Insects had it first: surfactants as a defence against predators

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Insects have evolved an astonishing array of defences to ward off enemies. Well known and widespread is the regurgitation of oral secretion (OS), fluid that repels attacking predators. In herbivores, the effectiveness of OS has been ascribed so far to the presence of deterrent secondary metabolites sequestered from the host plant. This notion implies, however, that generalists experience less protection on plants with low amounts of secondary metabolites or with compounds ineffective against potential enemies. Resolving the dilemma, we describe a novel defence mechanism that is independent of deterrents as it relies on the intrinsic detergent properties of the OS. The OS of Spodoptera exigua (and other species) was found to be highly amphiphilic and well capable of wetting the hydrophobic cuticle of predatory ants. As a result, affected ants stopped attacking and engaged in extensive cleansing. The presence of surfactants was sufficient to explain the defensive character of herbivore OS. We hypothesize that detergent is a common but unrecognized mode of defence, which provides a base level of protection that may or may not be further enhanced by plant-derived deterrents. Our study also proves that insects 'invented' the use of defensive surfactants long before modern agriculture had started applying them as insecticides.

Keywords: anti-predator defence; caterpillars; regurgitation; secondary metabolites; biosurfactants

1. INTRODUCTION

Many herbivorous insects such as grasshoppers or the larval stages of sawflies and Lepidoptera regurgitate their gut contents when disturbed (Grant 2006). Numerous studies suggest this behaviour to be an effective defence mechanism because the ejected oral secretion (OS) may have deterrent effects against vertebrate and invertebrate predators such as lizards, birds or ants (Eisner 1970; Peterson et al. 1987; Codella & Raffa 1995; Sword 2001). Enteric discharges from herbivores contain recently consumed plant material mixed with digestive and salivary secretions (Ortego et al. 1997), but their mode of defence has been attributed primarily to ingested plant secondary compounds (e.g. Sword 2001; Calcagno et al. 2004). Thus, defence seems to depend strongly on a herbivore’s food plant. Eastern tent caterpillars, for example, feed on plants containing cyanogenic glycosides. Benzaldehyde, a product of cyanogenesis, is incorporated into the OS and effectively protects the larvae from ant predation (Peterson et al. 1987). It has been suggested that plant secondary metabolites are important because most predators are not adapted to these compounds (Whitman 1990).

From an evolutionary point of view, complete reliance on plant-derived secondary chemicals should be detrimental for generalist herbivores because the insects are expected to be much more vulnerable on host plants that contain little or no defensive secondary metabolites. Therefore, we hypothesize that selection pressure acts on generalists to possess OS with insect- and/or plant-derived compounds that are ubiquitous (e.g. primary compounds) as this would make their defence against predators independent from certain plant species or families.

This notion was tested by investigating the defensive behaviour of Spodoptera exigua (Lepidoptera, Noctuidae), a popular model herbivore in plant defence studies. Its OS has been well studied as it contains fatty acid amides that elicit the emission of plant volatiles, thereby attracting the herbivore’s natural enemies (Turlings et al. 1990; Alborn et al. 1997; Maischak et al. 2007; Weech et al. 2008). Caterpillars of this moth are highly polyphagous. They feed on more than 50 plant species and play an important role as agricultural pests (Berdegue et al. 1998; Ehler 2004). The larvae are attacked by generalist predators, with fire ants being a key mortality factor (Ruberson et al. 1994; Stewart et al. 2001; Ehler 2004).

Myrmica rubra (Hymenoptera, Formicidae), a European fire ant species and the so-called red imported fire ant Solenopsis invicta (Hymenoptera, Formicidae) were used as model predators in this study. M. rubra is common throughout the Palearctic region of Eurasia and has become invasive in parts of North America. It inhabits open mesophilic and humid habitats, such as woodland edges, meadows, gardens and agricultural landscapes (Seifert 1996). Foragers collect food mainly in the vegetation. Prey items are overwhelmed by single scouts or, if too large, nest-mates are recruited by laying a pheromone trail (Evershed et al. 1982; Putyatina 2007). Solenopsis invicta, native to South America, is a notorious invasive species found in agricultural, urban and natural habitats in the United States, Australia and

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China (Zhang et al. 2007). Although considered a pest, S. invicta can significantly suppress defoliating herbivores and potentially benefit crop yield (Styrsky et al. 2006).

The experiments presented here highlight the role of surfactants in caterpillar OS as a hitherto undescribed physico-chemical defence mode that does not rely on variable plant toxin content.

2. MATERIAL AND METHODS

(a) Caterpillars and ants

Eggs of S. exigua (Hübner) were provided by Bayer CropScience, Monheim, Germany. Three groups of larvae were reared in plastic boxes (19 × 9 × 5.5 cm) in a climate chamber with a 15 L: 9 D photoperiod at 28/25°C (light/ dark) and 75% per cent rel. humidity. Each group received either artificial diet based on agar and cooked bean meal (modified from King & Leppa 1984), celery leaves (Apium graveolens var. Dulce) or tomato leaves (Lycopersicon esculentum var. Marmande) as food. Fresh, organically grown celery leaves were commercially obtained. Tomato plants were grown in pots with standardized potting soil (Einheitserde Typ P) in the greenhouse with supplemental light from sodium vapour lamps (400 W). Fourth instar larvae were used for all experiments and for collecting OS.

OS was obtained by gently holding a larva behind the head capsule with gloved fingers and allowing it to regurgitate voluntarily into a micropipet (volume 100 μl). Caterpillars were not squeezed or impaired in any other way. The amounts of discharged OS varied strongly but up to 12 μl could be obtained from a single larva. Within 30 min, OS of approx. 30 caterpillars was pooled and briefly centrifuged at 4°C to remove coarse undigested plant material. The resulting supernatant was frozen at −20°C until used in the experiments.

Three M. rubra (L.) nests with several queens per nest were excavated from the field near Würzburg (Germany) and transferred to the laboratory. The ants were reared in plastic bowls filled with humid soil. The invasive fire ant S. invicta (Buren) originated from Lake Okeechobee (Florida, USA). Three subcolonies with workers and brood were kindly provided by the ant-rearing facility of the Department of Behavioural Physiology and Sociobiology, University of Würzburg. The ants were kept in a plaster-of-Paris nest into which chambers had been moulded. The opening of a 1.5-ml vial. The vial was placed horizontally on the bottom of a Petri dish (9 cm diam.) and a single worker started attacking the caterpillar by biting and/or stinging it anywhere on the body. Caterpillars defended themselves by vigorous movements thereby regurgitating a droplet of enteric fluid. The droplet in the front of the mouth parts was used to repel the ant. Ants that came in contact with the OS stopped their attack and started intensive grooming. We measured the duration of grooming for the first ant that was hit by the fluid. The arena experiments were replicated after an observation time of 10 min with new pairings of caterpillars and ants (n = 10–11 for each treatment).

In addition to M. rubra, workers of S. invicta were used to test diet effects on caterpillar defence. Probably due to their small size (2.4 mm), this aggressive species attacked S. exigua only in larger numbers, making the observation of hit individuals difficult. Therefore, single ants were placed in a small Petri dish (5.5 cm diam.) that was painted with fluon on the sides to prevent escape. A 3-μl droplet of OS was applied to head and thorax with a pipette and the time spent grooming was recorded. As soon as the ant displayed normal walking behaviour, grooming was considered to be over. The experiment was stopped after 10 min and replicated with a new ant in a clean Petri dish (n = 15–16 for each treatment).

In a second bioassay, we tested whether OS from S. exigua had deterrent effects on ants. Each of the three OS types was diluted (1 : 1) with sucrose solution (10% w/v) and offered in a no-choice set-up to both ant species. Pure sucrose solution containing the same amount of sugar (5% w/v) was used as a control. The OS was tested on M. rubra by applying a droplet (5 μl) of test fluid on cotton wool that clogged the opening of a 1.5-ml vial. The vial was placed horizontally on the bottom of a Petri dish (9 mm diam.) and a single worker was introduced. The time spent drinking was recorded. A Petri dish with OS and another Petri dish with pure sucrose solution, each with a single worker, were observed in parallel. Petri dishes and ants were replaced after each observation (n = 15 for each treatment).

To measure any deterrent effects of OS on S. invicta a slightly different approach was used. Workers of this species refused to drink the sucrose solutions when kept isolated from their nest-mates. Therefore, a little ball of cotton wool was drenched with one of the test liquids (3 μl), stuck into a small plastic tube (10 mm length, 3–4 mm diam.) and placed into the foraging area of the nest. The cotton wool in the tube was accessible to the ants from both sides. Numbers of ants feeding after 5, 10 and 15 min were noted and then totalled. Only one type of OS was tested on a given experimental day. OS and sucrose solutions were tested in alternating sequences. Tube and cotton wool were replaced after each replicate (n = 10 for each treatment).

To establish whether surfactants in OS of S. exigua were necessary and sufficient for defence, single workers of

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M. rubra or S. invicta were placed into a Petri dish and a droplet of 5 μl (M. rubra) or 3 μl (S. invicta) of the test liquids was applied on to head and thorax of the ant. These amounts were used because we did not want the ants to drown. The following test liquids were used: (i) OS from caterpillars feeding on celery, (ii) demineralized water adjusted to a contact angle of 65° or 83° with the wetting agent Tween 20 (0.12% v/v, Sigma-Aldrich, Munich, Germany), and (iii) demineralized water. A preliminary assay had shown that ants survived on sugar water containing 0.12 per cent Tween 20 as well as on pure sugar water during three days of observation. The time spent grooming was recorded. A new ant was used each time (n=15–16 per treatment and ant species). One-way ANOVA followed by Tukey HSD test was used to analyse grooming duration in all bioassays. Square-root transformation was performed where necessary to meet the assumptions of variance analyses.

(d) Other species
The OS of the following other species were compared: three other generalist Noctuidae (S. frugipenda, Spodoptera littoralis, Helicoverpa armigera); Lynanthra dispar (Lymantriidae), which feeds on many tree species; Pieris brassicae (Pieridae), a specialist herbivore of brassicaceous plants; and larvae of the Colorado potato beetle (Leptinotarsa decemlineata, Chrysomelidae), which were also observed to regurgitate upon disturbance. All noctuid caterpillars were kept on Zea mays. Larvae of L. dispar were fed wheat germ artificial diet, P. brassicae was reared on Brassica oleracea and Colorado potato beetle larvae were kept on potato leaves. Contact angle measurements of all OS were performed as described above.

3. RESULTS
(a) OS is amphiphilic
Caterpillars of S. exigua raised on artificial diet, celery or tomato leaves, produced OS fluids that were easily distinguishable by their colours (artificial diet: brown; celery: light green; tomato: dark green). However, independent of food type, all OS types were highly amphiphilic and spread on a hydrophobic glass surface. Contact angle values among different OS types were almost identical (artificial diet: θ = 64.4 ± 0.5°, tomato: θ = 64.0 ± 0.6°, celery: θ = 65.5 ± 0.4°; ANOVA: F_{2,27} = 1.837, p = 0.178) but compared with water (θ = 96.7° ± 0.3°) the difference was highly significant (ANOVA: F_{5,36} = 1285.7, p < 0.001).

(b) Surfactants in OS provide defence
Ants that came in contact with caterpillar OS immediately engaged in extensive grooming activity. In the case of M. rubra, where workers were confronted with live caterpillars, the affected individual always stopped the attack and usually did not attack the larva again within observation time. There was no significant difference in grooming time evoked by exposure to the three OS types (figure 1; M. rubra, ANOVA: F_{2,29} = 0.549, p = 0.583; S. invicta, ANOVA: F_{2,44} = 2.858, p = 0.068). All tested OS had temporary, sublethal effects. Although most ants fully recovered after grooming, some workers remained with clotted antennae.

In no-choice feeding assays, none of the three OS types were found to deter feeding in M. rubra (figure 2a). Workers of this species spent as much time drinking from sugar-supplemented OS as from uncontaminated sucrose solution (Student’s t-test: artificial diet: t = 0.376, p = 0.709; celery: t = 0.113, p = 0.911; tomato: t = −0.235, p = 0.816). Total feeding time was shorter on the experimental day when OS from celery was tested. This was probably due to accidental feeding of M. rubra one day before the bioassay was carried out. By contrast, individuals of S. invicta significantly preferred sucrose solution over all types of S. exigua OS (Kruskal–Wallis ANOVA and median test: H = 46.618, p < 0.001; artificial diet: p = 0.001; celery: p = 0.039; tomato: p < 0.001, figure 2b). However, diet effects were not found since there was no difference in the number of ants feeding on the various OS types (artificial diet versus celery: p = 0.999; artificial diet versus tomato: p = 0.999; celery versus tomato: p = 0.999). No significant day effects were apparent as control solutions between all treatments were equally attractive (control_{artificial diet} versus control_{celery}: p = 0.999; control_{artificial diet} versus control_{tomato}: p = 0.999; control_{celery} versus control_{tomato}: p = 0.999).

Further bioassays investigated the necessity of surfactants for defence. We observed that applying a droplet of pure water to the anterior part of M. rubra did not result in grooming behaviour as the droplet rolled off and did not wet the insect’s cuticle (figure 3a). Similar observations were made with S. invicta. These smaller ants had to rid themselves of the droplet (figure 3b). The time needed for this was also counted as grooming time. When the contact angle was reduced to an intermediate level (θ = 83°), grooming times of both ant species were not significantly different compared with pure water.
rather than plant secondary metabolites was important for protecting the insect on a range of host plants including those of lower toxicity. Plants are highly heterogeneous in their chemical composition with large pounds may contribute to a more repugnant OS is not ruled out as only two out of 50 known host plants were ruled out as only two out of 50 known host plants were not differentiated between the offered OS. Caterpillar-derived and/or primary plant compounds may have rendered OS less palatable to S. invicta.

Clearly, the possibility that plant secondary compounds may contribute to a more repugnant OS is not ruled out as only two out of 50 known host plants were tested and some of them may indeed contain powerful detergents. However, our results suggest that the role of plant secondary metabolites in the OS of herbivores as a defence against natural enemies might be overestimated. This makes sense if it is assumed that selection pressure should be particularly on generalists to maintain a defence mechanism that protects the insect from a range of host plants including those of lower toxicity. Plants are highly heterogeneous in their chemical composition with large proportions of their primary and secondary metabolites.

4. DISCUSSION
This study describes a novel aspect in the defence of insect herbivores against predators by demonstrating the importance of surfactants in OS. Our results show that the ability to wet the predators' hydrophobic cuticle rather than plant secondary metabolites was important for defending S. exigua against ants. The effectiveness of different OS types did not depend on the ingested diet on which the insects had been reared because OS produced from artificial diet evoked the same grooming responses in ants as OS from two different host plants (figure 1). This suggests that potentially deterrent secondary compounds such as terpenes or alkaloids in tomato (Yahara et al. 2004; Simmons & Gurr 2005) or furanocoumarins in the leaves of celery (Lombart et al. 2001) were either not present in the OS or the ants did not mind them. The lack of secondary compound effects was further confirmed by deterrence tests with both ant species (figure 2). Workers of M. rubra readily accepted all OS types as a food source and did not discriminate between OS and sugar solution. For S. invicta, OS was less attractive than uncontaminated sucrose but workers did not differentiate between the offered OS. Caterpillar-derived and/or primary plant compounds may have rendered OS less palatable to S. invicta.

Figure 2. Feeding deterrence test. OS (black or grey bars) was diluted (1:1) with sucrose solution (10% w/v) and offered on cotton wool. Sucrose solution (5% w/v, white bars) was used as control. (a) Myrmica rubra: bars represent time (mean ± s.e.) spent drinking (n=15, Student's t-test), (b) S. invicta: bars represent numbers (mean ± s.e.) of ants feeding (n=10, Kruskall-Wallis ANOVA). OS was obtained from caterpillars reared on artificial diet (AD), celery (CE) or tomato leaves (TO).

Figure 3. Role of detergency in the defence against ants. (a) Myrmica rubra (n=15–16), (b) S. invicta (n=15–16). Ants were treated with a droplet of water (AQ), low-concentration surfactant (TW1), high-concentration surfactant (TW2) or oral secretion (OR). Contact angles (θ) of liquids are indicated below each column. Bars represent average time spent grooming (mean ± s.e.). Different letters represent significant differences (ANOVA followed by HSD test).

controls (M. rubra: ANOVA: \( F_{3,56} = 64.228 \), p<0.001, HSD test: p=0.999; S. invicta: ANOVA: \( F_{3,59} = 28.819 \), p<0.001, HSD test: p=0.362). The amount of surfactant in the water was obviously not sufficient to wet the ants (figure 4). Water with more surfactant and adjusted to a contact angle similar to OS (θ=66°) induced significant grooming activity compared with water (M. rubra, HSD test: p<0.001; S. invicta, HSD test: p<0.001). In M. rubra, OS treatment led to longer grooming periods than surfactant treatment (HSD test: p=0.005), while in S. invicta no significant difference was found for either fluid (HSD test: p=0.554).

(e) Amphiphilic OS in other species
All of the tested Lepidoptera, as well as the beetle larvae, had highly amphiphilic OS with similar contact angles: S. littoralis: θ=63.8°±0.6; S. frugiperda: 67.5°±0.5; H. armigera: θ=66.2°±0.8; L. dispar: θ=58.4°±0.4; P. brassicae: 52.0°±0.4; L. decemlineata: θ=56.0°±0.7.

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variation in quality and quantity of secondary compounds between different species, among individuals of the same species or even between different parts of an individual plant (Schoonhoven et al. 2005). Moreover, the storage of secondary chemicals is not the only survival strategy plants have evolved to cope with herbivory. Tolerance and regrowth, but also mechanical defences, may be more important in certain species. In a recent study, Agrawal & Fishbein (2008) showed that within a plant genus not only trade-off between resistance traits and regrowth ability exist but also that phylogenetically older species were more toxic than derived species. Given this variation, herbivores benefit if they do not exclusively rely on reusing plant secondary metabolites against their own enemies but can employ host-independent defences in the first place.

Here, we propose that surfactants in the OS of caterpillars are crucial for effective anti-predator defence against invertebrates by offering a base level of protection. Contact angle measurements showed that OS of S. exigua was highly amphiphilic, regardless of the diet fed to the caterpillars. Consequently, OS was able to spread over the ants’ hydrophobic cuticle and, unlike water, did not roll off. As a response, ants immediately commenced grooming that persisted for a few minutes. The exact causes that elicited the grooming response remain to be elucidated but it is conceivable that merely reducing the surface tension and thus allowing any kind of liquid to wet the ant should be enough to stop the predator’s attack and to induce grooming. Usually, the affected individual was reluctant to attack a second time. Compounds such as proteins in the OS may have had an additional impact on the ants. In comparison, ants did not respond differently to intermediate levels of surfactant than to pure water as the droplets did not spread over the cuticle (figures 3 and 4).

The analyses of five other lepidopteran and one beetle species revealed that amphiphilic OS could be found in all of the investigated larvae. Thus, we hypothesise that detergency may represent a general mode of defence in regurgitating Lepidoptera and possibly in many other insects.

In recent years, considerable and promising efforts have been made to isolate surfactants from biological materials (mostly bacteria) for application in cosmetics, pharmaceuticals, bioremediation, the food industry and agriculture (Lu et al. 2007). Biosurfactants (e.g. glycolipids or lipopeptides) are generally considered to be less toxic, more environmentally safe and more cost-effective than many synthetic, mainly petroleum-based surfactants, and consequently there is a growing demand for them (Rahman & Gakpe 2008). Possibly, insects could be a rewarding, yet unexplored, source of new surface-active compounds.

Surfactants have lately also been considered as less human-toxic and cheaper alternatives to conventional insecticides in agriculture (Oetting & Latimer 1995; Curkovic et al. 2007). Their activity has been attributed to several factors, such as drowning pest insects, destroying biological membranes, inhibiting enzymes or simply removing individuals from the foliage (Curkovic & Araya 2004). Our findings show, as is so often the case, that nature has invented a technique by means of natural selection long before humans started using surfactants against their own insect adversaries.

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