			
77_II_P_K	02.09.14	C	est.	*A. alfari*	77	V	Patch			
77_III_P_K	02.09.14	C	est.	*A. alfari*	77	V	Patch			
77_I_C_K	02.09.14	C	est.	*A. alfari*	77	V	Carton			

77_II_C_K	02.09.14	C	est.	*A. alfari*	77	V	Carton			94/CP001016
77_III_C_K	02.09.14	C	est.	*A. alfari*	77	V	Carton			no readouts
77_IV_C_K	02.09.14	C	est.	*A. alfari*	77	V	Carton			
77_V_C_K	02.09.14	C	est.	*A. alfari*	77	V	Carton			
112_I_P_K	20.08.14	C	est.	*A. alfari*	112	V	Patch			
112_II_P_K	20.08.14	C	est.	*A. alfari*	112	V	Patch			
48_L_N	12.08.14	$^{15}N_2$	est.	*A. constructor*	48	V	Larva	50		
62_L_N	29.08.14	$^{15}N_2$	est.	*A. alfari*	62	V	Larva	72.25		
75_L_N	01.09.14	$^{15}N_2$	est.	*A. alfari*	75	V	Larva	72.83		
76_L_N	01.09.14	$^{15}N_2$	est.	*A. alfari*	76	V	Larva	96		
77_L_N	02.09.14	$^{15}N_2$	est.	*A. alfari*	77	V	Larva	97		
48_Pup_N	12.08.14	$^{15}N_2$	est.	*A. constructor*	48	V	Pupa	50		
62_Pup_N	29.08.14	$^{15}N_2$	est.	*A. alfari*	62	V	Pupa	72.25		
75_Pup_N	01.09.14	$^{15}N_2$	est.	*A. alfari*	75	V	Pupa	96.33		
76_Pup_N	01.09.14	$^{15}N_2$	est.	*A. alfari*	76	V	Pupa	96		
77_Pup_N	02.09.14	$^{15}N_2$	est.	*A. alfari*	77	V	Pupa	97		
55_W_N	24.08.14	$^{15}N_2$	est.	*A. constructor*	55	V	Work.	72		
56_W_N	25.08.14	$^{15}N_2$	est.	*A. alfari*	56	V	Work.	72		
62_W_N	29.08.14	$^{15}N_2$	est.	*A. alfari*	62	V	Work.	72.25		
75_W_N	01.09.14	$^{15}N_2$	est.	*A. alfari*	75	V	Work.	72.83		
76_W_N	01.09.14	$^{15}N_2$	est.	*A. alfari*	76	V	Work.	96		
77_W_N	02.09.14	$^{15}N_2$	est.	*A. alfari*	77	V	Work.	96.88		
48_LIC_N	12.08.14	$^{15}N_2$	est.		48	V	Coc.	50		
49_LIC_N	13.08.14	$^{15}N_2$	est.		49	V	Coc.	91.75		
54_LIC_N	23.08.14	$^{15}N_2$	est.		54	V	Coc.	56.28		
56_LIC_N	25.08.14	$^{15}N_2$	est.		56	V	Coc.	72.08		
61_LIC_N	27.08.14	$^{15}N_2$	est.		61	V	Coc.	72.2		
St2_N	27.08.14	$^{15}N_2$	est.			V	Stepi.	72.2		
St3_N	27.08.14	$^{15}N_2$	est.			V	Stepi.	72.2		
St5_N	27.08.14	$^{15}N_2$	est.			V	Stepi.	72.42		
St4_N	27.08.14	$^{15}N_2$	est.			V	Stepi.	72.42		
St6_N	28.08.14	$^{15}N_2$	est.			V	Stepi.	72.25		
E10_N	29.08.14	$^{15}N_2$	est.			V	Epi.	73		
E6_N	28.08.14	$^{15}N_2$	est.			V	Epi.	72.25		
E7_N	28.08.14	$^{15}N_2$	est.			V	Epi.	72.25		
E8_N	29.08.14	$^{15}N_2$	est.			V	Epi.	73		
E9_N	29.08.14	$^{15}N_2$	est.			V	Epi.	73		
48_L_K	12.08.14	C	est.	*A. constructor*	48	V	Larva			
62_L_K	29.08.14	C	est.	*A. alfari*	62	V	Larva			
75_L_K	01.09.14	C	est.	*A. alfari*	76	V	Larva			
76_L_K	01.09.14	C	est.	*A. alfari*	76	V	Larva			
77_L_K	02.09.14	C	est.	*A. alfari*	77	V	Larva			
48_Pup_K	12.08.14	C	est.	*A. constructor*	48	V	Pupa			

62_Pup_K	29.08.14	C	est.	*A. alfari*	62	V	Pupa			
75_Pup_K	01.09.14	C	est.	*A. alfari*	75	V	Pupa			
76_Pup_K	01.09.14	C	est.	*A. alfari*	76	V	Pupa			
77_Pup_K	02.09.14	C	est.	*A. alfari*	77	V	Pupa			
48_W_K	12.08.14	C	est.	*A. constructor*	48	V	Work.			
62_W_K	29.08.14	C	est.	*A. alfari*	62	V	Work.			
75_W_K	01.09.14	C	est.	*A. alfari*	75	V	Work.			
76_W_K	01.09.14	C	est.	*A. alfari*	76	V	Work.			
48_LIC_K	12.08.14	C	est.		48	V	Coc.			
49_LIC_K	13.08.14	C	est.		49	V	Coc.			
54_LIC_K	23.08.14	C	est.		54	V	Coc.			
56_LIC_K	25.08.14	C	est.		56	V	Coc.			
61_LIC_K	27.08.14	C	est.		61	V	Coc.			
St2_N	27.08.14	C	est.			V	Stepi			
St3_N	27.08.14	C	est.			V	Stepi			
St5_N	27.08.14	C	est.			V	Stepi			
St4_N	27.08.14	C	est.			V	Stepi			
St6_N	28.08.14	C	est.			V	Stepi			
E10_N	29.08.14	C	est.			V	Epi.			
E6_N	28.08.14	C	est.			V	Epi.			
E7_N	28.08.14	C	est.			V	Epi.			
E8_N	29.08.14	C	est.			V	Epi.			
E9_N	29.08.14	C	est.			V	Epi.			
47_i10_P_N	11.08.14	$^{15}N_2$	fdg.	*A. constructor*	47	V	Fpatch	48.68	OTU3	
47_i13_P_N	11.08.14	$^{15}N_2$	fdg.	*A. alfari*	47	V	Fpatch	48.68		
47_i8_P_N	11.08.14	$^{15}N_2$	fdg.	*A. alfari*	47	V	Fpatch	48.68	OTU3	
53_i8_P_N	21.08.14	$^{15}N_2$	fdg.	*A. alfari*	53	V	Fpatch	72		
63_i3_P_N	28.08.14	$^{15}N_2$	fdg.	*A. xanthochroa*	63	V	Fpatch	72		
42_i8_P_N	11.08.14	$^{15}N_2$	fdg.	*A. xanthochroa*	42	S	Fpatch	48	OTU3	
45_i6_P_N	11.08.14	$^{15}N_2$	fdg.	*A. alfari*	45	S	Fpatch	48		
80_i6_P_N	07.09.14	$^{15}N_2$	fdg.	*A. constructor*	80	S	Fpatch	95.75		
82_i13_P_N	07.09.14	$^{15}N_2$	fdg.	*A. constructor*	82	S	Fpatch	94	OTU3	
82_i3_P_N	07.09.14	$^{15}N_2$	fdg.	*A. constructor*	82	S	Fpatch	94	OTU3	
42_i8_Qx_N	11.08.14	$^{15}N_2$	fdg.	*A. xanthochroa*	42	V	Queen	48		
47_i8_Qa_N	11.08.14	$^{15}N_2$	fdg.	*A. alfari*	47	V	Queen	48.68		
53_i8_Qa_N	21.08.14	$^{15}N_2$	fdg.	*A. alfari*	53	V	Queen	72		
55_i5_Qc_N	24.08.14	$^{15}N_2$	fdg.	*A. constructor*	55	V	Queen	72		
55_i7_Qc_N	24.08.14	$^{15}N_2$	fdg.	*A. constructor*	55	V	Queen	72		
45_i6_Qa_N	11.08.14	$^{15}N_2$	fdg.	*A. alfari*	45	S	Queen	48		
82_i13_Qc_N	07.09.14	$^{15}N_2$	fdg.	*A. constructor*	82	S	Queen	94		
82_i3_Qc_N	07.09.14	$^{15}N_2$	fdg.	*A. constructor*	82	S	Queen	94		
47_i10_P_K	11.08.14	C	est.	*A. constructor*	47	V	Fpatch			
47_i13_P_K	11.08.14	C	est.	*A. alfari*	47	V	Fpatch			

47_i8_P_K	11.08.14	C	est.	*A. alfari*	47	V	Fpatch			
63_i9_P_K	28.08.14	C	est.	*A. xanthochroa*	63	V	Fpatch			
78_i9_P_K	07.09.14	C	est.	*A. sp.*	78	V	Fpatch			
85_i3_P_K	07.09.14	C	est.	*A. sp.*	85	V	Fpatch			
86_i6_P_K	05.09.14	C	est.	*A. sp.*	86	V	Fpatch			
86_i7_P_K	05.09.14	C	est.	*A. sp.*	86	V	Fpatch			
79_i3_Qc_K	07.09.14	C	est.	*A. constructor*	79	V	Queen			
Qa_K	07.09.14	C	est.	*A. alfari*		V	Queen			
Qx_K	07.09.14	C	est.	*A. xanthochroa*		V	Queen			
Qa_K	07.09.14	C	est.	*A. alfari*		V	Queen			
Qc_K	07.09.14	C	est.	*A. constructor*		V	Queen			
37_i8_L_N	15.08.14	AS	fdg.	*A. xanthochroa*	37	S	Larva	137.5		
60_i19_L_N	25.08.14	AS	fdg.	*A. alfari*	60	S	Larva	186.4		
69_i5_L_N	31.08.14	AS	fdg.	*A. alfari*	69	S	Larva	167.4		
73_i10_L_N	31.08.14	AS	fdg.	*A. alfari*	73	S	Larva	167.2		
37_i8_P_N	15.08.14	AS	fdg.	*A. xanthochroa*	37	S	Fpatch	137.5		
59_i8_P_N	25.08.14	AS	fdg.	*A. alfari*	59	S	Fpatch	187		
60_i19_P_N	25.08.14	AS	fdg.	*A. alfari*	60	S	Fpatch	186.4		
69_i5_P_N	31.08.14	AS	fdg.	*A. alfari*	69	S	Fpatch	167.4		
73_i10_P_N	31.08.14	AS	fdg.	*A. alfari*	73	S	Fpatch	167.2		
37_i8_Pup_N	15.08.14	AS	fdg.	*A. xanthochroa*	37	S	Pupa	137.5		
59_i8_Pup_N	25.08.14	AS	fdg.	*A. alfari*	59	S	Pupa	187		
60_i19_Pup_N	25.08.14	AS	fdg.	*A. alfari*	60	S	Pupa	186.4		
69_i5_Pup_N	31.08.14	AS	fdg.	*A. alfari*	69	S	Pupa	167.4		
73_i10_Pup	31.08.14	AS	fdg.	*A. alfari*	73	S	Pupa	167.2		
37_i8_Qx_N	15.08.14	AS	fdg.	*A. xanthochroa*	37	S	Queen	137.5		
59_i8_Qa_N	25.08.14	AS	fdg.	*A. alfari*	59	S	Queen	187		
60_i19_Qa_N	25.08.14	AS	fdg.	*A. alfari*	60	S	Queen	186.4		
69_i5_Qa_N	31.08.14	AS	fdg.	*A. alfari*	69	S	Queen	167.4		
73_i10_Qa_N	31.08.14	AS	fdg.	*A. alfari*	73	S	Queen	167.2		
59_i8_W_N	25.08.14	AS	fdg.	*A. alfari*	59	S	Work.	187		
60_i19_W_N	25.08.14	AS	fdg.	*A. alfari*	60	S	Work.	186.4		
73_i10_W_N	31.08.14	AS	fdg.	*A. alfari*	73	S	Work.	167.2		
91_L_N	08.09.14	M	est.	*A. alfari*	91	S	Larva	72		
114_L_N	08.09.14	M	est.	*A. alfari*	114	S	Larva	72		
90_i9_P_N	08.09.14	M	fdg.	*A. alfari*	90	S	Patch	72		
90_i17_P_N	08.09.14	M	fdg.	*A. constructor*	90	S	Patch	72		
91_P_N	08.09.14	M	est.	*A. alfari*	91	S	Patch	72		
114_P_N	08.09.14	M	est.	*A. alfari*	114	S	Patch	72		
90_i17_Pup_N	08.09.14	M	fdg.	*A. constructor*	90	S	Pupa	72		
91_Pup_N	08.09.14	M	est.	*A. alfari*	91	S	Pupa	72		

114_Pup_N	08.09.14	M	est.	*A. alfari*	114	S	Pupa	72		
90_i9_Qa_N	08.09.14	M	est.	*A. alfari*	90	S	Queen	72		
90_i17_Qc_N	08.09.14	M	est.	*A. constructor*	90	S	Queen	72		
90_i9_W_N	08.09.14	M	fdg.	*A. alfari*	90	S	Work.	72		
90_i17_W_N	08.09.14	M	fdg.	*A. constructor*	90	S	Work.	72		
91_W_N	08.09.14	M	est.	*A. alfari*	91	S	Work.	72		
114_W_N	08.09.14	M	est.	*A. alfari*	114	S	Work.	72		

10 Curriculum vitae

Oberhauser Felix B., BSc.; Glatzgasse 4/12, 1190 Vienna

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Personal data:	Name:	Felix Benjamin Oberhauser, BSc.
	Born:	24.07.1989 in Linz, Upper Austria
	Name:	Glatzgasse 4/12, 1190 Vienna
	Telephone:	+43680/2047352
	E-mail:	Felix.benjamin.oberhauser@univie.ac.at
School education:	07/2000	Graduating elementary school in Hagenberg
	06/2008	Graduating secondary school in Freistadt
University education:	10/2009	Start of Psychology Bachelor in Salzburg
	10/2010	Start of additional Biology Bachelor in Salzburg
	12/2012	Earning the Bachelor's degree in Psychology
	03/2013	Start of the Behavior, Neurobiology and Cognition Master in Vienna
Grants:		Performance scholarships for Psychology Bachelor and Behavior, Neurobiology and Cognition Master; "Förderungsstipendium" and "KWA" scholarship from the University of Vienna for Master's thesis expenses
Scientific career:	3-4/2011	Practical work in the Psychiatry department of the Wagner-Jauregg mental hospital
	10-12/2011	Practical work in the Laboratory for Sleep and Consciousness Research of the University of Salzburg
	8-9/2014	Field research for the Master's thesis in La Gamba, Costa Rica
	9/2015	Poster presentation of Master's thesis results at the "Botaniker Tagung 2015" in Munich
	2014-current	Tutor of the Neurobiology department in physiology courses