UNIVERSIDAD MICHOACANA DE SAN NICOLÁS DE HIDALGO

INSTITUTO DE INVESTIGACIONES QUÍMICO BIOLÓGICAS

Ecological role of extrafloral nectar secretion and *Acacia* food body proteins and their relation with nitrogen fluxes in the *Acacia-Pseudomyrmex* mutualism

PRESENTA:

DOMANCAR ORONA TAMAYO

TESIS QUE PARA OBTENER EL GRADO

DE DOCTOR EN CIENCIAS BIOLÓGICAS

DIRECTORES DE TESIS:

Dr. en biotecnología y bioingeniería RODOLFO FARÍAS RODRÍGUEZ

Dr. phD. MARTIN HEIL

Morelia, Michoacan. Octubre de 2013

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Papel ecológico de la secreción del néctar extrafloral y proteínas de gránulos nutritivos de acacia y su relacion con el flujo del nitrógeno en la interacción *Acacia-Pseudomyrmex*

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Resumen

Los mutualismos son interacciones entre diferentes especies que brindan servicios y recompensas con beneficios en la adecuación de ambas partes. En la interacción *Acacia-Pseudomyrmex*, las plantas proveen recompensas a sus hormigas defensoras brindándoles gránulos nutritivos (FBs) un alimento exclusivo para la nutrición de larvas de hormiga, néctar extrafloral (NEF) del cual se alimentan las hormigas adultas y lugar de anidamiento en espinas huecas para toda la colonia. A cambio la hormiga defiende a la planta hospedera de insectos herbívoros y plantas competentes. Como en todos los mutualismos, la interacción *Acacia*-hormiga es propensa a la explotación por organismos que no son recíprocos, hormigas explotadoras como *Pseudomyrmex gracilis*, viven y se alimentan de las recompensas producidas por acacias mutualistas y no dan el pago que es el servicio de la protección. El objetivo del presente estudio fue entender como la producción de recompensas es optimizada en este mutualismo. Es así que se investigo: (i) como los FBs son protegidos del consumo por explotadores, (ii) como la producción de NEF es controlada y optimizada y (iii) como las hormigas mutualistas logran suplementar su demanda de nitrógeno con una dieta estrictamente basada en recompensas derivadas de la planta. En FBs de plantas de *Acacia hindsii* y *Acacia cornigera* se llevó a cabo una extracción de proteínas y una secuenciación masiva de estas biomoléculas, encontrándose la presencia de inhibidores de proteasas tipo Kunitz (IPs). Las larvas de hormigas mutualistas utilizan proteasas tipo quimotripsina y elastasa como principales enzimas proteolíticas (responsables de la digestión de los FBs), sin embargo, las actividades de estas enzimas digestivas en hormigas explotadoras fueron menores. Para poder dar una función

ecológica y relevante de los IPs contra algún insecto explotador, extractos de FBs fueron confrontados con las proteasas de escarabajos de *Prostephanus truncatus* y *Zabrotes subfasciatus* (potenciales explotadores de esta recompensa y modelos muy estudiados para evaluar IPs de diferentes plantas), en ambos casos los IPs ejercieron una fuerte inhibición proteolítica de sus enzimas. Estos resultados muestran que las enzimas de la hormiga explotadora son incapaces de digerir esta recompensa asimilándola solo parcialmente, mientras que la mutualista se encuentra totalmente adaptada para digerir completamente esta recompensa. Por otro lado, escarabajos de *P. truncatus* y *Z. subfasciatus* son ineficientes explotadores de FBs.

Otra de las recompensas presentes en este mutualismo es el NEF, el cual contiene diferentes biomoléculas, en el caso de acacias, está constituido por glucosa y fructosa (azucares principales), aminoácidos y proteínas (nectarinas: glucanasas, peroxidasas y quitinasas) clasificadas como proteínas relacionadas a patogénesis (Proteínas-PR). Encontramos que el NEF es secretado como un pico máximo de secreción en las mañanas (8:00-10:00 AM) con un cese rápido de nectar posterior al pico máximo de secreción, este patrón fue constante diariamente. Utilizando esta fisiología de secreción se investigó el proceso bioquímico directamente asociado con el proceso de secreción activa. Se colectaron muestras de hoja, floema, tejido del nectario y NEF previo a la secreción y durante la secreción del néctar y se analizaron las proteínas. Los resultados mostraron que proteínas del floema no se encuentran en el tejido del nectario o en el NEF, lo cual contradice la hipótesis de que el floema es secretado como néctar, indicando que el tejido del nectario es el responsable de la actividad biosintetica del NEF. Mapas proteómicos en doble dimensión de nectarios extraflorales colectados antes, durante y después de la

secreción del néctar, indican que existe acumulación de proteínas involucradas en metabolismo de carbohidratos, síntesis de aminoácidos y proteínas antes del proceso de secreción, consecuentemente, estas proteínas decaen su acumulación cuando termina la secreción del néctar, además la actividad de invertasa de pared celular fue muy activa previo a la secreción del néctar y su actividad disminuye cuando éste ya no es secretado. Al contrario, enzimas proteolíticas incrementan su actividad durante el proceso de secreción del néctar, y aumentaron mucho más su actividad cuando cesa la secreción, pareciendo estar involucradas en la degradación de proteínas biosinteticas e interrumpir la producción del néctar. Igualmente, se evaluó el nivel de expresión de genes de invertasa y proteínas-PR, observándose la expresión de estos genes sólo en el nectario extrafloral y no así en hojas. Entonces concluimos que el nectario extrafloral de plantas de *A. cornigera* es un órgano semiautónomo que contiene la maquinaria metabólica para la sintesis, producción y secreción del NEF en acacias.

Hormigas mutualistas son altamente dependientes del consumo de NEF, sin embargo; esta recompensa aparentemente tiene baja proporción de fuentes de nitrógeno. Para poder balancear esa carencia, bacterias endosimbióticas que residen en el tracto intestinal de estas hormigas contienen una maquinaria metabólica para suplementar fuentes de nitrógeno a partir de la fijación atmosférica o reciclaje del nitrógeno proveniente de material de desecho intestinal. La fijación de nitrógeno en hormigas mutualistas no pudo ser comprobada, debido a que las cantidades de asimilación de nitrógeno isotópico (^{15}N) no pudieron ser detectadas en la biomasa de esta hormiga. En cambio en la hormiga explotadora se econtraron niveles de ^{15}N, el cual fue incorporado y detectado en su biomasa. Sin embargo, cuando ambas hormigas fueron alimentadas con una solución de

urea, tanto la hormiga mutualista como la explotadora fueron capaces de inducir actividad de ureasa, esta actividad fue mayor en hormigas explotadoras, asimismo cuando las hormigas fueron alimentadas con urea isotópica (^{15}N-urea) y la subsecuente extracción de la hemolinfa para detectar incorporación de ^{15}N en los diferentes aminoácidos, se encontró que la hormiga explotadora es capaz de incorporar mayor cantidad de ^{15}N a los aminoácidos que las hormigas mutualistas, es asi que la enzima ureasa contribuye en el anabolismo de aminoácidos, los cuales podrían suplementar parte de la nutrición de estas hormigas. Mientras los animales carecen de una ureasa, éstos son apoyados por endosimbiontes bacterianos, que contienen la maquinaria metabólica que requiere la hormiga para suplementar su nutrición.

Nuestros análisis bioquímicos y moleculares, permitieron el descubrimiento de nuevos mecanismos que permiten un intercambio eficiente de recompensas en el mutualismo *Acacia-Pseudomyrmex* y que son protegidos contra explotadores.

Abstract

Mutualistic interactions among different species are usually based on the exchange of rewards and services and benefit the fitness of all involved partners. In the *Acacia-Pseudomyrmex* interaction, the host plant produces ant rewards such as food bodies (FBs, which serve as the exclusive food for the ant larvae), extrafloral nectar (EFN, which is the only food for ant workers) and nesting space in hollow thorns. In turn, the ant protects the host plant from herbivores and encroaching vegetation. Like all mutualisms, the ant-Acacia interaction is prone to exploitation by non-reciprocating organism such as exploiter ants as *P. gracilis*, that live on the Acacia plants and feed on the ant rewards but do not show any protective behavior. The goal of the present study was to understand how the reward production in this mutualism is optimized. In detail I aimed to investigate (i) how the FBs are protected from consumption by exploiters, (ii) how EFN production is optimized and controlled and (ii) how the 'vegetarian' mutualist ants manage to fulfill their nitrogen demands with a strictly plant-based diet. *Acacia hindsii* and *Acacia cornigera* FBs were analyzed for proteins. Consecutive sequencing indicated the presence of Kunitz-type protease inhibitors (PIs). Mutualistic ant larvae use chymotrypsin-1 and elastase as main proteolytic enzymes to digest the acacia FBs. By contrast, the activities of the same enzymes in the exploiter ants were much lower. Extracts obtained from FBs were confronted with proteases from potential exploiters (seed-feeding beetles such as *Prostephanus truncatus* or *Zabrotes subfasciatus*), which showed a strong inhibition of their proteases. These observations demonstrate that exploiter ants have proteases that are inefficient for the digestion of FBs and digest the reward only partially, whereas mutualistic

ants possess an enzymatic machinery that is highly suitable for the complete digestion of the reward. By contrast, seed-feeding beetles cannot consume the FBs because their proteolytic activity would be completely inhibited.

The EFN of ant-Acacias contains glucose and fructose as main sugars, amino acids, and proteins (nectarins: glucanases, peroxidases and chitinases, which are classified as pathogenesis-related proteins [PR-proteins]). EFN is secreted in a daily short peak in the morning hours (8:00-10:00 AM). I used this known secretion physiology to investigate biochemical processes directly associated with an active secretion process. Proteins of samples of leaves, phloem, nectary tissue and EFN collected before and during the active secretion were analyzed and compared. Phloem proteins were not found in the nectary tissue or in the EFN, which contradicts the hypothesis that nectar is secreted phloem sap and indicates significant biosynthetic activity in the nectary itself. Two-dimensional maps of the proteomes of the nectary tissue obtained before and during the secretion process indicated the accumulation of biosynthetic proteins involved in carbohydrates metabolism, amino acid and proteins synthesis, and of PR-proteins. These proteins dramatically diminished in their quantity or even disappeared after the nectar secretion ceased. Similarly, cell wall invertase activity was highest just before the nectar secretion and decreased during and - more strongly - after nectar secretion. By contrast, proteolytic enzymes increased during the nectar secretion and reached highest activity after nectar secretion. Their main function might be to degrade synthetic proteins and thereby terminate the nectar secretion. Moreover, the expression of important genes encoding, e.g., for invertase and PR-proteins was found only in nectary tissues. The nectary of *A. cornigera* represents a semi-

autonomous organ that contains the complete metabolic machinery for the synthesis, production and secretion of EFN.

Mutualistic ants feed only on FBs (as larvae) and EFN (as adults). However, apparently these rewards contain low amount of nitrogen. It has been hypothesized that endosymbiotic bacteria that reside in the ant midgut contain the metabolic pathways required to supplement the lack of nitrogen in arboreal ants. These bacteria could fix nitrogen from air or recycle nitrogen from metabolic waste product. The nitrogen fixation in mutualistic ants could not be proven here, because no assimilation of significant amounts of isotopic nitrogen (^{15}N) into the ant biomass could be detected. Even in the exploiter ants ^{15}N was incorporated into their body mass. By contrast, mutualistic or exploiters ants fed with urea showed a induction of their intestinal urease activity, however urease activity was most active in exploiter than the mutualist ants, by the way, when the exploiter ants were fed with isotopic ^{15}N-urea this was traced in high proportion in the amino acids from the hemolymph, than the amino acids of mutualistic ants that were traced in low rate. These result indicate that a bacterial enzyme that could have a main role in the amino acid anabolism of the ants. Whereas animals in general lack urease, several of the endosymbiotic bacteria were found to have the entire metabolic machinery that is required supply the ants with nutritious nitrogen obtain from metabolic waste products.

Molecular and biochemical analyses allowed the discovery of several new mechanisms that allow for an efficient exchange of rewards in the *Acacia-Pseudomyrmex* mutualism and that protect this mutualism against exploiters.

Chapter 1

General introduction

Picture by: Domancar Orona-Tamayo

Domancar Orona-Tamayo

1.1. Mutualisms

In nature, many interactions among plants and carnivorous insects result in beneficial effects for both organisms, due to the indirect defensive effects against herbivores that are exerted by the carnivores (Heil 2008). These interactions are based on the exchange of resources and services and represent mutualisms (Bronstein *et al.* 2006), i.e., interspecific interactions in which one partner provides a service to be rewarded by the other and which improve the net fitness of all partners involved (Bronstein 1994). Mutualisms have been widely described in nature, and their ecological and evolutionary importance is becoming well recognized. Most mutualistic interactions, however, can attract specific exploiters that consume the rewards produced by the host but do not pay the cost of reciprocating a service (Bronstein 2001). Thus, an important question in the ecology and evolution of mutualisms remains how the fluxes of rewards and services among the involved partners can be optimized and how the rewards that are produced to be traded against a specific service can be protected from exploitation.

1.2. Plant-ant mutualisms

Different plants that live in tropical zones interact with ants (Rico-Gray and Oliveira 2007). Myrmecophytic plants such as several *Acacia* species (Janzen 1966), *Cordia nodosa* (Solano *et al.* 2005), *Cecropia peltata* (Ayala *et al.* 2002), multiple species of *Macaranga* (Federle *et al.* 1998, Fiala and Maschwitz 1992a, Fiala *et al.* 1994,1996,1989) and *Piper* (Fischer *et al.* 2002) are known have associations with specialized ants from the genera *Pseudomyrmex*, *Azteca*, *Crematogaster* and *Pheidole* respectively. These ant-plant

associations provide valuable model systems to study mutualism (Heil *et al.* 2010). These interactions involve over 100 genera of angiosperms and 40 genera of ants (Davidson and McKey 1993) and represent a typical element in tropical forests. Obligate interactions between so-called myrmecophytes and their (usually highly specialized) ant inhabitants in particular are restricted to the tropics (Heil and McKey 2003). The obligate defensive mutualisms comprise host plants that provide nesting space (in hollow structures termed domatia) to their ants and that often also produce extrafloral nectar (EFN) or cellular food bodies (FBs) as food rewards. In other systems, scale insects form part of the mutualistic system and contribute to the nutrition of the ants (Gaume *et al.* 2000, Heckroth *et al.* 1998, Janzen 1966). In turn, the ants protect their host from herbivores, pathogens and encroaching vegetation. These myrmecophytes commonly are pioneer trees that can completely dominate secondary forests, likely because of the highly efficient defence against biotic stress that resident ant colonies provide to their hosts (Chamberlain and Holland 2009, Heil 2008, Koricheva and Romero 2012, Romero and Koricheva 2011, Rosumek *et al.* 2009).

1.3. Exploiting mutualisms

Ant-plant mutualisms are prone to exploitation by non-reciprocating exploiters, *i.e.*, species that inhabit the host and utilize the resources that usually serve to reward the symbiont, however, without paying the respective service (Bronstein 2001, Yu 2001). Exploiters safe the time and energy that mutualists spend on reciprocating and, therefore, are usually considered as competitively superior, at least as long resource availability remains unchanged. Exploiters can evolve from former mutualists that cease service provisioning

and then represent cheaters, or they can invade the mutualism starting from an originally independent lifestyle and then represent parasites of the mutualism (Bronstein 2001, Kautz *et al.* 2009, Segraves *et al.* 2005). Exploitation has been reported for a wide range of mutualisms, including nectar robbing (e.g. bees and birds) (Maloof and Inouye 2000, Roubik 1982), mycorrhizal fungi that uptake the plant carbon but transfer no nutrients to the plant (Smith *et al.* 1996), and strains of *Rhizobium* and *Bradyrhizobium* that transfer less or no nitrogen to the host than mutualistic strains (Wilkinson *et al.* 1996). In defensive ant-plant mutualisms, *Phyllobaenus* beetles exploit the shelter and food rewards produced by *Piper* plants (Letourneau 1983), a foraging spider (*Bagheera kiplingi*) that lives in the hollow spines of Mexican acacias uses plant-derived food body rewards for its nutrition (Meehan *et al.* 2009), specific ants inhabit the plant without providing a defensive service (Clement *et al.* 2008, Janzen 1975, Raine *et al.* 2004), and even ants that efficiently fend off herbivores can cheat their host by manipulating its reproductive efforts for their own benefits (Gaume *et al.* 2005, Izzo and Vasconcelos 2002, Yu and Pierce 1998).

Several mechanisms have been proposed for the stabilization of mutualisms against the exploitation. One of these is "partner choice", which allows the selection of suitable partners based on certain traits that are used as keys for partner identification before the symbiosis is being established (Bull and Rice 1991). Partner choice has been studied particularly well for the root-*Rhizobia* mutualism, in which *Rhizobia* strains must identify themselves as potential mutualists via the secretion of specific chemical signals (Nod factors) (Oldroyd 2001, Simms *et al.* 2006). Another mechanism is "host sanctions", which applies when the symbiosis has already been established. Here, the host monitors the action of its symbiont in order to punish exploiters, usually via a reduction in reward provisioning.

For example, plant roots cease the allocation of assimilates towards nodules formed by non N-fixing bacteria or mycorrhizal fungi that turn into pathogens (Kiers and Denison 2008, Kiers *et al.* 2003, 2011, West *et al.* 2002).

1.4. The models system used in this study

The ant-acacias (also called "Swollen thorn" acacias) represent a diverse and widespread group of neotropical myrmecophytes. Myrmecophytic *Acacia* (Mimosoideae, Fabaceae) plants live in obligate protection mutualisms with ants of the *Pseudomyrmex ferrugineus* group or exploiter ants (Janzen 1966, 1967, 1974, Ward 1993) (**Figure 1.1)**.

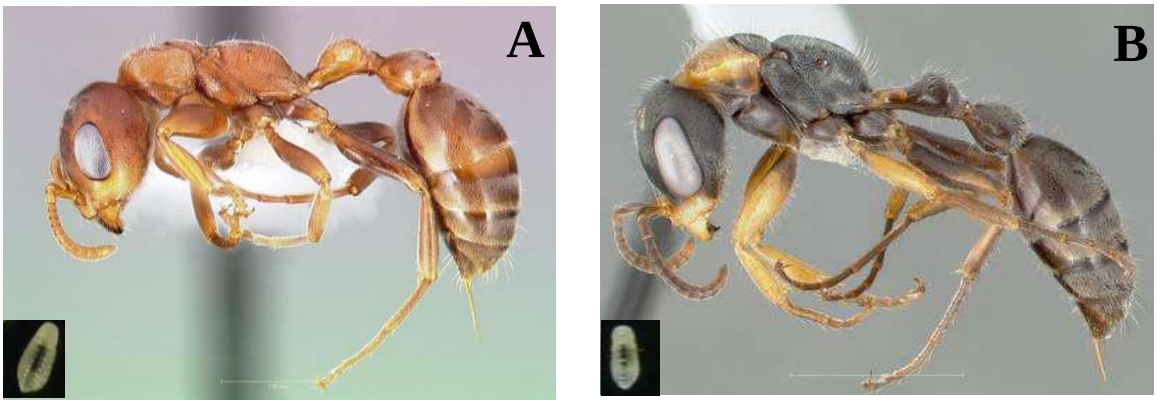

Figure 1.1. Vegetarian *Pseudomyrmex* ant worker. *Pseudomyrmex ferrugineus* (A) and *Pseudomyrmex gracilis* (B). Insets represent ant larvae of each ant species.

As mentioned before, *Acacia* plants provide nutritious rewards (FBs and EFN) and nesting space in hollow thorns (**Figure 1.2)** to the mutualistic ant species *P. ferrugineus* F. Smith, *P. mixtecus* Ward and *P. peperi* Forel. In return, the ants protect the plants from encroaching or competing vegetation (Heil *et al.* 2010, Janzen 1966, 1967, Ward 1993). These ants cannot be found nesting apart from their host plants (Clement *et al.* 2008) and

exclusively live in enlarged hollow thorns, which are cleaned through an entrance hole when the thorn is still green and relative soft (D. Orona-Tamayo, personal observation).

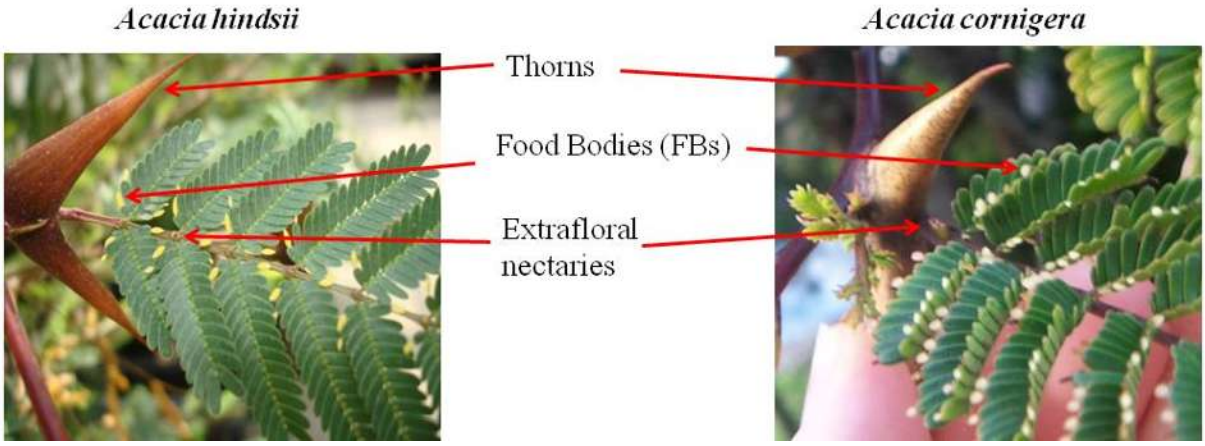

Figure 1.2. Ant rewards produced by Mesoamerican *Acacia*-ant plants.

1.5. Physiological adaptations in the *Acacia-Pseudomymex* interaction

1.6. Extrafloral nectar (EFN)

Nectar is a sweet and acqueous solution secreted by specific tissues named nectaries, and mediates the interactions of plants with pollinators and defenders. Whereas floral nectar serves for pollination, EFN is secreted outside of the flowers and is not involved in pollination (Bentley and Elias 1983, Heil 2011). Benefits for plants that secrete EFN include the attraction of ants, parasitoid wasps and generalist predators, which results in an indirect defense against herbivores (Bentley and Elias 1983, Bentley 1977a, Heil 2011). EFN secretion has been described for species that belong to more than 300 genera of plants (Bentley 1977b, Koptur 1992). Different biomolecules form the fraction of soluble solids that can be found in the EFN. The soluble solids mainly comprise monosaccharides, disaccharides and amino acids (Baker *et al.* 1978, Baker and Baker 1973, Heil 2011). In addition, other compounds such proteins, lipids, phenols and volatile organic compounds

have been reported in different nectars (Hillwig *et al.* 2010, Kessler and Baldwin 2007, Nicolson *et al.* 2007). The presence of free carbohydrates and nitrogenous compounds makes EFN an attractive nutritional resource for insects, however sugars and amino acids are mainly responsible for ant attraction (Baker and Baker 1973, Blüthgen and Fiedler 2004, González-Teuber and Heil 2009b, Heil *et al.* 2005, Lanza 1988). Interestingly, certain common compounds can mediate very specific interactions, for example, sucrose is very common in EFN of most species and usually attracts generalist ants. However, plants can exhibit adaptive changes in the chemical composition of EFN in order to filter the nectar consumers. In fact, the sucrose-free EFN of the myrmecophytic acacia plants is the product of a soluble invertase in this EFN and is unattractive to generalist or exploiter insects, by contrast, the specialized workers of *Pseudomyrmex* ants that live on myrmecophytic *Acacia* plants possess almost no invertase activity in their digestive tracts and only prefer sucrose-free EFN (**Figure 1.3)** (Boevé and Wäckers 2003, Heil *et al.* 2005, Kautz *et al.* 2009, Zhang *et al.* 2012).

Figure 1.3. Extrafloral nectaries. *A.cornigera* (A) and *A. hindsii* (B) extrafloral nectaries. *A. cornigera* EFN consumption by *P. ferrugineus* workers (C).

A class of nectar components that has received attention only recently is the nectarins (nectar proteins). Nectarins have been discovered first in the floral nectar (FN) of ornamental tobacco (*Nicotiana langsdorfii* x *Nicotiana sanderae).* Five nectarins have been biochemically characterized and are likely to protect FN from microbial contamination by non-sterile pollinators through the nectar redox cycle (Carter *et al.* 1999, 2007, Carter and Thornburg 2004). However, several nectarin proteins with sequence homology to those described for tobacco were found in other plant FNs, from species such as *Jacaranda mimosifolia* (Kram *et al.* 2008), *Petunia hibrida* (Hillwig *et al.* 2010, Hillwig *et al.* 2011) and *Cucurbita pepo* (Nepi *et al.* 2011). Most of these nectarins function as Pathogenesis Related Proteins (PR-proteins) (Heil 2011). That an anti-microbial protection of nectar represents an important mechanism illustrated by the fact that yeasts can be found in other nectars that are virtually free of protective proteins, such as the EFN of non-myrmecophytic *Acacia* plants (González-Teuber and Heil 2009a). Bacteria and yeasts are commonly reported for floral nectars which they use as a growing medium and chemically alter due to their own metabolism (Álvarez-Pérez *et al.* 2012, Canto and Herrera 2012, Canto *et al.* 2008, Herrera *et al.* 2009, 2010, Vannette *et al.* 2013). The recent discovery of chitinases and glucanases in the EFN of *Acacia* myrmecophytes (González-Teuber *et al.* 2009, 2010) indicate that EFN-infecting microorganisms might also represent common exploiters of ant-plant mutualisms.

1.7. Food Bodies (FBs)

Cellular food bodies (FBs) are nutritionally valuable rewards that are produced by plants to nourish their mutualistic ant defenders (**Figure 1.4)**. This type of reward is provided by many myrmecophytes, including the genera *Cecropia* (Folgarait *et al.* 1994), *Piper* (Fischer *et al.* 2002), *Macaranga* (Fiala and Maschwitz 1992b, Heil *et al.* 1998) and *Ochroma* (O'Dowd 1980). *Acacia* FBs are produced during the ontogenetic development on the leaflet tips (Rickson 1969, 1975) and are harvested by the ants and fed to their larvae (Janzen 1974).

Figure 1.4. Acacia FBs. *A. cornigera* (A) and *A. hindsii* (B) FBs. Recollection of *A. hindsii* FB by *P. ferrugineus* worker (C).

FBs are rich in lipids, proteins and contain essential amino acids (glycine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tryptophane, and valine) and their protein content exceeds the protein content of the leaf tissues (Canavoso *et al.* 2001, Chen 1985, Heil *et al.* 2004, Heil *et al.* 2010). Some polyunsaturated fatty acids have also been detected in FBs, and many studies have shown that either linoleic or linolenic acid adequately satisfy the nutritional needs of ants and are necessary for normal insect development (Arrese and Soulages 2010, Canavoso *et al.* 2001). For hymenopterans, linoleic and linolenic acid are considered as essential (Barbehenn *et al.* 1999, Canavoso *et*

al. 2001, Hagen *et al.* 1984). In summary, FBs provide their consumers with a valuable diet that is rich in essential compounds.

1.8. Bacterial associates of the ants

Obligate plant-ants that feed only on host-derived rewards are truly "vegetarian" and thus represent a particularly extreme case of nutritional specialisation. Ants are one of the most important insects group and dominate multiple different habitats, and a shift towards a more vegetarian life style as been suggested as a key factor explaining the dominance of ants in certain habitats (Davidson *et al.* 2003, Russell *et al.* 2009). In ant-plant mutualistic interactions, where the exchange of resource and services are the principal aspect, the host-derived rewards represent the only source of nutrition for the ants. Thus, workers invest high amounts of energy to patrolling and protect their partner to avoid negative effects on host performance and food reward production. Interestingly, defending this valuable source of energy against non-ant EFN-consumers is facilitated by the plant, which produces the EFN only during a very short diurnal peak (González-Teuber *et al.* 2012)

Although EFN is rich in proteins and supplemented by some nectarins, it appears unlikely that the nitrogen demands of the developing ant larvae can be fully fulfilled by this diet. A possible explanation would be the existence of ant-microbe mutualism that allows the ants some kind of N supplementation (Davidson *et al.* 2003, Zientz *et al.* 2005). Approximately 20% of all insects contain in their midguts intracellular endosymbiontic bacteria (Buchner 1965) and some insects completely depend on symbiotic bacteria for balancing the host nutrition (**Figure 1.5)** (Chandler *et al.* 2008, Eilmus and Heil 2009, Nardi *et al.* 2002).

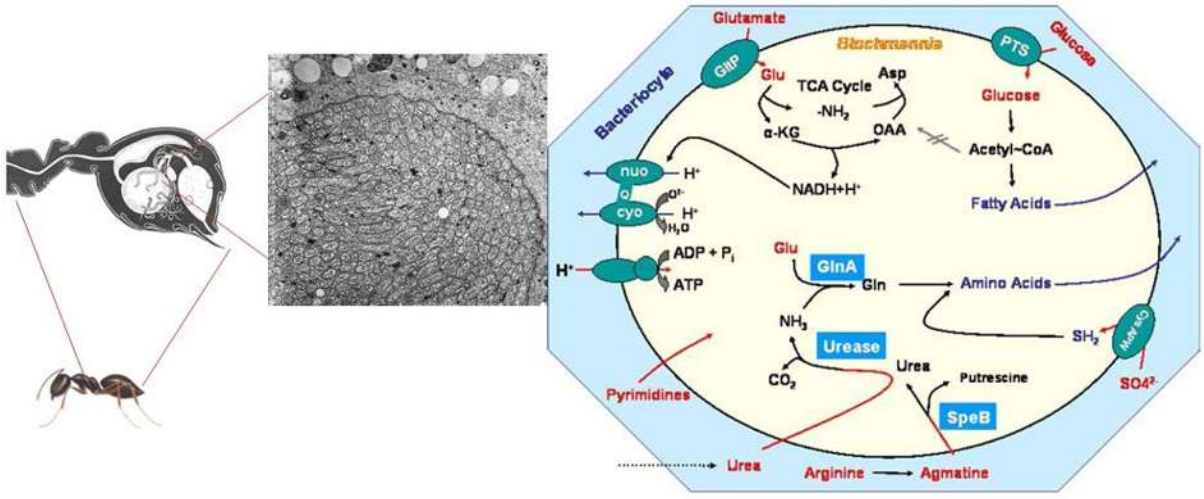

Figure 1.5. Hypothetical metabolic interactions between endobacteria-ant symbiosis (Modified from Zientz *et al.* 2005).

Ant endosymbionts might also play a secondary nutritional role by augmenting an existing metabolic process in the host, for example by providing more organic nitrogen (N) due to N-fixation or N-recycling (Cook and Davidson 2006, Heil *et al.* 2010, Wernegreen *et al.* 2003, Zientz *et al.* 2005). Different endosymbiotic bacteria such *Blochmannia*, *Buchnera*, *Rickettsia* and *Wolbachia* are common intracellular residents in ants and several authors suggested that these microbes may play specific roles in fixing, recycling or upgrading N (Anderson *et al.* 2012, Cook and Davidson 2006, Russell *et al.* 2009, Wernegreen *et al.* 2003). For example, the *Blochmannia* endosymbiont in *Camponotus floridans* ants provides essential amino acids and might also play a role in N recycling (Feldhaar *et al.* 2007). Similarly, *Tetraponera* ants contain gut bacteria that were suggested to be involved in N-fixation and recycling. However, final evidence for the specific role of these bacteria is still lacking.

1.9. Background

Mesoamerican Acacia-myrmecophytes represent a classical example of an ant-plant mutualism, because their mutualism with ants depends in a continuous exchange of resources and services. *Acacia* plants produce EFN and FBs and in addition provide hollow thorns as domatium for the mutualistic ant colony (Janzen 1966, 1974). The ants exclusively feed of these plant-derived food-rewards (ant larvae only of FBs and workers of EFN), which are being constitutively produced in high rates by the plant (Clement *et al.* 2008, Heil *et al.* 2009). Both partners seem to be highly adapted to this mutualism (Janzen 1967, Raine *et al.* 2002), because the plant produce biochemically adapted FBs and enlarged foliar nectaries that secrete EFN all year-round, whereas the ants are extremely aggressive towards plants´ enemies and patrol the plant surface permanently as a constitutive defense (Janzen 1966).

The chemical contents of these rewards, appear to be adapted to nourish mutualistic *Pseudomyrmex ferrugineus* ants. However, each morphological instar of the ants requires different biomolecules for their nutrition. How can the nutritional requirements of ant larvaes, workers and queens be fulfilled with a strictly vegetarian diet? Moreover, FBs due to their high contents of nutritionally valuable compounds appear prone to exploitation by other insects. It remains unknown, however, whether FBs contain defensive proteins or other defensive compounds as it has been described above for the EFN. The EFN of *Acacia* plants is rich in sugars (glucose and fructose) and amino acids and thus needs protection from microbial infestation. This function is supported by nectarins. However it is not known whether these molecules are synthesized in the nectary tissues or elsewhere in the

plant. Similarly, it is unknown how plants synthesize other nectar components and how the temporal patterns in its secretion (such as the above-mentioned diurnal peak in EFN secretion by *A. cornigera*) are controlled at the enzymatic level. Earlier studies used floral nectaries with an ontogenetically fixed secretion and hence were limited to time scales with an order of magnitude of days (Escalante-Pérez *et al.* 2012, Kram *et al.* 2009).

1.10. Hypothesis

Acacia FBs contain proteins that avoid the exploitation by non-adapted insects and the larvae of the specialized mutualistic ants posses the biochemical "key" to open this "lock".

The most important biomolecules that extrafloral nectar contains are synthesized in the nectary tissues.

Microbial endosymbionts contribute in the N-nutrition of ant workers.

1.11. Justification

It is currently unknown where and in which temporal pattern the most important components of the extrafloral nectar are synthesized, which kinds of proteins are found in the food bodies, how these proteins contribute to the nutrition *Pseudomyrmex* ants, how these food bodies are protected from exploitation, and how the ant workers can obtain a balanced diet with sufficient nitrogen although they feed only on extrafloral nectar.

1.12. Objectives

1.12.1. General Objective

Determine spatial-temporal patterns in the synthesis of extrafloral nectar components, some specific roles of proteins that are present in the food bodies and to investigate whether endosymbiotic bacteria contribute to the nutrition of ant workers.

1.12.2. Specific Objectives

Determine whether proteins involved in the defense of *Acacia* Food Bodies (FBs) against exploiters and their effects on digestive proteases present in the ant larvae.

Study spatiotemporal patterns in the proteome of the nectary tissues as metabolic factory for the synthesis of important biomolecules in the extrafloral nectar.

Determine the microbial contribution to the nitrogen supplementation in ant workers.

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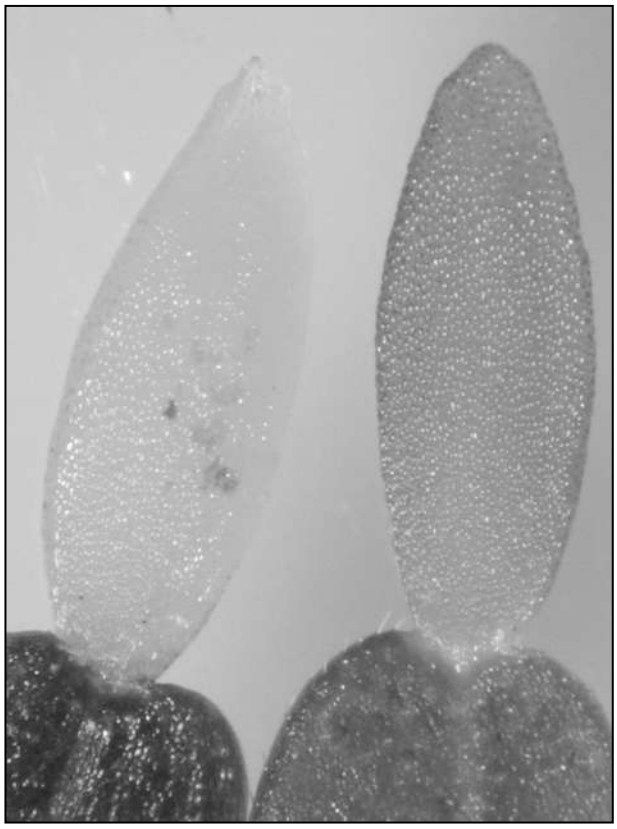
Chapter 2

Exclusive rewards in mutualisms: ant proteases and plant protease inhibitors create a lock–key system to protect *Acacia* food bodies from exploitation



Picture by: Domancar Orona-Tamayo

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2.1. Abstract

Myrmecophytic *Acacia* species provide nesting space, extrafloral nectar and food bodies (FBs) to ants of the *Pseudomyrmex ferrugineus* group, with which they live in an obligate mutualism. Here we report how the FBs are protected from exploitation by non-adapted generalist herbivores without diminishing their nutritional value to the mutualist ant larvae. Two-dimensional gel-electrophoresis of the FB proteomes of *Acacia hindsii* and *Acacia cornigera* and consecutive protein sequencing indicated the presence of several Kunitz-type protease inhibitors (PIs). Extracts obtained from the FBs exerted strong inhibitory effects on trypsin-like activity of potential exploiters, the seed-feeding coleopterans *Prostephanus truncatus* and *Zabrotes subfasciatus* and a related exploiter ant, *Pseudomyrmex gracilis*. Trypsin is the dominant proteolytic activity in the midguts of these coleopterans and elastase dominated in the exploiter ant. By contrast, proteolytic activity in the midguts of the mutualist ant larvae was dominated by chymotrypsin 1-like besides elastase-like enzymes, both of which were not inhibited by the FB PIs. *Acacia* FBs are specifically adapted to nourish the larvae of their mutualistic ants. However, whereas their consumption by non-adapted potential exploiters is impaired by defensive proteins, to which the congeneric exploiter ant appears to be adapted partly, but not completely. We suggest that the term 'exclusive rewards' can be used to describe situations similar to the one that has evolved in myrmecophytic *Acacia* species, which reward mutualists with FBs but safeguard the reward from exploitation by generalists by making the FBs difficult for the non-adapted consumer to use.

2.2. Introduction

Many interactions among plants and carnivorous insects result in beneficial effects for both organisms because of the indirect defensive effects against herbivores that are exerted by the carnivores (Heil 2008, Kessler and Heil 2011). These interactions are based on the exchange of resources and services and represent mutualisms (Bronstein *et al.* 2006). However, virtually all mutualistic interactions are exploited by organisms that consume the host-derived rewards without providing an adequate service, thereby reducing the fitness of the mutualists (Bronstein 1998, 2001, 2003). The exploitation of a wide range of mutualisms by other organisms has been reported, including nectar robbing by, bees and birds (Maloof and Inouye 2000, Roubik 1982), some mycorrhizal fungi (Smith *et al.* 1996) that uptake plant carbon but transfer no nutrients to the plant, and strains of *Rhizobium* and *Bradyrhizobium* (Wilkinson *et al.* 1996) that either transfer no nitrogen to the host or less than mutualistic strains. In defensive ant–plant mutualisms (Heil and McKey 2003), *Phyllobaenus* beetles exploit the shelter and food rewards produced by *Piper* plants (Letourneau 1990), a foraging spider (*Bagheera kiplingi*) living in the hollow spines of Mexican acacias uses plant-derived food body rewards for its own nutrition (Meehan *et al.* 2009), and specific parasitic ants (*Pseudomyrmex gracilis* and *P. nigropilosus*) make use of the host-derived rewards without rendering a defensive service (Clement *et al.* 2008, Janzen 1975).

Food bodies (FBs) are nutritionally valuable rewards that are produced by plants to nourish their mutualistic ant defenders. This type of reward is provided by many obligate ant-plants (myrmecophytes), including the genera *Cecropia* (Folgarait *et al.* 1994), *Piper* (Fischer *et*

al. 2002), *Macaranga* (Heil *et al.* 1998), *Ochroma* (O'Dowd 1980) and *Acacia* (Heil *et al.* 2004). We investigated whether the FBs produced by myrmecophytic *Acacia* (Mimosoideae, Fabaceae) species in Central America are specifically protected from consumption by species that represent potential exploiters and a real ant exploiter. Plants in several *Acacia* species in Mesoamerica and Africa live in an obligate protection mutualism with ants, although the details of the interaction differ between the American and the African clades (Goheen and Palmer 2010, Janzen 1967, Palmer *et al.* 2008, Ward 1993). Central American ant-acacias provide ants of the *Pseudomyrmex ferrugineus* (F. Smith) group (Janzen 1967, Ward 1993) with hollow thorns that serve as nesting space (domatia) and with food rewards (**Figure. 2.1**): extrafloral nectar (EFN) and FBs (Janzen 1974).

Figure 2.1. Leaves of myrmecophytic *Acacia* plants with food bodies (FBs). (A) *A. hindsii* and (B) *A. cornigera;* FBs are marked with arrows. Insets illustrate individual FBs. (C) *A. hindsii* FB harvested by a *Pseudomyrmex ferrugineus* worker.

In exchange, the ants protect their hosts from herbivores and encroaching or competing vegetation (Janzen 1967). *Acacia* FBs are produced during the normal leaf ontogeny on the leaflet tips (Clement *et al.* 2008, Rickson 1975, 1980) and fed to the ant larvae (Clement *et al.* 2008, Janzen 1966, 1967, 1974). They are rich in lipids and proteins and contain essential amino acids (Heil *et al.* 2004) and thus also represent a potentially attractive food

source for herbivores that feed on leaves (Abdulrazak *et al.* 2000) or seeds (Miller 1996) of *Acacia* or on other legumes (Singh and Emden 1979).

How are FBs protected from exploitation? Although the ants actively defend the leaves that bear the FBs, we assumed that FBs also require a direct, chemical protection. The EFN produced by *Acacia* myrmecophytes is protected from microbial exploiters by means of pathogenesis-related (PR) proteins (González-Teuber *et al.* 2009, 2010). Hence, it appeared likely that FBs would also be protected by defensive proteins. With the aid of proteomics techniques, we have detected numerous protease inhibitors (PIs) in the FBs (Wielsch *et al.* 2011). In the current study, we used in-gel activity assays (zymograms) and inhibitory assays to investigate whether these PIs can diminish the protein digestive activities in an ant exploiter and two non-ant species that represent potential exploiters of the FBs. *P. gracilis* (Fabricius) inhabits the hollow thorns and consumes FBs but does not show any detectable defending behaviour and thus acts as an exploiter (Clement *et al.* 2008). Since this exploiter could be expected to be at least partly adapted to any defensive components of the FBs, we also searched for potential exploiters that seemed likely to feed on the FBs if they would not be chemically protected. For this category, we chose the seed-feeding beetles *Prostephanus truncatus* (Horn) (Coleoptera: Bostrichidae) (Cowley *et al.* 1980) and *Zabrotes subfasciatus* (Boh) (Coleoptera: Bruchidae) (Cardona *et al.* 1992), two beetles physiologically very well studied (Hodges 1986, Teixeira and Zucoloto 2011) and commonly being used in bioassays to evaluate the activity of protease inhibitors (Aguirre *et al.* 2004, 2009, Castro-Guillén *et al.* 2012, Torres-Castillo *et al.* 2009). We also investigated the effect of these PIs on the proteolytic activity in the digestive tracts of the legitimate consumers: the larvae of *Pseudomyrmex ferrugineus* ants. We found that PIs in

Acacia FBs are active and inhibited the protein digestion in non-adapted herbivores and larvae of the exploiter ants. By contrast, the proteolytic activity in the digestive tracts of the ant larvae was not significantly affected. The PIs found in the *Acacia* FBs render a highly nutritive food reward an item that is difficult to digest for potential and real exploiters, whereas the legitimate consumers posses the biochemical 'key' to open this 'lock'. 'Reward exclusivity' can represent an effective strategy to protect valuable rewards from exploitation.

2.3. Materials and methods

2.3.1. Plant species and study site

For this study, we selected a high-reward species, *Acacia cornigera* (L.) Willdenow, and a low-reward species, *Acacia hindsii* (Bentham), that differ in the amount of FBs they produce (Heil *et al.* 2009) and in their chemical composition (Heil *et al.* 2004, 2010). FBs were collected in the south of Mexico near Puerto Escondido, Oaxaca (Pacific coast; ~15°55´ N and 097°09´ W, elevation 15 m). All the sites were pastures used for extensive cattle grazing and the plants used were shrubs (1.5–2.0 m in height) growing in full sun that did not appear to be infected by pathogens or damaged by herbivores.

2.3.2. Sample collection

All plants chosen for FB collection were inhabited by the ant mutualist *Pseudomyrmex ferrugineus*. To collect FBs, the main shoots were deprived of ants by cutting off the thorns and by mechanically removing ants before placing the shoots in gauze bags to protect the development of FBs. After isolation, a ring of sticky resin (Tangletrap, Tanglefoot Corp., http://www.tanglefoot.com) was applied to exclude ants. Three weeks later, all newly produced leaves were collected. FBs were removed and frozen immediately in dry ice for transportation to the laboratory. Ants (adults and larvae) were collected from independent plants by cutting off swollen thorns. Entire thorns, which contained adult ants and larvae, were then kept in 1 l plastic pots with adequate ventilation until the ants were dissected.

2.3.3. Total protein extraction of *Acacia* FBs and leaves

Tissues were ground in liquid nitrogen. To extract proteins, 0.1 g of sample was placed in 1 ml 10% TCA/acetone and then centrifuged at 16 000 *xg* for 3 minutes at 4°C. The samples were washed with 80% methanol/0.1 M of ammonium acetate and centrifuged and then washed in 80% acetone, centrifuged as before and resuspended in a mixture (1:1) of 0.4 ml of phenol (Tris-buffered, pH 8.0; Sigma St. Louis, MO) and 0.4 ml of dense SDS buffer (30% sucrose, 2% SDS, 0.1 M Tris-HCl, pH 8.0, 5% β-mercaptoethanol). The mixture was vortexed for 5 min and then centrifuged at 14 000 *xg* at room temperature for 5 min. The phenol phase was recovered and 0.4 ml of fresh SDS buffer was added twice and processed as before. Tubes that contained the phenol phase were filled with 0.1 M ammonium acetate, stored at –20°C for 30 min and centrifuged for 5 min. Pellets produced by precipitation were washed twice with methanol and once with 80% acetone (Wang *et al.* 2006). The

protein content was determined using the Bradford kit (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA) as the standard (Bradford 1976).

2.3.4. Extraction of soluble protease inhibitors from *Acacia* FBs

To 0.1 g of ground tissue we added 300 µl of a mixture of chloroform:methanol (2:1 v/v) and mixed for 30 min at 4°C. Samples were centrifuged at 10 000 *xg* for 30 min at 4°C and resuspended in 300 µl of the same solution, processed as before and then dried at 25°C for 6 h. Then, the dry samples were suspended in water (1:5 w/v) for 4 h at 4°C and centrifuged at 10 000 *xg* for 60 min at 4°C. The supernatants were recovered and stored at –70°C (Aguirre *et al.* 2004).

2.3.5. SDS-PAGE and Zymograms

The electrophoretic separation of proteins was realized by 13% SDS-polyacrylamide gel-electrophoresis (PAGE) (Laemmli 1970). Extracts (25 µg of protein per sample) were loaded onto the wells and separated on a vertical dual mini gel electrophoresis device (Bio-Rad, Hercules, CA) at 120 V and 20 µA. Gels were stained with Coomassie colloidal blue. Native electrophoresis was performed using 12% PAGE co-polymerized with 0.1% gelatine and midgut extract: the gels were loaded with 20 µg of protein per sample and the separation was performed at 4°C (100 V and 12 µA). Gels were immersed twice in an activation solution [2.5% of Triton X-100 in 0.05 M of Tris-HCl (pH 7.4)] for 10 min at 25°C and then submerged in reaction buffer [0.02% of Triton X-100; 0.2 M of NaCl; 0.005 M of $CaCl_2$ in 0.05M of Tris-HCl (pH 7.4)] for 4 h at 37°C. The gel was washed, stained

and destained. Clear bands in a blue background were identified as protease activity bands (Choi *et al.* 2001).

2.3.6. Two-dimensional gel electrophoresis and in-gel digestion of proteins

In order to investigate the FB proteome, the total protein content was separated by two-dimensional gel-electrophoresis (2-DE). 300 µg of protein per sample were re-suspended in 250 µl of rehydrating solution [7 M urea, 2 M thiourea, 2% CHAPS, 65 mM of DTT, 2% IPG buffer (pH 4–7)] and then each individual sample was loaded onto 13-cm IPG dry strip gels and allowed to rehydrate for 15 hours. The isoelectrofocusing (first dimension) of IPG strips was carried out according to the manufacturer's instructions (GE-Amersham) and the proteins were then separated by 13% SDS-PAGE two-dimensional gel electrophoresis. Protein spots of interest were cut from the gel matrix and tryptically digested (Shevchenko et al. 2006). Briefly, proteins were in-gel reduced by 10 mM dithiothreitol and alkylated by 55 mM iodoacetamide. Destained, washed and dehydrated gel pieces were rehydrated for 60 min in a 0.5 µM solution of bovine trypsin in a 25 mM ammonium bicarbonate buffer at 4°C and then digested overnight at 37°C. Tryptic peptides were then extracted from the gel matrix (50% ACN / 5% formic acid) and dried down in a vacuum centrifuge.

2.3.7. LC-MS/MS and data analysis

For LC-MS analysis, samples were reconstructed in 10 µl aqueous 1% formic acid. Depending on staining intensity, 1.5–6.0 µl of samples were injected on a nanoAcquity nanoUPLC system. LC-MS/MS analysis was carried out as previously described (González-Teuber *et al.* 2009, 2010). Briefly, the peptides were desalted and concentrated

on a Symmetry C18 trap-column (20 × 0.18 mm, 5 µm particle size) using a mobile phase of 0.1% aqueous formic acid at a flow rate of 15 µl min^{-1} and then eluted on a nanoAcquity C18 column (100 mm × 100 µm ID, BEH 130 material, 1.7 µm particle size) using an 10 min increasing acetonitrile gradient (0.1% formic acid) at a flow rate of 0.500 µl min^{-1}.

The eluted peptides were on-line transferred via a nano electrospray source into a Synapt HDMS tandem mass spectrometer (Waters) operated in V-mode with a resolving power of at least 10 000. The data were collected under data-dependent acquisition using MassLynx version 4.1 software (Waters); the acquisition cycle consisted of a survey scan covering the range of m/z 400–1500 Da followed by MS/MS fragmentation of the four most intense precursor ions collected over a 1 sec interval in the range of 50–1700 m/z. To compensate for mass shifts in MS and MS/MS fragmentation mode, human Glu-Fibrinopeptide B [650 fmol/µl, 0.1% formic acid/acetonitrile (1:1 v/v)] was infused every 30 seconds at a flow rate of 0.5 µl min^{-1} through the reference NanoLockSpray source.

The acquired data were processed by baseline subtraction, smoothing and deisotoping using ProteinLynx Global Server Browser version 2.4 (Waters) and pkl-files of MS/MS spectra were generated. For data analysis we applied stringent and homology-based database searching in a combined approach. MS/MS spectra were first searched against a comprehensive NCBInr database (updated January, 28, 2011 installed on a local server) using MASCOT version 2.3. Mass tolerances for precursor and fragment ions were 15 ppm and 0.03 Da, respectively. Other search parameters were: instrument profile, ESI-Trap; fixed modification, carbamidomethyl (cysteine); variable modification, and oxidation (methionine); up to 1 missed cleavage were allowed. Hits were considered as confident if at

least three peptides were matched with ion scores above 25, or proteins were identified by one or two peptides with a score of 50 or better.

In parallel, the peptide fragment spectra were searched against a subdatabase containing common contaminants (human keratins and trypsin); spectra that remained unmatched were interpreted *de novo* to yield peptide sequences. For *de novo* sequencing a mass deviation of 0.005 was allowed and sequences with a ladder score exceeding 30 were subjected to homology-based searching using the MS BLAST program (Shevchenko *et al*. 2001) installed on a local server. MS BLAST searches were performed against a complete NCBInr database downloaded on August, 10, 2011 using described settings (González-Teuber *et al*. 2009, 2010).

2.3.8. Extraction of proteases from the midguts of ant larvae and beetles

Protease enzymes of mutalistic and exploiter ants, were extracted from fourth-instar larvae taken from colonies that lived on *A. hindsii* or *A. cornigera* shrubs. We only selected larval midguts that were visibly filled with FBs. Larvae were dissected in an 'Insect Ringer' solution (10.4 g NaCl, 0.32 g KCl, 0.48 g $CaCl_2$, 0.32 g $NaHCO_3$ in 1 l of water). Undigested FB fragments were discarded. A single replicate comprised 50 ant larval midguts, which were placed in 400 µl of 0.15 M NaCl, homogenized and centrifuged at 15 000 *xg* for 30 min at 4°C, before storage at –70°C. Midguts of fifty third-instar larvae of each of the two beetle species (*P. truncatus* and *Z. subfasciatus*) were subjected to the same protocol. Beetle larvae were cultivated on seeds of maize (*Zea mays*) and bean (*Phaseolus*

vulgaris), respectively, at 28°C and 60% relative humidity under a 12:12 h light–dark photoperiod (Aguirre *et al.* 2004).

2.3.9. Quantification of the proteolytic enzymes in the midguts of ant larvae

The activities of trypsin, chymotrypsin isoforms and elastase from ant larvae, and of trypsin and chymotrypsin from beetle larvae, were quantified in a microplate multi-reaction assay with specific chromogenic substrates (Sigma, St. Louis, MO). N-benzoyl-DL-arginine *p*-nitroanilide (Bz-R-*p*NA) substrate was used for trypsin-like activity, N-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine *p*-nitroanilide (Suc-AAPF-*p*NA) and N-glutaryl-L-phenylalanine *p*-nitroanilide (Glt-F-*p*NA) for the two isoforms of chymotrypsin-like activity, N-succinyl-L-alanyl-L-alanyl-L-alanine *p*-nitroanilide (Suc-AAA-*p*NA) substrate for elastase-like activity and the commercial inhibitor SKTI (soybean Kunitz trypsin inhibitor) was used as a positive control (Erlanger *et al.* 1961). All substrates were used at final concentrations of 0.01 M dissolved in DMSO, adjusted to a final volume of 240 µl with buffer (Tris-HCl 0.1 M; pH 7.4). For each sample, 10 µg of protein was loaded and the mixture was pre-incubated for 15 min at 37°C, after which time 20 µl of the specific substrate was added. A change of absorbance was recorded every 5 min for 30 min. A blank was prepared with 220 µl of buffer and 20 µl of each substrate.

2.3.10. PI activity of *A. hindsii* and *A. cornigera* FBs against proteases of ant and beetle larvae

To assess the inhibitory effects of the FB PIs, the trypsin-like, chymotrypsin-like and elastase-like activities in the ant larval midgut extracts and the trypsin-like and chymotrypsin-like activities in the beetle larval midgut extracts were quantified as described above in the presence of *A. hindsii* FB PIs or *A. cornigera* FB PIs. 10 µg of protein extract was mixed with 10 µg of FB protein extracted as described above. Samples were mixed and pre-incubated for 15 min at 37°C before adding 20 µl of substrate. Changes in the absorbance were measured at 405 nm in a µQuant® microplate-reader. Samples that lacked PIs were used as controls. Protease activities were expressed as the µM concentration of *p*-nitroaniline produced in 1 min in relation to insect protein concentration used in the reaction. A standard curve with different concentrations of *p*-nitroaniline was performed and linear regression was applied to the standard curve to obtain the µM of *p*-nitroaniline corresponding to each reaction.

2.3.11. Statistical analysis

Total protein quantification and protease activities were examined using global LSD post hoc tests after univariate analysis of variance (ANOVA). These statistical analyses were performed using Statistical Package for the Social Sciences 17.0 (SPSS Inc. Chicago, USA).

2.4. Results

2.4.1. SDS-PAGE patterns and 2-DE in *Acacia* FBs

The total protein content in the FBs of *A. hindsii* and *A. cornigera* amounted to 24.9 ± 3.5 and 22.8 ± 3.8 µg mg^{-1} dw, respectively, whereas the leaves contained 8.8 ± 0.2 and 8.4 ± 0.8 µg mg^{-1} dw of protein, respectively (**Table 2.1**). The FBs of both species contained significantly more protein than the leaves (for both species: $p < 0.001$, according to Student t-test, $n = 4$ for each tissue type and species).

Table 2. 1. Comparison of total protein content in *A. hindsii* and *A. cornigera* FBs and leaves.

	FBs		**Leaves**	
	A. hindsii	***A. cornigera***	***A. hindsii***	***A. cornigera***
Protein (µg mg^{-1}dw)	24.9 ± 3.5	22.8 ± 3.8	8.8 ± 0.2	8.4 ± 0.8

One-dimensional SDS-PAGE revealed clear differences between FBs and leaves for both species. Protein patterns also differed between the FBs of *A. cornigera* and those produced by *A. hindsii*, although most of these differences at the level of one-dimensional electrophoresis appeared to be of a quantitative nature (**Figure 2.2**). Major bands in the FBs ranged from 10–100 kDa and several strong bands that quantitatively dominated the FB proteomes were absent from the leaf proteomes (**Figure 2.2**). The leaf proteomes of the two species were similar and ranged from 15 to 100 kDa (**Figure 2.2**).

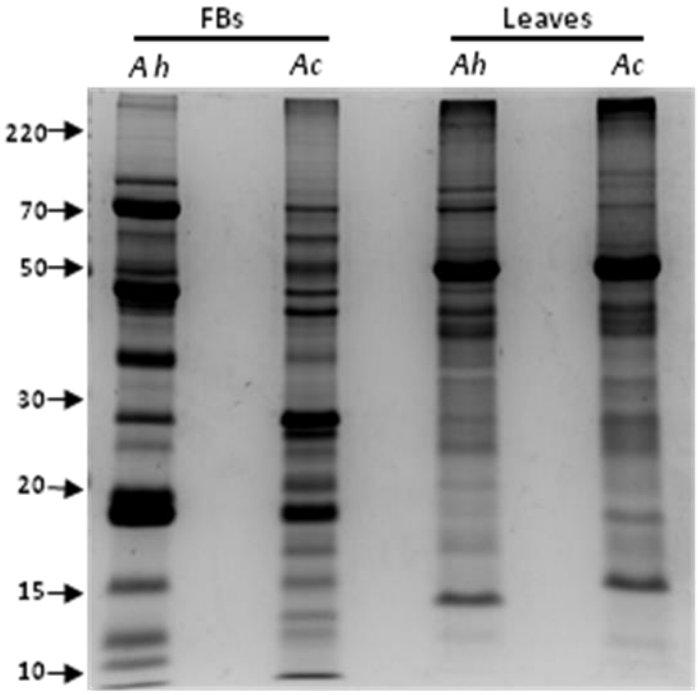

Figure 2.2. SDS-PAGE profiles of the proteomes of *A. hindsii* (*Ah*) and *A. cornigera* (*Ac*) FBs and leaves. Total protein contents were separated in 13% gel concentration and stained with Coomassie blue.

Two-dimensional electrophoresis of the FB proteomes confirmed that the molecular masses of most of the FB proteins ranged from 10 to 100 kDa and that there were clear differences between the FBs of *A. hindsii* and *A. cornigera* FBs (**Figure 2.3 A, B**). Individual spots from both proteomes were sequenced with LC-MS/MS. An MS-BLAST search of the resulting peptides indicated the presence of multiple PIs. Specifically, hits for 22 enzymes belonging to the family of Kunitz-type PIs and two Patatin-like proteins were found for proteins in *A. hindsii* FBs, whereas *A. cornigera* FBs contained 16 Kunitz-type PIs and one Patatin-like **(Table 2.2** and **Figure 2.3 A, B).**

Table 2. 2. Annotation results of Kunitz-type PIs from *A. hindsii* and *A. cornigera* FB tissues.

A. hindsii FB spots	Description	Accession number	Organism	Peptide hits	MS BLAST score
1	Patatin T5	XP_002510258	*Ricinus communis*	2	128
2	Patatin precursor	XP_002523555	*Ricinus communis*	1	68
3	Trypsin isoinhibitor DE5	1208243A	*Adenathera pavonina*	6	329
4	Trypsin inhibitor DE5	1208243A	*Adenathera pavonina*	2	136
5	Trypsin inhibitor DE5	1208243A	*Adenathera pavonina*	3	177
6	Trypsin isoinhibitor DE5	1208243A	*Adenathera pavonina*	2	138
7	Trypsin inhibitor DE5	1208243A	*Adenathera pavonina*	3	217
8	Trypsin isoinhibitor DE5	1208243A	*Adenathera pavonina*	5	287
9	Kunitz-type trypsin inhibitor	P32733	*Prosopis juliflora*	1	95
10	Trypsin inhibitor DE5	1208243A	*Adenathera pavonina*	6	290
11	Kunitz-type trypsin inhibitor	P32733	*Prosopis juliflora*	3	202
12	Trypsin inhibitor	P86451	*Enterolobium contortisiliquum*	3	164
13	Trypsin inhibitor	P86451	*Enterolobium contortisiliquum*	3	174
14	Kunitz-type trypsin inhibitor alpha chain	P32733	*Prosopis juliflora*	1	65
15	Trypsin inhibitor BvTI	P83595	*Bauhinia variegata*	3	159
16	Trypsin inhibitor	P86451	*Enterolobium contortisiliquum*	3	158
17	Kunitz-type trypsin inhibitor	P32733	*Prosopis juliflora*	3	185
18	Kunitz-type trypsin inhibitor	P32733	*Prosopis juliflora*	3	172
19	Kunitz-type trypsin inhibitor	P32733	*Prosopis juliflora*	2	153
20	Kunitz-type trypsin inhibitor	P32733	*Prosopis juliflora*	3	176
21	Kunitz-type trypsin inhibitor alpha chain	P32733	*Prosopis juliflora*	1	90
22	Kunitz-type trypsin inhibitor	P32733	*Prosopis juliflora*	2	144
23	Patatin precursor	EEF38753	*Ricinus communis*	3	148
24	Kunitz-type trypsin inhibitor	P32733	*Prosopis juliflora*	1	73

A. cornigera FB spots	Description	Accession number	Organism	Peptide hits	MS BLAST score
1	Kunitz-type trypsin inhibitor	P32733	*Prosopis juliflora*	2	152
2	Kunitz-type trypsin inhibitor	P32733	*Prosopis juliflora*	1	90
3	Kunitz-type trypsin inhibitor	P32733	*Prosopis juliflora*	2	138
4	Kunitz-type trypsin inhibitor	P32733	*Prosopis juliflora*	2	130
5	Kunitz-type trypsin inhibitor	P32733	*Prosopis juliflora*	1	90
6	Kunitz-type trypsin inhibitor	P32733	*Prosopis juliflora*	2	144
7	Patatin precursor	EEF38753	*Ricinus communis*	3	153
8	Kunitz-type trypsin inhibitor	P32733	*Prosopis juliflora*	2	133
9	Trypsin inhibitor	P86451	*Enterolobium contortisiliquum*	3	177
10	Kunitz-type trypsin inhibitor	P32733	*Prosopis juliflora*	3	133
11	Kunitz-type trypsin inhibitor	P32733	*Prosopis juliflora*	2	168
12	Kunitz-type trypsin inhibitor	P32733	*Prosopis juliflora*	2	174
13	Trypsin isoinhibitor DE5	1208243A	*Adenathera pavonina*	3	191
14	Kunitz-type trypsin inhibitor	P32733	*Prosopis juliflora*	2	138
15	Kunitz-type trypsin inhibitor	P32733	*Prosopis juliflora*	2	113
16	Trypsin inhibitor	P86451	*Enterolobium contortisiliquum*	2	143
17	Trypsin inhibitor	P86451	*Enterolobium contortisiliquum*	3	183

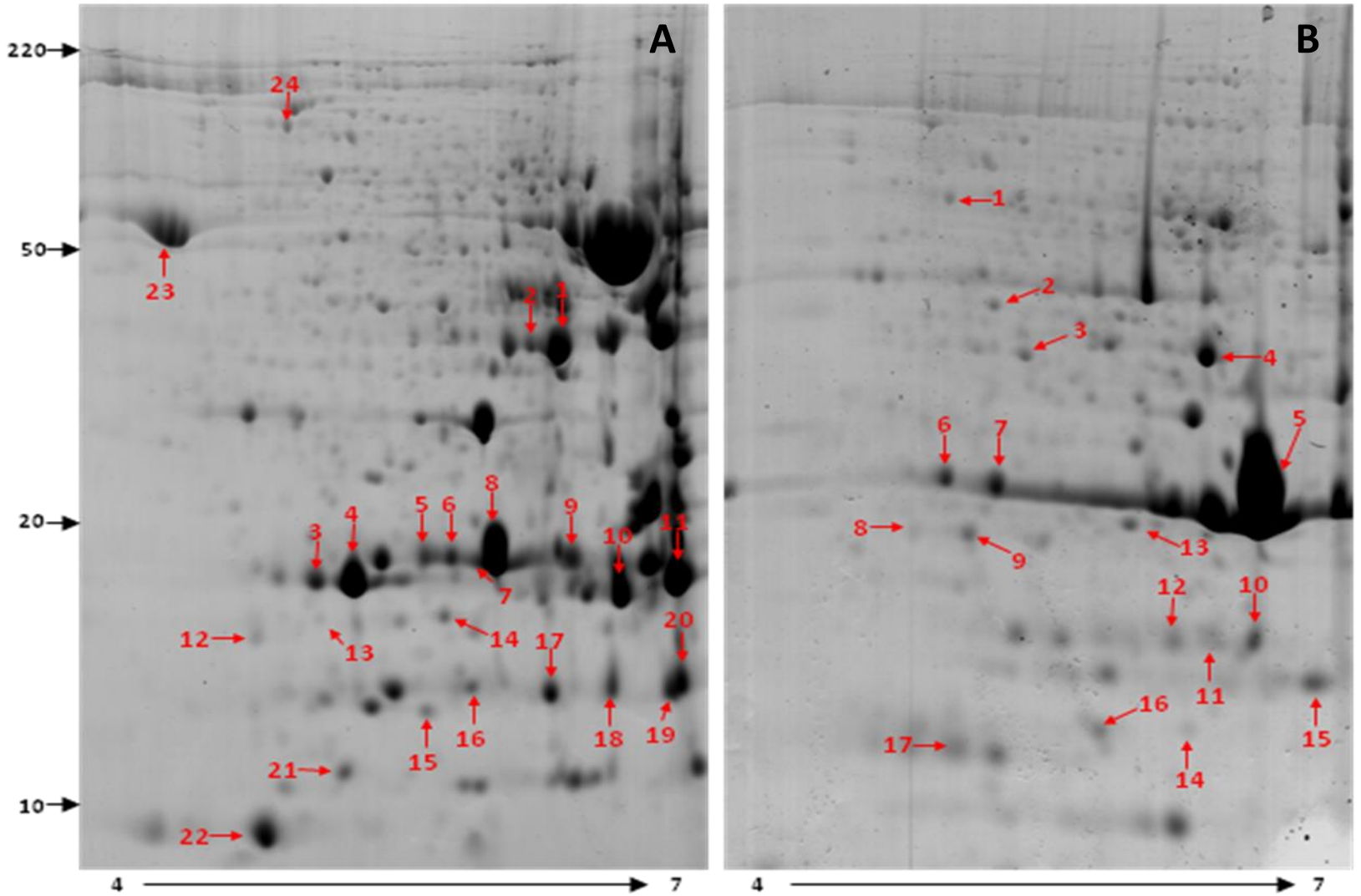

Figure 2.3. 2-DE profiles of the proteomes of (A) *A. hindsii* and (B) *A. cornigera* FBs. Proteins identified in this study are indicated by arrows and numbers (see Table 2.2 for protein identity and description).Total protein contents were separated in 13% gel concentration and stained with Coomassie blue.

2.4.2. Effects of *Acacia* FB PIs against serine proteases from *P. truncatus* and *Z. subfasciatus* larvae

When *A. hindsii* or *A. cornigera* FB PIs were added to the midgut extracts of larvae of *P. truncatus* and *Z. subfasciatus*, strong inhibitory effects on the trypsin-like activity in both species became apparent.

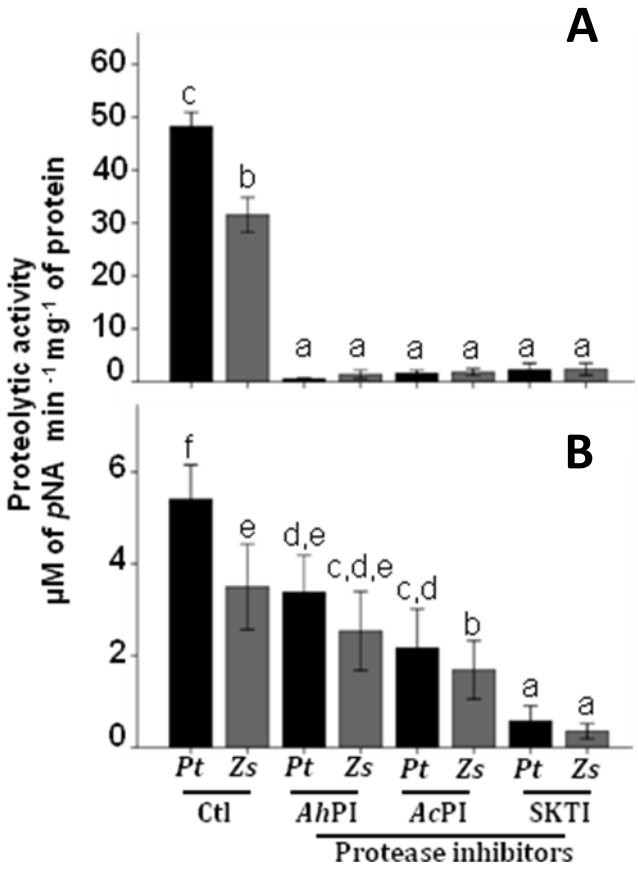

Figure 2.4. Effect of *A. hindsii* and *A. cornigera* FB PIs on serine proteases of *P. truncatus* (*Pt*) and *Z. subfasciatus* (*Zs*). (A) Trypsin-like activity detected with Bz-R-*p*NA substrate. (B) Chymotrypsin 2-like activity detected with Suc-AAPF-*p*NA substrate. Proteolytic activities were quantified in the presence of *A. hindsii* or *A. cornigera* FB PIs. Abbreviations: Ctl, control; *Ah*PI, PIs from *A. hindsii*; *Ac*PI, PIs from *A. cornigera* FBs; SKTI, soybean Kunitz trypsin inhibitor. $n = 7$ samples, each comprising fifty animals. Bars indicate the means ± standard errors; different letters above the bars indicate significant differences among conditions ($P < 0.05$ according to ANOVA and Tukey test).

Whereas, for example, the trypsin-like activity of control *P. truncatus* larvae was *ca*. 48 µM of *p*-nitroaniline liberated per min and mg protein, activity dropped to <1 µM *p*-nitroaniline liberated per min and mg protein in response to exposure to the FB extract (**Figure 2.4 A**). Similar effects were observed for *Z. subfasciatus*. By contrast, chymotrypsin-like activity in both species were less inhibited (only by *ca*. 30% on average), although these differences were also statistically significant (**Figure 2.4 B**).

2.4.3. Zymography assays

Native electrophoresis in PAGE with gelatine demonstrated the presence of several enzymes with proteolytic activity in the midgut extracts obtained from ant larvae from mutualistic and exploiter ants that lived on *A. hindsii* or *A. cornigera* plants (**Figure 2.5**). At least three distinct bands with masses of 22–30 kDa were observed in *P. Ferrugineus*, and change two different bands with masses of 25 and 50 kDa were found in *P. gracilis* larvaes. by contrast, no proteolytic activity was detected in extracts obtained from the FBs of both plant species (**Figure 2.5**), which demonstrates that the proteolytic activity demonstrated in our assays derives exclusively from the insects, rather than from their vegetarian diet.

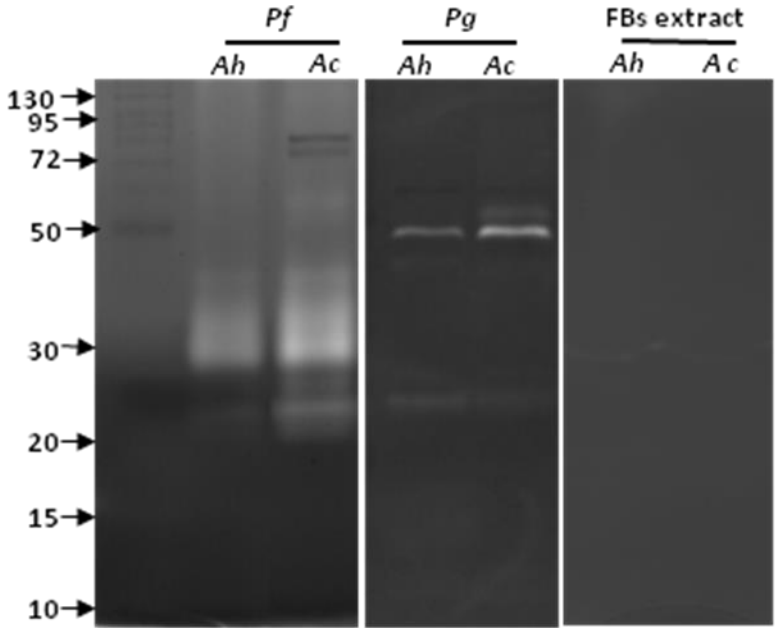

Figure 2.5. PAGE zymography of extracts of mutualistic and exploiter ant larval midguts and crude extract of *Acacia* FBs. *Pf* and *Pg*: *P. ferrugineus* or *P. gracilis m*idgut extract in *Ah*: Larvae from colonies that lived on *A. hindsii* or *Ac*: Larvae from colonies that lived on *A. cornigera.*

2.4.4. Screening the protease activities of *Pseudomyrmex* larval midguts

To characterize the main serine proteases in the midguts of *Pseudomyrmex* ant larvae, the use of different specific *p*-nitroaniline substrates revealed four types of serine proteases for mutualistic and exploiter ants that lived in *A. hindsii* or *A. cornigera* plants: trypsin-like activity, elastase-like activity and two isoforms of chymotrypsin-like activities (**Figure 2.6**).

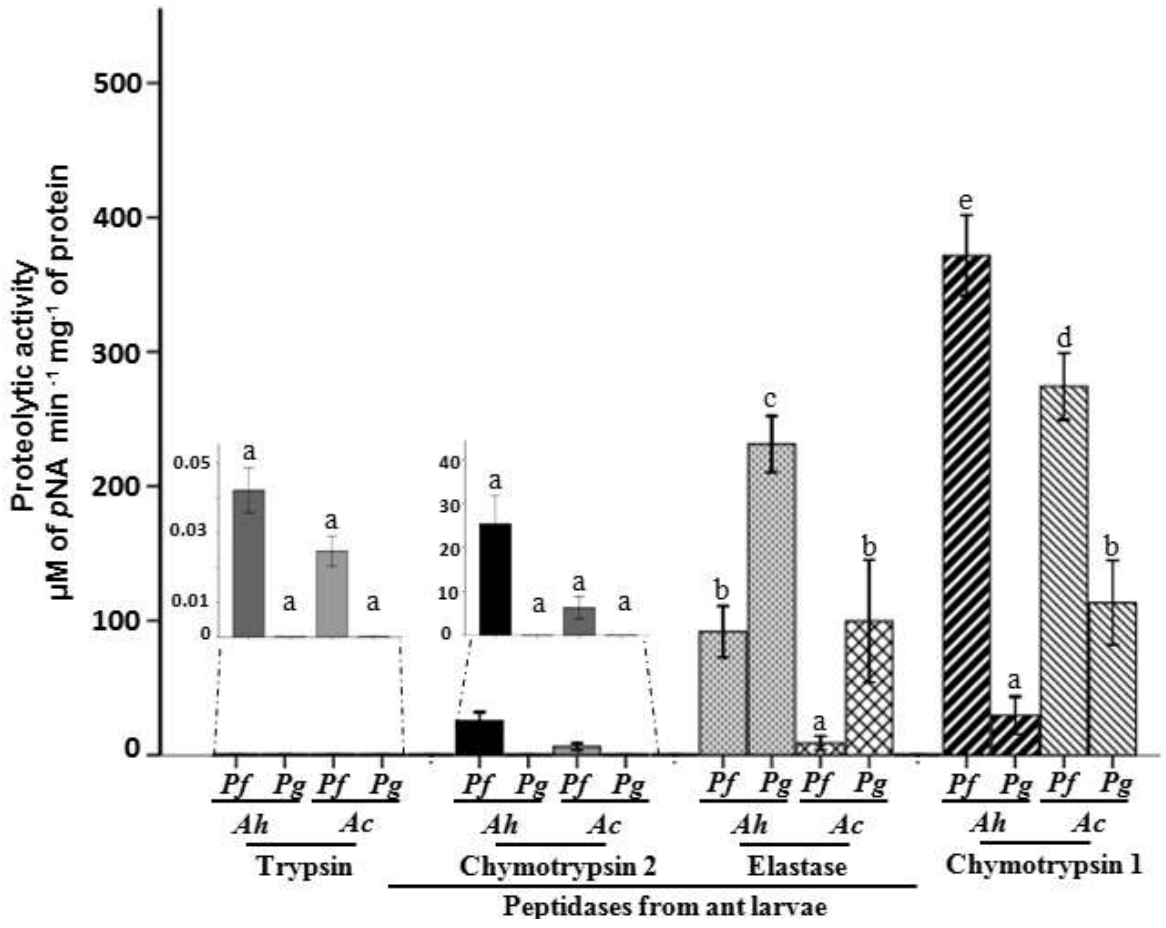

Figure 2.6. Activities of the major serine peptidases from mutualistic *P. ferrugineus* (*Pf)* and exploiter *P. gracilis* (*Pg*) ant larval midguts. Trypsin-like activity detected with Bz-R-*p*NA substrate, chymotrypsin 2-like activity detected with Suc-AAPF-*p*NA substrate, elastase-like activity detected with Suc-AAA-*p*NA substrate and chymotrypsin 1-like activities detected with Glt-F-*p*NA substrate were quantified in fourth instar larvae. *Ah*: Ant lived and fed from *A. hindsii*; *Ac*: Ant lived and fed from *A. cornigera*. n = 7 samples, each comprising fifty animals. Bars means ± standard errors, different letters above bars mark significant differences among conditions ($P<0.05$ according to ANOVA and Tukey test).

Elastase 1-like and chymotrypsin 1-like activity were the dominant serine proteases and the activity of these enzymes was significantly higher in larvae that had been nourished by *A. hindsii* FBs than in larvae from *A. cornigera* plants. However elastase was significantly

higher in larvae of the exploiter than the mutualist ants, whereas chymotrypsin 1-like activity dominated in the mutualists. By contrast, trypsin-like activity was *ca.* 1000 times lower than chymotrypsin 1-like activity and did not differ significantly between larvae collected from the two host species or between the two ant species (**Figure 2.6**).

2.4.5. Effects of *Acacia* FB PIs on serine proteases in ant larval midguts

No significant effect of any of the FBs on any of the individual proteolytic activities tested could be detected (**Figures 2.7 and 2.8**). By contrast, all four types of proteolytic activities in both ant species (trypsin-like, chymotrypsin 1-like, chymotrypsin 2-like and elastase-like) were significantly inhibited by commercial, soybean-derived PIs (SKTI, **Figures 2.7 and 2.8**).

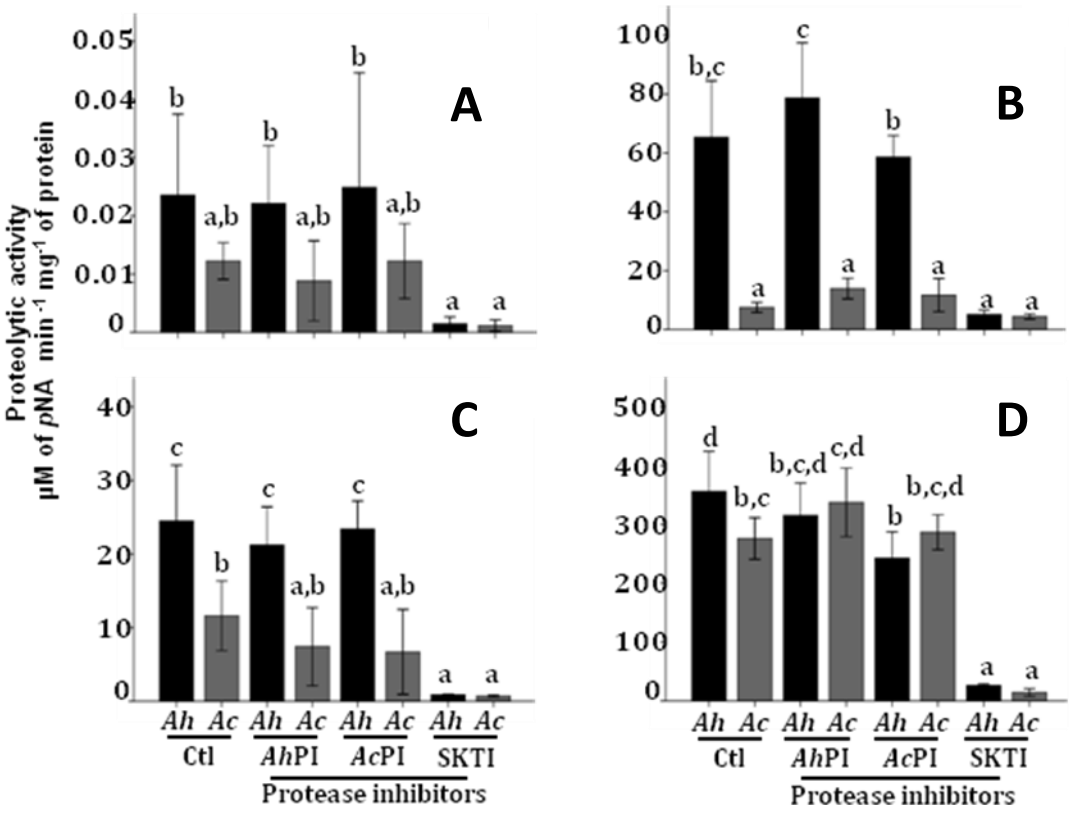

Figure 2.7. Effect of *A. hindsii* and *A. cornigera* FBs protease inhibitors against serine peptidases of mutualistic *P. ferrugineus* ant larvae. (A): trypsin-like activity as quantified with Bz-R-*p*NA substrate. (B): elastase-like activity as quantified with Suc-AAA-*p*NA substrate. (C): chymotrypsin 2-like activity as quantified with Suc-AAPF-*p*NA substrate. (D): chymotrypsin 1-like activity as quantified with Glt-F-*p*NA substrate. Proteolytic activities were measured in the presence of PIs from *A. hindsii* or *A. cornigera* FBs. Abbreviations: Ctl, control; *Ah*PI: *A. hindsii* FBs protease inhibitors. *Ac*PI: *A. cornigera* FBs protease inhibitors. SKTI: Soybean Kunitz trypsin inhibitor. *Ah*: Ant lived and fed from *A. hindsii*; *Ac*: Ant lived and fed from *A. cornigera*. n = 7 samples, each comprising fifty animals. Bars means ± standard errors, different letters above bars mark significant differences among conditions ($P<0.05$ according to ANOVA and Tukey test).

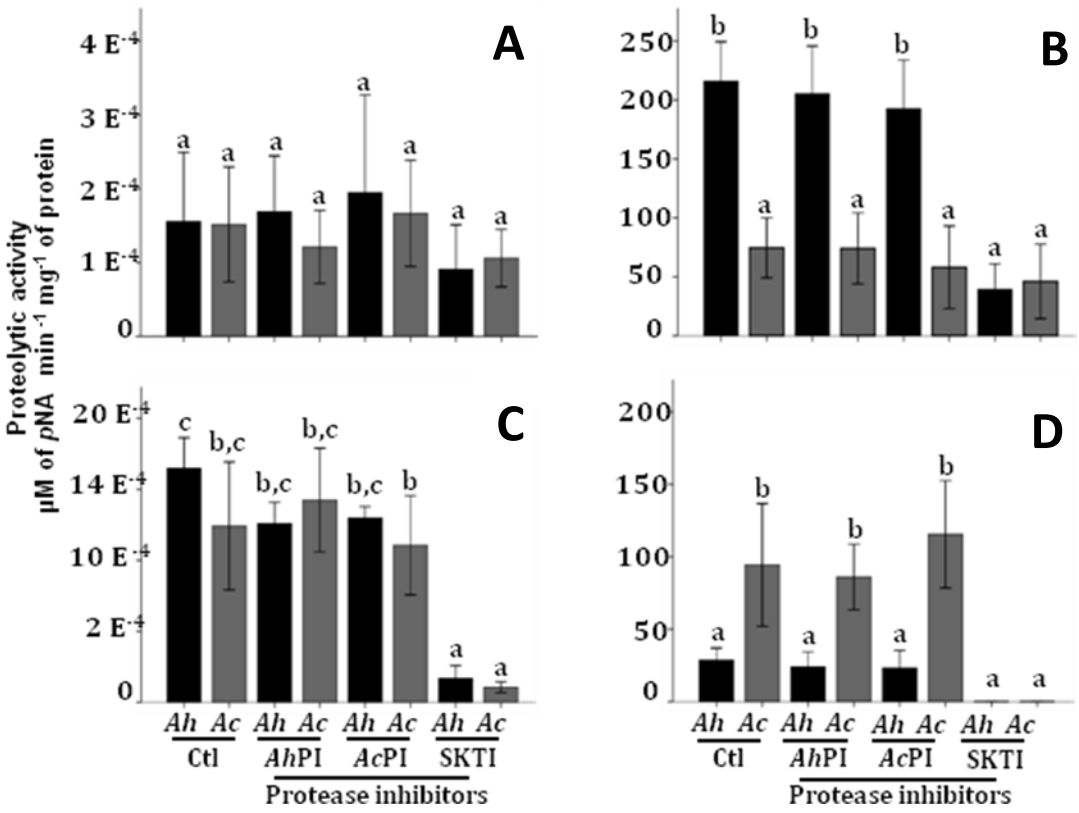

Figure 2.8. Effect of *A. hindsii* and *A. cornigera* FBs protease inhibitors against serine peptidases of exploiter *P. gracilis* ant larvae. (A): trypsin-like activity as quantified with Bz-R-*p*NA substrate. (B): elastase-like activity as quantified with Suc-AAA-*p*NA substrate. (C): chymotrypsin 2-like activity as quantified with Suc-AAPF-*p*NA substrate. (D): chymotrypsin 1-like activity as quantified with Glt-F-*p*NA substrate. Proteolytic activities were measured in the presence of PIs from *A. hindsii* or *A. cornigera* FBs. Abbreviations: Ctl, control; *Ah*PI: *A. hindsii* FBs protease inhibitors. *Ac*PI: *A. cornigera* FBs protease inhibitors. SKTI: Soybean Kunitz trypsin inhibitor. *Ah*: Ant lived and fed from *A. hindsii*; *Ac*: Ant lived and fed from *A. cornigera*. n = 7 samples, each comprising fifty animals. Bars means ± standard errors, different letters above bars mark significant differences among conditions ($P<0.05$ according to ANOVA and Tukey test).

2.5. Discussion

Food bodies (FBs) produced by Central American acacias or other myrmecophytes represent highly attractive targets for exploiters because they are generally rich in lipids, carbohydrates, amino acids and proteins (Andrade-Buono *et al.* 2008, Fischer *et al.* 2002,

Heil *et al.* 1998, 2004, O'Dowd 1980). Interestingly, specific components of their protein fraction, i.e. the fraction that greatly contributes to the nutritive value of these FBs, also represent the key to their protection from exploiters. *Acacia* FBs contain protease inhibitors (PIs) that effectively reduced the proteolytic activity in the digestive tracts of bruchid seed beetles (*P. truncatus* and *Z. subfasciatus*) and an exploiter ant, *Pseudomyrmex gracilis.* By contrast, the legitimate mutualistic consumers maintained a high level of proteolytic activity in their intestines that mainly consisted of chymotrypsin 1-like and chymotrypsin 2-like and elastase-like activities (**Figure 2.6**). Larvae of *P. gracilis* exhibited mainly elastase-like and to a lower degree chymotrypsin 1-like activity and thus appear partly, but not completely, adapted to consume *Acacia* FBs. We suggest that plant PIs and ant proteases form a lock–key system that converts the FBs into an exclusive food source for the mutualistic ants.

In general, two principal mechanisms have been described by which mutualisms can be protected from exploiters. Partner choice applies before the mutualism is established and usually means that hosts actively select the species of symbionts that are allowed to enter the interaction (Bever *et al.* 2009, Noë and Hammerstein 1994, Sachs *et al.* 2004, Simms *et al.* 2006). By contrast, host sanctions occur when the mutualism has already been established and apply when the host ceases to provide rewards to a partner that does not behave adequately (Charlotte *et al.* 2012, Kiers *et al.* 2003). These mechanisms are particularly well studied for the root–Rhizobia interaction (Clarke *et al.* 1992, Fisher and Long 1992, Kiers and Denison 2008, Kiers *et al.* 2011, van Rhijn and Vanderleyden 1995, West *et al.* 2002). For ant-plants, both mechanisms have been demonstrated at the anatomical level. Partner choice can occur via specifically shaped entrances to domatia

(Brouat *et al.* 2001, Charlotte *et al.* 2012) and slippery stem surfaces (Federle *et al.* 1997) that restrict access to the legitimate, defending ant species. Host sanctions have been described in two cases of myrmecophytes that shed domatia when they were not protected (Edwards *et al.* 2006, Izzo and Vasconcelos 2002).

A further possible mechanism is to make the reward exclusive ('Exclusive rewards'): specific anatomical or biochemical characteristics can make a reward less attractive, accessible or suitable for generalists that represent potential exploiters. Floral nectar can be protected from unspecialized consumers by the evolution of particularly long nectar spurs (Darwin 1862) or might contain alkaloids or non-proteinogenic amino acids to make it less suitable for non-adapted consumers (Adler 2000). Floral and extrafloral nectar can also contain PR-proteins to protect the reward from infestation by microorganisms (González-Teuber *et al.* 2009, 2010, Thornburg *et al.* 2003). The EFN of *Acacia* myrmecophytes also contains a soluble invertase that keeps it free of sucrose and, hence, is unattractive for potential insect exploiters (Heil *et al.* 2005); workers of the specialized *Pseudomyrmex* ants lack this enzyme and, hence, prefer the resulting, sucrose-free EFN (Heil *et al.* 2005, Kautz *et al.* 2009). Here, we demonstrate that the biochemical composition of *Acacia* FBs also shows characteristics that are consistent with their 'exclusiveness'.

The particular anatomical features of long-spurred orchids that make their nectar an 'exclusive reward' represent the paramount example of a co-evolutionary process (Anderson and Johnson 2008, Darwin 1862, Johnson and Steiner 1997). However, do 'exclusive rewards' always represent – and need to be – the result of co-evolution? The protein content of the *Acacia* FBs was considerably higher than that of the leaves from which they are ontogenetically derived (**Table 2.1**). Apart from these quantitative differences, SDS-PAGE

demonstrated that the FB proteomes were highly distinct from those of the leaves and significantly different between the two *Acacia* species, whereas the leaf proteomes were highly similar (**Figures 2.2, 2.3**). FBs serve the 'external' function of being a reward for the ants, whereas leaves serve multiple 'internal' functions that are unrelated to defensive mutualism. Thus, the proteomes of the FBs appear to be evolutionarily more flexible and indeed might be prone to rapid, (co-) evolutionary adaptations.

In principle, insects can adapt to PIs in their food by shifting their proteolytic digestive enzymes to types that are less sensitive to the specific PIs that dominate in their respective food sources (Broadway 1995). To understand whether the 'lock–key' system that we describe here is likely to represent the result of a specific, co-evolutionary process, we searched for the typical composition of PIs in related non-ant plants and for the typical composition of proteases in non-plant ants. BLAST database searches revealed the presence of 22 and 16 Kunitz-type PIs in *A. hindsii* and *A. cornigera*, respectively (**Table 2.2**). These PIs mainly act against serine proteases such as trypsin, chymotrypsin and elastase (Lawrence and Koundal 2002, Macedo *et al.* 2004, Pouvreau *et al.* 2003, Srinivasan *et al.* 2006). Kunitz-type PIs form a large family and are common in various organs of multiple plant taxa (Hendriks *et al.* 1991, Jofuku and Goldberg 1989, Lawrence and Koundal 2002, Oliva *et al.* 2010), including seeds of legumes such as *Acacia* (Babu *et al.* 2012, Ee *et al.* 2009, Habib and Fazili 2007, Kortt and Jermyn 1981, Weder 1985). The spots identified as PIs in the FB proteomes had molecular masses (Mw) of 10–25 kDa and specific isoelectric points of 4.5–7.0, characteristics that are consistent with those of PIs in seeds of *Acacia confusa* (Lin *et al.* 1991), *Acacia victoria* (Ee *et al.* 2009), *Acacia senegal* (Babu and Subrahmanyam 2010), *Acacia nicolitica* (Babu *et al.* 2012) and soybean (*Glycine max*)

plants (Oliva *et al.* 2010). Thus, *Acacia* FBs contain PIs that are likely to be common in the entire family of legumes. Although the localization of several of these PIs in non-reproductive tissue appears to be unique, the biochemical characteristics of these PIs show no sign of a highly specific adaptation.

To corroborate the biochemical activity and, thus, a potential protective role of these PIs of the *Acacia* FBs, we studied their effects on the digestive proteases in larvae of an exploiter ant and the beetles *P. truncatus* and *Z. subfasciatus.* Trypsin and chymotrypsin proteases are the major digestive enzymes in Coleoptera, with trypsins usually representing the more important class (Houseman and Thie 1993, Johnson and Rabosky 2000, Lemos *et al.* 1990). Whereas the PIs of *Acacia* FBs had only slightly inhibitory effects on the chymotrypsin-like activity in the midguts of the beetle larvae (**Figure 2.4 B**), they exhibited strong inhibitory effects on the dominant, trypsin-like activity (**Figure 2.4 A**). In fact, in a recent study, trypsin and chymotrypsin were identified as the most active proteases in *P. truncatus*, and trypsin-like activity was more sensitive than chymotrypsin to plant PIs extracted from the seeds of species such as tepary bean (*Phaseolus acutifolius*), soybean and chan (*Hyptis suaveolens*) (Castro-Guillén *et al.* 2012). Similarly, chymotrypsin-like activities in *Z. subfasciatus* were not inhibited by natural PIs from soybean and common bean (*Phaseolus vulgaris*) (Magalhães *et al.* 2007, Silva *et al.* 2001). Thus, both the dominance of trypsin-like activity and the relatively low sensitivity of chymotrypsin-like activity appear to be common features of beetles. By contrast, larvae of *P. ferrugineus* showed mainly chymotrypsin-like activity (**Figure 2.6**), which was not detectably inhibited by FB PIs (**Figures 2.7 and 2.8**). These ant proteases are not generally insensitive to PIs, as demonstrated by their strong inhibition by the commercial SKTIs in both cases (**Figures**

2.7 and 2.8). Thus, the larvae of the mutualist ants, which represent the legitimate consumers, possess specifically those proteases that are the least sensitive to the PIs in their specific food source.

Does the dominance of chymotrypsins in the midguts of these ant larvae represent a specific adaptation to feeding on these FBs, or might the activities observed by us at the phenotypic level simply represent the product of ant proteases that have been under the influence of the FB PIs during the entire life of the larva? Serine proteases have also been observed by zymography in the larvae of the leaf-cutter ant (*Acromyrmex subterraneus*) and four bands of protease activities had similar molecular masses to those observed in the mutualist ants (Erthal *et al.* 2007). Similarly, elastase 1 and chymotrypsin 1 and 2 have been observed in 4th instar larvae of the fire ant (*Solenopsis invicta*) (Meyer *et al.* 2002, Whitworth *et al.* 1998) and elastase and chymotrypsin 1 dominated the proteolytic activity in the larvae of the exploiter ant *P. gracilis* (**Figure 2.6**). Even in the digestive tracts of spiders, the dominant proteases are of the chymotrypsin-type and are not inhibited by several plant-derived Kunitz-type PIs (Mommsen 1978, Tugmon and Tillinghast 1995).

In conclusion, the few ant species and other arthropod carnivores that have been investigated to date show similar patterns in their proteases to those of the mutualist and the proteases that usually dominate in ant digestive tracts generally show a low sensitivity to Kunitz-type PIs (**Figures 2.7 and 2.8**). PIs of the Kunitz-type are common in legumes, although they usually accumulate in reproductive tissues rather than in leaves. Thus, the dominance of chymotrypsin and elastase in *Pseudomyrmex* ants and the insensitivity to the Kunitz-type inhibitors in the *Acacia* FB tissue does not necessarily proof a direct co-evolution, although the strong dominance of the least sensitive type of protease in the

mutualist indicates that the mutualist might have secondarily adapted to achieve an optimized use of its food reward. Although co-evolutionary adaptations cannot be excluded, these results make it more likely that this is an interaction between pre-adapted partners: legume PIs have low inhibitory activity on typical ant proteases in general. Thus, a legume–ant mutualism can make use of this coincidence to make the rewards 'exclusive' with no need for any co-evolutionary history, although co-evolutionary processes might then strengthen the interaction. However, independently of whether the ant and plant enzymes have co-evolved, 'exclusive rewards' reduce the risk that FBs will be robbed by non-defending exploiters, without reducing their digestibility for the legitimate consumers. Exclusivity represents an effective means by which rewards produced for exchange among mutualists can be protected from non-adapted exploiters.

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Chapter 3

Short-term proteomic dynamics reveal metabolic factory for active extrafloral nectar secretion by *Acacia cornigera* ant-plants

Picture by: Martin Heil

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3.1. Abstract

Despite the ecological and evolutionary importance of nectar, mechanisms controlling its synthesis and secretion remain largely unknown. It is widely believed that nectar is 'secreted phloem sap', but current research reveals a biochemical complexity that is unlikely to stem directly from the phloem. We used the short daily peak in the production of extrafloral nectar by *Acacia cornigera* to investigate metabolic and proteomic dynamics before, during and after 2h of diurnal secretion. Neither hexoses nor dominating nectar proteins (nectarins) were detected in the phloem before or during nectar secretion, excluding the phloem as the direct source of major nectar components. Enzymes involved in the anabolism of sugars, amino acids/proteins and nectarins, such as invertase, β-1,3-glucanase and thaumatin-like protein, accumulated in the nectary directly before secretion and diminished quantitatively after the daily secretion process. The corresponding genes were expressed almost exclusively in nectaries. By contrast, protein catabolic enzymes were mainly present and active after the secretion peak, and may function in termination of the secretion process. Thus the metabolic machinery for extrafloral nectar production is synthesized and active during secretion and is degraded thereafter. Knowing the key enzymes involved and the spatiotemporal patterns in their expression will allow elucidation of mechanisms by which plants control nectar quality and quantity.

3.2. Introduction

Nectar plays multiple roles in plant pollination (floral nectar, FN) and in the indirect defence of plants against herbivores (extrafloral nectar, EFN) (Brandenburg *et al.* 2009, Heil 2008, 2011). In addition to its chemical composition, the quantity of nectar secreted also represents an important trait that is positively correlated with pollination success or the resulting indirect defence (Brandenburg *et al.* 2012, Heil *et al.* 2009). Plants are therefore capable of adjusting nectar secretion rates to the current needs and may even re-absorb unconsumed nectar (Búrquez and Corbet 1991, Escalante-Perez *et al.* 2012, Heil *et al.* 2000, Nepi *et al.* 2001, 2011b, Nicolson 1995, Pederson *et al.* 1958, Ziegler and Lüttge 1959). However, little is known about the mechanisms that underlie the genetic control or phenotypic plasticity of nectar secretion rates, or where in the plant dominant nectar components others than sugars are synthesized.

The classical idea that nectar represents 'secreted phloem sap' (Agthe 1951, de la Barrera and Nobel 2004, Heil 2011, Lüttge 1961) is challenged by several observations. First, nectaries are commonly characterized by a highly sophisticated ultrastructure consisting of typical secretory parenchyma, and many of them lack a direct connection to the vascular system (Escalante-Pérez and Heil 2012, Pacini and Nepi 2007). Second, floral nectaries in many species accumulate starch grains, which are degraded during anthesis (Horner *et al.* 2007, Kram *et al.* 2009). Gene expression analyses confirmed a shift from starch anabolism to catabolism when floral nectaries of Arabidopsis and ornamental tobacco start to secrete FN (Kram, Xu and Carter 2009, Ren *et al.* 2007a, 2007b), and an extracellular invertase that is essential for both starch accumulation and nectar secretion in Arabidopsis flowers is

exclusively expressed in nectaries (Bender *et al.* 2012, Kram *et al.* 2009, Ruhlmann *et al.* 2010). Similarly, many genes related to exocytosis, hormone metabolism and sugar metabolism were over-expressed in poplar extrafloral nectaries compared to the leaf tissue (Escalante-Perez *et al.* 2012). Third, proteins found in the phloem sap of leek (*Allium porrum*) showed little overlap with those in the nectar (Peumans *et al.* 1997). It has been shown that *NECTARIN* genes are expressed in the nectary tissue of ornamental tobacco (Carter and Thornburg 2003, 2004). Some of these *NECTARIN* genes are under the control of an MYB305 transcription factor, which is required for full secretion activity (Liu and Thornburg 2012, Liu *et al.* 2009). All these observations suggest a much stronger metabolic contribution of the nectary itself than was originally considered.

Nectar secretion is a highly dynamic process that depends on the ontogenetic stage of the nectar-secreting structure (Liu and Thornburg 2012, Ren *et al.* 2007a, 2007b) and on environmental factors such as consumption rate (Corbet and Delfosse 1984, Gill 1988, Heil *et al.* 2000, Pyke 1991), and, in the case of EFN, herbivory and current light conditions (Bixenmann *et al.* 2011, Heil *et al.* 2001, Radhika *et al.* 2010). However, most of the studies performed so far were based on comparisons between nectaries and leaf tissue. Therefore, they did not allow identification of the spatiotemporal dynamics of the complete metabolic machinery that is required for nectar production. In order to circumvent the problems that arise from the lack or small size of nectaries in most genetically tractable model species, we used a proteomics approach in combination with biochemical analyses and degenerate primers to study the role of the extrafloral nectaries of the ant-plant, *Acacia cornigera*, in the synthesis of the major nectar components: sugars (Heil *et al.* 2005b),

amino acids (González-Teuber and Heil 2009) and nectarins (Carter and Thornburg 2004, González-Teuber *et al.* 2009, 2010, Nepi *et al.* 2011a, 2012, Zha *et al.* 2012). We observed highly dynamic processes in the accumulation and activity of key metabolic enzymes in the nectary tissue, demonstrating that the entire metabolic machinery required for the synthesis of nectar is established and active directly before and during the hours of peak nectar secretion.

3.3. Materials and methods

3.3.1. Plant material and study site

Acacia cornigera L. Willdenow (Mimosoidea, Fabaceae) (Janzen 1974) plants were investigated in the south of Mexico (approximately ≈15° 55' N and 097° 09' W). All plants grew in full sun, had not been visibly damaged by pathogens or herbivores and were inhabited by the mutualist ant *Pseudomyrmex ferrugineus* (Ward 1993). For an initial screening of the diurnal secretion pattern, the two youngest branches on 12 plants were identified: one of these branches received an aqueous 1 mM solution of JA, the other received the same amount of distilled water. The next day, EFN secretion was quantified every 2 h as the total amount of soluble solids (Heil *et al.* 2004). This experiment was repeated at two sites on three further days (n = 5 plants each). The resulting EFN secretion curve determined the sampling times for all biochemical, enzymatic, proteomic and gene expression studies (n = 3 biologically independent samples for all experiments). Samples of nectary tissue, leaves, EFN and phloem exudates were collected on dry ice before (t = 0:

06:00–08:00 am), during (*t* = 1: 08:00–10:00am) and after secretion (*t* = 2: 10:00–12:00am), and then stored in liquid nitrogen.

3.3.2. Collection of phloem exudates and EFN

Extrafloral nectar (EFN) was collected as described previously (Heil *et al.* 2004). For phloem collection, the cut rachis of each leaf was immediately transferred into the pre-incubation chamber of a home-made Plexiglas device (Deeken *et al.* 2008) containing 1 mM Na_2EDTA buffer, pH 7.5 and protease inhibitor (Roche complete mix), osmotically adjusted to 260 mOsmol with sorbitol. The petioles were re-cut under EDTA buffer to prevent sieve-tube embolism (Deeken *et al.* 2008). The pre-incubation chamber was washed by sucking EDTA buffer through the chamber and replacing it with fresh buffer. Leaves were illuminated with natural light, and incubated at ambient temperature in CO_2- and H_2O-saturated air (0.1 M $NaHCO_3$). After 1.5 h bleeding, the EDTA buffer containing phloem exudates was removed, frozen and subsequently lyophilized.

3.3.3. Enzymatic activities

The activity of *A. cornigera* cell-wall invertase from nectaries was assayed as described previously (Ruhlmann *et al.* 2010) with modifications. Briefly, 0.05 g of ground sample was mixed with 500 µl ice-cold 50 mM HEPES/NaOH (pH 8.0, containing 5 mM $MgCl_2$, 2 mM EDTA, 1 mM $MnCl_2$ and 1 mM $CaCl_2$). Samples were incubated on ice for 10 min and then centrifuged at 13 000 xg for 10 min at 4°C. The supernatant was discarded and pellets containing the cell walls with associated invertases were washed three times with 500 µl extraction buffer by resuspension and centrifugation as above. Finally, pellets were washed

with 500 µl of 80 mM sodium acetate, pH 4.8. Invertase activity was measured as described previously (Heil *et al.* 2005a) with modifications. Briefly, 300 µl of 80 mM sodium acetate (pH 4.8) were added to the pellets, and the mixture was incubated at 37 °C. Every 5 min, 20 µl of samples were taken and mixed with 200 µl of HK reaction solution (Glucose (HK) Assay Kit. Sigma-Aldrich). After reaching steady state (Heil *et al.* 2005a), 100 µl of an aqueous 100 mM solution of sucrose was added, and the absorption was measured at 340 nm in a µQuant® microplate reader every 5 min for 30 min. For quantification of trypsin- and chymotrypsin-like activity, soluble proteins were extracted by grinding 0.1 g tissue in 300 µl ice-cold 50 mM phosphate buffer (pH 6.0) with 0.02% of polyvinylpyrrolidone. The mixture was incubated for 15 min at 4 °C in a mixer; after this time, samples were centrifuged (10 000 xg) for 15 min at 4°C, and supernatants were placed in new tubes and stored at -70°C. Then the activity of serine proteases such as trypsin and chymotrypsin was estimated in photometric assays using the chromogenic substrates: *N*-α-benzoyl-D,L-arginine-*p*-nitroanilide (BApNA) for trypsin-like activity and *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (SAAPPpNA) for chymotrypsin-like activity (all materials were purchased from Sigma-Aldrich). All substrates were used at a final concentration of 0.01 M dissolved in DMSO (4.35 mg ml^{-1}). The final volume of the reaction mixture was adjusted to 220 µl by varying the volume of the buffer (0.1 M Tris/HCl, pH 7.4). Aliquots of 20 µg protein were used, sample and the mixture was pre-incubated for 15 min at 37°C. Then 20 µl of the specific substrate was added, and changes in absorbance were measured at 405 nm using a µQuant® microplate reader every 10 min for 1 h.

3.3.4. Protein extraction for 2-DE

For analysis of phloem proteins (Giavalisco *et al.* 2006), we used 25 mg of lyophilized phloem exudate to which 800 µl of a mixture of acetone/methanol/dithiothreitol (90%,10%, 10 mM) and 200 µl water were added (final volume 1 ml). Samples were incubated overnight at -20°C and then centrifuged at 14 000 xg at 4°C for 15 min. Pellets were washed twice in 100% of acetone and centrifuged as above.

Nectary tissue was ground in liquid nitrogen. To extract proteins (Wang *et al.* 2006), 0.1 g of sample was placed in 1 ml 10% TCA/acetone and centrifuged at 16 000 xg for 3 min at 4°C. The pellet was washed with 80% methanol/0.1 M ammonium acetate and centrifuged again. The pellet was then washed three times in a 1:1 mixture of 0.4 ml phenol (Tris-buffered, pH 8.0; Sigma-Aldrich) and 0.4 ml of dense SDS buffer (30% sucrose, 2% SDS, 0.1 M Tris-HCl, pH 8.0, 5% β-mercaptoethanol) and once each with 100% methanol and 80% acetone. After centrifugation, the recovered proteins were dissolved in 2-DE rehydration solution containing protease inhibitor (Roche complete mix).

3.3.5. Two-dimensional gel electrophoresis

The protein content was quantified using a Bradford protein concentration kit (Bio-Rad,). Protein (300 µg of protein per sample) was re-suspended in 250 µl rehydrating solution (7 M urea, 2 M thiourea, 2% CHAPS, 65 mM of dithiothreitol, 2% IPG buffer, pH 3–10), loaded onto 13 cm IPG dry strips, and allowed to rehydrate for 15 h. The proteins were then

separated by two-dimensional gel electrophoresis. Protein bands of interest were cut from the gel matrix, and tryptic digestion was performed as described previously (Shevchenko *et al.* 2006).

3.3.6. LC-MS/MS and data analysis

Protein digests were analysed by nanoelectrospray liquid chromatography/tandem mass spectrometry (nanoLC-MS/MS) on a Synapt HDMS quadrupole time-of-flight mass spectrometer (Waters) (González-Teuber *et al.* 2009, 2010). Data were processed using PLGS software version 2.4 (Waters) with baseline subtraction, smoothing and deisotoping of acquired spectra. MS/MS spectra were searched against a sub-database containing common background proteins (human keratins and trypsin) to exclude the proteins that produce these spectra from *de novo* sequencing. The search parameters were: mass tolerances for precursor and fragment ions, 15 ppm and 0.03 Da, respectively; instrument profile, ESI-Trap; fixed modification, carbamidomethyl (cysteine); variable modification, oxidation (methionine); up to one missed cleavage was allowed. Spectra that remained unmatched by database searching were interpreted *de novo* to yield peptide sequences. A 0.002 Da mass deviation for *de novo* sequencing was allowed and sequences with a ladder score exceeding 40 were selected for homology-based searching using an MS BLAST program (Shevchenko *et al.* 2001) installed on an in-house server. MS BLAST searches were performed against the comprehensive NCBInr database (updated on 10 August 2011) using previously described settings (Shevchenko *et al.* 2001). In parallel, pkl files of MS/MS spectra were generated and searched against the NCBInr database (updated 11 September 2011, installed on a local server) using MASCOT version 2.3 and the above

described search parameters. Hits were considered as confident if at least three peptides were matched with ion scores above 30, or proteins were identified by one or two peptides with a score of 55 or better.

3.3.7. cDNA synthesis

RNA was extracted using a buffer consisting of 2% w/v CTAB (Sigma), 2% w/v polyvinylpyrrolidone K-25 (Sigma), 100 mM Tris/HCl (pH 8.0), sodium EDTA (pH 8.0), 2.0 M NaCl 25 mM. All solutions used, except Tris/HCl, were prepared using Millipore purified water, treated with (600 µl) diethylpyrocarbonate (Sambrook *et al.* 1989) and autoclaved. Extraction buffer was added to 50 mg of frozen ground plant material with β--mercaptoethanol to a final concentration of 2% v/v. This mixture was extracted twice using an equal volume of chloroform:isoamyl alcohol (24:1). The water phase was cleaned using LiCl, sodium acetate and ethanol precipitation steps. The mRNA was subsequently isolated using a Dynabeads mRNA DIRECT kit (Invitrogen) and used for first-strand cDNA generation using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions.

3.3.8. PCR and sequencing with degenerate primers

Degenerate primers (**Table S5 online**) were designed using Vector Nti software. Degenerate PCR was performed using Platinum PCR Supermix (Invitrogen) with a ramp program as follows: 3 mi denaturation starting at 95°C, followed by 40 cycles of 30 seconds of denaturation at 94°C and, annealing at 48°C with a ramp of 0.2°C sec^{-1} and an

increase of 8 sec per cycle, and 1 min elongation at 72°C. The PCR products were analysed by agarose 2% electrophoresis and sent for sequencing.

3.4. Results

3.4.1. The study system

The *A.cornigera* plants are obligate ant-plant that bears conspicuous extrafloral nectaries on its petioles (**Figure 3.1 A**). While the plants remain inhabited by mutualistic ants, these nectaries secrete large quantities of a biochemically complex EFN with a predictable and sharp diurnal peak, i.e., only between 8:00 and 10:00 AM (**Figure 3.1 B**). Secretion rates decrease dramatically from one day to the next as soon as EFN consumption ceases (Heil *et al.* 2000, 2004). Although production of EFN in most species represents a jasmonic acid (JA)-dependent induced response to herbivory (Heil 2011), EFN secretion by *A. cornigera* is not induced by herbivory or JA application (Heil *et al.* 2004) (**Figure 3.1 B**). We used this independence of nectar secretion on external factors that are related to herbivory and its short diurnal peak, to search for enzymes and nectarins that accumulate in the nectary in a temporal pattern directly related to the active secretion.

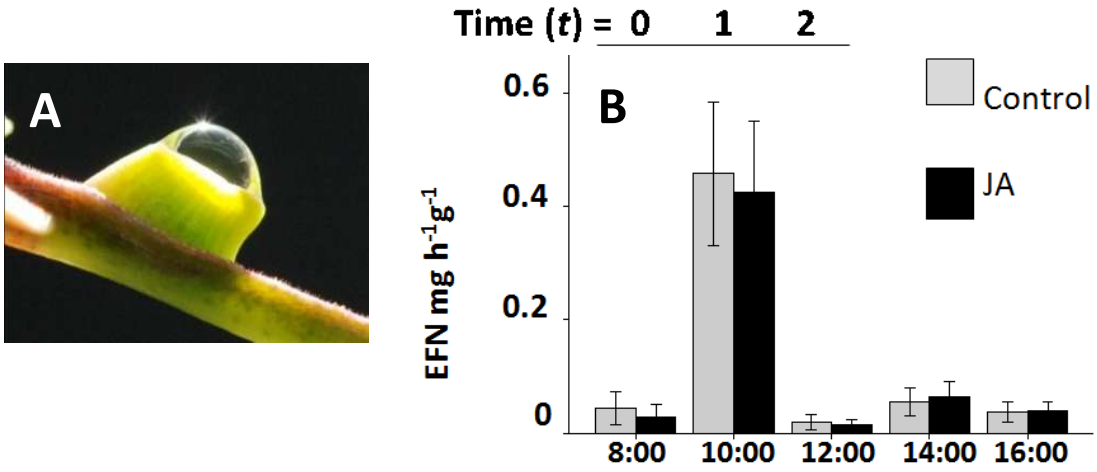

Figure 3.1. Extrafloral nectary and secretion of EFN from *A.cornigera.* Nectary tissue with EFN drop (A). Mean diurnal secretion activity ± SE (mg soluble solids secreted per hour and g leaf dry mass) of extrafloral nectar (EFN) (*n*=12) (B).

3.4.2. Nectar is not secreted phloem sap

Invertase (EC 3.2.1.26, β-fructofuranosidase, catalysing the hydrolysis of sucrose to glucose and fructose) was suggested many years ago as an enzyme with a general role in nectar secretion (Lüttge 1961). The highest level of invertase activity in the *A. cornigera* extrafloral nectary was observed in the hours directly before the secretion process began (at time *t* = 0, **Figure 3.2**). Gas chromatography/electron impact mass spectrometry (GC-EIMS) confirmed the dominance of sucrose in the phloem (2.0 ± 0.8 mg g^{-1} dry mass, *n* = 3) whereas the EFN contained fructose and glucose, but no sucrose (180 ± 4.1 mg g^{-1} fructose and 400 ± 27 mg g^{-1} glucose, *n*=3). By contrast, the amino acid profiles of phloem exudates and EFN were similar at the qualitative and quantitative level (**Table S1 online**).

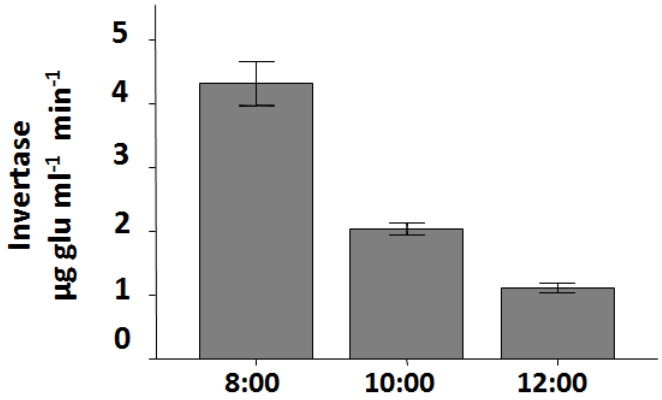

Figure 3.2. Mean invertase activity ± SE (µg glucose released per minute per ml, measured at 340 nm), *n*=5 replicates before (*t*=0), during (*t*=1) and after (*t*=2) active EFN secretion.

Next, we compared the proteomes of leaves, phloem exudates, nectary and EFN using one-dimensional SDS-PAGE, tryptic digestion and subsequent analysis of the resulting peptides using nano-electrospray liquid chromatography-tandem mass spectrometry (nanoLC-MS/MS). Although approximately 100 times more phloem sample than EFN sample was loaded onto the gel, absolute protein quantities in the phloem sample were very low (**Figure 3.3**). None of the peptides obtained gave hits to any *A. cornigera* nectarin (González-Teuber *et al.* 2009), and only two of the phloem proteins were also detected in the nectary tissue (**Tables S2 and S3 Online**). Thus, whereas little overlap was found among the proteomes of leaves, phloem exudate and nectar, the proteome of the secreted EFN represented essentially a subset of the nectary proteome (**Figure 3.3**), i.e. most (if not all) nectarins were found in the nectary tissue before secretion. Finally, the EFN proteome was highly similar among control plants and JA-treated plants (**Figure 3.3**), demonstrating its independence from general gene expression patterns in the rest of the plant.

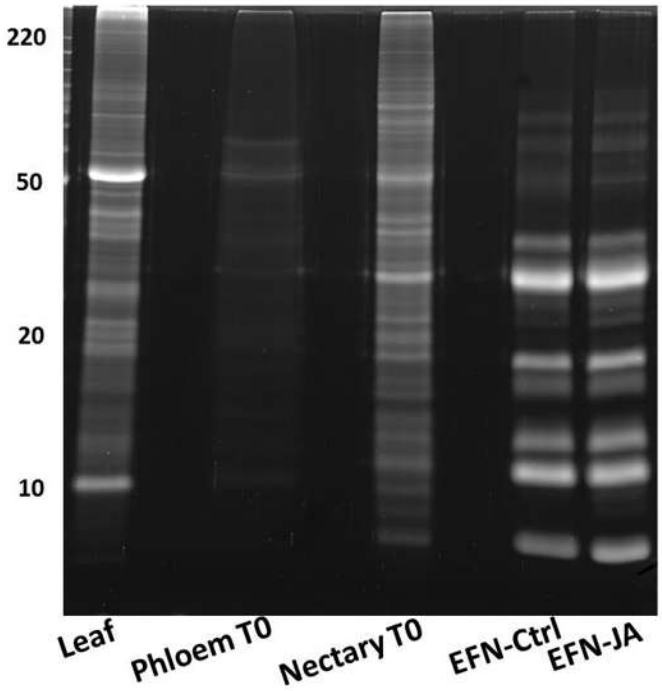

Figure 3.3. Proteomes obtained via SDS-PAGE for leaf tissue, phloem exudates, nectary tissue and secreted EFN with and without prior application of JA. 10 µg of protein for each sample were loading per well.

3.4.3. Proteomic dynamics during peak secretion

We followed the development of the nectary proteome by extracting proteins from the nectary tissue and separating them via two-dimensional gel electrophoresis (2DE; **Figures 3.4-3.6**). Tandem mass spectrometric (MS/MS) analysis of 592 quantitatively dominant protein spots was performed independently for the three time points and revealed 203 annotated proteins (**Figures 3.4** and **3.7**), the majority of which represented enzymes of carbohydrate metabolism (9% of the spots; e.g. invertase and sucrose synthase) and metabolism of amino acids and proteins (26%, e.g., methionine synthase, glutamine synthetase, proteolytic enzymes).

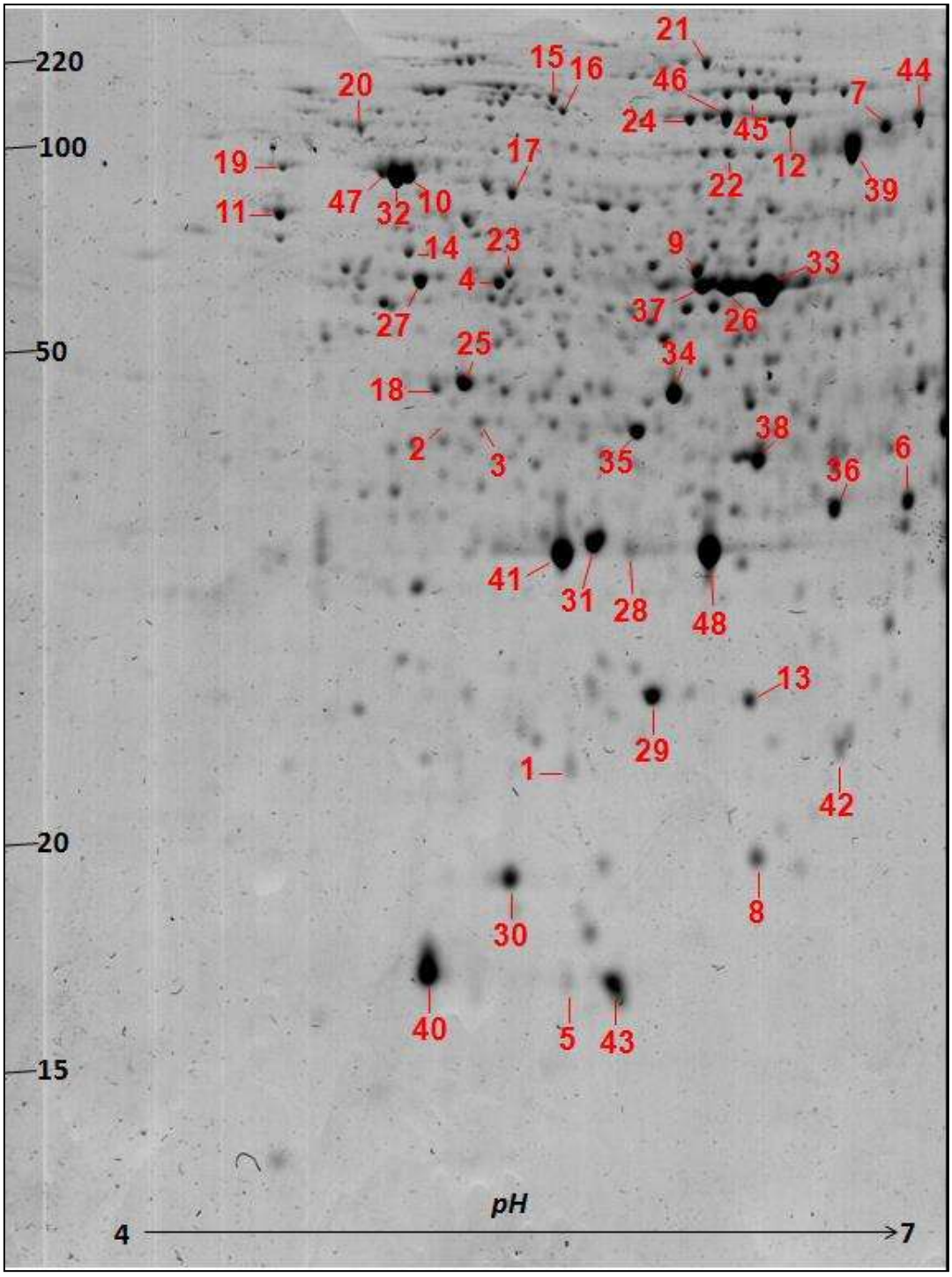

Figure 3.4. Diurnal proteome of the *A. cornigera* extrafloral nectary directly before active EFN secretion (*t*=0).

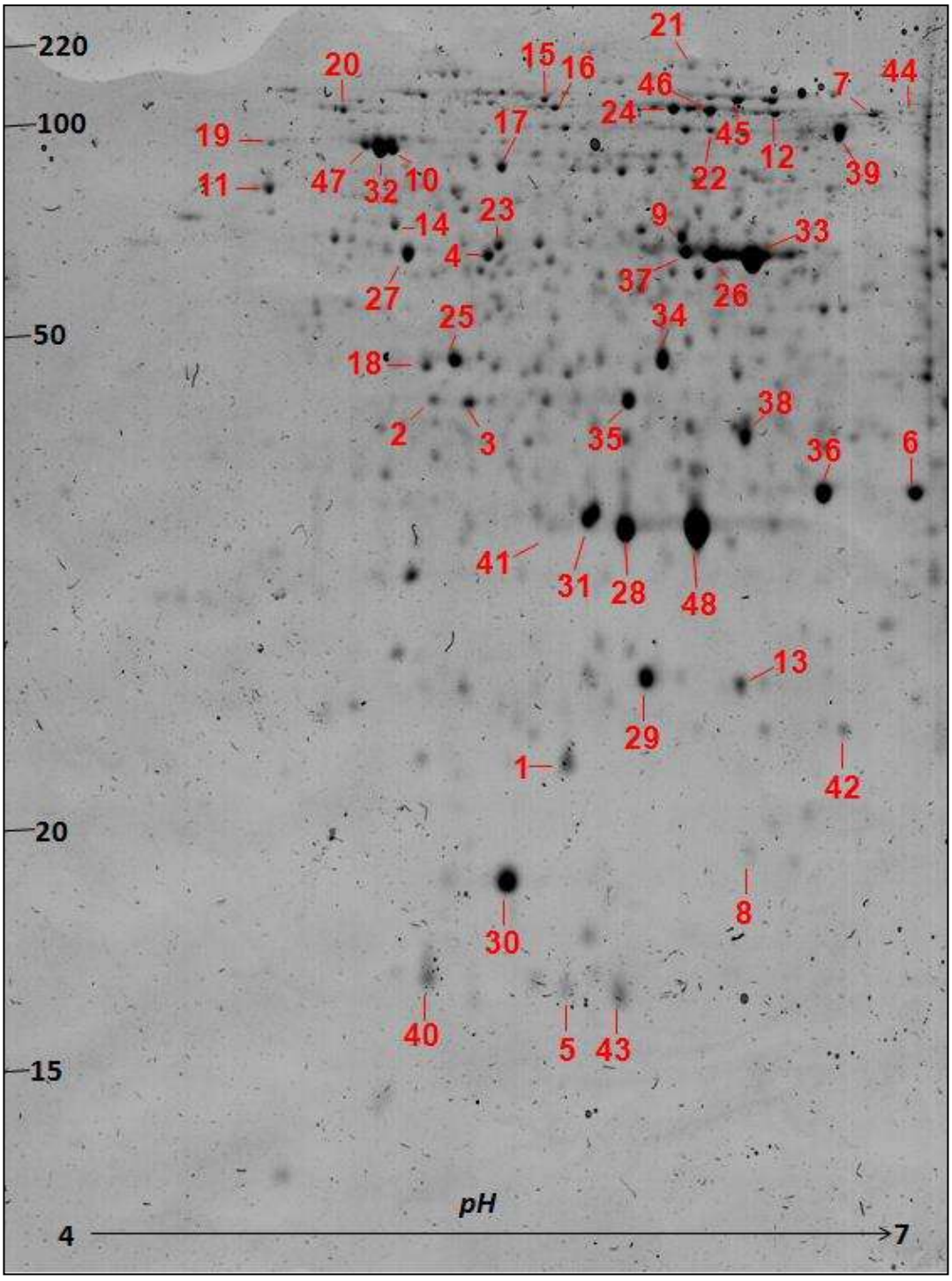

Figure 3.5. Diurnal proteome of the *A. cornigera* extrafloral nectary directly during active EFN secretion (*t*=1).

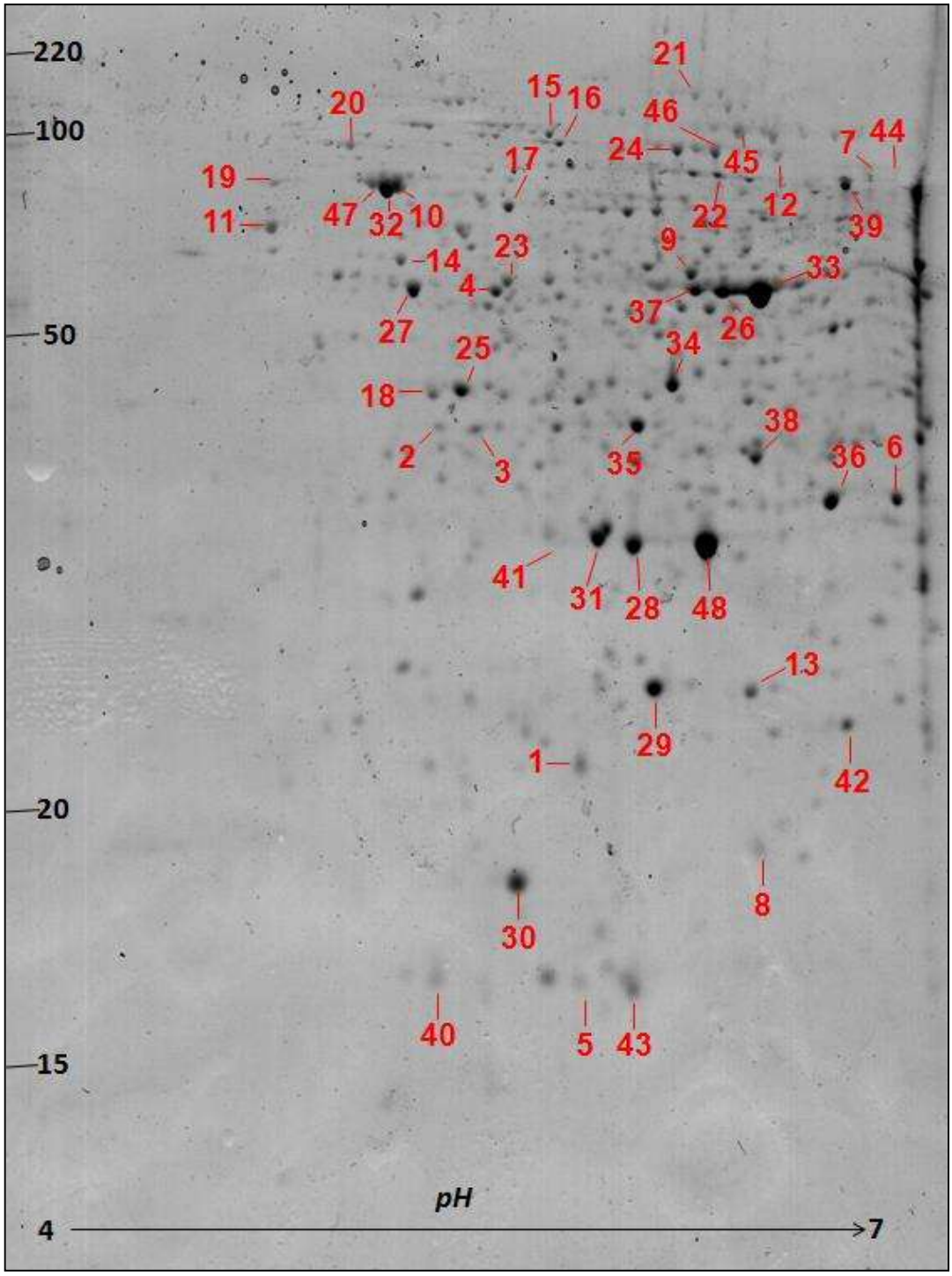

Figure 3.6. Diurnal proteome of the *A. cornigera* extrafloral nectary directly after active EFN secretion (*t*=2).

Further quantitatively important functional groups included proteins related to other metabolic processes (9%) and defence and stress (23%, e.g., heat shock protein 70, trypsin inhibitor) (**Figure 3.7**). Pathogenesis-related (PR) proteins (van Loon and van Strien 1999) were particularly common (**Figure 3.8 A, B**), which explains their dominance in the secreted EFN (González-Teuber *et al.* 2009, 2010). An invertase with 90% similarity to At CWINV4 was detected and temporal patterns in its absorbance/optical density (**Figure 3.8 A**) resembled those of invertase activity (**Figure 3.2**).

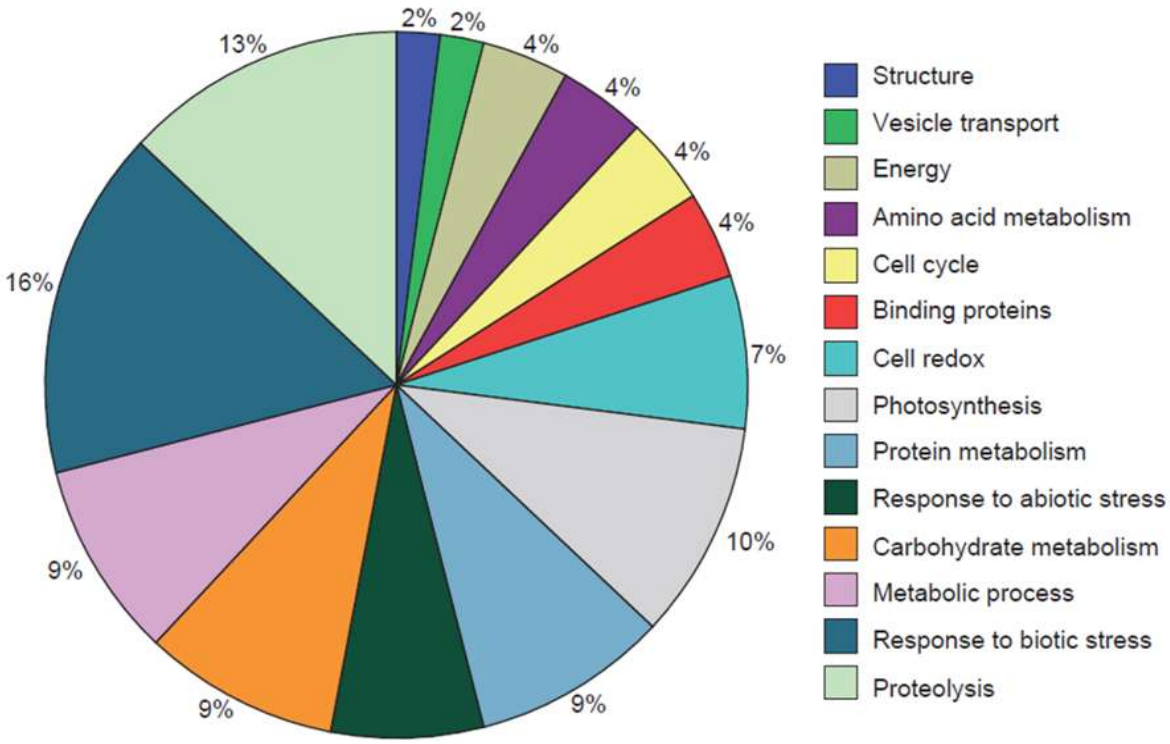

Figure 3.7. Functional categories of nectary proteins. Pie chart illustrating the major functional classes of proteins in the *A. cornigera* extrafloral nectary (percentages of annotated proteins).

The optical densities of the 48 dominant protein spots were quantified using the program 2D Image Master in Coomassie-stained gels and were found to be highest before secretion (t = 0), diminished during secretion (t = 1) and reduced after secretion (t = 2) (**Figure 3.8 A, B** and; **Table S4 Online**).

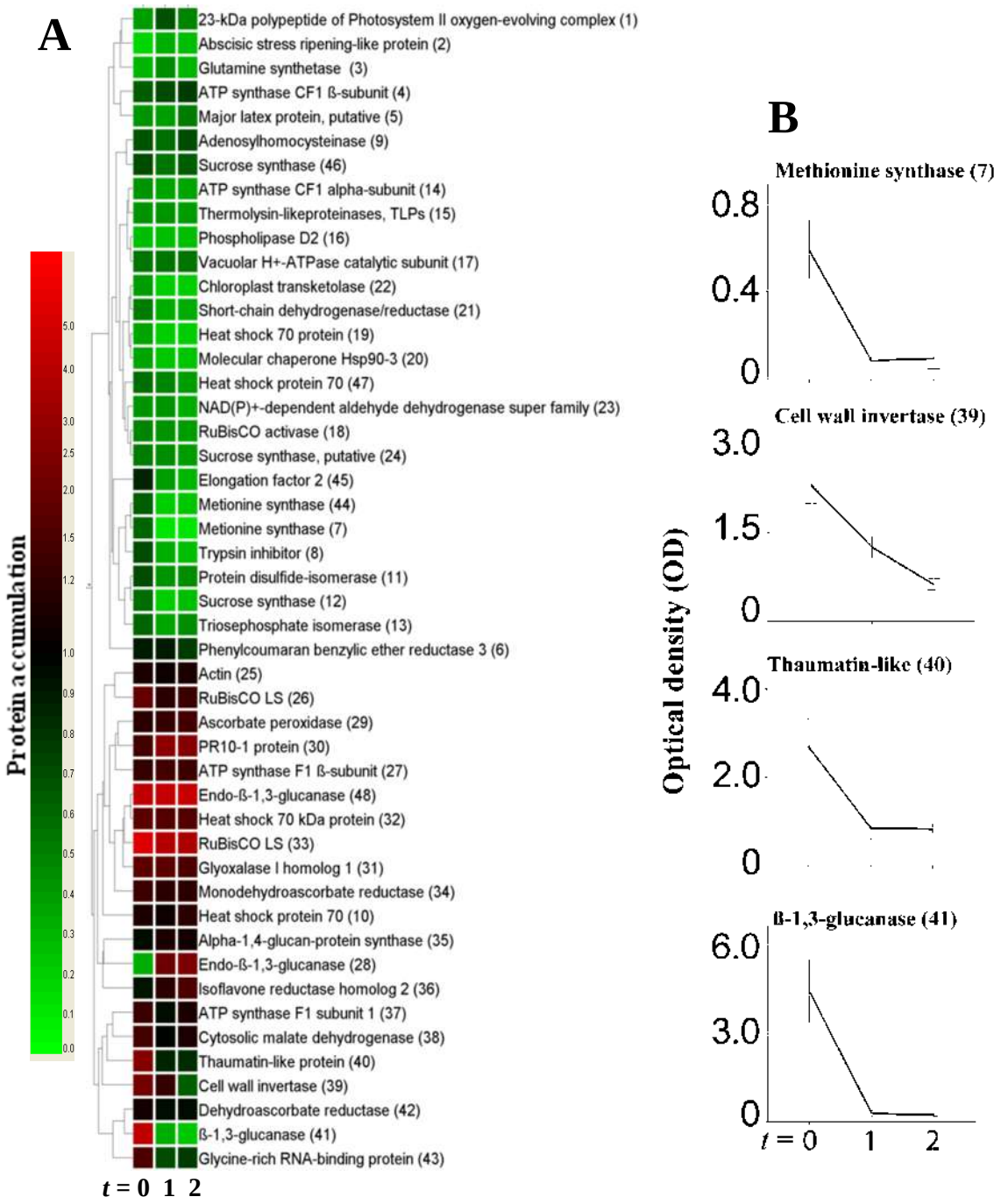

Figure 3.8. Protein accumulation before, during and after active EFN secretion. Heat map of optical densities (*n*=3 per time point) for the functionally most important proteins (A). Optical densities (OD, means ± SE) of representative proteins involved in amino acid metabolism and sucrose hydrolysis or dominant nectarins (B).

In general, a significant reduction in optical densities during the secretion process was observed for enzymes involved in sucrose hydrolysis (cell-wall invertase) and amino acid metabolism (methionine synthetase) and for secreted nectarins (González-Teuber *et al.* 2009, 2010), such as thaumatin-like protein and β-1,3-glucanase (**Figure 3.8 A, B** and **Table S4 Online**). By contrast, enzymes involved in the degradation of proteins remained quantitatively constant until after the secretion peak. The nectary proteome was mainly composed of enzymes that are directly involved in the synthesis of important nectar components (hexoses, amino acids and nectarins) and the secreted nectarins themselves, many of which accumulated immediately before the active secretion process and quantitatively decreased during and after the active secretion process.

3.4.4. Proteolytic activity

The 2DE and subsequent MS/MS analysis revealed numerous proteolytic enzymes (13%, e.g. proteases, ubiquitin-related proteins and a subunit of the proteasome, see **Table S3 Online**), many of which remaining at stable or slightly increasing abundances over the secretion peak. Enzymes with proteolytic activity are common in plants and play multiple roles, including defence against herbivores and pathogens, mobilization of storage proteins, liberation of amino acids and the degradation of proteins (Muntz *et al.* 2001, Schaller 2004, Vierstra 1996). We quantified trypsin- and chymotryspin-like proteolytic activity in the nectary tissue (**Figure 3.9**) and observed that trypsin-like activity increased significantly over the secretion period ($P < 0.001$ for an effect of time on activity, univariate ANOVA, $n = 5$, all three times differed at $P < 0.05$ according to an Least Significant Difference (LSD)

posthoc test) (**Figure 3.9 A**). Chymotrypsin-like activity increased from $t = 0$ to $t = 1$ and then remained stable ($P < 0.001$ for an effect of time on activity, univariate ANOVA, $n = 5$; $t = 0$ different at $P < 0.05$ from $t = 1$ and $t = 2$ according to a Least Significant Difference (LSD) posthoc test) (**Figure 3.9 B**). Thus, the proteolytic activity increased during secretion and was highest after the secretion peak.

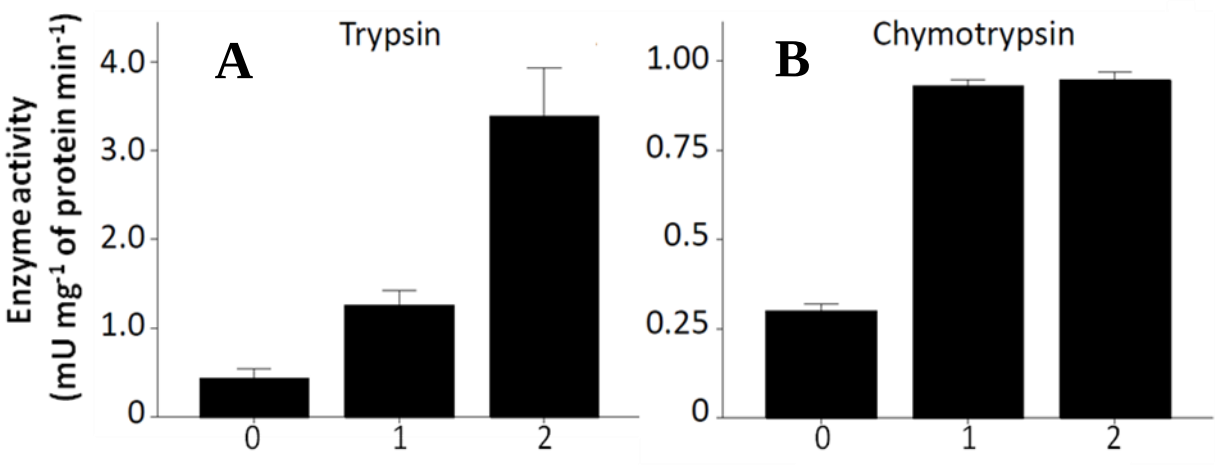

Figure 3.9. Proteolytic activity in the nectary tissue. Trypsin-like activity (A) Chymotrypsin-like activity (B) was quantified before (t=0), during (t=1) and after (t=2) the diurnal secretion peak. Values are means ± SE (n=5).

3.4.5. Tissue-specific gene expression

We aimed to identify the site of synthesis of important sugar metabolic enzymes (sucrose synthetase and invertase) and some nectarins [invertase ((Heil *et al.* 2005b) and ß-1,3-glucanase, PR10 and thaumatin-like protein ((González-Teuber *et al.* 2009)] by demonstrating expression of the corresponding genes in the nectary tissue. The isolation of specific members of gene families in species with no genetic information available typically involves use of degenerate primers for regions that are conserved across many members of the gene family of interest. We selected six functionally important genes (the

sugar metabolic genes invertase *CWIN4* and sucrose synthase *SS1*, and the dominant nectarins β-1,3-glucanase, endo-β-1,3-glucanase, PR10 and thaumatin; **Table S5 Online**). Degenerate PCRs based on mRNA extracted from nectary and leaf tissue indicated that all of these genes were expressed in the nectary tissue, and most of them exclusively so (**Figure 3.10**). Only *CWIN4* was expressed in the leaves as well, and only before EFN secretion (**Figure 3.10**). The identity of all PCR products was confirmed by sequencing (**Table S6 Online**). Genes encoding major nectarins and sugar metabolic genes are expressed in the nectary itself rather than in the leaves and are at their highest levels during active secretion.

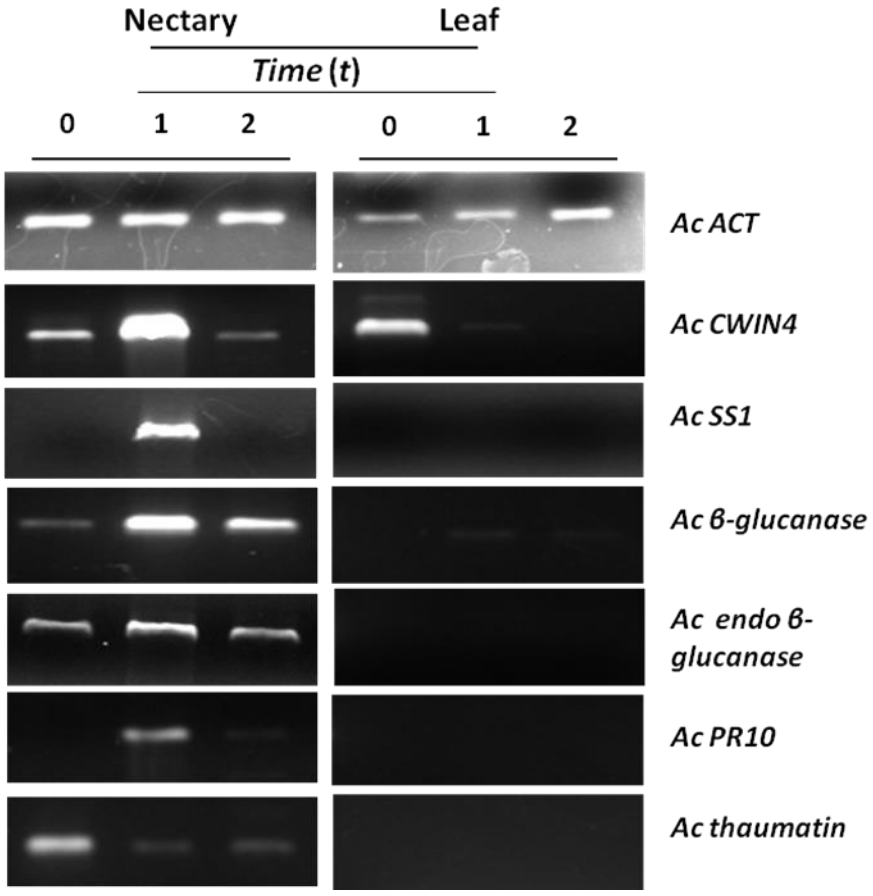

Figure 3.10. Expression of functionally important genes. Expression (bands obtained via PCR) is shown for the housekeeping gene actin (*ACT*), genes central to sugar metabolism (invertase, *CWIN4*; sucrose synthase,

SS1) and genes encoding the PR proteins β-1,3-glucanase, endo-β-1,3-glucanase, PR10 and thaumatin-like protein), in nectary tissue and in leaves before (t=0), during (t=1) and after (t=2) the diurnal secretion peak. All experiments were repeated (n=3).

3.5. Discussion

The secretion of nectar represents a common plant trait of high ecological, evolutionary and economic importance (Brandenburg *et al.* 2009, Heil 2011). However, the mechanisms by which plants adjust nectar secretion rates at the genetic or phenotypic level to environmental factors and current requirements remain to be elucidated. Our understanding of the enzymatic and genetic mechanisms that control these adaptive responses remains rudimentary, most likely because research into the mechanisms that control nectar production is impaired by the lack of conspicuous nectaries in most genetically tractable species. The highly predictable short-term changes in secretion activity (**Figure 3.1 B**) make the extrafloral nectaries of *A. cornigera* an ideal model system to study any changes in the nectary proteome and gene expression patterns that are directly related to the secretion activity at a high temporal resolution.

Nectar and phloem sap significantly differed in their chemical composition, particularly in terms of hexoses and nectarins. These differences between EFN and phloem exudate exclude the phloem as a direct source of the sugars and proteins that are secreted into the EFN. A cell-wall invertase of Arabidopsis (*At-CWINV4*) is the only gene known to encodes a metabolically active enzyme with an essential role in FN secretion (Ruhlmann *et al.* 2010). We found the highest invertase activity directly before active secretion (**Figure 3.2**),

and its expression in the nectary tissue was highest during the concurrent secretion process (**Figure 3.10**). Transcriptomic studies also found invertases to be overexpressed as compared to leaf tissue in both floral and extrafloral nectaries (Kram *et al.* 2009, Escalante-Perez *et al.* 2009). All these observations underline the importance of invertases in the secretion of nectar, probably because they are required for unloading of sucrose from the phloem and for secretion of hexose-rich nectars (Heil 2011). Moreover, the stability of the EFN proteome after JA treatment (**Figure 3.3**) was in accordance with the insensitivity of the EFN quantities to external application of JA (Heil *et al.* 2004). Hundreds or thousands of genes change their expression in response to exogenous JA in most plant tissues (Chini *et al.* 2007, Thines *et al.* 2007), and *A. cornigera* leaves exhibit a functioning octadecanoid signalling pathway, elevating their endogenous JA levels in response to damage (Heil *et al.* 2004). All these observations underline the independence of the regulatory pathways in the nectary from those in the rest of the leaf.

Taken together, our results demonstrate that the nectary represents a metabolically independent organ and that most synthetic processes that are required for production of important nectar components occur in the nectary itself. Therefore, we focused on the proteomic dynamics in the nectary tissue and found numerous biosynthetic enzymes responsible for the synthesis of amino acids, sugars and proteins, and the secreted nectar proteins themselves. Most anabolic enzymes were present at high optical densities before secretion and decreased during and after the secretion peak (**Figures 3.4-3.6 and 3.7** and **Figures 3.10.S1-S3**), but proteolytic enzymes showed the opposite pattern (**Figure 3.9**). The presence of numerous proteolytic enzymes (13%, e.g. proteases, ubiquitin-related proteins and a subunit of the proteasome, see **Table S3 Online**) is consistent with the

proteolytic activity in the nectary, which reached highest overall values after the secretion peak (**Figure 3.9**). Several proteolytic genes appeared down-regulated in poplar extrafloral nectaries in comparison with leaves, whereas a serine carboxypeptidase (PtpAffx.80486.1.A1_at) was upregulated (Escalante-Perez *et al.* 2012). Similarly, multiple serine proteases and ubiquitin-like proteins were over-expressed in floral nectaries of Arabidopsis (Kram *et al.* 2009). Proteolytic enzymes may play multiple roles in the secretion process, including protection from infection and the liberation of amino acids. However, their high activity in the hours directly after the secretion peak is more consistent with the interpretation that proteolysis is involved in subsequent removal of enzymes from the nectary tissue (**Figure 3.9**). Thus, the activity of proteolytic enzymes in the extrafloral nectaries of *A. cornigera* appears to be involved in the termination of nectar production.

We present here proteomic information from nectary tissue at various stages of the diurnal secretion peak, consider the synthesis of three classes of major nectar components and report on short-term dynamics in proteomic patterns that are directly related to the active nectar secretion process. How representative are these findings for the mechanisms that underlie nectar secretion in general? Our observations in the extrafloral nectary of *A. cornigera* are in agreement with previous analyses of floral nectaries, which demonstrated the essential role of an extracellular invertase in nectar secretion in Arabidopsis flowers (Kram *et al.* 2009, Ruhlmann *et al.* 2010) and indicated that *NECTARIN* genes are expressed in the nectary tissue of ornamental tobacco (Carter and Thornburg 2003, 2004, Liu and Thornburg 2012, Liu *et al.* 2009). However, these studies used floral nectaries with an ontogenetically fixed secretion program, and hence were limited to time scales with an

order of magnitude of days. For example, the phase dominated by starch anabolism in ornamental tobacco flowers lasts approximately 8 days (Liu and Thornburg 2012).

Using extrafloral nectaries with a short diurnal secretion peak, we demonstrate that gene expression, protein accumulation and invertase activity in the nectary are highly dynamic and coincide at the timescale of hours with the concurrent secretion activity. We conclude that accumulation of metabolically important enzymes and nectarins in the nectary immediately before the active secretion process is required for the production of extrafloral nectar. The proteomic approach helped to identify metabolically important enzymes in a non-model plant growing in the wild, and degenerate primers were successfully used to demonstrate a tissue-specific expression of the corresponding genes in a species for which no genetic information is available. Similar approaches applied to other species will provide a more complete understanding of the metabolic processes that control nectar quantity and quality in the multitude of plant species for which this process is essential.

Supporting information online

Table S1. Relative composition of sugars and amino acids in phloem exudate and extrafloral nectar of *Acacia cornigera.*

Table S2. Proteins detected in phloem exudate of *A. Cornigera.*

Table S3. Annotation results for proteins from the nectary proteome at t = 0, 1 and 2.

Table S4. Development in optical densities of nectary proteins from before secretion to after secretion.

Table S5. Sequences of degenerate primers and actin primers.

Table S6. Sequences and annotation results for degenerate PCR product.

3.6. Acknowledgements

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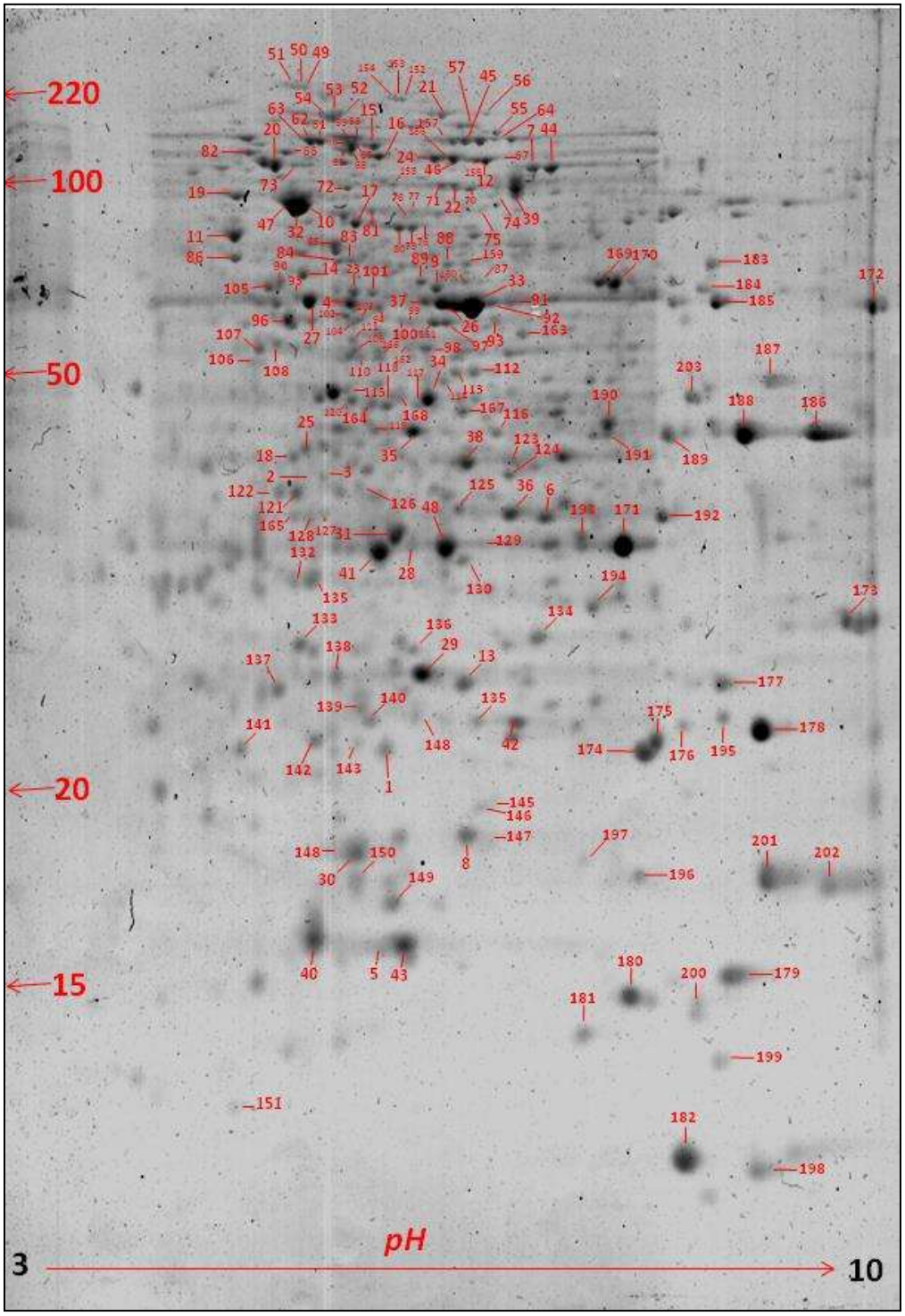

Figure 3.10.S1. Proteome of the *Acacia cornigera* extrafloral nectary directly before the secretion peak.

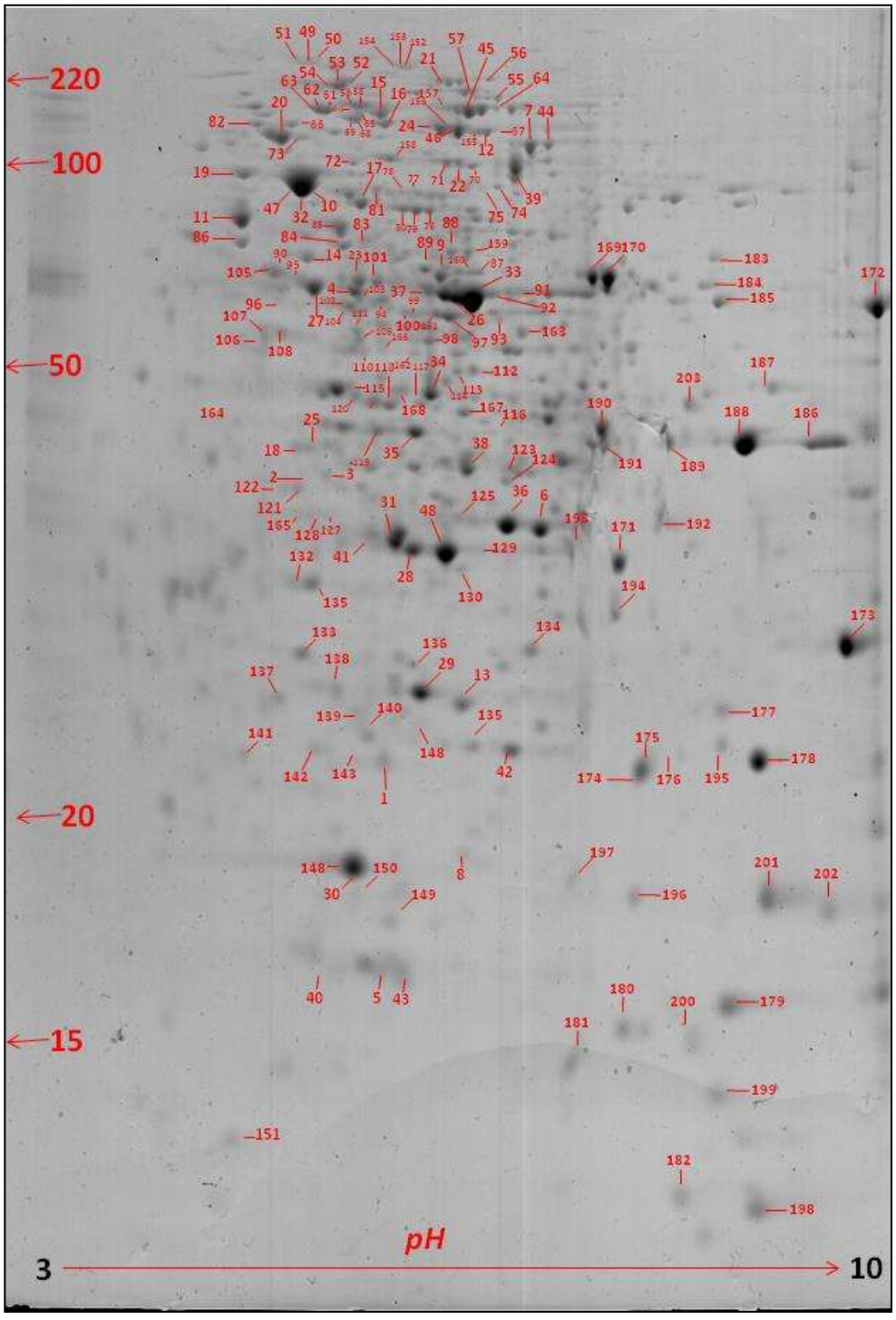

Figure 3.10.S2. Proteome of the *Acacia cornigera* extrafloral nectary during the secretion peak.

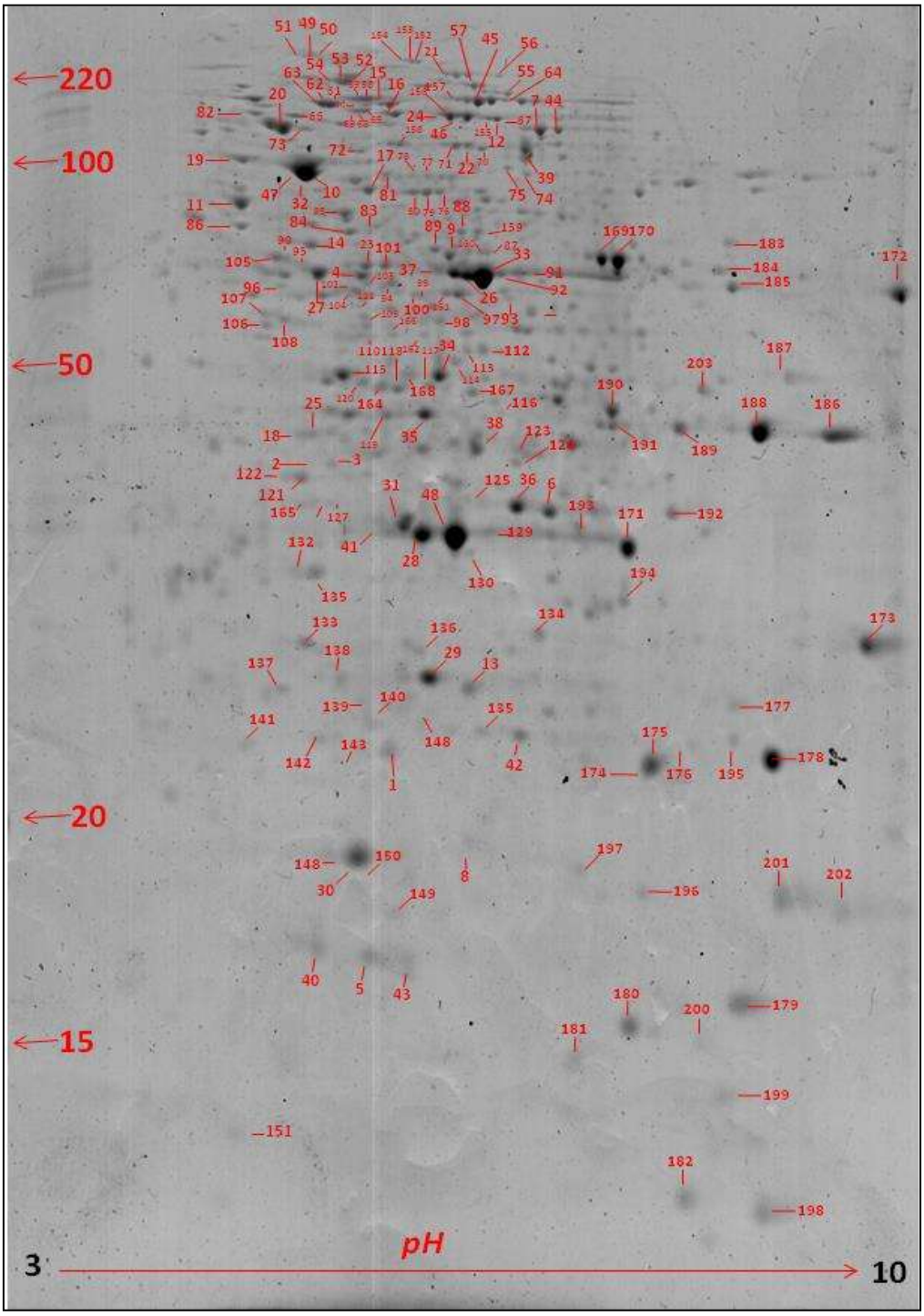

Figure 3.10.S3. Proteome of the *Acacia cornigera* extrafloral nectary immediately after the secretion peak.

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Chapter 4

Endosymbiotic bacteria in arboreal ants recycle nitrogen from the intestine

Screenshoot from: Kluge–pflanzen, Mexiko Akazien

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4.1. Abstract

Insects such as arboreal ants that life on nitrogen-poor diets commonly host endosymbiotic bacteria that probe positive for nitrogen dehydrogenase (*nifH*). These bacteria are believed to supplement their host with N fixed from the atmosphere. We used two plant-ant species (*Pseudomyrmex ferrugineus* and *P. gracilis*) that differ in their diet: *P. ferrugineus* is strictly vegetarian whereas *P. gracilis* also preys on insects. We investigated whether endosymbiotic bacteria contribute to the N-metabolism of these ants and whether the underlying mechanism is N-fixation, or rather recycling. The presence of *nifH* in several strains of endosymbiotic bacteria was confirmed with PCR. A positive ethylene assay indicated a functioning N-fixing activity under laboratory conditions, for one host species. However, larvae and workers of both species that were kept in a $^{15}N_2$-enriched atmosphere did not assimilate detectable amounts of ^{15}N into their body mass. By contrast, zymograms indicated high urease activity in the intestines and feeding the ants with ^{15}N-urea resulted in the appearance of ^{15}N-marked amino acids in their hemolymph. Thus, ^{15}N from intestinal urea appeared in ant metabolites. Bacteria that count with the genetic machinery for N-fixation also require urease activity. We conclude that endosymbiotic bacteria that probe positive for *nifH* can supply their host with N recycled from the intestine, a process for which the conditions in the intestine of insects might be more favorable than for the fixation of atmospheric N.

4.2. Introduction

Ants are one of the most important insect groups and from ecologically dominant guilds in multiple ecosystems (Davidson *et al.* 2003, Holway *et al.* 2002, Hölldobler and Wilson 1990, Philpott and Armbrecht 2006, Russell *et al.* 2009). Although ants with few exceptions are considered as predominantly predatory organisms, their biomass commonly outbalances the biomass of their putative prey organisms; a phenomenon that has been termed 'Tobin's ant biomass paradoxon' (Davidson *et al.* 2003, Davidson and Patrell-Kim 1996, Tobin 1995, Wilson and Hölldobler 2005). Explanations were based on the hypothesis that tropical arboreal ants are more 'vegetarian' than commonly assumed and stated that many of them might predominantly feed on plant secretion, such as extrafloral nectar and phloem sap, and on insect-derived excretions such as honeydew (Davidson *et al.* 2003). This assumption concerning the ecological dominance of 'vegetarian' ants is challenged when considering nitrogen balances. Floral and extrafloral nectar contain amino acids and in some cases also proteins (Heil 2011) and honeydew also can contain amino acids (Blüthgen *et al.* 2004, Woodring *et al.* 2004). However, most of the mentioned food sources that are derived directly or indirectly from plants and accessible to ants appear to be too low in N as to allow for building the biomass of ants, and of their larvae in particular. This dilemma received a possible explanation from the observation that the evolution of arboreal ants is tightly linked to the presence of characteristic endosymbiotic bacteria (Russell *et al.* 2009), many of which probe positive for nitrogen dehydrogenase (*nifH*). This enzyme catalyzes the limiting step in the bacterial fixation of atmospheric nitrogen (Hoffman *et al.* 2009, Kneip *et al.* 2007, Seefeldt *et al.* 2009). Therefore, bacteria carrying this gene might supply their host with additional N. Several recent studies on ant

endosymbionts found bacteria that probed positive for *nifH* (Eilmus and Heil 2009, Pinto-Tomas *et al.* 2009, Russell *et al.* 2009). Therefore, these authors commonly speculated that the fixation of N by endosymbiotic bacteria might contribute to the N-metabolism of arboreal ants and thereby solve 'Tobin´s ant biomass paradoxon'.

In fact, approximately 20% of all insects harbor in their midguts, intracellular endosymbiontic bacteria (Buchner 1965). Many insects depend on symbiotic bacteria for balancing the nutrition of their host (Chandler *et al.* 2008, Eilmus and Heil 2009, Nardi *et al.* 2002) and endosymbiotic bacteria such *Blochmannia, Buchnera, Rickettsia* and *Wolbachia* are common intracellular residents in ants and might fulfill specific roles, such as the fixation, recycling or upgrading N (Anderson *et al.* 2012, Cook and Davidson 2006, Russell *et al.* 2009, Wernegreen *et al.* 2003). However, endosymbiotic bacteria live inside the body of their host (i.e., within cells or in the intestine). Therefore, their access to atmospheric N appears to be very limited. In fact, only few studies have experimentally demonstrated that insect endosymbionts can actually fix N in the living insect and that the host organism has access to this N. Here, we tested the hypothesis that endosymbiotic bacteria supply their host with N that has been recycled from the host's intestine, rather than fixed *de novo* from the atmosphere, and that this function can be fulfilled by bacteria that carry *nifH*. We used two congeneric ant species that differ in their degree of dependency on plant-derived food sources. *Pseudomyrmex ferrugineus* is a mutualistic plant-ant that protects myrmecophytic acacia plants from herbivores, pathogens and encroaching vegetation (Heil *et al.* 2010, Janzen 1966, 1967) and has a strictly vegetarian lifestyle: this ant feeds exclusively on cellular food bodies (FBs) and extrafloral nectar (EFN) that are produced by its host plant (Clement *et al.* 2008). By contrast, *P. gracilis*

represents an exploiter of this mutualism (Heil *et al.* 2009, Kautz *et al.* 2009). The ants can nest facultatively on acacia hosts and feed on the host-derived rewards, but they forage also off the pants and their diet regularly includes insect prey (Clement *et al.* 2008). We screened these ants for endosymbiotic bacteria that carry *nifH*, subjected living ants to the acetylene assay that is commonly used to demonstrate an active nitrogenase enzyme (Breznak *et al.* 1973, Morales-Jiménez *et al.* 2009, 2013, Pinto-Tomas *et al.* 2009) and exposed living ants to a ^{15}N-rich atmosphere. The results demonstrated the presence of bacteria with *nifH* and a positive acetylene assay but no assimilation of atmospheric N into the metabolism of the ants. By contrast, we found that the same bacteria had an active urease and that ^{15}N-marked urea could be processed in living ants, which obtained organic (amino acid-) ^{15}N when being fed ^{15}N-marked urea. We conclude that the recycling of N, rather than its *de novo* fixation from the atmosphere, represents the biologically relevant process in this system and that bacteria that carry *nifH* are commonly also able to recycle N from urea present in the intestine of their host insects.

4.3. Material and methods

4.3.1. Sampling site and ants

The study site was visited in summer of 2011 and 2012. Site of collection were conducted 15 km of Puerto Escondido in the coastal area of Oaxaca, México (15° 55.596′ N and 97° 9.118′ W, elevation 15 m). *Pseudomyrmex ferrugineus* (F. Smith) inhabits *Acacia hindsii* or *A. cornigera* plants and feed only of host-derived food rewards produced by

myrmecophytic plants. However *Pseudomyrmex gracilis* (Fabricius 1804) can displace to the mutualistic ants, and can inhabit both myrmecophytes. To collect mutualistic or exploiter ants were selected from *A. hindsii* and *A. cornigera* plants by cutting off swollen thorns: entire thorns, which contained adult ants and larvae, were then kept in plastic pots with adequate ventilation and carry to the laboratory for analysis.

4.3.2. In vivo Acetylene Reduction Assay (AR)

AR is a functional assay for nitrogen fixation, AR was conducted by placing individual ant larvae or workers of *P. ferrugineus* or *P. gracilis* were placing an individual vessel (10 ml). Acetylene was injected to a final concentration of 10% (v/v) by replacement of an identical volume of air, and samples were incubated at 30°C for 10 min and ethylene production was measured by gas chromatography.

4.3.3. *In vivo* $^{15}N_2$ Assay

Ant larvae and adult workers were placed into an airtight vessel of 1l, and was subjected to atmosphere replacing with a mixture of 10 or 5% $^{15}N_2$ (v/v) and the animals were exposed by 1 week and colonies as control group were exposed only to regular air. Each colony exposed was fed with 4% of sugar solution (glucose/fructose). At final of isotopic gas exposure time, samples were analyzed as Pinto-Tomas *et al.* (2009). In short, samples were frozen and dried at 60 °C and homogenized. To analyze N isotopic ratios, 1 mg of each

samples were placed in quartz tubes and charged with 5 g of CuO, 0.5 g Cu and 250 mg of Ag, samples in tubes were sealed and combusted at 800 °C by 6 h. After this time, samples were vacuum and destilled at -78 and -196 °C. Isotopic N was trapped on 5 Å molecular sieves at the last temperature. Finally the sieves were heated to 250 °C in a Finnegan MAT Delta E Isotopic Ratio Mass Spectrometer. The isotopic ratio was determined as percentage of ^{14}N and ^{15}N compared with an air as standard to give a δ value:

$$\delta = 1000[\{(^{15}N\text{sample}/^{14}N\text{sample})/(^{15}N\text{standard}/^{14}N\text{standard})\}-1]$$

4.3.4. Feeding experiments and hemolymph collection

50 individual workers from individual shrub collection (*A. hindsii* and *A. cornigera*) were isolated and placed in individual plastic pots. After a starvation period of 3 days, the ants were feed with 200 µl of 4% (w/v) glucose and fructose containing 1% urea and experimental groups were feed with the same sugars and ^{15}N-labelled urea (98 atom % ^{15}N. Sigma-Aldrich, México). After 8 h of feeding, the ants were anesthetized with cold and cut to obtain three pieces (head, thorax and gaster). Hemolymph was obtain by press the piece samples with forceps and was recovered with a 2 µl microcapillary tube from head and thorax, and gaster was discarded to avoid contamination. ≈25 µl of hemolymph samples were collected from each replicate and were stored to -70 °C (Feldhaar *et al.* 2007).

4.3.5. Midgut extract of ant workers after urea feeding

Midguts from ant workers were obtained by dissection in an 'Insect Ringer' solution (10.4 g NaCl, 0.32 g KCl, 0.48 g $CaCl_2$, 0.32 g $NaHCO_3$ in 1 l of water). A single replicate comprised 50 ant worker midguts, which were placed in 200 µl of 25 mM phosphate buffer (pH 6.5), containing 25 mM of NaCl and 0.5 EDTA and were homogenized and centrifuged at 15 000 *xg* for 15 min at 4°C, and stored at –70°C.

4.3.6. Urease zymograms

Native electrophoresis was performed under non-denaturing conditions using 7.5% PAGE (Laemmli 1970). Gels were loaded with 20 µg of protein per sample and the separation was performed at 4°C (100 V and 12 µA). After electrophoresis, gels were immersed in an activation solution of 50 mM sodium acetate (pH 6.5), containing 1 mM EDTA and incubated 30 min. Gels were then transferred to a buffer of 20 mM sodium acetate and 1 mM EDTA and incubated for 30 min. Gels were equilibrated in 1 mM EDTA and 0.5% (w/v) cresol red for 30 min. For the enzymatic reaction was initiated when the gels were transferred into solution containing 1 mM EDTA, 0.5% cresol red and 1.5% (w/v) urea and for termination of urease reaction, they were transferred into a solution of 0.1 M lead acetate and 3-5 min of incubation, the activity bands appear as opaque white bands (Shaik-M *et al.* 1980).

4.3.7. Gas chromatography-mass spectrometry (GC-MS) analysis of amino acids from ant workers hemolymph

20 µl of ant hemolymph was derivatized by adding 20 µl of piridine and 80 µl N, O-bis (trimethylsilyl)-trifluoroacetamide (BSTFA) and the mixtures were incubated at 80 °C by 30 min in a Thermomixer® (Eppendorf). After this time, 1 µl of derivative samples were injected and analyzed by GC-MS system (Agilent 7890A; Agilent technologies Santa Clara, CA, USA) coupled to a mass-selective detector (Agilent 5975C; Agilent technologies Santa Clara, CA, USA) with a capillary column (60 m X 250 mm X 0.25 µm coating; Agilent technologies Santa Clara, CA, USA) that include a helium flux of 1 ml/min under next conditions: 70 °C initial temperature (5 min), ramp at 5 °C /min to 310 by 15 min. The MS injector was set at 250 °C. Identification of amino acids was done using NIST 2.0 library of 2008. To obtain the relation of ^{15}N incorporated in free amino acids detected from ant hemolymph, abundance of the major isotope peak of each chromatograms was detected and divided with the molecular mass ion of each amino acid.

4.3.8. Endosymbiont isolation and culture conditions

The ant workers were surface sterilized with 70 % ethanol and 15 % of bleach solution (5.25 % sodium hypochloride stock solution) for 1 min, and consecutive washes in sterilized ddH_2O and dissected in sterile 'Insect Ringer' solution (mentioned as before). 10 midguts were placed in 1 ml of MR medium (grams per liter: 0.3 KH_2PO_4; 0.3 Na_2HPO_4; 0.1 $MgSO_4$- $7H_20$; 0.05 $CaCl_2$. $2H_20$; 4 glycerol; 0.25 NH_4Cl; 0.01 H_3BO_3; 0.001 $ZnSO_4$ $2H_20$; 0.001 $FeCl_3$ $6H_20$; 0.0005 $CuSO_4$ $5H_20$; 0.0005 $MnCl_2$ $4H_20$; 0.0005 Na_2MoO_4-

$2H_20$; 0.0001 biotin (pH 6.8)) (O'Gara and Shanmugam 1976) and serial dilutions were performed (1000-10,000-fold) in MR medium and then spread on agar plates and incubated at 28 °C.

4.3.9. Ant midgut and endosymbiont DNA isolation

10 midguts were homogenized in liquid nitrogen. We added 200 µl of extraction buffer (50 mM Tris-HCl pH 8.0, 50 mM EDTA, 0.1 M 2-mercaptoethanol, 2% sodium dodecyl sulfate) supplemented with 25 µl of proteinase K (10 mg/ml), the mixture was incubated at 65 °C for 10 min. After this time, 100 µl of potassium acetate (5 M) was added and samples were mixed carefully and incubated in ice for 10 min. Samples were centrifuged (18,000xg, at 4 °C for 10 min), and then aqueous solution was collected. DNA was purified adding 1 volume of phenol/chloroform/isoamyl alcohol (25:24:1), mixtures were centrifuged as before and 0.6 volumes of ice-cold isopropanol (100 %) was added to each tube, and samples were precipitated by incubation at -20 °C for 30 min, finally samples were centrifuged and pellets were washed with 1 ml of 70 % ethanol and were diluted with water. For DNA from isolated bacteria, a single colony from each isolate was inoculated into 3 ml of liquid MR medium, incubated 12 hr at 28 °C. 1 ml of each cultures were centrifuged (18,000xg, at 4 °C for 10 min) and were washed twice with phosphate-buffered saline (PBS), and pellet were used for DNA extraction with the QuickGene DNA tissue kit was used in combination with QG-mini80 instrument (Fujifilm, Japan) and the respective protocol (provided by the manufacturer) was followed by default.

4.3.10. PCR of *nifH* and *UreC* gene from ant midgut and isolated bacteria

DNA extracts were amplified by Touchdown PCR (TD-PCR) using a temperature range of 50-60 °C based of *Tm*. The following TD-PCR conditions: Phase 1 consist of initial denaturation performed at 95 °C for 3 min, followed by 10 cycles where denaturation was at 95 °C for 30 sec, and an annealing temperature of 65-45 °C for 45 sec, the annealing temperature was decreasing 2° C per cycle. Elongation was 72 °C for 30 sec, the next phase 2, consist of a denaturing temperature of 95°C for 30 seconds, an annealing temperature of 58°C for 45 seconds, and an elongation temperature of 72°C for 30 sec by 25 cycles. The final extension (termination) was at 72°C for 10 minutes and 4 °C (Korbie and Mattick 2008). Amplified PCR products were resolved by electrophoresis in 1.2 % agarose gel. PCR amplifications were undertaken in 25 µl of final volume that containing 10X PCR buffer, 10 mM dNTPs mix, 50 mM $MgCl_2$, *Taq* DNA polymerase (Invitrogen) and 1:20 dilution of the DNA template solution prepared as before. The sequence of primers of *UreC* gene used for PCR was the next: UreCF 5´-CTGAAGCTGCACGAGGACTGGG-´3 and UreCR 5´-GTCGTGCAGGATGTCCTCGGC-´3. *nifH* gene: NifHF 5´-TGCGAYCCSAARGCBGACTC-´3 and NifHR 5-´ATSGCCATCATYTCRCCGGA-´3.

4.4. Results

4.4.1. Acetylene reduction

When the individual larvae or worker of *P. ferrugineus* ants were under acetylene atmosphere, they present a low acetylene reduction to ethylene (**Figure 4.1**), however the acetylene reduction activity by ant larvae of *P. gracilis* increase the ethylene detection in

high amount (**Figure 4.1**), but no significant activity was present in workers of the same species respectively.

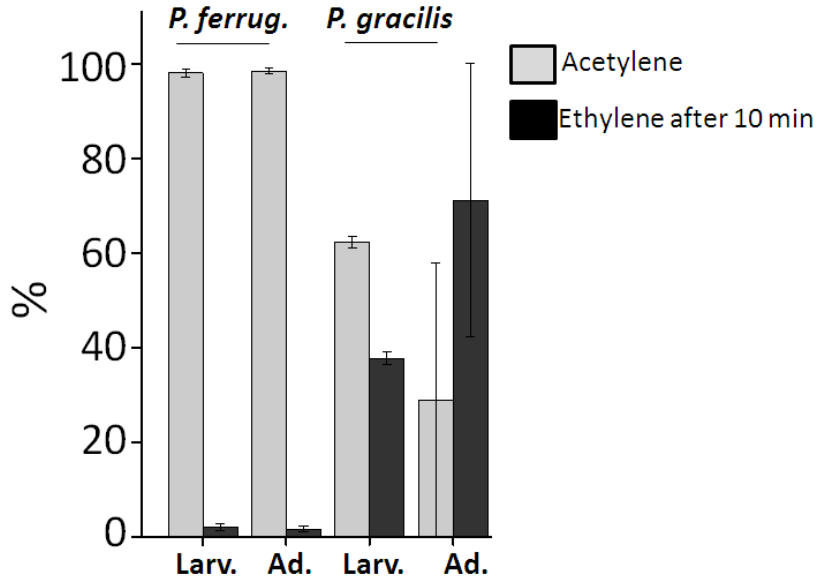

Figure 4.1. Acetylene reduction assay in larvae and workers of *P. ferrugineus* or *P. gracilis* ants. N_2-fixation activity measured in: Larvae (Larv) and adult workers (Ad).

4.4.2. $^{15}N_2$ -enrichment experiments under laboratory and field conditions

The results of the measured of isotopic $^{15}N_2$ in larvae or workers of obligate *P. ferrugineus* ants no showed enrichment in their body mass after one week of exposure to enrichment atmosphere of $^{15}N_2$ (**Figure 4.2**), the larvae and worker showed no significant incorporation of this isotope. Under same condition, larvae of *P. gracilis* present significant isotopic incorporation in their bodies mass compared with their control, by the way, workers of *P. gracilis* present incorporation, but this result not showed significant difference (**Figure 4.2**).

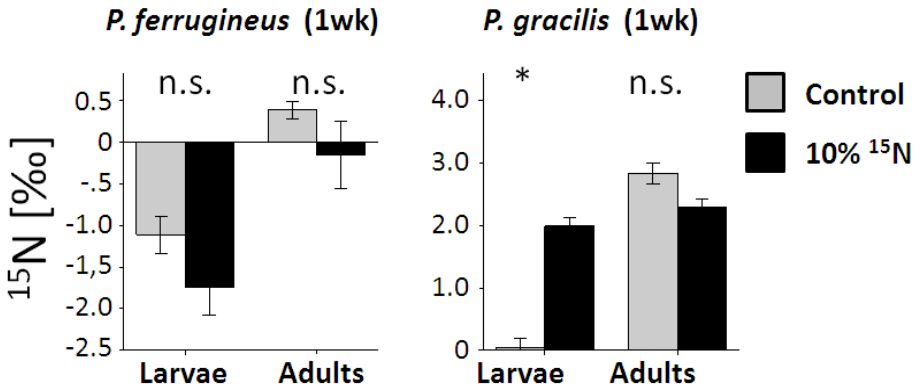

Figure 4.2. ^{15}N-enrichment experiment in larvae and worker of *P. ferrugineus* and *P. gracilis* ants under laboratory conditions. Colonies were incubated per 1 week.

Under field conditions, larvae of mutualistic ants that lived in natural condition on the plant, showed no incorporation of ^{15}N in their body mass, and after one week of ^{15}N exposition, they do not present significant incorporation of isotope and the control present the same situation (**Figure 4.3**), with respect of ant workers, they present insignificant patters of incorporation (**Figure 4.3**). Similar results were showed in larvae of *P. gracilis*, when the larvae were exposed to isotopic gas, the larvae present the same level than the ant larvae before the isotopic exposition (**Figure 4.3**).

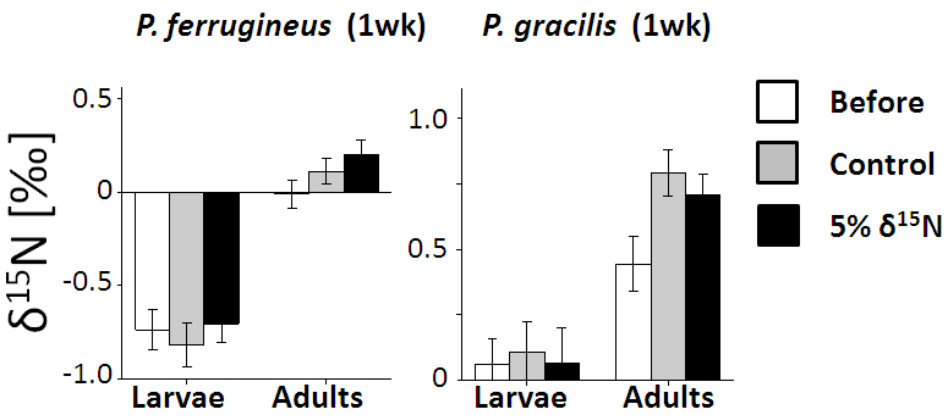

Figure 4.3. ^{15}N-enrichment experiment in larvae and worker of *P. ferrugineus* and *P. gracilis* ants under field conditions. Colonies were incubated per 1 week.

4.4.3. Isolation of bacterial and *nifH* and *ureC* genes identification from midgut of ant workers

Five strains from midguts of *P. gracilis* were isolates using N-free media and *nifH* was detected in all strains isolated (**Figure 4.4 A**), and also, DNA extracted of midguts from *P. ferrugineus* and *P. gracilis* present *nifH* amplification (**Figure 4.4 A**). *ureC* gene was amplified from isolated bacteria and ant midgut (**Figure 4.4 B**), samples of ant workers were isolated after time feeding. In all samples the *nifH* gene band was very clear with a 450 pb and *ureC* gene was of 404 pb.

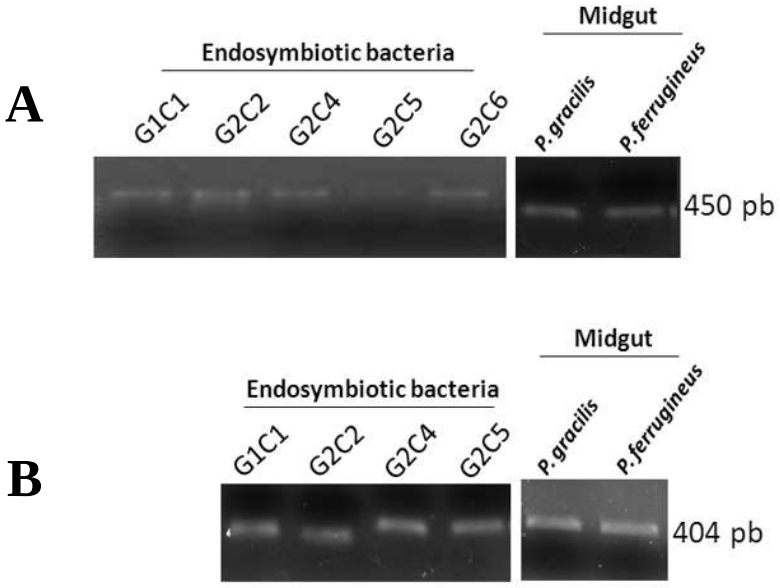

Figure 4.4. PCR amplification of endosymbiotic isolates from *P. gracilis*. Different lines present PCR amplification from isolates bacteria and genomic DNA from *P. gracilis* and *P. ferrugineus* ant workers. (A): *nifH* gene amplification; (B): *ureC* gene amplification.

4.4.4. Urease zimography from ant worker midgut after feeding with urea

To verify if urease enzyme is active in ant workers after the urea feeding, the midgut samples were homogenized and loaded in native gel. A single band (≈90 kDa) appeared in midgut extract of mutualistic *P. ferrugineus* and two intensive bands (≈ 90 and 180 kDa) were found in samples of *P. gracilis* workers respectively (**Figure 4.5**). Only the lower band (90 kDa) was similar in size and the exact same position in the gel for both ant species. Based on electrophoresis migration, urease enzyme is present in both ant species, however is mostly active in generalist ants.

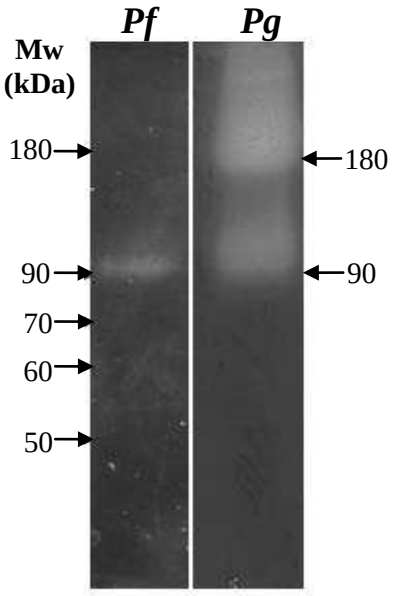

Figure 4.5. Native PAGE of urease activity. Samples were separed in a 7.5 % of polyacrilamide gel electrophoresis. *Pf*: *P. ferrugines* and *Pg*: *P. gracilis* midgut extracts. Arrow indicates the relative molecular weight of the bands.

4.4.5. Amino acid hemolymph analysis with ^{15}N-urea

To investigate if urease in digestive tract of ant workers can facilitate the ^{15}N-incorporation into amino acids that circulate in the ant hemolymph, obligate and generalist ants were fed with ^{15}N-labelled urea or control urea. Ant hemolymph amino acid detection with GC-MS, revealed high percent of ^{15}N-incorporation in several amino acids as: Proline, Alanine, Threonine, Glycine, Glutamine, Valine, Serine and Aspartic acid (**Table 4.1**). Glycine, Serine, valine and Threonine are essential amino acids for the ants and were found in all ant workers (**Table 4.1**). However, ^{15}N incorporation showed different incorporation for mutualistic ants (*P. ferrugineus*), when *P. ferrugineus* lived in *A. hindsii* their ^{15}N incorporation varies from two until ten times of ^{15}N isotope when this mutualistic ant lived in *A. cornigera* plants, however, exploiter ant workers present dramatically high percent of

^{15}N-incorporation in the hemolymph amino acids, except in Glutamine or Aspartic acid that we not found in any replicate (**Table 4.1**).

Table 4.1. ^{15}N incorporated in amino acids from hemolymph in ant workers.

	Control urea[1]			**^{15}N-Urea[2]**		
	Pseudomyrmex			***Pseudomyrmex***		
AA[3]	***Pf Ac***	***Pf Ah***	***Pg Ah***	***Pf Ac***	***Pf Ah***	***Pg Ah***
Proline	0.0047	0.0030	0.0022	1.1351	11.2981	15.3068
Alanine	0.0023	0.0076	0.0066	4.7862	9.3388	19.2921
Threonine*	0.0049	0.0046	0.0041	3.6364	4.5834	12.3193
Glycine*	0.0047	0.0046	0.0014	5.9149	12.9058	13.9379
Glutamine	0.0029	0.0059	N.D	2.3356	4.8716	N.D
Valine*	0.0043	0.0042	0.0007	1.3219	13.1265	7.9618
Serine*	0.0038	0.0039	0.0054	2.2179	15.9139	9.2989
Aspartic	0.0022	N.D	N.D	3.6259	N.D	N.D

Ant fed with urea[1] without ^{15}N and ^{15}N-labelled urea (98 atom % ^{15}N)[2]. Amino acids found in ant hemolymph[3] in *Pf*: *P. ferrugineus* or *Pg*: *P. gracilis*. *Ac*: *A. cornigera*; *Ah*: *A. hindsii*. *Essential amino acids for the insects. ND: not determined.

4.5. Discussion

Studies on different organism were published over the last four decades and reported the presence of intestinal or other types of endosymbiotic bacteria in insects that carry *nifH* (Buchner 1965, French *et al.* 1976, Kneip *et al.* 2007, Lilburn *et al.* 2001). By contrast, reports on an actual fixation of N from the atmosphere by endosymbiotic bacteria are scarce or absent. In fact, the conditions in the intestine (low vapor pressure of gaseous N) might disfavor the fixation of N. What is, then, the ecological role of these bacteria? Here we present evidence that ants that carry endosymbiotic bacteria with *nifH* are capable of recycling N from intestinal urea and that this N shows up in metabolites of the ant host. When living ants were fed ^{15}N-urea they exhibited ^{15}N-marked amino acids in the

hemolymph, a liquid that contain endosymbiotic bacteria but with unknown function (Gunawan *et al.* 2008). Thus, the ^{15}N from the urea was found in molecules that could be metabolites of the ants. Urease is a common enzyme of bacteria but has not been described for insects so far (Balasubramanian *et al.* 2013, McDonald *et al.* 1980, Mobley and Hausinger 1989, Wiebke-Strohm *et al.* 2012). Thus, we conclude that endosymbiotic bacteria were responsible for this recycling effect.

Arboreal ants only consume plant-derived food sources for their nutrition, specifically, acacia-ant plants, produce food rewards as cellular FBs and EFN but, FBs are only for nourish the ant larvae and EFN is consumed by adult ants (Janzen 1966, 1967, 1974). However, most ants can utilize a large variety of food sources for their ecological and evolutionary success, by these reasons is difficult to study them with respect to nutritional ecology (Feldhaar *et al.* 2010).

Nitrogen fixation has been probed in insects that lived in nitrogen-poor diets as termites species (Benemann 1973, Breznak *et al.* 1973), bark beetles (Kuranouchi *et al.* 2006, Morales-Jiménez *et al.* 2009, 2013) and fruit fly (Behar *et al.* 2005) with acetylene reduction activity assays. In this work, live larvae and workers of *P. ferrugineus* ants have been exposed to an acetylene atmosphere, and we not detected acetylene reduction in AR assay, by the way, ant larvae of *P. gracilis* produce highly amount of ethylene and occurs the same pattern for adult workers. However similar results were obtained, when we used isotopic ^{15}N-atmosphere, larvae of the generalist ants can incorporate isotopic N in their body mass under field or laboratory conditions (**Figures 4.3-4.4**). These findings show that mutualistic *P. ferrugineus* ant workers not obtain nitrogen or N_2-fixation is null from midgut endosymbiont, by contrast, generalist *P. gracilis* obtain supplemental nitrogen in

high percent by these diazotrophs. *In vivo* assays in *Atta cephalotes* ant colonies, presented that the ants only reduce minimal acethylene, and the ants acquire additional nitrogen from symbiont fixers (Pinto-Tomas *et al.* 2009). Insect gut endosymbionts play an essential role in the insect adaptation as: food types, compound detoxification, synthesis of vitamins and amino acids and defence against pathogens proliferation (Brownlie and Johnson 2009, de Souza *et al.* 2009, Ishak *et al.* 2011, Lai *et al.* 1996, Scott *et al.* 2008, Shi *et al.* 2013, Zientz *et al.* 2004). To locate the specific endosymbiotic fixers, midgut of ant workers were plated, and five strains were isolated and were checked to *nifH* gene. *nifH* gene was found in all strains isolated and both and midgut workers, these findings reveal that strains can putatively be N_2-nitrogen fixers at least in part. Strikingly bacterial associated to mutualistic *P. ferrugineus* have been described, and different taxas were found, in addition many of these bacteria detected belong to purportedly N-fixing to the host (Eilmus and Heil 2009), however the individual percentage of N_2-fixers in the midgut of this ant is missing.

It has been discussed that endosymbionts of ant midgut provide any form of N, but in the last years appoint to the N-recycling contributing significantly in the body mass of the ant hosts (Anderson *et al.* 2012, Feldhaar *et al.* 2007, 2010). To analyze the contribution of ureases, we conducted urease zymography after mutualistic and generalist ant workers were fed with urea. Generalist worker ants showed highly urease activity and mutualistic workers only presented low activity of this enzyme in the zymogram gel (**Figure 4.5**), one band of ≈ 90 kDa was similar in the samples of mutualistic and generalist ant workers, but a second band was unique in generalist workers with molecular weight of ≈ 180 kDa (**Figure 4.5**). The plant, fungal and bacterial ureases have been reporter similar molecular weight of 90 and 200 kDa (Hirayama *et al.* 2000, Kurahashi *et al.* 2005, Mobley and Hausinger 1989,

Precious *et al.* 1987), similar as our findings. A specific insect urease enzyme is not reported, and then with isolated bacteria from ant worker midguts, we confirm the presence of *ureC* gene. These results may be explained the presence of the bacterial urease activity in the midgut be result in nitrogen contribution of these ants.

Urease function was indirectly evaluated with independent ant colonies, that were fed with ^{15}N-urea or urea as control, after the period of urea metabolism, ant hemolymph were obtained and processed by GC-MS, and we observed that mutualistic ant incorporate different signature in its amino acids of hemolymph, mutualistic ants that lived in *A. hindsii* plants obtain high incorporation of isotopic N than mutualistic ant that lived in *A. cornigera* plants, by contrast generalist ant worker increase more the signature of ^{15}N in their amino acids than the mutualistic ants (**Table 4.1**). Essential amino acids as threonine, serine and glycine were found in all samples, and these amino acids are involucrate in vital function of the ants. Urease enzyme is important for ants, because when urea is produced by metabolic process by the host, act as substrate for the micro-organisms, the microbial urease hydrolyze urea and is converted to CO_2 and ammonium, next, glutamine synthetase is required for the incorporation of ammonium and this molecule is necessary for the synthesis of amino acids which may be required by the ant host (Zientz *et al.* 2005, 2006). Our results of feeding experiments can be support the idea that endosymbiont provide to the ants host with essential amino acid release in the ants hemolymph for their nutrition. Similar results were obtained when *Camponotus ants* were fed with isotopic urea and apparently the endosymbiotic bacteria upgrading the diet of the ant by N-recycling via a functional urease (Feldhaar *et al.* 2007, Shetty 1982).

Mutualistic ants apparently not need fixing nitrogen, because the ant larvae consumed a balanced FBs, rich in amino acids and proteins, and the ant larvae secreted different proteases for optimal digestion and development (Orona-Tamayo *et al.* 2013). Ant workers consume the EFN rich in carbohydrates and amino acids that produce high amount of energy for their ant behavior, but in part, nectarin proteins could be support the N-balance in the mutualistic workers, and this nutrition is supported by nitrogen recycling. By the way, generalist *P. gracilis* workers consume FBs and EFN, but also foragers insect for their nutrition, and could be explain why this ant only obtain less amount of nitrogen by recycling.

4.6. Conclusions

Bacterial diversity harbored in the midgut of the mutualistic and generalist ants, depend strictly on the hosts lifestyle, habitat and diet. This appears to be possible because *P. ferrugineus* ants depend of the derived plant food rewards strictly and *P. gracilis* relies less of this food resources and reduce the dependence of the rewards produced by the host plant. Both species are congeneric and inhabit the same hosts, causing different ecological lifestyles and their nutrimental conditions depend of each food that consume and digest.

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Chapter 5

Stabilizing mutualisms threatened by exploiters: new insights from ant–plant research

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5.1. Abstract

Mutualisms are commonly threatened by parasites and cheaters: species that exploit the host–derived resources without providing an adequate service. Here, we summarize mechanisms for the stabilization of obligate defensive ant–plant mutualisms, a typical element of tropical lowland forests. Host plants exert partner choice and can sanction non–defending ants by shedding the domatia that serve as nesting space or ceasing the production of ant rewards. Hosts can also restrict the exploitation of the ant rewards by means of specific biochemical traits that decrease their quality for non–adapted generalist exploiters and, thus, convert them into exclusive rewards. Reward provisioning can even shift the competitive balance between mutualists and exploiters in favour of the mutualists. In turn, plant–ants show adaptations in their colony structure and changes in their digestive capacities that enhance their efficiency in the use of the host–derived resources. Founding queens use plant odours for host choice behaviour, and ants not supplied with adequate amounts of EFN decrease their defensive service and thereby exert partner sanctions. Theoretical models and empirical research into mutualisms usually focus on actions that are taken by the host. Using ant–plants as model systems, we are now discovering the importance of contributions that come from the symbiont. This discovery indicates the potential for multiple reciprocal interactions between phenotypically plastic hosts and symbionts, which contribute significantly to what is still considered a miracle: the stability of mutualisms in the presence of exploiters.

5.2. Introduction

Ant–plant interactions represent a typical element in tropical forests. Facultative interactions between ants and plants exist in virtually all terrestrial ecosystems besides arctic regions and high mountains, but obligate interactions between so–called myrmecophytes and their (usually highly specialized) ant inhabitants are restricted to the tropics (Heil and McKey 2003). The obligate defensive mutualisms on which we focus here comprise host plants that provide nesting space (in hollow structures termed domatia) and usually also produce extrafloral nectar (EFN) or cellular food bodies (FBs) as food rewards. In some systems, scale insects form part of the mutualistic system and contribute to the nutrition of the ants (Defossez *et al.* 2009, Gaume *et al.* 2000, Heckroth *et al.* 1998, Janzen 1966). In turn, the ants protect their host from herbivores, pathogens and encroaching vegetation. These myrmecophytes commonly are pioneer trees that can completely dominate secondary forests, likely because of the highly efficient defence against biotic stress that resident ant colonies provide to their hosts (Chamberlain and Holland 2009, Heil 2008, Koricheva and Romero 2012, Romero and Koricheva 2011, Rosumek *et al.* 2009).

Like all mutualisms, ant–plant mutualisms are prone to exploitation by non–reciprocating exploiters, *i.e.*, species that inhabit the host and utilize the resources that serve to reward the symbiont, however without paying for the service (Bronstein 2001, Yu 2001). Exploiters save the time and energy that mutualists spend on reciprocating. Therefore, many authors consider them as competitively superior, at least as long as resource availability remains unchanged. Exploiters can evolve from former mutualists that cease service provisioning and then represent cheaters, or they can invade the mutualism starting from an originally independent lifestyle and then represent parasites of the mutualism (Bronstein 2001, Kautz

et al. 2009a, Segraves *et al.* 2005). Exploitation has been reported for a wide range of mutualisms, including nectar robbing (e.g. bees and birds) (Maloof and Inouye 2000, Roubik 1982), domatia exploiters (Gaume *et al.* 2006, Shenoy and Borges 2008), mycorrhizal fungi that uptake the plant carbon but transfer no nutrients to the plant (Smith *et al.* 1996), and strains of *Rhizobium* and *Bradyrhizobium* that transfer less or no nitrogen to the host than mutualistic strains (Wilkinson *et al.* 1996). In defensive ant–plant mutualisms, *Phyllobaenus* beetles exploit the shelter and food rewards produced by *Piper* plants (Letourneau 1983), a foraging spider (*Bagheera kiplingi*) that lives in the hollow spines of Mexican acacias uses plant–derived food body rewards for its nutrition (Meehan *et al.* 2009), several ants inhabit the plant without providing a specific defensive service (Clement *et al.* 2008, Janzen 1975, Raine *et al.* 2004), and even ants that efficiently fend off herbivores can damage their host by manipulating its reproductive efforts for their own benefits (Gaume *et al.* 2005a, Izzo and Vasconcelos 2002, Yu and Pierce 1998).

Research into the stability of mutualisms has traditionally focused on pollination and the root – Rhizobia and root – mycorrhiza mutualism. However, ant–plant interactions are increasingly being used to study the underlying mechanisms (Bronstein 1998, Heil and McKey 2003). In this review, we first give a short introduction to the different mechanisms that have been suggested for the stabilization of mutualisms, then present exploiters that are commonly reported for ant–plant mutualisms and lastly discuss in detail which stabilizing mechanisms have been rejected, corrected, confirmed, or even discovered, in the research into ant–plant mutualisms. We conclude with a short outlook discussing how likely the mechanisms that have been described for ant–plant interactions are to stabilize also other, non ant–plant and non tropical mutualisms.

5.2.1. Concepts in the stabilization of mutualisms

Theoretical research distinguishes among five main strategies by which mutualisms can be stabilized against exploitation. Unfortunately, different authors make different assumptions and different use of central terms, for which reason these concepts are under heavy and controversial discussion (Kiers *et al.* 2011a, Weyl *et al.* 2010, 2011). In the following, we shortly introduce the most commonly reported stabilizing mechanisms as they have been described for other mutualisms and define the terms as we will use them in the present article.

5.2.2. Long-spurred orchids and exclusive rewards

Mechanical barriers can represent the primary means to restrict access to a reward and protect it from exploitation. The long nectary spurs of *Angraecum sesquipedale*, a Malagasy orchid, make floral nectar inaccessible to most insects and thus turn it into an exclusive reward for a specialized, long–tongued hawkmoth (Darwin 1862), thereby increasing the probability of pollen transfer (Rodríguez-Gironés and Santamaría 2007). This orchid represents a classical example of the outcome of a co–evolutionary process (Martins and Johnson 2007). Subsequent studies confirmed the importance of anatomical structures such as long corolla tubes or nectar spurs for the effective protection of floral nectar from nectar robbers (Ellis and Johnson 2010, Johnson and Steiner 2000, Micheneau *et al.* 2009, Sletvold and Ågren 2010).

5.2.3. Partner choice

Partner choice refers to the selection of suitable future partners before the symbiosis is being established and is usually based on specific characteristics of the potential symbiont (Bull and Rice 1991). Partner choice allows host individuals to differentially reward cooperative vs. uncooperative partners in advance of any possible exploitation (Bull and Rice 1991). This phenomenon has been studied particularly well for the root–Rhizobia mutualism, in which Rhizobia strains must identify themselves as potential mutualists via the secretion of specific chemical signals (Nod factors) (Oldroyd 2001, Oldroyd and Downie 2008, Simms *et al.* 2006). The importance of partner choice mechanisms, however, has also been demonstrated for other mutualism, e.g. for the cleaner fish–host interaction (Bshary and Grutter 2002), plant–pollinator (Bull and Rice 1991), plant–mycorrhiza (Kiers *et al.* 2011b) and the ant–fungus interaction (Mueller *et al.* 2004, Zhang *et al.* 2007).

5.2.4. Host sanctions

Host sanctions apply when the symbiosis has already been established. Here, the host monitors the action of its symbiont in order to punish exploiters, usually via a reduction in reward provisioning. For example, plant roots cease the allocation of assimilates towards nodules formed by non N–fixing bacteria or mycorrhizal fungi that turn pathogenic (Kiers and Denison 2008, Kiers *et al.* 2011b, 2003, West *et al.* 2002). A recent study demonstrated that host sanctions can even function when one host is interacting with multiple symbionts of different quality, which demonstrates an impressive level of specificity of this strategy, at least in the fig–fig wasp mutualism (Jandér *et al.* 2012).

5.2.5. Partner fidelity feedback

The concept of mutualism is based on the major idea that each partner benefits from the interaction with the other partner. The benefits provided by partner 1 to partner 2 automatically feed back to partner 1 always when the quantity or quality of the resources provided to partner 1 depend on the performance or vigour of partner 2 (Connor 1986, Sachs *et al.* 2004). Therefore, several authors assume that natural selection favours mutualists over cheaters simply because partner fidelity feedback inevitable reduced the fitness of cheaters (Weyl *et al.* 2010).

5.2.6. Competitive screening

Partner choice as well as host sanctions require that hosts can evaluate the identity or the actions of their symbionts, whereas partner fidelity feedback occurs as an inevitable consequence of the negative effects that the failure to reciprocate has on the other partner. Recently, partner screening has been proposed as an alternative mechanism for the stabilization of mutualisms (Archetti *et al.* 2011a, 2011b). Partner screening means that the host imposes a 'contract' that consists of appropriate costs and benefits of being a mutualist. The decision of the potential symbiont whether or not to make this investment depends on the net benefits that it can gain in the mutualism, thereby causing the potential symbionts to screen themselves according to their quality as mutualists. Only the adapted mutualists might gain sufficient benefit from engaging in the interaction to make it attractive for them to pay the cost of entry. This model does not require hosts to have any direct information on the quality of the symbionts.

5.2.7. Exploiters of ant-plant mutualisms

Ant–plant mutualisms are transmitted horizontally, usually comprise more than one species of both host and symbiont and depend commonly on rewards that are presented openly on the plant surface. All these characteristics make ant–plant mutualisms particularly prone to invasions by taxonomically and functionally different exploiters. In this respect, ant–plant mutualisms resemble the general case, because the vast majority of exploiters are generalist foragers, such as pollen and nectar robbers, which transiently feed on rewards when these are available (Addicott 1986, Jackson 2009, West *et al.* 1996). In the following sections, we shortly summarize the types of exploiters that most commonly have been reported for ant–plant mutualisms.

5.2.8. Non-defending ants

The most important service that plant–ants provide to their hosts is the defence against biotic stressors such as herbivores and pathogens. Facultative as well as obligate ant–plant mutualisms can include more than one ant species, and ants vary in their patrolling behaviour and defensive efficiency (Bruna *et al.* 2004, Debout *et al.* 2005, Fiala *et al.* 1994, Frederickson 2005, Gaume *et al.* 2005b, 2006, Hossaert-McKey *et al.* 2001, Palmer *et al.* 2002, 2003, Xu and Chen 2010). Although under certain circumstances it can be beneficial for the plant to associate with a variety of different species of ants (Labeyrie *et al.* 2001, Palmer *et al.* 2010), the differences in their defensive quality make some ants more desirable symbionts than others. Species such as *Camponotus planatus* (Raine *et al.* 2004),

Pseudomyrmex nigropilosus and *P. gracilis* (Clement *et al.* 2008, Janzen 1975) inhabit *Acacia* myrmecophytes without providing them with an efficient protection (**Figure 5.1**), *Cataulacus mckeyi* exploits *Leonardoxa africana* (Gaume and McKey 1999), and several species of *Cephalotes* and *Crematogaster* act as non–defending parasites of *Cordia alliodora* (Tillberg 2004).

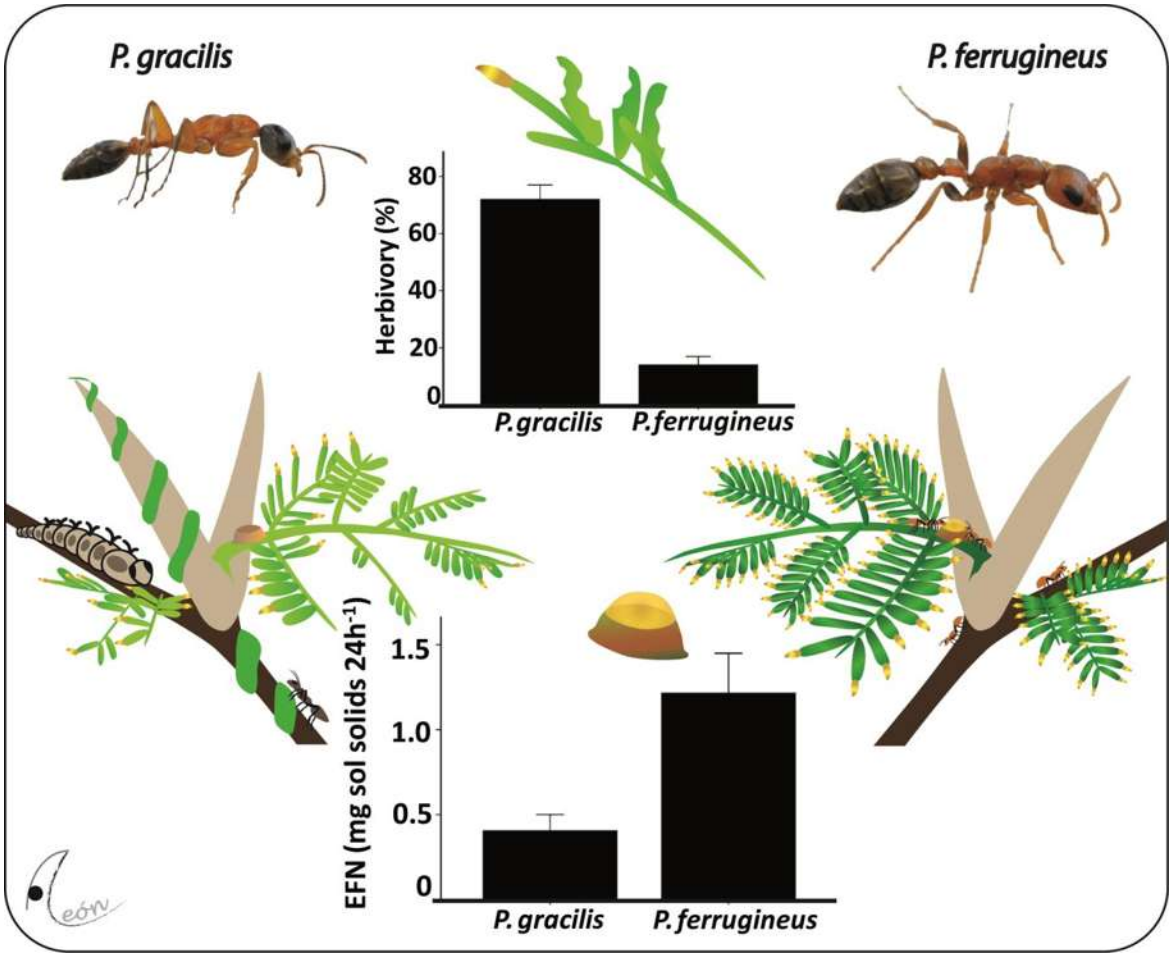

Figure 5.1. An exploiter ant and its effect on the host plant: Mesoamerican ant acacias can be inhabited by exploiter ants (*P. gracilis*, left panel) which lack a specific defensive behaviour and therefore cause high rates of leaf damage. By contrast, mutualistic *P. ferrugineus* ants protect their host against herbivory. Both ant species feed on food bodies (FBs) and extrafloral nectar (EFN), although plants inhabited by the exploiter, *P. gracilis*, appear to reduce reward production and thereby exert host sanctions. Host sanctions by bull's horn acacias: Mesoamerican ant acacias reduce the production of ant rewards [extrafloral nectar, EFN: mean secretion as milligrams (mg) of soluble (sol) solids] when being inhabited by the parasitic ant, *P. gracilis* (left

panel), whereas high amounts of rewards are provided to the mutualist, *P. ferrugineus* (right panel) (Clement *et al.* 2008, Heil *et al.* 2009).

However most ants are foragers and protect their food sources, and particularly the surroundings of their nest, from potential competitors or invaders. Thus, the presence of any type of ant colony on a plant will usually cause some protective effect, as compared to the completely ant–free plant. It therefore remains an open question whether these ants represent true exploiters or should better be understood as 'less efficient mutualists'. Still the parasitic ants can replace the good defenders under certain circumstances (Heil 2013) and their presence reduces the probability at which a host will be colonized by a high–quality mutualist (Clement *et al.* 2008, 2009). We argue, therefore, that non–defending ants are correctly understood as exploiters of obligate ant–plant mutualisms.

5.2.9. Castration parasites

In some ant–plant interactions, the ant castrates the host, meaning that it physically removes the reproductive organs (Baudoin 1974, Frederickson 2005). Although final experimental evidence is missing, this behaviour is commonly interpreted as shifting the allocation patterns in the plant from reproductive to vegetative growth and, thus, to the production of more ant rewards. For example, workers of *Allomerus octoarticulatus* protect the new leaves of *Cordia nodosa* from herbivory, which directly benefits the ants themselves because they live in stem domatia, one of which is produced with every new shoot. The ants, however, destroy flowers and thereby in most cases reduce the reproduction of their host (Edwards and Yu 2008, Szilágyi *et al.* 2009). Moreover, since

Allomerus prevents their host from associating with the good mutualist, ants of the genus *Azteca*, its presence imposes a high cost to the host and *Allomerus* clearly represents a parasite of the *Cordia–Azteca* mutualism (Frederickson 2009, Yu and Pierce 1998). Interestingly, the same ant species (*Allomerus octoarticulatus*) has been reported as an obligate mutualist of *Hirtella* plants, which use the abortion of leaves and domatia as strategy of development: this species apparently employs host sanctions to prevent its inhabitant from cheating (Izzo and Vasconcelos 2002). In fact, ants due to their general aggressive behaviour will usually reduce pollinator visits to the flowers of their host plants, for which reason it appears likely that all ant–defended plants require strategies to control ant–pollinator conflicts (Adler 2000, Gaume *et al.* 2005a, Ghazoul 2001, Heil 2011, Hernández-Cumplido *et al.* 2010, Holland *et al.* 2011, Raine *et al.* 2002, Willmer and Stone 1997).

5.2.10. Exploiters of food rewards

Most myrmecophytes provide their ants with FBs, EFN, or both. FBs contain lipids and proteins and both types of ant rewards are rich in carbohydrates, thus appearing to be adapted to specifically serve as the major or exclusive food source for animals (Fischer *et al.* 2002, Heil *et al.* 1998).Thus, ant rewards represent highly attractive targets for all types of exploiters: animals that simply use these rewards for their own nutrition, without interacting in any other way with the plant. Whereas 'non–defending ants' might provide their host plant with at least some protective effect, these exploiters simply reduce the amount of rewards that are available to the mutualist ants. A classic example in this category is a species of *Phyllobaenus* beetles that prey on ant inhabitants of *Piper* plants

and also feed on the FBs that are produced by these plants, even being capable of chemically inducing the production of this reward (Letourneau 1990). Similarly, the spider, *Bagheera kiplingi*, preys on *Pseudomyrmex* ants that inhabit *Acacia* myrmecophytes and also feeds on the FBs (Jackson 2009, Meehan *et al.* 2009). Moreover, bees and wasps might act as robbers of EFN (González-Teuber *et al.* 2012).

Less conspicuous but perhaps quantitatively more important are microorganisms: yeasts and bacteria that infest EFN (González-Teuber and Heil 2009). Yeasts are commonly reported for floral nectars, which they use as a growing medium and chemically alter due to their own metabolism (Canto and Herrera 2012, Canto *et al.* 2008, Herrera *et al.* 2009, 2010). Therefore, many types of nectar contain specific hydrolytic proteins to prevent them from microbial infestation (Carter and Thornburg 2000, 2004, Thornburg *et al.* 2003). The recent discovery of chitinases and glucanases in the EFN of *Acacia* myrmecophytes (González-Teuber *et al.* 2009, 2010) indicates that EFN–infecting microorganisms might also represent common exploiters of ant–plant mutualisms.

5.2.11. Protecting ant-plant mutualisms from exploiters

Mutualistic ants show multiple adaptations allowing them to more successfully compete for, and ultimately dominate, their host plants. The pruning of lianas and encroaching vegetation provides us with a nice example of how the symbiont can help to make its rewards exclusive. *Pseudomyrmex dendroicus*, a mutualist of *Triplaris americana* plants, prunes the shoots and petioles of plants in contact with its host and thereby reduces the danger of invasion by *Crematogaster* exploiters (Davidson *et al.* 1988). Similarly,

Crematogaster (*Decacrema*) ants living on waxy *Macaranga* ant–plants prune more intensively when they live on a less waxy species on which most ants could walk easily (Federle *et al.* 2002).

However, exclusive rewards usually represent an adaptation of the host. The workers of the exploiter ant, *Camponotus planatus*, which inhabits the thorns of *Acacia mayana* and feeds on its EFN, do not harvest the FBs (Raine *et al.* 2004). This example nicely illustrates that, in spite of their high nutrient contents, ant rewards are not invariably suitable for specialised mutualists and generalist exploiters. In fact, FBs of mesoamerican ant acacias contain protease inhibitors (PIs) (Wielsch *et al.* 2011) that successfully inhibit protease activity in the digestive tracts of seed–feeding beetles and likely other non–specialist exploiters (**Figure 5.2**)

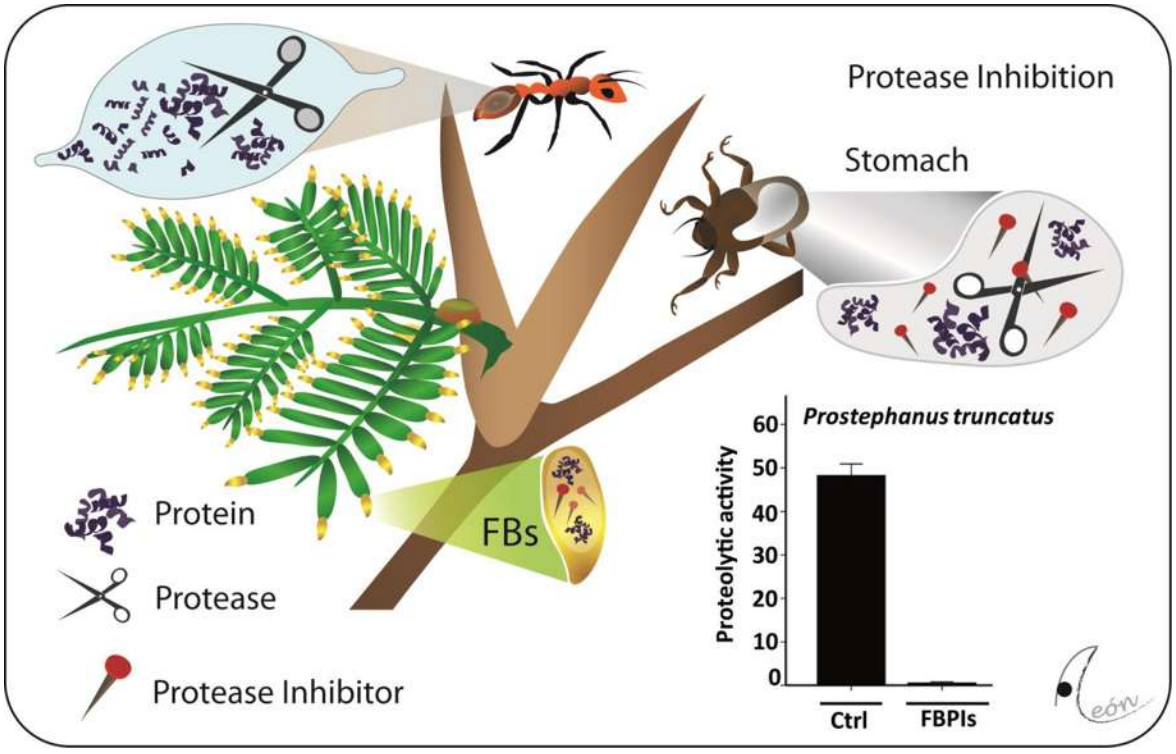

Figure 5.2. *Acacia* food bodies are exclusive rewards: The food bodies (FBs) produced by Mesoamerican ant–acacias contain protease inhibitors (PIs) that reduce proteolytic activity (graphical inset; Ctl: control;

FBPIs: Food Body Protease Inhibitors) in the digestive tracts of non–adapted potential exploiters whereas they do not affect the digestive capacities of the mutualist ant, *P. ferrugineus* (Orona-Tamayo *et al.* 2013a, Wielsch *et al.* 2011).

By contrast, the proteases of the mutualist, *Pseudomyrmex ferrugineus*, appeared insensitive to this inhibitory activity (Orona-Tamayo *et al.* 2013a). As mentioned above, the same *Acacia* species protect their EFN from nectar–infecting microorganisms with pathogenesis–related proteins such as glucanases and chitinases (González-Teuber *et al.* 2009, 2010). This EFN is further protected by a secreted invertase that keeps it free of sucrose and, thus, unattractive for local generalist ants (Heil *et al.* 2005). The mutualist ant, in turn, lacks invertase in the adult stage (Kautz *et al.* 2009a) and thus prefers sucrose–free EFN in direct choice tests (Heil *et al.* 2005). Finally, the ant acacias secrete the EFN only during a few hours per day, which facilitates its protection from exploiters (González-Teuber *et al.* 2012, Raine *et al.* 2002). These short peaks are physiologically achieved by the concurrent synthesis of the entire enzymatic machinery for nectar synthesis and secretion, directly before and during the hours of active secretion (Orona-Tamayo *et al.* 2013b).

In summary, the rewards produced by mesoamerican ant acacias show multiple chemical and physiological characteristics that make them an almost exclusive food source for the resident mutualists, although the existence of exploiter ants and FB–robbing spiders shows that no protection is perfect. Unfortunately, comparable research into the detailed composition of other ant rewards is lacking, which makes it impossible to decide whether exclusive rewards represent a general characteristic of myrmecophytes. Phenomena such as toxic floral nectars (Adler 2000, Adler and Irwin 2005) and specific ant–attractants in

elaiosomes (Boieiro *et al.* 2012, Brew *et al.* 1989, Fischer *et al.* 2008, Hughes *et al.* 1994), however, make it tempting to assume that all ant rewards possess a chemical composition that enhances their level of exclusiveness.

5.2.12. Partner choice and host choice in the establishment of ant-plant mutualism

Choosing the right partner represents a crucial step in the establishment of all mutualisms. Evidently, both partners have an interest in taking this decision correctly. Morphological structures such as self–opening entrances to domatia occur in some myrmecophytes (Brouat *et al.* 2001, Moog *et al.* 2002) and can have features such as entrance slit sizes that exclude most ants from entering the pre–formed domatia (Brouat *et al.*2001). The physico–chemical characteristics of the cuticular waxes of *Macaranga* myrmecophytes (Dragota and Riederer 2007, Müller and Riederer 2005) also contribute to partner choice because only specific mutualistic ants can walk on the stems and branches that are covered by these waxes (Federle *et al.* 1997, 2002).

Research into ant–plant mutualisms has also discovered the phenomenon of host choice, which is exerted by the symbiont. Colony–founding ant queens ('foundresses') shed their wings as soon as they have decided on a specific nesting site (Hölldobler and Wilson 1990). Thus, foundresses of plant–ants cannot easily correct their decision for a certain host. Myrmecophytes (hosts) often are closely related to non–myrmecophytes (non–hosts) [see (Bänfer *et al.* 2004, Blattner *et al.* 2001) for a phylogeny of myrmecophytic *Macaranga* species and (Heil *et al.* 2004) for Mesoamerican acacias]. Even myrmecophytes in the same genus, which in principle represent suitable hosts for a certain ant species, can strongly

differ in their quality as a host (Heil *et al.* 2009), and nutrient availability and light conditions further define how much ant rewards an individual plant can produce (Bixenmann *et al.* 2011, Heil *et al.* 2002). Thus, foundresses of plant–ants must be able to distinguish hosts from non–hosts and good hosts from bad hosts. Because plant ants usually swarm at night and find their host over comparably large distances (Türke *et al.* 2010), many of them use volatile organic compounds (VOCs) in their host–finding behaviour. The queens of both *Azteca* and *Allomerus* ants are attracted to the VOCs emitted by *Cordia nodosa* plants, which suggests that they use olfactory cues for the long range detection of their host plants (Edwards *et al.* 2006a). Similarly, *Crematogaster* foundresses are able to distinguish among the odours emitted from myrmecophytic and non–myrmecophytic *Macaranga* plants (Jürgens *et al.* 2006), and *Pheidole* queens use volatiles to distinguish their *Maieta* hosts from other, sympatric myrmecophytes (Dáttilo *et al.* 2009). *Pseudomyrmex triplarinus* ants use cuticular compounds to distinguish their host tree *Triplaris americana* from non–host plant in the context of their pruning behaviour (Weir *et al.* 2012). These signals could also be involved in host recognition for colony founding, although they are unlikely to serve as long-distance signals.

The fact that foundresses use plant odours to localize their hosts raises the question regarding the degree to which they can judge the quality of a plant as a future host. Recent olfactometer experiments demonstrated that foundresses of *Pseudomyrmex ferrugineus* can use VOCs to distinguish their host *Acacia* plants from non–host species (**Figure 5.3**). VOCs also allowed them to prefer a high–reward *Acacia* species over a low–reward species and healthy plants with high levels of reward production over damaged plants that produce lower amounts of EFN and FBs (Razo-Belmán and Heil 2012). In conclusion, queens of

plant–ants clearly show host choice behaviour that is based on VOCs, at least to some degree.

5.2.13. Host sanctions? Plants shed domatia when they are not being defended

Because obligate ant–plants nest in domatia which are localised in hollow shoots, thorns, or leaf pouches, the growth of the ant colony and thus its potential reproductive success depend strongly on the vegetative growth rate of their host plant (Fonseca 1993, 1994, Frederickson and Gordon 2009).

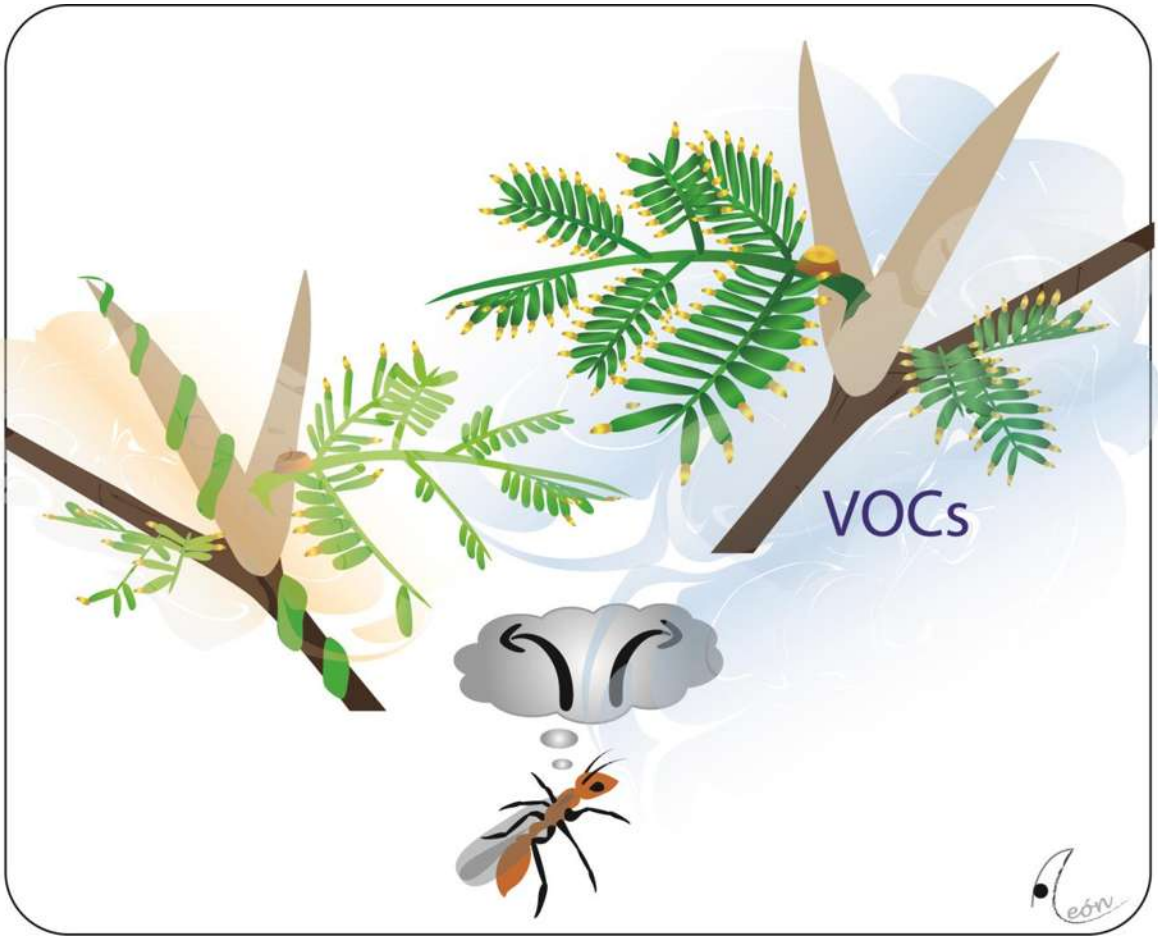

Figure 5.3. Host choice by foundresses of mutualistic ants: Colony–founding queens of the mutualistic ant, *P. ferrugineus*, use volatile organic compounds (VOCs) that are emitted from their host to choose healthy individuals of a high–reward host species (Razo-Belmán and Heil 2012).

Although this situation apparently enhances the temptation to cheat (see 'castration mutualisms'), it also provides the plant with a possibility to punish inefficient exploiters. *Hirtella myrmecophila* produces leaf–pouches in the young parts of the branches and *Allomerus octoarticulatus* ants use these structures as domatia. *Hirtella* plants can use the abortion of domatia in older leaves as a strategy of host sanction mechanism (Izzo and Vasconcelos 2002). Similarly, simulated herbivory caused *Cordia nodosa* plants to shed stem domatia and thereby punishes non–defending inhabitants (Edwards *et al.* 2006b). However, it has been questioned (Weyl *et al.* 2010) whether these responses represent true sanction mechanism or should be better understood as effects of partner fidelity feedback (see below).

5.2.14. Competitive screening

Myrmecophytes in most genera can associate with more than one ant species and direct competition among ant colonies represents a crucial determinant of the colony that can dominate a certain host (Davidson and Snelling 1989, Debout *et al.* 2005, Palmer 2003, Palmer *et al.* 2003, Raine *et al.* 2004). In adaptation to this situation, some species of plant–ants have evolved cooperative colony founding (Izzo *et al.* 2009) or secondary polygyny (Dalecky *et al.* 2005, Feldhaar *et al.* 2000, 2005, Kautz *et al.* 2009b), the first adaptation facilitating colony establishment, the second one likely allowing an individual colony to occupy a given host longer than it would be allowed by the lifetime of a single queen.

Competition among foundresses represents an important determinant in the ant–plant mutualism, which opens the question whether myrmecophytes can employ competitive

screening to associate preferably with good partners. An ideal test of screening would be to manipulate the cost of entry into the host and observe the subsequent evolutionary trajectories of mutualists and parasites (D. Yu, pers comm.). In practice, the mesoamerican ant–acacias with their positive assortment of high–reward hosts with high–quality mutualists (Heil *et al.* 2009) represent a natural experiment of this type. Indeed, observations over seven months demonstrated that mutualists are more likely than exploiters to finally dominate young plants of a high–reward species. Even within one species, the frequency at which mutualists could outcompete the exploiters was positively correlated to the initial rate of reward production by the individual plants (Heil 2013). Because more aggressive ants generally represent the better defenders (Xu and Chen 2010) and because the aggressiveness of most ant workers is limited by energy supply (see below), ant–plants can use the fact that the adapted mutualists are more efficient in making use of the specific food rewards to shift competitive balances in favour of the mutualists.

5.2.15. Sanctions, reciprocal rewarding or partner fidelity feedback? Lessons from ant-plant research

Most myrmecophytes reduce the amounts of FBs or EFN produced when they are not well defended by their resident ants. In some cases, the presence of ants *per se* can induce FB production via an as–yet unknown chemical mechanism (Heil *et al.* 1997, Risch and Rickson 1981). Similarly, mesoamerican acacias reduce EFN secretion when they are inhabited by the exploiter ant, *Pseudomyrmex gracilis* (Heil *et al.* 2009). Alternatively, the harvesting of the FBs can directly enhance production rates (Folgarait *et al.* 1994), as it is also commonly being reported for nectar (Heil 2011): plants respond to an accumulation of

EFN on the nectary with a decrease in its *de novo* production (Bixenmann *et al.* 2011, Heil *et al.* 2000) or perhaps even with its re–absorption (Escalante-Pérez *et al.* 2012). The crucial involvement of invertase (catalyzing the hydrolysis of sucrose to yield glucose and fructose) in the secretion of both floral and extrafloral nectar (Orona-Tamayo *et al.* 2013b, Ruhlmann *et al.* 2010) provides a possible mechanism for the re–absorption of monosaccharides that accumulate on the outside of a nectary.

The ants, however, are not passive consumers in these systems. Colony growth of the ants responds positively to FB supply (Itino *et al.* 2001), and plant–ants that are supplied with more EFN immediately enhance their aggressive behaviour and, thus, their protective efficacy (González-Teuber *et al.* 2012). That is, both ant–plants and plant–ants exhibit phenotypic plasticity in the provisioning of rewards and services that amount to an efficient reciprocal rewarding system.

In most mutualisms, partner choice appears to be realized by the host. By contrast, studies on ant–plant mutualisms commonly describe the phenomenon of host choice, which is exerted by the symbiont (**Figure 5.3**). Moreover, the efficiency of ant–mediated defence depends on the amount (and, likely, the quality) of rewards that ants receive (**Figure 5.4**), a situation which can be described as partner sanctions. Consequently, research into ant–plant mutualisms demonstrates that actions and characteristics of the symbiont strongly contribute to the stable association of good hosts with high quality symbionts.

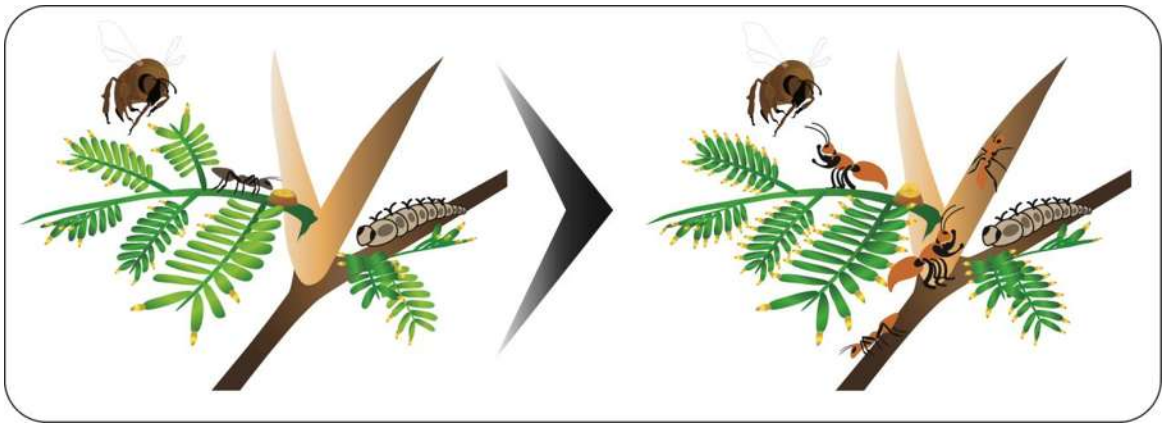

Figure 5.4. Partner sanctions by mutualistic ants: Adapted mutualist ants (*P. ferrugineus*) respond to the level of reward provisioning by their *Acacia* host and defend their host more strongly against nectar robbers (such as bees) and herbivores (such as caterpillars) and – likely – exploiter ants when they receive more rewards, particularly extrafloral nectar (EFN) (Clement *et al.* 2008, Heil *et al.* 2009).

In the above sections we summarized the different phenomena that make ant rewards less suitable for non–adapted exploiters, reduce the production of rewards when the legitimate consumer is absent (**Figure 5.1**), or in which hosts seem to sanction cheating ants by actively shedding domatia. Unfortunately, the underlying physiological mechanisms remain to be discovered: we are not aware of any study that has found a compound, or trait, that is used by a myrmecophyte to identify its resident ant or to directly measure its actions, although plants in principle can perceive animal–derived compounds such as egg–deposition glue (Hilker and Meiners 2006) or pheromones (Helms *et al.* 2013).

Independently of the detailed physiological mechanisms, however, it remains an open question whether the above–described phenomena represent true host sanctions or specific adaptations that make the rewards exclusive, or whether they are consequences of partner fidelity feedback and ecological fitting. Partner fidelity feedback means that fitness–relevant traits of host and symbiont depend on each other and that this reciprocal

dependency is enough to prevent cheating: each partner has a benefit from maintaining the other one in a good physiological state. By contrast, host sanctions apply when a (costly) punishment is required to prevent symbionts from cheating (Weyl *et al.* 2010). In fact, ant–plant mutualisms are likely to depend mainly on partner fidelity feedback. On the one hand, plants require healthy leaves to maintain the photosynthesis that allows for future growth, including the production of any type of tissue or organ, independently of whether or not they are myrmecophytes. Thus, undefended plants might simply lack the physiological capacities to invest in the production of new domatia or ant rewards. Moreover, plants commonly shed leaves as soon as these are too heavily damaged by herbivores or microorganisms. In fact, controlled cell death represents a paramount strategy in plant resistance to pathogens. On the other hand, ant workers are commonly limited by energy acquisition. Thus, increased EFN secretion rates have been related to higher survival rates of ant workers (Lach *et al.* 2009) and also increased the activity and aggressiveness of ants in facultative and in obligate ant–plant interactions (González-Teuber *et al.* 2012, Ness 2006, Ness *et al.* 2009, Sobrinho *et al.* 2002).

In summary, both the decrease in the production of ant rewards and domatia by undefended plants and the lower aggressiveness in ant colonies that receive less EFN are likely intrinsic physiological responses rather than specific adaptations to the mutualistic interaction. Even the biochemical 'lock–key system' that is formed by Kunitz–type PIs in the FBs of ant acacias and the chymotrypsin 1–like proteases in the ants, which are insensitive to these PIs, is not likely to represent the outcome of a co–evolutionary process (Orona-Tamayo *et al.* 2013a). Kunitz–type PIs are common in seeds of members of the Fabaceae and all of the few ant species that have been investigated so far posses chymotrypsin–like proteases,

which are generally less sensitive to Kunitz–type PIs (Orona-Tamayo *et al.* 2013a). Thus, although secondary co–evolutionary adjustments can strengthen the reciprocal matching, ecological fitting as defined by (Janzen 1985) is more likely to explain a large part of this phenomenon. Partner fidelity feedback (also termed 'closed loop of fitness–relevant traits', see (Heil *et al.* 2009) turns out to represent an important stabilizing process, and its functioning does not necessarily require any specific adaptations. Mutualisms are generally formed among species from different kingdoms, each of which can produce something easily and at low costs that is required (and difficult to produce) by the other (Bronstein 2009, Bronstein *et al.* 2006, Leigh 2010). Think only of all the transportation mutualisms: plants are unlikely to evolve mobility, whereas animals are similarly unlikely to evolve photosynthesis as an efficient source of metabolically accessible energy; thus, the trade 'energy for mobility' serves all partners involved (Boucher *et al.* 1982, Bronstein 1994, Fleming and Holland 1998, Roopin *et al.* 2008, Wagner *et al.* 1979) and each partner benefits from maintaining the other one in a good physiological state.

5.3. Conclusions

Research into symbiotic ant–plant mutualisms has significantly contributed to our knowledge on factors that stabilize symbiotic mutualisms. Factors and mechanisms that have been described for ant–plant mutualisms include chemical characteristics and signalling mechanisms that facilitate the production of exclusive rewards, host choice and partner sanctions (that is, control mechanisms exerted by the symbiont), and closed loops among fitness–relevant vital traits of host and symbiont that lead to stable partner fidelity feedback mechanisms. How likely are these mechanisms to apply also to other types of

mutualisms and what should be done to further improve our knowledge on ant – plant mutualism?

One obvious benefit of studying ant–plant mutualisms lies in the fact that, although the mutualisms commonly are obligate in the long run, both partners can be separated in order to study each of them with and without the mutualistic interaction. Whereas it appears almost impossible to deprive a legume of its Rhizobia once nodules have been formed, plant–ants can be removed easily. Thus, both the formation and the dissolution of the mutualism can be experimentally simulated and its consequences for both partners studied independently. It might be no coincidence that many examples of partner sanctions and host choice stem from this research field. Furthermore, the rewards generally are easily accessible and thus available for direct analysis.

Tropical ant–plants provide researchers with benefits than are difficult to find in other mutualisms. Research into ant–plant mutualisms tells us that more caution is required before we assume specific strategies and adaptations to explain phenomena that contribute to the stability of mutualisms. A deeper mechanistic understanding and comparative research will be required to distinguish adapted (co-evolved) strategies from ecological fitting and partner fidelity feedback. Most obligate ant–plants are found in the same genera with non–myrmecophytic species and the same situation applies to plant–ants as well, allowing for direct comparative approaches. Do non–myrmecophytic *Hirtella* or *Cordia* species shed heavily damaged and infected leaves as easily as the myrmecophytes? Do plant –ants show nutritional adaptations to their vegetarian lifestyle that cannot be observed in other arboreal ants? Are plant –ants biochemically and behaviourally adapted to defend their host, or are they simply expanding the use of compounds and behaviours used for their

own protection to include their host as well? The possibility to separate both partners without destroying their physical integrity and the existence of symbiotic and non–symbiotic species in the same genera on the side of both ants and plants, make ant–plants highly suitable systems to study questions of general interest in the establishment and maintenance of mutualisms at the ecological and evolutionary level.

However, there are no reasons to assume that mechanisms which stabilize an ant–plant mutualism are in principle different from those that stabilize other mutualisms. In fact, the recent discovery that pollinators can sanction against cheating flowers which produce less nectar but have unchanged morphological and olfactory traits (Brandenburg *et al.* 2012) demonstrates that partner sanctions function also in other types of mutualisms. Research into ant–plant mutualisms has proven highly successful in the identification and understanding of multiple mechanisms that are required to stabilize mutualisms in the presence of non–reciprocating exploiters.

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Chapter 6

Discussion and Conclusion

Picture by: Domancar Orona-Tamayo

Domancar Orona-Tamayo

Summary

The goals of my study were to determine spatial-temporal patterns in the synthesis of the main components of *Acacia* extrafloral nectar, the principal roles of defensive proteins present in the food bodies and the contribution of endosymbiotic bacteria to the nutrition of the ants. Thereby, I aimed to discover molecular mechanisms that contribute to the stabile functioning of an obligate ant-plant mutualism in which the specialized mutualist ants make no use of any host plant-independent food sources. My main results are: I found how the FBs are protected from exploitation by non-adapted generalist herbivores without diminishing their nutritional value to the mutualist ant larvae (**Chapter 2)**. I discovered that the nectary tissue contains the entire metabolic machinery for extrafloral nectar production, which is synthesized and active during concurrent secretion and becomes degraded thereafter, in a diurnal circle (**Chapter 3**). Knowing the key enzymes involved in the secretion of extrafloral nectar and the spatiotemporal patterns in their expression will allow the elucidation of the mechanisms by which plants control nectar quality and quantity. Finally, I could not confirm the hypothesis that endosymbiotic bacteria of mutualistic *Pseudomyrmex ferrugineus* fixing nitrogen from the atmosphere, however in generalist *Pseudomyrmex gracilis* apparently exist a nitrogen fixation at least in part and I discovered that both ants can recycling nitrogen by a functional urease enzyme that contribute in the N support in both ants as the biologically relevant function (**Chapter 4**).

6.1.1 Protease inhibitors in Acacia Food Bodies, a lock-key system

Food bodies (FBs) produced by Central American acacias or other myrmecophytes represent highly attractive targets for exploiters because they are generally rich in lipids, carbohydrates, amino acids and proteins (Andrade-Buono *et al.* 2008, Fischer *et al.* 2002, Heil *et al.* 2004, Heil *et al.* 1998, O'Dowd 1980). Interestingly, specific components of their protein fraction, i.e. the fraction that greatly contributes to the nutritive value of these FBs, also represent the key to their protection from exploiters. Using a proteomic approach I discovered that *Acacia* FBs contain protease inhibitors (PIs) (**Figure 2.3; Table 2.2**). Activity assays demonstrated that these PIs can effectively reduce the proteolytic activity in the digestive tracts of bruchid seed beetles (*P. truncatus* and *Z. subfasciatus*) (**Figure 2.4**) and an exploiter ant, *Pseudomyrmex gracilis.* By contrast, the legitimate mutualistic consumers maintained a high level of proteolytic activity in their intestines that mainly consisted of chymotrypsin 1-like and chymotrypsin 2-like and elastase-like activities **(Figure 2.6**). Larvae of *P. gracilis* exhibited mainly elastase-like and to a lower degree chymotrypsin 1-like activity and thus appear partly, but not completely adapted to consume *Acacia* FBs (**Figure 2.6**). Based on these observations we suggest that plant PIs and ant proteases form a lock–key system that converts the FBs into an exclusive food source for the mutualistic ants (Orona-Tamayo *et al.* 2013a). Possible mechanisms have been described by which mutualisms can be protected from exploiters. Partner choice applies before the mutualism is established and usually means that hosts actively select the species of symbionts that are allowed to enter the interaction (Bever *et al.* 2009, Noë and Hammerstein 1994, Orona-Tamayo and Heil 2013, Sachs *et al.* 2004, Simms *et al.* 2006). By contrast, host sanctions occur when the mutualism has already been established and apply when the host ceases to provide rewards to a partner that does not behave adequately (Charlotte *et al.* 2012, Kiers *et al.* 2003). A further possible mechanism is to make the

reward exclusive ('Exclusive rewards'): specific anatomical or biochemical characteristics can make a reward less attractive, accessible or suitable for generalists that represent potential exploiters. We demonstrate now that the biochemical composition of *Acacia* FBs also shows characteristics that are consistent with their 'exclusiveness' (Orona-Tamayo *et al.* 2013a).

6.1.2. Extrafloral nectary tissue: Metabolic factory for the synthesis of main nectar components

Nectar plays multiple roles in plant pollination (floral nectar, FN) and in the indirect defence of plants against herbivores (extrafloral nectar, EFN) (Brandenburg *et al.* 2009, Heil 2008, 2011). In addition to its chemical composition, the quantity of nectar secreted also represents an important trait that is positively correlated with pollination success or the resulting indirect defence (Brandenburg *et al.* 2012, Heil *et al.* 2009). However, little is known about the mechanisms that underlie the genetic control or phenotypic plasticity of nectar secretion rates, or where in the plant dominant nectar components others than sugars are synthesized. Nectar secretion is a highly dynamic process that depends on the ontogenetic stage of the nectar-secreting structure (Liu and Thornburg 2012, Ren *et al.* 2007a, Ren *et al.* 2007b) and on environmental factors such as consumption rate (Corbet and Delfosse 1984, Gill 1988, Heil *et al.* 2000, Pyke 1991), and, in the case of EFN, herbivory and current light conditions (Bixenmann *et al.* 2011, Heil *et al.* 2001, Radhika *et al.* 2010). We observed highly dynamic processes in the accumulation and activity of key metabolic enzymes in the nectary tissue, demonstrating that the entire metabolic machinery required for the synthesis of nectar is established and active directly before and during the

hours of peak nectar secretion (**Figure 3.8**). *A. cornigera* plants bear conspicuous extrafloral nectaries on their petioles. These nectaries secrete large quantities of a biochemically complex EFN in a predictable and sharp diurnal peak (González-Teuber *et al.* 2012, Orona-Tamayo *et al.* 2013b) (**Figure 3.1**). Although earlier researchers hypothesised nectar to represent secreted phloem sap, our analysis demonstrated that nectar and phloem sap significantly differed in their chemical composition, particularly in terms of hexoses and nectarines (**Figure 3.3**). The proteomes of leaves, phloem, nectary tissues, and EFN differed strongly from each other and the EFN proteome represented a subset of the nectary proteome, but showed no overlap with the phloem proteome (**Figure 3.3**). Therefore we expected strong metabolic activity in the nectar-producing organ.

A central enzyme that is required for an active nectar secretion is invertase (Heil 2011, Ruhlmann *et al.* 2010). Indeed, the highest level of invertase activity in the *A. cornigera* extrafloral nectary was observed in the hours directly before the secretion process began (**Figure 3.2**). These observations underline the importance of invertase in the secretion of nectar, probably because this activity is required for unloading of sucrose from the phloem and for the formation of hexose-rich nectars (Heil 2011). Furthermore, synthetic enzymes and nectarines accumulated in the nectary tissue before active secretion process (**Figure 3.8**). Taken together, our results demonstrate that the nectary represents a metabolically independent organ and that most synthetic processes that are required for production of important nectar components occur in the nectary itself.

Protein sequencing of nectary tissues revealed that the majority of these proteins represented numerous biosynthetic enzymes responsible for the synthesis of amino acids, sugars and proteins, and the secreted nectar proteins themselves: Enzymes of carbohydrate

metabolism and the metabolism of amino acids and proteins. Further important functional groups included proteins related to other metabolic processes and defence or stress (**Figure 3.7**). Most anabolic enzymes were present at high optical densities before the secretion and decreased during and after the secretion peak (**Figure 3.8**), whereas proteolytic enzymes showed the opposite pattern (**Figure 3.9**). The presence of numerous proteolytic enzymes (proteases, ubiquitin-related proteins and a subunit of the proteasome) is consistent with the proteolytic activity in the nectary, which reached highest overall values after the secretion peak: trypsin and chymotrypsin increased and their high activity in the hours directly after the secretion peak supports the interpretation that proteolysis is involved in subsequent removal of enzymes from the nectary tissue and that the activity of these proteolytic enzymes in the extrafloral nectaries of *A. cornigera* controls the termination of nectar production.

6.1.3. Nitrogen fixation as secondary needs in *P. ferrugineus*

Obligate plant-ants that feed only on host-derived rewards are truly "vegetarian" and thus represent a particularly extreme case of nutritional specialisation. Ants are one of the most important insects group and dominate multiple different habitats, and a shift towards a more vegetarian life style as been suggested as a key factor explaining the dominance of ants in certain habitats (Davidson *et al.* 2003, Russell *et al.* 2009). In ant-plant mutualistic interactions, where the exchange of resource and services is the principal aspect, the host-derived rewards represent the only source of nutrition for the ants. Thus, workers invest high amounts of energy into patrolling and protection of their partner, to avoid negative

effects on host performance and food reward production. Although FBs are comparably rich in proteins and these are supplemented by some nectarines, it appeared unlikely that the nitrogen demands of the developing ant larvae can be fully fulfilled by this diet. A possible explanation would be the existence of an ant-microbe mutualism that allows some kind of N supplementation for the ants (Davidson *et al.* 2003, Zientz *et al.* 2005). Different endosymbiotic bacteria such *Blochmannia*, *Buchnera*, *Rickettsia* and *Wolbachia* are common intracellular residents in ants, situation which suggests that these microbes may play specific roles in fixing, recycling or upgrading N (Anderson *et al.* 2012, Cook and Davidson 2006, Russell *et al.* 2009, Wernegreen *et al.* 2003). However, the assimilation of atmospheric N by endosymbiotic bacteria and the subsequent incorporation of this N into the body mass of the ant hosts have never been demonstrated directly. Indeed, the larvae and workers of *P. ferrugineus* ants that were exposed to an acetylene atmosphere did not reduce acetylene to ethylene in detectable amounts, which indicates that – if present at all - the nitrogenase enzyme is inactive (**Figure 4.1**). By contrast, workers of the generalist *P. gracilis* produced a high amount of ethylene, indicating an active reducing activity as exhibited by nitrogenase (**Figure 4.1)**. More importantly, no or only an unsignificantly low incorporation of atmospheric N into the body mass of larvae or workers could be detected in experiments in which living animals were kept in an ^{15}N- enriched atmosphere (**Figures 4.2** and **4.3**). These findings show that mutualistic *P. ferrugineus* only obtain no significant amounts nitrogen from the atmosphere and generalist *P. gracilis* ant workers can incorporate high amount of N from the atmosphere. I discovered an alternative, biologically relevant function of endosymbiotic bacteria in ants that carry *nifH*. When mutualistic and generalist ant workers were fed with urea, they exhibited urease activity in their midguts (**Figure 4.5**). Ants fed with ^{15}N-marked urea exhibited increased of ^{15}N levels in amino

acids that were obtained from the hemolymph (**Table 4.1**). As the hemolymph should be free of bacteria, these results indicate that N from urea in the intestine can be recycled and converted to organic N that is accessible for the metabolism of the ants. Many of the amino acids into which ^{15}N from urea was incorporated are considered essential for ants, which indicates that the bacterial urease activity in the midgut of these ants can help to provide the ant host with essential amino acids synthesized using N that has been recycled from waste products in the intestine.

6.2. Conclusions

Mutualism between *Acacia-Pseudomyrmex* interaction is more than rewards and services. This interaction involves genetically, biochemical, physiological and behavior adaptations that contribute in the maintenance of this mutualism. *P. ferrugineus* the mutualistic ant, protected *Acacia* plants effectively against different enemies, monopolizes the resources for their convenience, by contrast non-defending ant species appeared physiologically less adapted to the host-derived food rewards.

Myrmecophytic *Acacia* plants produce different rewards to nourish mutualistic ant. Plant food rewards as FBs and EFN are chemically adapted to their specific function in feeding ant and both are protected from different exploiters. FBs contained higher concentration of protein than the leaves from which they are originate, a considerable fraction from acacia species contained a protease inhibitors (PIs) of Kunitz-type family, that exerts inhibition effects against serine proteases (or serine peptidases). FBs are exclusive food source for ant

larvae and the ant needs some digestive adaptation. The ant midgut is dominated by adapted chymotrypsin-1 and elastase proteases as the main digestive arsenal to hydrolyze the proteins in FBs reward, in spite of this reward is loaded with PIs, these did not inhibit the digestive machinery of the ant, by contrary, exploiter ant larvae (*P. gracilis*) only can digest FBs partially, which indicates, the exploiter ant forager other food items. These PIs were extremely effective to neutralizing trypsin enzyme, the main peptidase from two potential exploiter *P. truncatus* and *Z. subfasciatus*. Based on these arguments, FBs are exclusive food reward only for biochemical adapted organisms as mutualistic ants.

By other way, EFN from acacias is a complex solution that contain different biomolecules e.g. sugars, amino acids and nectarines, however, the synthesis of these compounds, and their rate of secretion have ecological relevance but are uncertain. *A. cornigera* secreted large quantities of EFN in a short daily peak on the morning and EFN ceases near to the midday and restart to the next day. Phloem does not contains hexoses, and proteins (nectarines) are absent, excluding the phloem as direct source of major nectar components. A very important enzyme with a role in nectar secretion is cell wall invertase. This enzyme hydrolyzes sucrose producing glucose and fructose, and these hexoses are the main sugars presented in EFN. Nectaries tissues contain high level of this enzyme hours before the EFN secretion, and decreased when the nectar ceases the secretion. Similarly, other important enzymes involved in metabolism of amino acids, sugars, proteins and nectarines as glucanases, and thaumatin-like proteins, were accumulated hours before the nectar secretion and diminished after secretion process. However, corresponding genes for invertase and nectarines were expressed only in nectar tissue, and also proteins related to catabolic process as trypsin and chymotrypsin were activated only during the main peak of

secretion and their main function was the termination of secretion process. Thus nectary tissue of *A. cornigera* contains the complete metabolic machinery for EFN synthesis, production and secretion.

EFN as a reward is chemically adapted to the nutritional need of ant workers, however, a nitrogen misbalance occur in mutualistic ants, to avoid this effect, endosymbiotic bacteria that reside in the ant midgut, can supplement the ants with nitrogen from the atmosphere or recycling. The presence of a *nifH* gene was found in isolated bacteria from the ant midguts, but in mutualistic ants, nitrogen fixation did not occur, contrary to what happens in exploiter ants the nitrogen fixation was present. By contrast, endosymbiotic bacteria produce high urease activity in the ant intestines for recycle waste products and contribute in amino acid metabolism for the nutrition of vegetarian and exploiter ants. Mutualistic and exploiter ants carry with endosymbiotic bacteria that supply their host with nitrogen recycled from midgut a pathway more suitable than the nitrogen fixation.

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Future directions

With my results and conclusions obtained from this work, I determine that I need other studies that are necessaries to sustain events of stabile between *Acacia-Pseudomyrmex* interaction.

To mutualistics *P. ferrugineus* or exploiter *P.gracilis* ant larvae, are necessary analyze other different digestive enzymes than serine proteases, such as cystein, metallo and aspartic proteases could be accomplish the complete digestive arsenal, by other way is necessary dilucidate in which magnitude is found lipase enzyme involved in lipid degradation and the same manner investigate sacarolityc enzymes.

Is necessary accomplish my results with the digestive capacity in *Acacia* exploiter, such as the vegetarian spider *Bagheera kiplingi* that living in the hollow spines of Mexican acacias, the spider uses as main food the *Acacia* FBs, but sometimes consume EFN or preys on ant larva. By similar, other exploiter ant species such as *Camponotus planatus* and *Pseudomyrmex nigropilosus* inhabit the thorns of *Acacia* myrmecophytes and feed on its EFN but they do not harvest the FBs to their ant larvae, because the ant larvae of these species lack of a digestive machinery to digest FBs. Analyze different gut protease of these ant larvae to known if protease inhibitors exert a inhibition effect on its digestive proteases.

I need to perform a transcriptomic analysis and evaluate genes involved in the synthesis and production of EFN four hours before the EFN secretion in *A. cornigera* nectaries, and these results should be contrasted with genes from samples after the EFN secretion.

About the nitrogen recycling performed by mutualistic or exploiter ants, I need to isolate gene (i.e urease gene) involved in this physiological event and sequencing to corroborate the function, by the way, I need to isolate bacteria of ant midguts and detect the gene expression of the same gene when the ants were fed.

Anexum

In this section are shown the publications generated in collaboration with different working groups during development of this thesis.

Heil, M., **Orona-Tamayo, D**., Eilmus, S., Kautz, S. and González-Teuber, M. (2010) Chemical communication and coevolution in an ant-plant mutualism. *Chemoecology*, **20**, 63-74.

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Orona-Tamayo, D., Eilmus, S., Molina-Torres, J., Adame-Álvarez, R.M., Ramírez-Chavéz, E., Farías-Rodríguez, R. and Heil, M. Endosymbiotic bacteria in arboreal ants recycle nitrogen from the intestine.

Millán-Cañongo, C., **Orona-Tamayo, D**. and Heil, M. Optimized defense by extrafloral nectar (EFN): A physiological explanation by ecological adaptation.

Orona-Tamayo, D., Quintana-Rodríguez, E., Millán-Cañongo, C., Molina-Torres, J and Heil, M. Increase of indirect defenses by jasmonic acid in *Ricinus communis* under field conditions.