Let the best one stay: screening of ant defenders by *Acacia* host plants functions independently of partner choice or host sanctions

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Summary

1. Multiple plant species are engaged in defensive mutualisms with members of the third trophic level. However, mutualisms are prone to exploitation by low-quality symbionts that do not provide the adequate service to their host. Can mutualisms proceed only when hosts identify their symbionts in advance or continuously monitor their activity, or are there other mechanisms to avoid the invasion of mutualisms by exploiters?

2. High-reward species amongst Mesoamerican *Acacia* myrmecophytes are dominantly colonized by defending mutualistic ants, whereas about 50% of the low-reward hosts are inhabited by non-defending exploiters. I followed the development of recently founded ant colonies on a high-reward and a low-reward *Acacia* host species over 7 months, to investigate whether reward production correlates with a preferred maintenance of defending ants on the respective hosts.

3. Ant diversity decreased sooner on high-reward than on low-reward hosts, and mutualistic ants were more likely to finally dominate the high-reward hosts. I observed an increased frequency of mutualists replacing parasites at high initial rates of reward production. Apparently, higher nectar provisioning by the host plants shifted the competitive balance between mutualistic and parasitic ants. Independently of the causal reason for the different secretion rates, producing more nectar thereby favours the maintenance of defending mutualists on high-reward hosts.

4. *Synthesis*. The aggressiveness that enables ants to outcompete other ants also underlies their defensive effect against herbivores. I conclude that hosts can preferably associate with high-quality mutualists without measuring their effectiveness. Mutualisms remain stable when partner screening is based on traits that are relevant for the mutualistic interaction, with no need for the host to have information on the quality or identity of the symbiont.

Key-words: *Acacia*, ant–plant interaction, exploiter, extrafloral nectar, parasite of mutualism, partner screening, plant–herbivore interactions, *Pseudomyrmex*

Introduction

Understanding the evolution and maintenance of interspecific cooperation is a difficult task, because mutualisms are continuously threatened by exploiters, that is, individuals which make use of the host-derived rewards without rendering an adequate service (Bronstein 2001; Yu 2001). Partner choice and host sanctions are two prominent examples of mechanisms that have been suggested to stabilize mutualisms (Bull & Rice 1991; West *et al.* 2002). Hosts can identify their future partner in advance and associate only with high-quality

mutualists (partner choice), or they can monitor the quality of the service that is provided by the partner after having established the symbiotic interaction and cease reward allocation towards exploiters (host sanctions). However, both strategies require that hosts can directly judge on the identity or the actions of their partners. Recently, Marco Archetti (Archetti 2011; Archetti *et al.* 2011) proposed partner screening as an alternative mechanism for the stabilization of mutualisms. Partner screening means that the host imposes a 'contract' that consists of an appropriate cost of entry and benefit of being a mutualist, thereby causing the potential symbionts to screen themselves according to their quality as mutualists. Simply speaking, only the adapted mutualists gain sufficient benefit from engaging in the mutualism to make it attractive

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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. An ideal test of screening would be to manipulate this cost of entry into the host and observe the subsequent evolutionary trajectories of mutualists and parasites (D. Yu, pers. comm.). In practice, a manipulable system is difficult to find. However, here I report on an ant–plant community in Mexico, which amounts to a natural experiment of this type.

In Mesoamerica, myrmecophytic *Acacia* plants are engaged in a protective mutualism with ant species in the genus *Pseudomyrmex*: the plants provide nesting space in hollow thorns and food rewards (cellular food bodies and extrafloral nectar, EFN) to their ant inhabitants, which function as a defence against herbivores, pathogens and encroaching vegetation (Janzen 1974). Exploiting, non-defending ants can inhabit these plants and feed on food bodies and EFN. Because these exploiters prevent mutualist queens from successful colony founding, they represent a significant threat to the stability of the mutualism (Janzen 1975; Clement *et al.* 2008; Kautz *et al.* 2009a). We recently documented that two of four sympatric host species are high-reward hosts, which produce *ca.* three times more food rewards and more nesting space than the low-reward hosts. Intriguingly, this ant–plant community is characterized by a positive assortment of host reward level with ant quality. More than 90% of the high-reward plants are occupied by mutualistic ant species, whereas 50% of the low-reward hosts are inhabited by non-defending, parasitic ant species (Heil *et al.* 2009). I conclude that host investment pays off in this system, but which mechanism creates the bias in favour of the mutualists? This study was realized to investigate whether partner screening can explain the preferential association of high-reward hosts with mutualistic symbionts.

Materials and methods

The study was conducted in Oaxaca, South Mexico, at a site near Puerto Escondido (Pacific coast; ~15°55′N and ~097°09′W). Plant species were identified following Janzen (1974) and ants according to Ward (1993). The site had been cleared in spring 2009 and presented a high number of young *Acacia* shrubs that had recently re-grown from roots of cut plants. During a first census (Sept. 2009), I searched for plants 0.5–1 m high of the high-reward species, *A. cornigera* and the low-reward species, *A. hindsii*, that were inhabited by at least two different *Pseudomyrmex* species. Plants that were dominated by a single colony at this date were not considered. Five *Pseudomyrmex* species occurred on these plants, of which *P. ferrugineus*, *P. mixtecus* and *P. peperi* are mutualists, whereas *P. gracilis* and *P. nigropilosus* are exploiters (Janzen 1975; Clement *et al.* 2008; Kautz *et al.* 2009a). All *Pseudomyrmex* ants observed on the plant surface were considered, independently of whether they were workers or founding queens. The occupancy by ants was rated for every species on an ordinal scale following Clement *et al.* (2008) as: 0 = no ant present, 1 = a single ant present, 2 = few (2–10) ants present on < 25% of leaves, 3 = ants on 25–50% of leaves, 4 = ants on 50–75% of leaves, 5 = entire plant occupied/patrolled. I then quantified the EFN secretion of the main shoot of each plant as described earlier (Heil *et al.* 2009). The site was revisited after 7 months (April 2010), and all remaining plants were censused as described earlier. To obtain a quantitative impression of the diversity of the ant communities on the different hosts, Shannon-Weaver diversity indices were calculated for every plant and census date as:

$$H' = -\sum_{i=1}^{s} pi \times \ln (pi) \qquad \text{eqn 1}$$

with H' = Diversity, S = number of species and p_i = the proportion of ants of species i relative to the overall number of ants on the plant, using the above occupancy index as a measure of ant frequency.

During both censuses, EFN secretion was quantified as a measure of the investment of the plant into its ants. Extrafloral nectar secretion was quantified as amounts of soluble solids as described previously (Heil *et al.* 2004). In short, I selected the main shoot of every plant and isolated the region comprising the youngest five leaves, applying a ring of sticky resin (Tangletrap®, Tanglefoot Company, Grand Rapids, Michigan, USA) below the fifth leaf. Entrance holes of thorns localized in this part of the shoot were blocked with ca 3 mm³ of Tangletrap, and all ants that remained on the surface of the isolated part were removed mechanically. Then, the youngest five leaves were placed in a mesh bag to exclude flying EFN consumers. After 24 h, all nectar that had accumulated on the nectaries of these five youngest leaves was collected with pipettes and quantified, using a portable refractometer (ATAGO hand refractometer) and microcapillaries. To remove EFN quantitatively, 20 μL of pure water was then applied on all nectaries. The resulting solution was removed and measured as described earlier, and the whole procedure was repeated until a concentration < 1% had been reached. Values (volume × concentration) from all collections conducted for the nectaries on one shoot were summed up, to calculate plant investment in the EFN secretion as μg soluble solids per day. Finally, the Tangletrap was removed mechanically with tweezers and paper. After 2 days, ant activity on the treated shoots could not be distinguished from the activity on untreated plants (MH, pers. observation).

Results

The mean number of ant species occupying individual plants decreased over time. For instance, of the 52 plants that housed at least two ant species in Sept 2009, 36 were inhabited by one colony of one ant species in April 2010. This effect was stronger for *A. cornigera* (16 of 19 plants (= ca 85%) inhabited by one colony at the second census) than for *A. hindsii* (20 of 33 = ca 60%). Consequently, ant diversity on the host plants decreased from September 2009 to April 2010 (Fig. 1). Univariate ANOVA revealed a significant effect on ant diversity of census date (SS = 1.982, F = 11.080, P < 0.001). The decrease in diversity was stronger for *A. cornigera* than for *A. hindsii*, but not significantly so; neither 'host species' nor the 'host species × date' interaction terms were retained in the minimal model (P > 0.10 for both factors). However, and importantly, the identity of the retained ant species differed between high- and low-reward acacias. On most of the *A. cornigera* plants, the remaining ant colonies were mutualists; parasites only inhabited two of 19 plants, without dominating a single one (occupancy index for parasites all < 3 in the second census; first census: parasitic ants inhabited six plants). By contrast, 18 *A. hindsii* plants were inhabited, and eight of these were dominated (occupancy index ≥ 4), by parasitic ants in the second
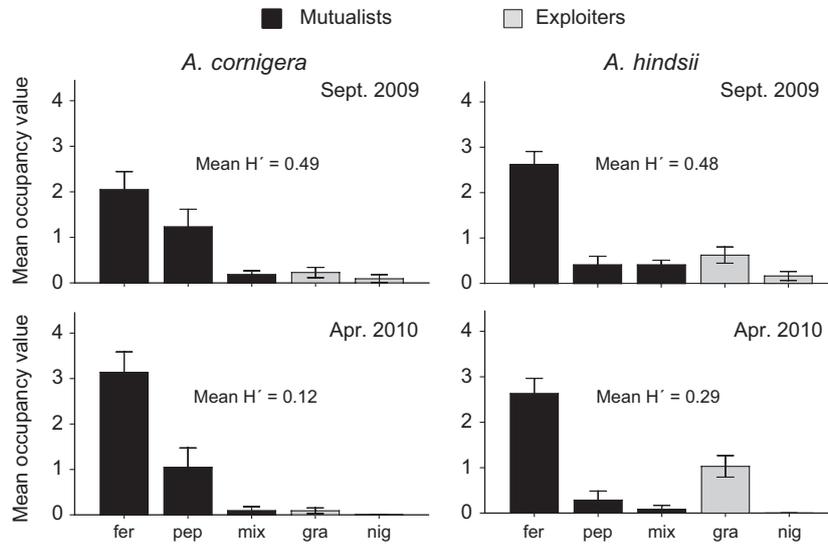
Fig. 1. Occupancy of *Acacia* hosts by the different ant species. The occupancy by ants at both census dates is illustrated separately for the high-reward host (*Acacia cornigera*) and the low-reward host (*A. hindsii*). Mutualist ant species (fer = *Pseudomyrmex ferrugineus*, pep = *Pseudomyrmex peperi*, mix = *Pseudomyrmex mixtecus*) are indicated by black bars, parasitic ants (grac = *Pseudomyrmex gracilis*, nig = *Pseudomyrmex nigropilosus*) are indicated by grey bars. Bars represent means ± SE, sample size *n* = 19 for A*cacia cornigera* and 33 for *Acacia hindsii*.

census (first census: 16 plants inhabited by parasites, and three were dominated).

Extrafloral nectar secretion levels were a strong predictor of occupancy by mutualist vs. parasitic ants in the second census (Fig. 2): the greater the EFN level, the higher the occupancy index by mutualistic ant species ($P < 0.001$, $n = 52$) and the lower the occupancy index by parasitic species ($P = 0.002$). In the first census, EFN secretion levels were only non-significantly positively correlated with the occupancy index of mutualistic ants ($P > 0.05$) and not correlated with the occupancy index of parasitic ants (Fig. 2A).Thus, the differences in EFN secretion between plants inhabited by parasites and by mutualists were much stronger during the second census (Fig. 2B). Intriguingly, the development in the occupation by ants (calculated as 2nd value–1st value) was significantly correlated with the EFN secretion as measured at the beginning of the experiment (Fig. 3). However, whereas this correlation was positive ($t = 2.646$ and $P = 0.011$) for the mutualists, the development in occupancy by parasites was negatively correlated with initial EFN secretion ($t = -3.464$ and $P < 0.001$). Thus, the EFN secretion in the beginning of the observational phase was a strong predictor of the probability at which mutualistic or parasitic ants were able to maintain themselves, or ultimately dominate, on a certain host plant.

Discussion

Hosts that have limited information on the quality of their partners cannot apply sanction mechanisms to enforce mutualistic behaviour. In the present study, the competition amongst ant symbionts reduced their diversity on all *Acacia* host plants, and this tendency was particularly beneficial for the high-reward host species, *A. cornigera*: a large majority of these plants were dominated by one single mutualist ant
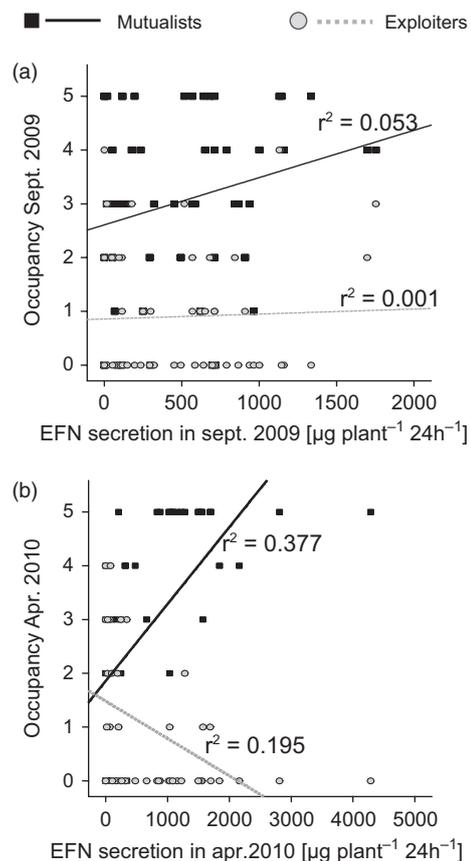

Fig. 2. Relations between extrafloral nectar (EFN) secretion and current occupancy. The occupancy indices by mutualist (black squares) and parasitic (grey circles) ants are depicted in dependency of the current rate of EFN secretion (in micrograms per plant and 24 h), separately for both census dates. Regression lines are indicated independently for mutualistic (solid black line) and parasitic (dotted grey line) ants. $N = 52$ plants.
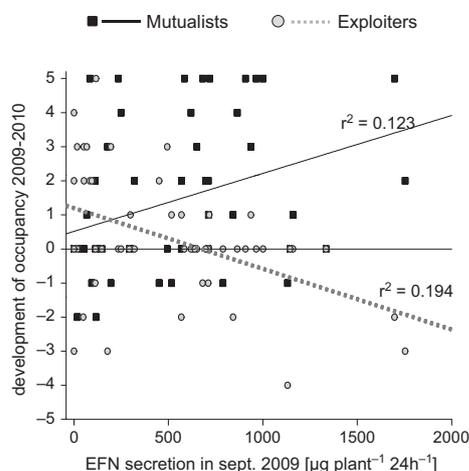
Fig. 3. Predictive value of extrafloral nectar (EFN) secretion for future occupancy by mutualist vs. parasitic ants. The development in the occupancy by mutualist (black squares) and parasitic (grey circles) ants was calculated as difference between the final (April 2010) and the initial (September 2009) occupancy index and is depicted here in dependency of the initial rate of EFN secretion (in micrograms per plant and 24 h). Regression lines are indicated independently for mutualistic (solid black line) and parasitic (dotted grey line) ants. $N = 52$ plants.

colony, in the end of the observational period. Most interestingly, initial EFN secretion rates predicted how likely the mutualists were to win during the observed time span of 7 months. Quantitative variations in reward provisioning by the host can shift the competitive balance between mutualistic and parasitic symbionts. Are these patterns a consequence of a functioning partner screening *sensu* Archetti (Archetti 2011; Archetti *et al.* 2011) and what are the resulting evolutionary implications?

Several prerequisites make partner screening likely to happen: (i) the host is not able to directly assess the quality of the different symbionts, (ii) there is a cost for the symbionts to enter the interaction and (iii) the mutualist can gain a higher benefit from the interaction than the exploiter. Available evidence indicates that these central prerequisites are met in the present system. First, ant-free *Acacia* saplings are quickly inhabited by colony-founding queens of both mutualistic and parasitic species (Kautz *et al.* 2009b; and this study). Thus, the hosts are apparently not able to avoid initial colony founding by exploiters. Second, foundresses must gnaw entrance holes into an empty thorn, remove the pith, must continuously leave the colonized thorn to harvest EFN and food bodies until the first workers are available, and the foundresses or the workers must outcompete other emerging colonies to monopolize their host plant. Thus, the symbionts clearly pay a high cost of entering the interaction. Interestingly, in this context, the mutualist *P. ferrugineus* appears to be more competitive, because colonies of *P. ferrugineus* usually dominate the entire host plant, whereas colonies of the exploiter, *P. gracilis*, tend to share hosts (Kautz *et al.* 2012). Host sharing can enhance the possibilities for competitively inferior ants for successful colonization (Izzo *et al.* 2009).

Finally, mutualists such as *P. ferrugineus* and *P. peperi* reach much larger colony sizes than *P. gracilis*, when living on an *Acacia* host (Clement *et al.* 2008; Kautz *et al.* 2009b). Thus, we have a case of a host that 'cannot actively choose', there is a cost of establishing the mutualism, and the potential benefits are higher for the mutualist than for the exploiter. It remains uncertain, however, whether the plants are able to monitor the identity, the activity or the defensive efficiency of the ants.

How can plants assure the preferred association with mutualists without information on the identity or efficacy of their symbionts? In contrast to the theoretical conditions applied by Archetti *et al.* (2011), the filtering process the present system occurs when the ants have already colonized the hosts. Thus, 'competition-based screening' can occur both before and after the initial colonization process. Competition plays an important role in the coexistence of different plant-ants (Palmer, Stanton & Young 2003; Heil *et al.* 2009). Ants compete via aggressive behaviour and these 'wars of attrition' are an important filter in ant-*Acacia* mutualisms (Palmer 2004). Increased EFN secretion rates have been related to higher survival rates of ant workers (Lach, Hobbs & Majer 2009) and can increase ant activity and aggressiveness (Sobrinho *et al.* 2002; Ness 2006; Ness, Morris & Bronstein 2009). Because the mutualists are more strongly adapted than the exploiters to make use of the plant-derived food sources (Heil, Büchler & Boland 2005; Clement *et al.* 2008; Kautz *et al.* 2009a), higher-reward provisioning by the host favours mutualists relatively more than parasites (Heil *et al.* 2009). In consequence, *P. ferrugineus* ants protect their plant more aggressively from non-defending EFN exploiters when they receive higher amounts of sugar (González-Teuber *et al.* 2012). Intriguingly, it is this aggressive capacity that makes mutualistic ants good defenders of their host plants, not only against EFN thieves and competing ants, but also against herbivores. In conclusion, high-reward hosts remain colonized preferably by defending ants even when the plant has no means to monitor the defending activity exerted by the ants. An additional stabilizing element that applies to this particular study system, however, results from the fact that better defended plants maintain more intact leaf area and thus can produce more EFN. This situation leads to a closed loop of positive feedback mechanisms between ant activity and host quality in the *Acacia-Pseudomyrmex* mutualism (Heil *et al.* 2009).

It remains unanswered whether screening processes as encountered here necessarily require, or lead to, co-evolution between symbiont and host. On the one hand, the mechanism as described here functions as soon as plants secrete varying amounts of nectar, irrespective of the reason of this variability. Therefore, the functioning screening mechanism does not necessarily represent the outcome of a co-evolutionary process. On the other hand, we can assume that the beneficial effects of aggressive ants on high-reward hosts do inflict a selective pressure on nectar secretion rates, for the following reasons; (i) the nutrition of defending insects is the only known primary function of EFN, (ii) nectar secretion rates are controlled by the plant at several different levels, including

the genetic one (Heil 2011) and (iii) nectary traits are heritable, at least in other species (Mitchell 2004; Rudgers 2004; Wooley *et al.* 2007; Kaczorowski, Juenger & Holtsford 2008). Thus, although co-evolution is no prerequisite for a functioning screening mechanism, the feedback between investment into, and effect of, the EFN-mediated defence can cause co-evolution, thereby strengthening the mutualistic interaction.

Most importantly, the present study reports on a mechanism that leads to a stable association of good mutualists with high-reward hosts at the ecological time scale. The screening process does not require host sanctions and is not necessarily the consequence of co-evolution, although it is likely to lead to co-evolutionary processes. Thus, the mechanism by which the filtering process takes place in the current study system comprises aspects that have been described as 'competitive screening' by Archetti *et al.* (2011). However, the filtering process is based on a trait that is directly and causally correlated with a trait of vital interest for the host, which represents an aspect not considered in the economic model. Is this mechanism likely to be a common one? Ants that win aggressive encounters are usually the best defenders of ant-plants. Likewise, bacteria that possess an active luziferase are more likely to successfully pass the biochemical hurdles created by their squid host (Visick *et al.* 2000). *Nod* signals, by which Rhizobia qualify themselves to enter host-plant roots, contain nitrogen (Cohn, Day & Stacey 1998); these molecules are therefore cheaper to produce for N-fixing bacteria than for non-fixing strains. We should start to screen other mutualisms for further examples in which the trait by which a potential partner qualifies as a mutualist is causally related to its quality. All these mutualistic systems are likely to be stable without the action of host sanction mechanisms.

Acknowledgements

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