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LETTER

Partner manipulation stabilises a horizontally transmitted mutualism

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Abstract

Mutualisms require protection from non-reciprocating exploiters. *Pseudomyrmex* workers that engage in an obligate defensive mutualism with *Acacia* hosts feed exclusively on the sucrose-free extrafloral nectar (EFN) that is secreted by their hosts, a behaviour linking ant energy supply directly to host performance and thus favouring reciprocating behaviour. We tested the hypothesis that *Acacia* hosts manipulate this digestive specialisation of their ant mutualists. Invertase (sucrose hydrolytic) activity in the ant midguts was inhibited by chitinase, a dominant EFN protein. The inhibition occurred quickly in cell-free gut liquids and in native gels and thus likely results from an enzyme–enzyme interaction. Once a freshly eclosed worker ingests EFN as the first diet available, her invertase becomes inhibited and she, thus, continues feeding on host-derived EFN. Partner manipulation acts at the phenotypic level and means that one partner actively controls the phenotype of the other partner to enhance its dependency on host-derived rewards.

Keywords

Ant–plant interaction, cheater, exploiter, host choice, host sanction, mutualism, partner manipulation.

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INTRODUCTION

Mutualisms, positive interactions between different species, are ubiquitous and represent an important source of biodiversity and ecosystem functioning (Thompson 2006): virtually all multicellular organisms engage in one or several mutualistic interactions. However, mutualisms are prone to exploitation (Bronstein 2001; Yu 2001; Bronstein *et al*. 2006). Exploiters do not invest in reciprocation and, therefore, should be competitively superior to the mutualists (Doebeli *et al*. 2004; Archetti *et al*. 2011). Mainly two mechanisms have been discussed that favour mutualistic over exploiting behaviour across a diversity of horizontally transmitted mutualisms (i.e. mutualisms that are established anew in every generation). Partner choice allows hosts to actively select their future symbionts (Bshary & Grutter 2002; Simms *et al*. 2006; Leigh 2010; Archetti *et al*. 2011), whereas sanctions allow hosts to base reward provisioning on actual mutualistic behaviour once the interaction has been established (Kiers *et al*. 2003; Bshary & Grutter 2005; Kiers & Denison 2008). These mechanisms are particularly well studied for the legume–rhizobia, plant–mycorrhiza and fig–fig wasp interactions (West *et al*. 2002; Kiers *et al*. 2003, 2011; Jandér *et al*. 2012).

However, additional mechanisms are required to optimise the exchange of rewards and services once a mutualism has been established (Leimar & Hammerstein 2010). Reciprocal rewarding systems that form positive feedback-loops among fitness-relevant traits of both partners can favour the stable association of high-quality hosts with good symbionts (Heil *et al*. 2009; Kiers *et al*. 2011) and hosts can use reward provisioning to favour specialised mutualists over less adapted exploiters (Heil 2013). Here, we introduce partner manipulation as a further mechanism that favours reciprocation: the host manipulates the phenotype of the symbiont to enhance its degree of dependency on host-derived rewards. We used Central American *Acacia* myrmecophytes that provide *Pseudomyrmex ferrugineus* (F. Smith) ants with nesting space and food rewards: extrafloral nectar (EFN, Fig. 1a) (González-Teuber & Heil 2009; Heil 2011) and cellular food bodies (Janzen 1974; Heil *et al*. 2004a, 2010; Orona-Tamayo *et al*. 2013a). In exchange, the ants protect their hosts from herbivores and encroaching or competing vegetation. These myrmecophytes secrete an invertase (β – 1,3 fructofuranosidase, which cleaves sucrose into glucose and fructose) into their EFN (González-Teuber *et al*. 2009) that keeps the EFN free of sucrose (Heil *et al*. 2005b). Adult workers of *P. ferrugineus* in nature possess almost no invertase activity in their digestive tract (Heil *et al*. 2005b; Kautz *et al*. 2009). Therefore, they prefer a sucrose-free diet (Heil *et al*. 2005b), obtain carbohydrates only when feeding on the EFN produced by their specific host and, hence, gain immediate benefit when they increase plant performance via an efficient protection behaviour. This phenomenon is highly advantageous for the host, whereas the proximate benefit for the ant partner remained unclear. These ants, as part of their host-defending behaviour, prone lianas that come into contact with their host plant and attack scale insects, tree hoppers and other herbivores that show up on the plant. In consequence, the ants regularly have access to sucrose-containing liquids such as phloem sap and honeydew. Thus, it appeared counterintuitive

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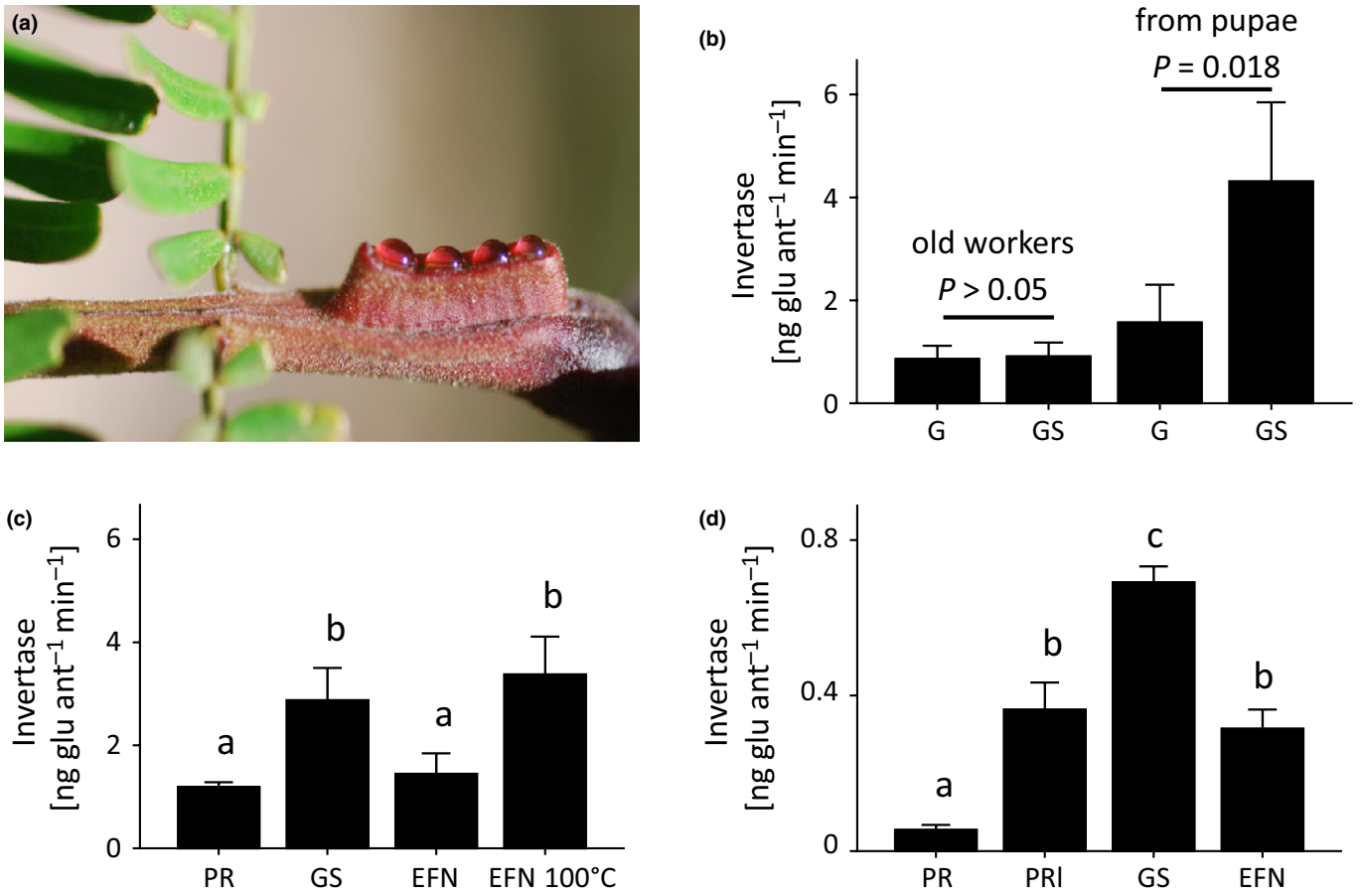

Figure 1 Extrafloral nectar of *Acacia* inhibits invertase in mutualistic ant workers. (a) Extrafloral nectary of *A. cornigera*. (b) *Pseudomyrmex ferrugineus* workers freshly eclosed from pupae exhibited higher invertase activity than older workers and invertase significantly ($P = 0.018$ Wilcoxon test, $n = 6$) increased in freshly eclosed workers when fed with 1 : 1 mixture of glucose and sucrose (GS) instead of glucose only (G). (c) Workers recently eclosed from pupae fed with PR proteins (PR), sugar solution (GS), fresh EFN or boiled EFN exhibited significantly ($P < 0.001$, ANOVA) different invertase activities ($n = 5$). (d) Older workers fed with PR proteins at high (PR) or low (PRl) concentration, sugar solution (GS) or fresh EFN exhibited significantly ($P < 0.001$, ANOVA) different invertase activities ($n = 5$). Bars represent means, error bars indicate 1 SEM, and bars marked with different letters are significantly different ($P < 0.05$, LSD *post hoc* test).

that they lose an enzyme required to digest a sugar that is readily available to them.

Interestingly, larvae of the mutualist species exhibit invertase activity in their digestive tract and this activity can be induced by sucrose in their diet (Kautz *et al.* 2009). The invertase, thus, has not been lost in the mutualist species *per se*, but rather disappears during ontogeny. Therefore, we hypothesised that the host manipulates the digestive capacities of its ant at the phenotypic level. First, we investigated at which ontogenetic stage the invertase becomes lost. Because larvae feed on food bodies, whereas adults feed exclusively on EFN (Clement *et al.* 2008), we then tested whether the EFN contains the invertase-inhibiting factor. Besides sugars and amino acids (González-Teuber & Heil 2009), EFN of *Acacia* myrmecophytes contains multiple pathogenesis-related (PR) proteins, which mainly have chitinase and ß-1,3-glucanase activity (González-Teuber *et al.* 2009, 2010). Thus, these two classes of PR proteins were investigated for their potential inhibitory activity. We also aimed at identifying the underlying physiological mechanism and therefore worked with purified proteins. We found that a direct interaction between a plant-derived nectar protein and the ant invertase can explain the inhibition of the ant's digestive enzyme and conclude that *Acacia* hosts actively manipulate the phenotype of their ant symbionts to force them into reciprocating behaviour.

MATERIAL AND METHODS

Biological material

All studies were carried out with ants that had been caught in their natural environment in Puerto Escondido in Southern Mexico (*c.*15°55′ N and 097°09′ W). Ants were collected from *Acacia cornigera* plants [Mimosoideae, Fabaceae, determined after Janzen (1974)]. The ant species used was *Pseudomyrmex ferrugineus* [determined after Ward (1993)]. Extrafloral nectar was collected as described earlier (Heil *et al.* 2004b).

Experimental alimentation of ants

Ants were fed with natural EFN or an artificial nectar consisting of an aqueous solution that contained amino acids quantita-

tively and qualitatively resembling the EFN of *A. cornigera* (González-Teuber & Heil 2009; Heil 2011) (alanine at 1.85 mM L^{-1}, asparagine at 3.37 mM L^{-1}, aspartic acid at 0.18 mM L^{-1}, glutamine at 1.19 mM L^{-1}, glutamic acid at 1.92 mM L^{-1}, glycine at 0.09 mM L^{-1}, histidine at 2.77 mM L^{-1}, isoleucine at 0.86 mM L^{-1}, leucine at 1.4 mM L^{-1}, lysine at 0.04 mM L^{-1}, methionine at 0.4 mM L^{-1}, phenylalanine at 13.13 mM L^{-1}, proline at 1.24 mM L^{-1}, threonine at 0.45 mM L^{-1}, tryptophan at 1.49 mM L^{-1}, serine at 0.94 mM L^{-1}, and valine at 1.71 mM L^{-1}), and either 30% (w/v) glucose and sucrose 1 : 1 (GS) or just 30% (w/v) glucose (G). In the treatments with PR proteins, this solution also contained chitinase from *Streptomyces griseus* and/or β-glucanase from *Aspergillus niger*, both purchased from Sigma-Aldrich (http://www.sigmaaldrich.com/mexico.html; C6137 for chitinase and 49101 for β-glucanase). The solution with PR proteins contained 4 units of chitinase and 22 units of β-glucanase (PR) or 0.4 units of chitinase and 2.2 units of β-glucanase per mL (PRl, resembling natural activities: González-Teuber *et al.* 2009). Ants were collected from plants by cutting off entire thorns, kept within their thorns (containing also brood) in glass vials (4–8 thorns per group that contained ca. 150–200 workers) and fed *ad libitum* for 3–5 days (100 μL solution per vial). In all experiments, ants from the same colony were distributed in several vials and subgroups of each colony were subjected to all treatments to control for differences among biologically independent colonies. The numbers of replicates as indicated in the different experiments represent the numbers of independent colonies from which workers had been obtained for each experiment.

Feeding schemes

To identify the developmental stage during which invertase activity is lost, pupae were collected and freshly eclosed workers were fed artificial nectar either containing glucose and sucrose or glucose only (see above). Older workers caught from the plant surface were fed the same two solutions.

Next, we investigated whether the protein fraction of the EFN represents the source of the invertase-inhibiting activity. Workers freshly eclosed from pupae were fed fresh EFN, boiled EFN (100 °C for 3 min to denaturalise all proteins) or artificial nectar (containing glucose and sucrose) to which commercial chitinase and glucanase had been added. We also prepared artificial nectars with two different concentrations of these PR-proteins and fed them to older workers, to investigate whether higher activities of PR proteins exert a stronger inhibitory effect.

In a final feeding assay, we aimed at separating putative effects caused by the chitinase vs. the glucanase and fed older workers with artificial nectar containing only chitinase, only glucanase, both PR proteins (as the positive control), or no PR proteins (as the negative control).

Quantification of invertase activity

For each analysis of invertase activity in the guts of ant workers, six workers per vial were selected randomly and dissected (Heil *et al.* 2005a). Their gut contents were pooled and invertase activity was determined photometrically as molecules of glucose liberated per minute from sucrose (Heil *et al.* 2005a). To separate gut tissue from the liquid content, guts were opened in 50 μL droplets of phosphate buffer (Heil *et al.* 2005a) and gut tissue was rinsed twice in buffer before being further processed. In order to exclude intact cells (prokaryotic or eukaryotic ones) as a putative source of the activities of interest, the liquid gut content (buffer with the pooled liquids of 5–10 guts) was passed through filters ('Rotilabo-Spritzenfilter', pore size 0.20 μm, diameter 13 mm: Carl Roth GmbH Germany; www.carl-roth.com) before further processing (i.e. incubating with pure chitinase or fresh EFN).

Polyacrylamide gel electrophoresis and zymography of invertase activity

Zymography (in-gel activity assays) serves to detect and quantify the activity of enzymes that have been separated electrophoretically in a native gel. We used zymography to unambiguously identify specific proteins as the source of the invertase activity and of the inhibitory activity, respectively. For zymogram assays, liquid gut contents were prepared in the field as described above; the invertase activity was determined in an aliquot as described above, and the rest of the sample was transported to the laboratory on dry ice. Total protein concentration was determined (Bradford 1976) using ovalbumin as a standard (Bio-Rad; www.biorad.com). Sixty microgram of protein of each sample were added (1 : 1) to electrophoresis sample buffer containing: 0.5 M Tris-HCl, pH 6.8, 0.25% glycerol, 10% (w/v) SDS and 1% (w/v) bromophenol blue and loaded in 7.5% gel concentration. Proteins were separated at a constant voltage of 110 V; gels were stained with Coomassie stain and then subjected to destaining with a mixture of 40% methanol and 10% acetic acid.

For zymograms, 40 μg of samples were mixed with sample buffer and loaded in 7.5% gel concentration and run at 120 V and 12 mA without boiling the samples (Laemmli 1970). Runs were performed completely at 4 °C and buffers were precooled. After the run, gels were rinsed twice in 100 mL of 100 mM of sodium acetate (pH 6.0) for 15 min at room temperature and then incubated for 30 min at 37 °C in 0.5 M of sucrose in the same buffer. After this time, gels were revealed by heating at 90 °C in a solution of 1 N of NaOH containing 0.1% 2,3,5-triphenyltetrazolium chloride (TTC): invertase activity was observed as a red band in a colourless background (Gabriel & Wang 1969).

Sample preparation for nanoLC-MS/MS analysis

We aimed to characterise the enzyme exhibiting the invertase activity in the digestive tract of the ants. Therefore, the region of the gel that exhibited invertase activity in the zymography assays was cut from the gel matrix and tryptically digested (Shevchenko *et al.* 2006). Tryptic peptides were extracted from the gel matrix (75% ACN/5% formic acid) and extracts were dried in a vacuum centrifuge. For LC-MS analysis samples were reconstructed in 10 μL aqueous 1% formic acid. Protein digests were analysed using a nanoAcquity nanoUPLC system (Waters: www.waters.com) interfaced online to

a Synapt HDMS quadrupole time-of-flight mass spectrometer (Waters) as described earlier (González-Teuber *et al.* 2009; Orona-Tamayo *et al.* 2013b).

Data were analysed as described earlier (González-Teuber *et al.* 2009; Orona-Tamayo *et al.* 2013b). Briefly, the peptide fragment spectra were first searched against a subdatabase containing common contaminants such as human keratins and trypsin using Protein Lynx Global Server (PLGS) (Waters, Milford, MA, USA; http://www.waters.com). Spectra that remained unmatched by database searching were interpreted *de novo* to yield peptide sequences and were subjected to sequence similarity searching using the MS-BLAST program installed on an in-house server. MS-BLAST searches were performed against the NCBInr database (downloaded from http://www.ncbi.nlm.nih.gov/ on August, 10, 2011). In parallel, pkl-files of MS/MS spectra were generated and searched against NCBInr database (updated September, 11, 2011, installed on a local server) using MASCOT version 2.4 using described searching parameters (González-Teuber *et al.* 2009; Orona-Tamayo *et al.* 2013b). Conventional database searching could not be implemented because no sequenced genomes exist for the species studied here. Therefore, proteins were identified based on their homology. Our approach relies on the prediction of peptide sequences directly from the MS/MS spectrum (*de novo* sequencing) and subsequent homology-based database searching, using an MS BLAST algorithm suited for MS data and optimised for peptide sequences as they are originating from *de novo* prediction (González-Teuber *et al.* 2009). With this approach, we could identify the proteins with the closest sequence in the database and thereby identify the putative organism producing the invertase (specifically: the ant itself vs. intestinal bacteria or yeasts).

RESULTS

Extrafloral nectar inhibits invertase

The older workers that we collected from the plant surface exhibited low invertase activity in their digestive tracts, which was not inducible by sucrose in the diet of the ants (Fig. 1b). In contrast, workers freshly eclosed from pupae showed higher levels of invertase activity than the older workers and this activity increased in workers fed a diet containing sucrose (Fig. 1b). Feeding EFN to workers freshly eclosed from pupae significantly decreased their invertase activity, an effect that was not observed in workers fed boiled nectar (100 °C for 3 min) (Fig. 1c). Feeding ant workers with a protein-free solution of sugars and amino acids resulted in significantly higher invertase activity than feeding them with natural, fresh nectar, which contains active PR proteins (Fig. 1c,d). Similarly, pure PR-proteins (chitinase and β-1,3-glucanase) in the diet inhibited the invertase activity of the workers in a dosage-dependent manner (Fig. 1d).

Localisation and identification of the invertase

The liquid content of the guts of workers recently eclosed from pupae exhibited significantly higher sucrose-hydrolysing activity than the gut tissue. Hence, this activity is almost completely restricted to the gut lumen (Fig. 2a). Next, we detected that pure chitinase caused an inhibitory effect as strong as the PR protein mixture, whereas pure β-glucanase did not detectably inhibit the invertase (Fig. 2b). Zymogram assays confirmed the reduction of the sucrose-hydrolysing activity in ants fed with PR proteins and demonstrated unambiguously that the invertase activity is caused by an active protein, rather than any other type of chemical process (Fig. 2c). Tryptic digestion and consecutive tandem mass spectrometric (MS/MS) analysis of the active protein and annotation of the resulting fragments (Table 1) revealed the best hit to be a maltase from the ant *Camponotus floridanus* (Bonasio *et al.* 2010).

Chitinase alone inhibits invertase *in vitro*

Cell-free gut liquid of older workers from the plant surface was incubated with fresh EFN or purified chitinase from *S. griseus*. An incubation period of 30 min with EFN significantly reduced sucrose-hydrolytic activity, and pure chitinase completely inhibited this activity (Fig. 3a). Zymogram assays confirmed this direct *in vitro* inhibition of the ant invertase (Fig. 3b) and revealed that the same inhibitory effect also occurred when invertase from baker's yeast (*Saccharomyces cerevisiae*) was incubated with purified chitinase (Fig. 3c).

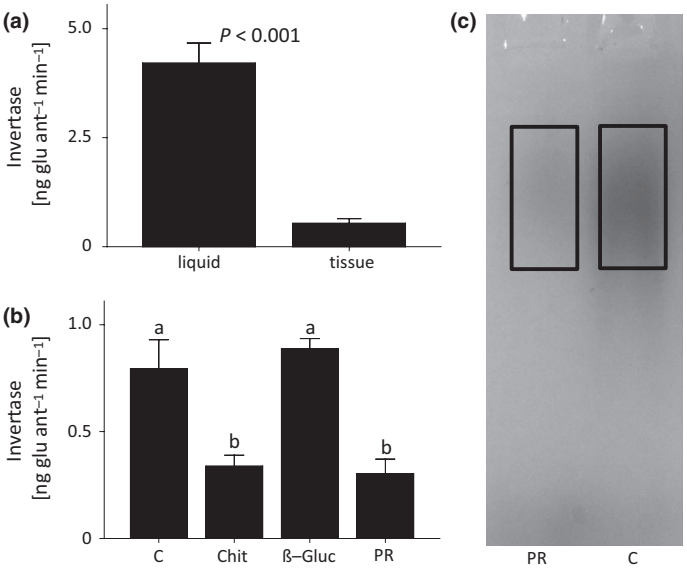

Figure 2 Localisation and identification of the invertase. (a) Invertase activity is localised in the gut liquid. Separating gut tissue from the lumen demonstrated that the invertase is localised in the liquid (workers freshly eclosed from pupae, $n = 8$, Wilcoxon pair test). (b) Chitinase but not ß-glucanase inhibits invertase. Invertase activity in older workers fed with pure chitinase (Chit) was as low as in workers fed both PR-proteins and significantly lower than in workers fed with sugar solution (control, C) or pure ß-glucanase (ß-Gluc) ($n = 5$). Bars represent means, error bars indicate 1 SEM, and bars marked with different letters in panel b indicate significant differences ($P < 0.05$; LSD *post hoc* test). (c) Zymogram assays confirmed lower invertase activity in workers fed PR proteins than in control animals fed sugar solution (C). Marked squares were cut from the gel for tryptic digestion and consecutive MS/MS analysis.

Table 1 Annotation of the ant invertase by MS-BLAST. Tryptic digestion produced 83 peptides of which eight gave significant hits in the MS-BLAST search after removing common contaminants such as human and animal keratins, trypsin (Orona-Tamayo *et al*. 2013b). The two best hits are presented.

Protein	Description	Accession	Organism	Peptide hits MS-BLAST	MS-BLAST score
P1	Maltase 1	EFN64560	*Camponotus floridanus*	8	513
P2	Probable maltase H-like	XP_003397344	*Bombus terrestris*	2	143

DISCUSSION

Specialisation and the resulting mutual dependency on the performance of the other partner is a paramount mechanism for the stable functioning of mutualisms (Kiers *et al*. 2011). For example, most mutualistic plant–ants that inhabit myrmecophytes such as the 'Swollen thorn *Acacia*' cannot live independently of their host plant. However, no physiological mechanisms have ever been discovered that originally cause such extreme specialisation. Although specialisation is usually considered at the evolutionary (i.e. genetic) level (Poisot *et al*. 2011), it can also occur within the framework of phenotypic plasticity. Partners in symbiotic mutualisms can undergo dramatic phenotypic changes when entering a symbiosis and these changes often appear disadvantageous for the symbiont, at least when we consider its possibilities to return to a free-living life style. For example, endophytic fungi are commonly sterile, i.e. they cannot reproduce sexually during their mutualistic phase (Yuan *et al*. 2010). Similarly, rhizobia cannot leave the nodules formed in the roots of certain plant species once they have differentiated into bacteroids (Oono & Denison 2010), because bacteroids are non-dividing and, thus, not viable outside the nodule (Mergaert *et al*. 2006). Interestingly, the plant controls the differentiation process that converts rhizobia into bacteroids (Mergaert *et al*. 2006) and thus 'traps' the differentiated rhizobia in the nodule.

In this study, we propose partner manipulation as a mechanism that favours reciprocation in horizontally transmitted mutualisms: the host manipulates the phenotype of the symbiont to enhance its degree of specialisation. Adult *P. ferrugineus* plant–ants posses almost no invertase activity in their digestive tract, consequently discriminate against sucrose in their diet and thus feed only on the sucrose-free EFN that is provided by their specific host plants (Heil *et al*. 2005b). Invertase activity disappears in the mobile adults but is present in the larvae. Hence, the energy supply of specifically the protecting developmental stage of the ant is tied to the performance of the host (Kautz *et al*. 2009). We have now discovered that invertase activity is lost when workers have already eclosed from the pupae and start to feed on EFN, because the EFN directly inhibits the gut invertase of the ant. Thus, the behavioural and physiological adaptation of *Pseudomyrmex* plant–ants to feed on host-derived rewards is strengthened at the phenotypic level by a negative feedback-mechanism that is

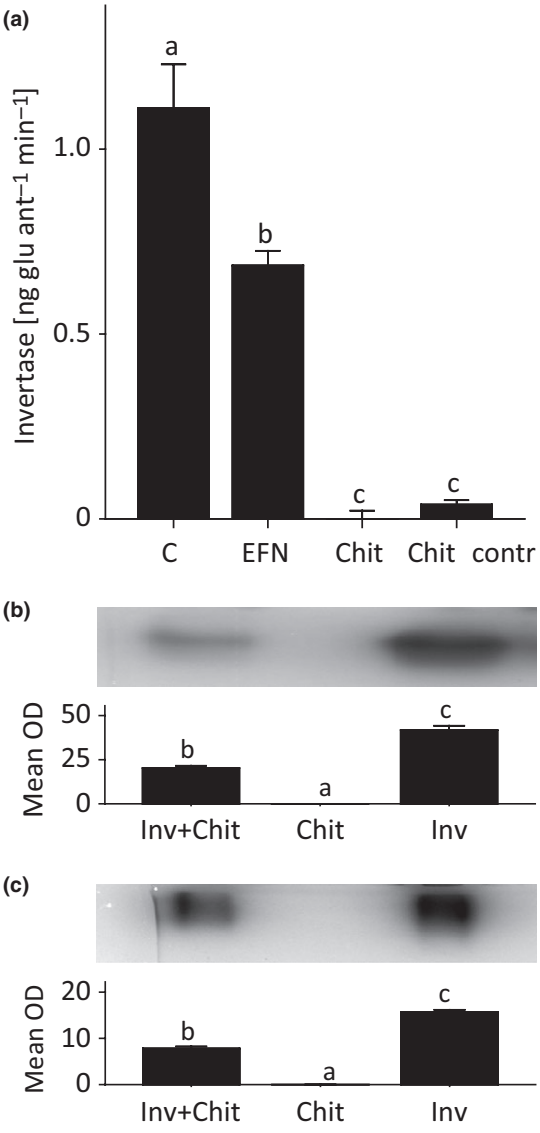

Figure 3 Chitinase activity directly inhibits invertase *in vitro*. (a) Invertase activity was inhibited when filtered gut liquids (control, C) were incubated 30 min with fresh extrafloral nectar (EFN) or chitinase from *S. griseus* (Chit). Negative control: chitinase from *S. griseus* without gut liquid (Chit contr). (b) Incubating gut liquid of *P. ferrugineus* with purified chitinase from *S. griseus* significantly inhibited invertase activity, whereas chitinase alone exhibited no visible activity. Graph: mean optical densities (ODs) for all three conditions ($n = 4$). (c) The same inhibition effect was observed when incubating purified invertase from baker's yeast with chitinase from *S. griseus* (mean ODs: $n = 5$). Bars represent means, error bars indicate 1 SEM, and bars marked with different letters are significantly different ($P < 0.05$, LSD *post hoc* analysis).

exerted by their host. A freshly eclosed worker is likely to feed first on EFN, or receive EFN from her nestmates via social feeding. Age-dependent casts were reported for the related acacia-ant, *P. spinicola*, and only the oldest workers were involved in host defence and left the plant to prune the surrounding vegetation (Amador-Vargas 2012). Own observations with marked *P. ferrugineus* workers confirmed that animals caught from opened spines or the plant surface were mainly re-observed on the plant surface over the next 5 days

(workers from opened spines exclusively so), whereas ants caught from abroad the plant were more likely to show up again on the surrounding vegetation (Fig. S1). We conclude that the food that is most easily available to the freshly eclosed workers is EFN produced by their host plant. Once a young worker feeds on EFN, her invertase decreases, she starts to discriminate against sucrose in her diet and consequently continues feeding on sucrose-free EFN, which then re-enforces the inhibition effect. In fact, freshly eclosed workers did not distinguish between artificial solutions with and without sucrose, but they discriminated against sucrose after having ingested EFN, or sugar solution with PR proteins, for 1 day (Fig. 2). Partner manipulation appears to be entirely under the control of the host.

Because boiled EFN exerted no inhibitory activity (Fig. 1c), we hypothesised that enzymes in the EFN cause this effect. The dominant enzymes in *Acacia* EFN are PR proteins, which protect the EFN from microbial infection (González-Teuber *et al*. 2009, 2010). Sequencing and annotation indicated that these PR proteins are typical plant proteins (González-Teuber *et al*. 2009, 2010), and subsequent studies of the proteome of the nectary tissue combined with reverse-transcription polymerase chain reaction (RT-PCR) confirmed that they are synthesised by the plant itself (Orona-Tamayo *et al*. 2013b). We prepared artificial nectar containing a commercial chitinase (from *Streptomyces griseus*) and β-glucanase (from *Aspergillus niger*) at two concentrations, the lower one resembling activities as found in natural EFN (González-Teuber *et al*. 2009), and also tested both PR proteins individually. We found that PR proteins in an artificial diet inhibited the invertase activity of the workers in a dosage-dependent manner (Fig. 1d) and that the effect was caused by chitinase alone (Fig. 2b). Thus, the inhibition of the ant invertase appears to be caused by enzymes with chitinase activity in general, rather than by a specific *Acacia* protein.

In principle, this effect could be caused by various mechanisms: (1) the invertase that we measure in the digestive tract of the ants could be produced by intestinal bacteria or yeasts, which are damaged by the chitinase, (2) the invertase could be localised in the peritrophic matrix, which becomes damaged by chitinases in the ingested food, (3) chitinase in the EFN could inhibit the expression of the gene encoding for the invertase; or (4) the enzymes could interact directly. Tryptic digestion and MS/MS analysis revealed as the best hit to the sucrose-cleaving enzyme of *P. ferrugineus* a maltase from *Camponotus* ants (Bonasio *et al*. 2010). Invertases are seldom reported from insects (but see Mita *et al*. 2004; Pauchet *et al*. 2008) and then were discussed to represent the products of horizontal gene transfer from microorganisms (Mita *et al*. 2004). Moreover, enzymes in the carbohydrate metabolism are often promiscuous (i.e. bind to more than one substrate or catalyse more than one reaction) and minor changes in their amino acid sequence can change their substrate specificity (Henrissat 1991). Therefore, it was not surprising to find another disaccharide-hydrolysing enzyme as the best hit to the ant invertase. More importantly, the twenty best hits in our MS-BLAST search were to animal-derived enzymes, rather than proteins of microbial origin. In summary, pending further analysis, this result makes it likely that the invertase is produced by the ant itself. To exclude histological effects on the peritrophic matrix or effects at the level of gene expression, we obtained gut liquid, which is the only source of invertase activity in the gut (Fig. 2a), and filtered it through membrane filters with 0.20 μm pore size to obtain a cell-free fraction. We observed the inhibition of invertase within < 30 min of incubation (Fig. 3a) and in proteins that had been separated in a gel (Fig. 3b). Thus, mechanisms that depend on living bacteria or fungi and mechanism that require gene expression in living cells can be discarded, leaving a direct enzyme–enzyme interaction as the most likely reason of the observed inhibitory effect.

An outstanding question is how a direct inhibition of invertase by a chitinase functions at the biochemical level. The independency of the effect of the detailed nature of the interacting enzymes (Fig. 3b,c) indicates that it is likely to depend on conserved motifs of the enzyme classes, rather than representing a co-evolved interaction between a specific plant chitinase and the ant invertase. Interestingly, chitinase from *S. griseus* (Kezuka *et al*. 2006) and common plant chitinases (Kezuka *et al*. 2010) possess a chitin-binding site containing two tryptophan molecules that are exposed from the surface in an antenna-like structure (Kezuka *et al*. 2006, 2010). This binding site could interact with the hydrophobic part of the active hydrolytic centre of invertases, which also contains three tryptophans (Lammens *et al*. 2008), and then would block the reactive centre of the invertase (P. Bayer pers. comm).

Independently of the biochemical mechanism, our results demonstrate that the plant host in the ant–acacia mutualism manipulates the digestive capacities of the mobile stage of the symbiotic ants to enhance their dependence on the host-derived food rewards. Because the ants, in consequence, cannot gain energy from the ingestion of non-host food sources, this manipulation of their digestive capacity ties their food availability directly to their defensive behaviour. When ants defend their host efficiently they maintain its photosynthetic capacity and, therewith, its capacity to produce the only food source that the workers can digest: EFN. We conclude that the phenotypic changes that symbionts undergo after engaging in a symbiotic interaction should be investigated in more detail to distinguish responses that are controlled by the changing partner itself from those that are under the control of the host. We propose that partner manipulation represents a mechanism that enhances the degree of specialisation of the symbiont, thereby makes reciprocation more beneficial and, thus, stabilises a mutualism.

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STATEMENT OF AUTHORSHIP

MH designed the experiments, collected the samples and wrote the first draft, ABB and DOT performed the enzymatic assays, NW and AS identified the invertase, and MH, DOT and NW contributed to the revisions of the text.

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