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

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Abstract

Myrmecophytic *Acacia* species produce food bodies (FBs) to nourish ants of the *Pseudomyrmex ferrugineus* group, with which they live in an obligate mutualism. We investigated how the FBs are protected from exploiting nonmutualists. Two-dimensional gel electrophoresis of the FB proteomes and consecutive protein sequencing indicated the presence of several Kunitz-type protease inhibitors (PIs). PIs extracted from *Acacia* FBs were biologically active, as they effectively reduced the trypsin-like and elastase-like proteolytic activity in the guts of seed-feeding beetles (*Prostephanus truncatus* and *Zabrotes subfasciatus*), which were used as non-adapted herbivores representing potential exploiters. By contrast, the legitimate mutualistic consumers maintained high proteolytic activity dominated by chymotrypsin 1, which was insensitive to the FB PIs. Larvae of an exploiter ant (*Pseudomyrmex gracilis*) taken from *Acacia* hosts exhibited lower overall proteolytic activity than the mutualists. The proteases of this exploiter exhibited mainly elastase-like and to a lower degree chymotrypsin 1-like activity. We conclude that the mutualist ants possess specifically those proteases that are least sensitive to the PIs in their specific food source, whereas the congeneric exploiter ant appears partly, but not completely, adapted to consume *Acacia* FBs. By contrast, any consumption of the FBs by non-adapted exploiters would effectively inhibit their digestive capacities. 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 nonadapted consumer to use.

Keywords: ant–plant interaction, co-evolution, exploiter, indirect defence, peptidase inhibitor, protein digestion

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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 & 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

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exploitation of mutualisms by other organisms has been reported for a wide range of interactions, including nectar robbing by bees and birds (Roubik 1982; Maloof & Inouye 2000), 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 & 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 (Janzen 1975; Clement *et al.* 2008).

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) and *Acacia* (Heil *et al.* 2004), and also by other plants, for example, in the genus *Ochroma*, that produce FBs to attract nonsymbiotic ants as facultative mutualists (O'Dowd 1980). We investigated whether the FBs produced by myrmecophytic *Acacia* (Mimosoideae, Fabaceae) species in Central America are specifically protected from consumption by potential exploiters. 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 (Janzen 1967; Ward 1993; Palmer *et al.* 2008; Goheen & Palmer 2010). 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 (Fig. 1): extrafloral nectar (EFN) and FBs (Janzen 1974). 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 (Rickson 1975, 1980; Clement *et al.* 2008) and fed to the ant larvae (Janzen 1966, 1967, 1974; Clement *et al.* 2008). 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 other legumes (Singh & Emden 1979).

How are FBs protected from exploitation? Although the ants actively defend the leaves that bear the FBs, we hypothesized that FBs also require a direct, chemical protection. The EFN produced by *Acacia* myrmecophytes

Fig. 1 Leaves of myrmecophytic *Acacia* plants with food bodies (FBs). (a) *Acacia hindsii* and (b) *Acacia cornigera*; FBs are marked with arrows. Insets illustrate individual FBs (4 × *100 ×). (c) *A. hindsii* FB harvested by a *Pseudomyrmex ferrugineus* worker.

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 confirm that these PIs are biologically active and to investigate whether these PIs can diminish the protein digestive activities in an ant exploiter and two nonant species

that represent potential exploiters of the FBs. *Pseudomyrmex gracilis* (Fabricius) inhabits the hollow thorns and consumes FBs but does not show any detectable defending behaviour and thus acts as facultative exploiter (Clement *et al.* 2008). As 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 these lacked chemical protection. 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 & Zucoloto 2011) and commonly being used in bioassays to evaluate the activity of PIs (Aguirre *et al.* 2004, 2009; Torres-Castillo *et al.* 2009; Castro-Guillén *et al.* 2012). We also investigated the effect of the FB PIs on the proteolytic activity in the digestive tracts of the legitimate consumers: the larvae of *P. ferrugineus* ants. We found that PIs in *Acacia* FBs are active and thus convert a highly nutritive food reward into an item that is difficult to digest for nonadapted potential 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.

Materials and methods

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 the chemical composition of these FBs (Heil *et al.* 2004, 2010). The plant species were determined according to Janzen (1974).[1] FBs were collected in southern 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.

[1]In order to avoid confusion, we continue using the name *Acacia* because it is well established in the literature, although the Nomenclature Session of the 17th International Botanical Congress in Melbourne (2011) suggested to separate the polyphyletic genus *Acacia* in the new genera *Senegalia* and *Vachellia* (the latter comprising all African and American clades, including the 'swollen-thorn acacias' of both continents).

Sample collection

All plants chosen for FB collection were inhabited by the ant mutualist *Pseudomyrmex ferrugineus*. All plant 'individuals' used in this study grew at distances of more than 10 m from each other and therefore were likely to represent genetically different individuals. 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 developing of FBs. After isolation, a ring of sticky resin (Tangletrap, Contech Inc http://www.contech-inc.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 individual plants that had not been used for FB collection, by cutting off swollen thorns and determined according to Ward (1993). Entire thorns, which contained adult ants and larvae, were then kept in 1 L plastic pots with adequate ventilation until the ants were dissected.

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 *g* for 3 min 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 *g* 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).

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 *g*

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 *g* for 60 min at 4 °C. The supernatants were recovered and stored at −70 °C (Aguirre *et al.* 2004).

SDS-PAGE and zymograms

The electrophoretic separation of proteins was performed 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 copolymerized 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 0.05 M 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).

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

To investigate the FB proteome, the total protein content was separated by two-dimensional gel electrophoresis (2-DE). Three hundred micrograms of protein per sample was resuspended in 250 μL of rehydrating solution [7 M urea, 2 M thiourea, 2% CHAPS, 65 mM of DTT and 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 h. 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.

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 was injected on a nano-Acquity 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 and then eluted on a nanoAcquity C18 column (100 mm × 100 μm ID, BEH 130 material, 1.7 μm particle size) using a 10-min increasing acetonitrile gradient (0.1% formic acid) at a flow rate of 0.500 μL/min A.

The eluted peptides were online 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 s at a flow rate of 0.5 μL/min 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 28 January 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 as follows: instrument profile, ESI-Trap; fixed modification, carbamidomethyl (cysteine); variable modification and oxidation (methionine); up to one missed cleavage was 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 10 August 2011 using described settings (González-Teuber *et al.* 2009, 2010).

Extraction of proteases from the midguts of ant larvae and beetles

Protease enzymes of mutualistic and exploiter ants were extracted from fourth-instar ant larvae taken from colonies that lived on *Acacia hindsii* or *Acacia 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 $CaCl_2$ and 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 ***g*** for 30 min at 4 °C, before storage at −70 °C. Midguts of fifty-third-instar larvae of each of the two beetle species (*Prostephanus truncatus* and *Zabrotes 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).

Quantification and characterization of the proteolytic activities 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 soybean Kunitz trypsin inhibitor (SKTI) 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 preincubated 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.

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. Protein extract of 10 μg was mixed with 10 μg of FB protein extracted as described above. Samples were mixed and preincubated 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.

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).

Results

SDS-PAGE patterns and 2-DE in Acacia FBs

The total protein content in the FBs of *Acacia hindsii* and *Acacia cornigera* amounted to 24.9 ± 3.5 and 22.8 ± 3.8 μg/mg dw, respectively, whereas the leaves contained 8.8 ± 0.2 and 8.4 ± 0.8 μg/mg dw of protein, respectively (Table 1). The FBs of both species contained significantly more protein than the leaves (for both species: $P < 0.001$, according to Student's *t*-test, $n = 4$ for each tissue type and species). One-dimensional

Table 1 Total protein content in *Acacia hindsii* and *Acacia cornigera* food bodies and leaves

	FBs		Leaves	
	A. hindsii	*A. cornigera*	*A. hindsii*	*A. cornigera*
Protein (μg/mg dw)	24.9 ± 3.5	22.8 ± 3.8	8.8 ± 0.2	8.4 ± 0.8

Values are given as mean ± SD in μg/mg dry mass.

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 (Fig. 2). Major bands in the FBs ranged from 10 to 100 kDa, and several strong bands that quantitatively dominated the FB proteomes were absent from the leaf proteomes (Fig. 2). The leaf proteomes of the two species were similar and ranged from 15 to 100 kDa (Fig. 2).

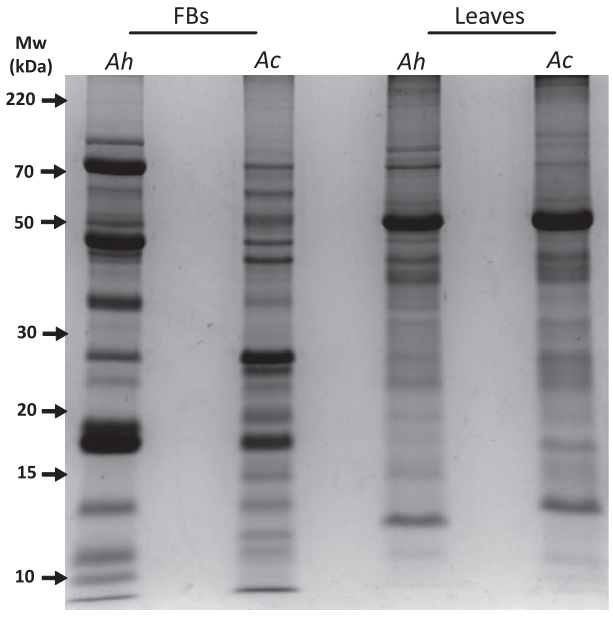

Fig. 2 SDS-PAGE profiles of the proteomes of *Acacia hindsii* (*Ah*) and *Acacia cornigera* (*Ac*) food bodies and leaves. Total protein contents were separated in 13% gel concentration and stained with Coomassie blue.

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 (Fig. 3a,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 proteins 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 PI (Table 2 and Fig. 3a,b). None of these PIs could be identified in the leaf proteomes.

Effects of Acacia FB PIs against serine proteases from Prostephanus truncatus and Zabrotes 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. Whereas, for example, the trypsin-like activity of control *P. truncatus* larvae was *c.* 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 (Fig. 4a). Similar effects were observed for *Z. subfasciatus*. By contrast, chymotrypsin-like activity in both species was less inhibited (only by *c.* 30% on average), although these differences were also statistically significant (Fig. 4b).

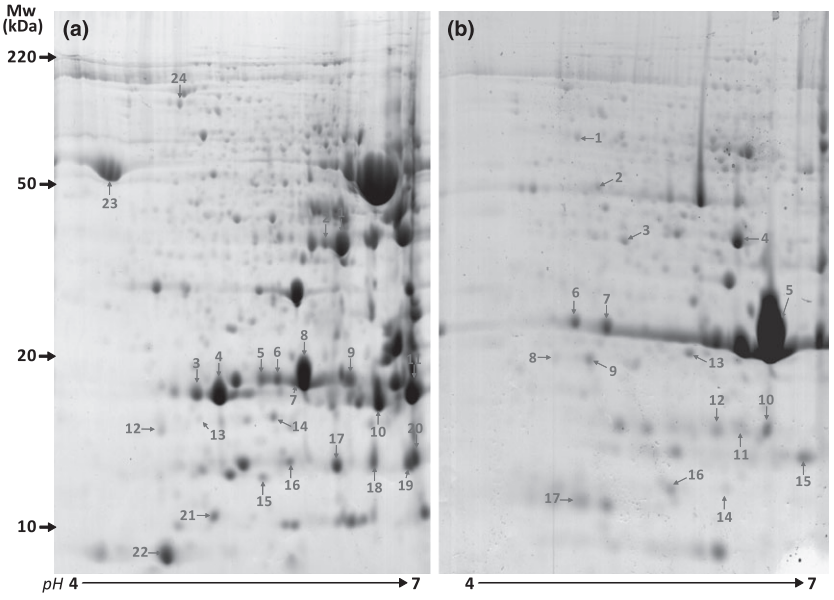

Fig. 3 2-DE profiles of the proteomes of (a) *Acacia hindsii* and (b) *Acacia cornigera* food bodies. Proteins identified in this study are indicated by arrows and numbers (see Table 2 for protein identity and description). Total protein contents were separated in 13% gel concentration and stained with Coomassie blue.

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

A. hindsii FB spots	Description	Accession no.	Organisms	Peptide hits	MS BLAST score	*A. cornigera* FB spots	Description	Accession no.	Organisms	Peptide hits	MS BLAST score
1	Patatin T5	XP_002510258	*Ricinus communis*	2	128	1	Kunitz-type trypsin inhibitor	P32733	*Prosopis juliflora*	2	152
2	Patatin precursor	XP_002523555	*R. communis*	1	68	2	Kunitz-type trypsin inhibitor	P32733	*P. juliflora*	1	90
3	Trypsin isoinhibitor DE5	1208243A	*Adenanthera pavonina*	6	329	3	Kunitz-type trypsin inhibitor	P32733	*P. juliflora*	2	138
4	Trypsin inhibitor DE5	1208243A	*A. pavonina*	2	136	4	Kunitz-type trypsin inhibitor	P32733	*P. juliflora*	2	130
5	Trypsin inhibitor DE5	1208243A	*A. pavonina*	3	177	5	Kunitz-type trypsin inhibitor	P32733	*P. juliflora*	1	90
6	Trypsin isoinhibitor DE5	1208243A	*A. pavonina*	2	138	6	Kunitz-type trypsin inhibitor	P32733	*P. juliflora*	2	144
7	Trypsin inhibitor DE5	1208243A	*A. pavonina*	3	217	7	Patatin precursor	EEF38753	*R. communis*	3	153
8	Trypsin isoinhibitor DE5	1208243A	*A. pavonina*	5	287	8	Kunitz-type trypsin inhibitor	P32733	*P. juliflora*	2	133
9	Kunitz-type trypsin inhibitor	P32733	*P. juliflora*	1	95	9	Trypsin inhibitor	P86451	*Enterolobium contortisiliquum*	3	177
10	Trypsin inhibitor DE5	1208243A	*A. pavonina*	6	290	10	Kunitz-type trypsin inhibitor	P32733	*P. juliflora*	3	133
11	Kunitz-type trypsin inhibitor	P32733	*P. juliflora*	3	202	11	Kunitz-type trypsin inhibitor	P32733	*P. juliflora*	2	168
12	Trypsin inhibitor	P86451	*E. contortisiliquum*	3	164	12	Kunitz-type trypsin inhibitor	P32733	*P. juliflora*	2	174
13	Trypsin inhibitor	P86451	*E. contortisiliquum*	3	174	13	Trypsin isoinhibitor DE5	1208243A	*A. pavonina*	3	191
14	Kunitz-type trypsin inhibitor alpha chain	P32733	*P. juliflora*	1	65	14	Kunitz-type trypsin inhibitor	P32733	*P. juliflora*	2	138
15	Trypsin inhibitor BvTI	P83595	*Bauhinia variegata*	3	159	15	Kunitz-type trypsin inhibitor	P32733	*P. juliflora*	2	113
16	Trypsin inhibitor	P86451	*E. contortisiliquum*	3	158	16	Trypsin inhibitor	P86451	*E. contortisiliquum*	2	143
17	Kunitz-type trypsin inhibitor	P32733	*P. juliflora*	3	185	17	Trypsin inhibitor	P86451	*E. contortisiliquum*	3	183
18	Kunitz-type trypsin inhibitor	P32733	*P. juliflora*	3	172						
19	Kunitz-type trypsin inhibitor	P32733	*P. juliflora*	2	153						

Table 2 *Continued*

A. hindsii FB spots	Description	Accession no.	Organisms	Peptide hits	MS BLAST score	*A. cornigera* FB spots	Description	Accession no.	Organisms	Peptide hits	MS BLAST score
20	Kunitz-type trypsin inhibitor	P32733	*P. juliflora*	3	176						
21	Kunitz-type trypsin inhibitor alpha chain	P32733	*P. juliflora*	1	90						
22	Kunitz-type trypsin inhibitor	P32733	*P. juliflora*	2	144						
23	Patatin precursor	EEF38753	*R. communis*	3	148						
24	Kunitz-type trypsin inhibitor	P32733	*P. juliflora*	1	73						

FB, food bodies; PIs, protease inhibitors.

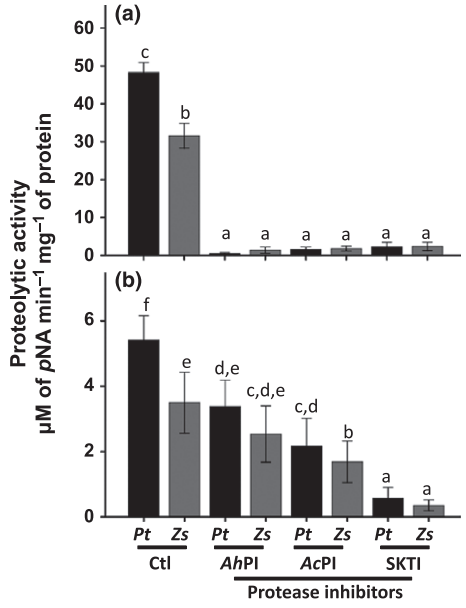

Fig. 4 Effect of *Acacia hindsii* and *Acacia cornigera* food bodies (FB) protease inhibitors (PIs) on serine proteases of *Prostephanus truncatus* (*Pt*) and *Zabrotes subfasciatus* (*Zs*). (a) Trypsin-like activity detected with Bz-R-*p*NA substrate. (b) Chymotrypsin 2-like activity detected with Suc-AAPF-*p*NA substrate. Proteolytic activities were quantified in the presence of *A. hindsii* or *A. cornigera* FB PIs. Abbreviations: CtI, 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 represent means ± standard errors; different letters above the bars indicate significant differences among conditions ($P < 0.05$ according to ANOVA and Tukey test).

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 (Fig. 5). At least three distinct bands with masses of 22–30 kDa were observed in *Pseudomyrmex ferrugineus*, and two different bands with masses of 25 and 50 kDa were found in the larvae of *Pseudomyrmex gracilis*. By contrast, no proteolytic activity was detected in extracts obtained from the FBs of both plant species (Fig. 5), which demonstrates that the proteolytic activity found in our assays derives exclusively from the insects, rather than from their vegetarian diet.

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

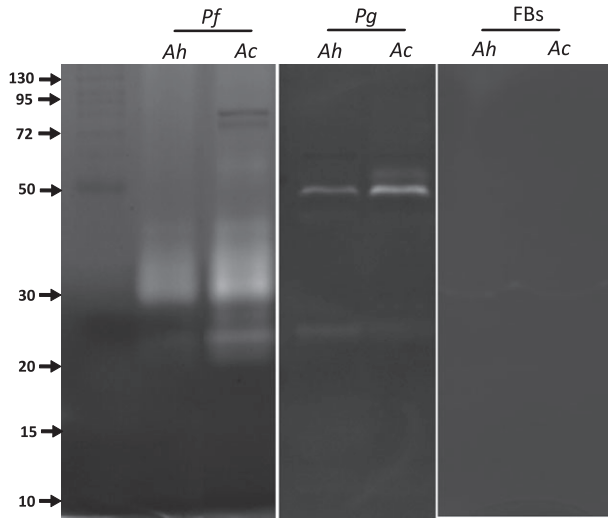

Fig. 5 Polyacrylamide gel electrophoresis zymography of extracts of mutualistic and exploiter ant larval midguts and crude extract of *Acacia* food bodies. *Pf* and *Pg*: *Pseudomyrmex ferrugineus* or *Pseudomyrmex gracilis* midgut extract in *Ah*: Larvae from colonies that lived on *Acacia hindsii* or *Ac*: Larvae from colonies that lived on *Acacia cornigera*.

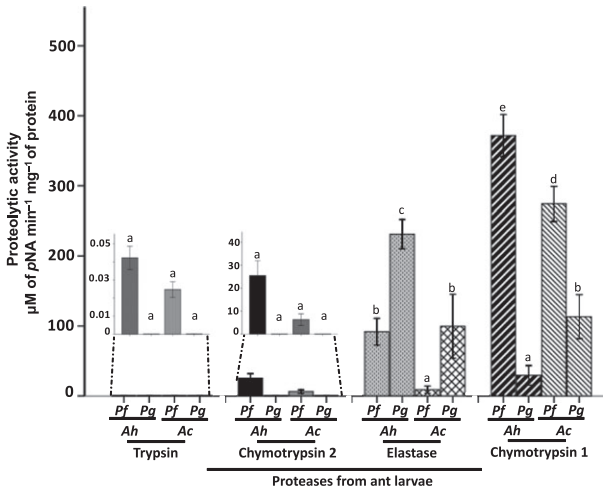

Fig. 6 Activities of the major serine peptidases from mutualistic *Pseudomyrmex ferrugineus* (*Pf*) and exploiter *Pseudomyrmex gracilis* (*Pg*) ant larval midguts. Trypsin-like activity detected with Bz-R-*p*NA substrate, chymotrypsin 2-like activity detected with Suc-AAPF-*p*NA substrate, elastase-like 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 *Acacia hindsii*; *Ac*: Ant lived and fed from *Acacia cornigera*. $n = 7$ samples, each comprising 50 animals. Bars represent means ± standard errors; different letters above bars mark significant differences among conditions ($P < 0.05$ according to ANOVA and Tukey test).

serine proteases for mutualistic and exploiter ants: trypsin-like activity, elastase-like activity and two isoforms of chymotrypsin-like activities (Fig. 6). Elastase 1-like activity 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 *c.* 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 (Fig. 6).

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

No significant effect of any of the FBs could be detected on any of the individual proteolytic activities tested in the larvae of both ant species (Figs S1 and S2, Supporting information). 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, Figs S1 and S2, Supporting information).

Discussion

Food bodies are produced by Central American ant-acacias or other plants to attract or nourish ant mutualists, but they also represent highly attractive targets for exploiters because they are generally rich in lipids, carbohydrates, amino acids and proteins (O'Dowd 1980; Heil *et al.* 1998, 2004; Fischer *et al.* 2002; Andrade-Buono *et al.* 2008). Interestingly, specific components of their protein fraction, that is, the fraction that greatly contributes to the nutritive value of these FBs, can also represent the key to their protection from exploiters. *Acacia* FBs contain numerous proteins that were annotated as potential PIs (Wielsch *et al.* 2011). Because annotation of proteins from a nonmodel species is difficult, we used nonadapted animals to corroborate the biological activity of these PIs. Whereas PIs extracted from *Acacia* FBs effectively reduced the trypsin-like and chymotrypsin-like proteolytic activity in the digestive tracts of seed-feeding beetles, the legitimate mutualistic consumers maintained a high level of proteolytic activity in their intestines that was dominated by chymotrypsin 1-like activity (Fig. 6). Chymotrypsin 1-like activity was found to be insensitive to the FB PIs. We suggest that plant PIs and ant proteases form a lock–key system that converts the FBs into an exclusive food source for ants.

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 (Noë & Hammerstein 1994; Sachs *et al.* 2004; Simms *et al.* 2006; Bever *et al.* 2009). 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 (Kiers *et al.* 2003; Jandér & Herre 2010; Jandér *et al.* 2012). These mechanisms are particularly well studied for the root–rhizobia interaction (Clarke *et al.* 1992; Fisher & Long 1992; van Rhijn & Vanderleyden 1995; West *et al.* 2002; Kiers & Denison 2008; Kiers *et al.* 2011). 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) and slippery stem surfaces (Federle *et al.* 1997) that restrict access to the legitimate, defending ant species. Host sanctions have been discussed in two cases of myrmecophytes that shed domatia when they were not protected (Izzo & Vasconcelos 2002; Edwards *et al.* 2006).

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 nonproteinogenic amino acids to make it less suitable for nonadapted consumers (Adler 2000). Floral and EFN can also contain PR proteins to protect the reward from infestation by microorganisms (Thornburg *et al.* 2003; González-Teuber *et al.* 2009, 2010). The EFN of *Acacia* myrmecophytes 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 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 result of a co-evolutionary process (Darwin 1862; Johnson & Steiner 1997; Anderson & Johnson 2008). 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 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 (Figs 2 and 3). FBs serve the 'external' function of being a reward for the ants, whereas leaves serve multiple 'internal' functions that are unrelated to the 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 nonant-plants and for the typical composition of proteases in nonplant ants. BLAST database searches revealed the presence of 22 and 16 Kunitz-type PIs in *Acacia hindsii* and *Acacia cornigera*, respectively (Table 2). These PIs mainly act against serine proteases such as trypsin, chymotrypsin and elastase (Lawrence & Koundal 2002; Pouvreau *et al.* 2003; Macedo *et al.* 2004; Srinivasan *et al.* 2006). Kunitz-type PIs form a large family and are common in various organs of multiple plant taxa (Jofuku & Goldberg 1989; Hendriks *et al.* 1991; Lawrence & Koundal 2002; Oliva *et al.* 2010), including seeds of legumes such as *Acacia* (Kortt & Jermyn 1981; Weder 1985; Habib & Fazili 2007; Ee *et al.* 2009; Babu *et al.* 2012). 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 & Subrahmanyam 2010), *Acacia nilotica* (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 these PIs in nonreproductive 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, we studied their effects on the digestive proteases in larvae of an exploiter ant and the beetles *Prostephanus truncatus* and *Zabrotes subfasciatus*. Trypsin and chymotrypsin proteases are the major digestive enzymes in Coleoptera, with trypsins usually representing the more important class (Lemos *et al.* 1990; Houseman & Thie 1993; Johnson & Rabosky 2000). Whereas the PIs of *Acacia* FBs had only slightly inhibitory effects on the chymotrypsin-like activity in the midguts of the beetle larvae (Fig. 4b), they strongly inhibited the dominant, trypsin-like activity (Fig. 4a). 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 tepary bean (*Phaseolus acutifolius*), soybean (*G. max*, both: Fabaceae) and chan (*Hyptis suaveolens*; Lamiaceae) (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*) (Silva *et al.* 2001; Magalhães *et al.* 2007). Thus, both the dominance of trypsin-like activity and the low sensitivity of chymotrypsin-like activity to Kunitz-type PIs appear to be common features of beetles. By contrast, larvae of *P. ferrugineus* showed mainly chymotrypsin-like activity (Fig. 6), which was not detectably inhibited by FB PIs (Figs S1 and S2, Supporting information). These ant proteases are not generally insensitive to PIs, as demonstrated by their strong inhibition by the commercial SKTIs (Figs S1 and S2, Supporting information). 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 result from the fact that the ant proteases have been under the influence of the FB PIs during the entire life of the larvae? 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 fourth-instar larvae of the fire ant, *Solenopsis invicta* (Whitworth *et al.* 1998; Meyer *et al.* 2002), and elastase and chymotrypsin 1 dominated the proteolytic activity in the larvae of the exploiter ant, *Pseudomyrmex gracilis* (Fig. 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 & Tillinghast 1995). If this finding can be generalized to all spiders, it might explain the capacity of *Bagheera kiplingi* spiders to feed on *Acacia* FBs (Meehan *et al.* 2009).

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 (Figs S1 and S2, Supporting information). 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 do 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 we observe an interaction between preadapted partners, which resembles 'ecological fitting' (Janzen 1985): 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 nonadapted 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 nonadapted exploiters.

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D.O.T., R.F.R. and M.H. designed the research. D.O.T. and M.H. collected field samples. D.O.T. and N.W. performed the research. A.S. provided new analytical tools. D.O.T., A.B.L., R.F.R. and M.H. analysed the data. D.O.T. and M.H. wrote the manuscript.

Data accessibility

The data underlying Figures 4, 6, S1, and S2 are available as supplemental files, and the Annotation results are presented in Table 2.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Effect of *Acacia hindsii* and *Acacia cornigera* FBs protease inhibitors against serine peptidases in the larvae of mutualistic *Pseudomyrmex ferrugineus* ants.

Fig. S2 Effect of *Acacia hindsii* and *Acacia cornigera* FBs protease inhibitors against serine peptidases in the larvae of exploiting *Pseudomyrmex gracilis* ants.

Data S1 Effect of *Acacia hindsii* and *Acacia cornigera* FBs protease inhibitors against serine peptidases of mutualistic *Pseudomyrmex ferrugineus* ant larvae.

Data S2 Effect of *Acacia hindsii* and *Acacia cornigera* FBs protease inhibitors against serine peptidases of exploiter *Pseudomyrmex gracilis* ant larvae.