

Insects had it first: surfactants as a defence against predators

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Postprint version for open access.

Original publication: Rostás & Blassmann (2009) Proceedings of the Royal Society B, 276:633-638.

Running title: Defensive surfactants in oral secretion.

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Abstract

Insects have evolved an astonishing array of defences to ward off enemies. Well-known and widespread is the regurgitation of oral secretions (OS), fluids that repel 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 OS' intrinsic detergent properties. 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 detergency is a common but unrecognised 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 like 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 (Codella & Raffa 1995; Eisner 1970; Peterson *et al.* 1987; 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. Calcagno *et al.* 2004;

Sword 2001). Thus, defence seems to depend strongly on an 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 fifty 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, nestmates are recruited by laying a pheromone trail (Putyatina 2007, Evershed *et al.* 1982). *S. invicta*, native to South America, is a notorious invasive species found in agricultural, urban, and natural habitats in the United States, Australia and 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 which does not rely on variable plant toxin content.

2. MATERIAL AND METHODS

(a) *Caterpillars and ants*

Eggs of *Spodoptera 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 L15:D9 photoperiod at 28°/25°C (light/dark) and 75 % rel. humidity. Each group received either artificial diet based

on agar and cooked bean meal (modified from King & Leppla 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 standardised 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 microcapillary (volume 100 μ l). Caterpillars were not squeezed or impaired in any other way. The amounts of discharged OS varied strongly but up to 12 μ l could be obtained from a single larva. Within 30 min, OS of approx. thirty caterpillars were 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 *Myrmica 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 open 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. Both ant species were supplied with diluted honey (1:1 v/v) and killed larvae of *Spodoptera frugiperda*. Two to three days prior to the experiments, ants were starved to enhance their responsiveness.

(b) Contact angle measurements

Contact angles of all tested liquids were measured on a standardised hydrophobic surface (silanised microscope glass slides) to assess their amphiphilic properties. The contact angle is the angle at which a liquid/vapour interface meets the solid surface. On a hydrophobic surface, hydrophilic liquids have a lower affinity and thus higher contact angle values. Measurements were performed on a video-based optical contact angle goniometer (OCA15 plus, DataPhysics Instruments, Filderstadt, Germany) using the sessile drop method (http://www.dataphysics.de/english/messmeth_sessil.htm). Single droplets of 5.5 μ l were applied and photographed after 30 s for contact angle measurements. Ten

replicates were carried out for each type of liquid.

(c) Behavioural tests

Bioassays were conducted to test the effects of *S. exigua* OS on ants and to find out whether the effectiveness of OS can be enhanced by secondary plant metabolites present in the caterpillar's host plants. Two different experimental setups were established, owing to the fact that both ant species behave differently when kept isolated from their nest mates. Generally, *S. invicta* displayed aggressive behaviour only in large numbers.

In the first experiment a single caterpillar that had been reared on artificial diet, celery or tomato, respectively, was exposed to eight workers of *M. rubra* in a Petri dish (9 cm diam.). After several seconds, usually one or two individuals from the ant group 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 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- μ l-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 setup to both ant species. Pure sucrose solution containing the same amount of sugar (5% w/v) was used as a control. The OS were tested on *M. rubra* by applying a droplet (5 μ l) 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 μ l), 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 *M. rubra* or *S. invicta* were placed into a Petri dish and a droplet of 5 μ l (*M. rubra*) or 3 μ l (*S. invicta*) of the test liquids was applied onto 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) demineralised 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) demineralised water. A preliminary assay had shown that ants survived on sugar water containing 0.12% 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 three other generalist Noctuidae (*S. frugiperda*, *S. littoralis*, *Helicoverpa armigera*), *Lymantria dispar* (Lymantridae), 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, were compared. 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) *Oral secretion is amphiphilic*

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

(b) *Surfactants in oral secretion provide defence*

Ants that came in touch 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: $F_{2,44} = 2.858$, $p = 0.068$).

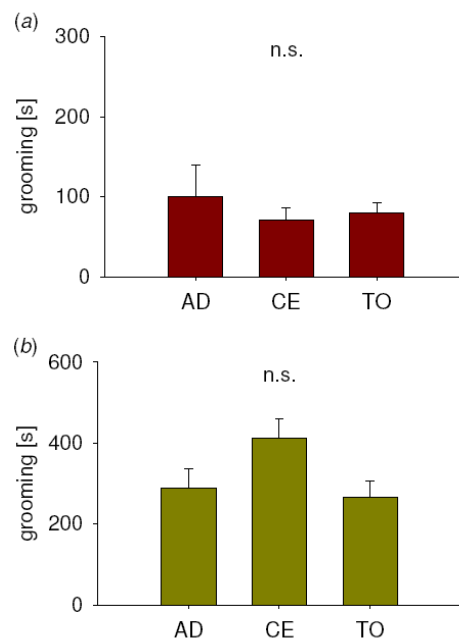


Figure 1. Effect of *Spodoptera exigua* oral secretion on the grooming responses of ants. (a) *Myrmica rubra* (n = 10-11), (b) *Solenopsis invicta* (n = 15-16). Oral secretion was obtained from caterpillars reared on AD = artificial diet, CE = celery or TO = tomato leaves, respectively. Bars represent average time spent grooming (means \pm s.e.). n.s. = not significant (ANOVA).

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 most likely due to

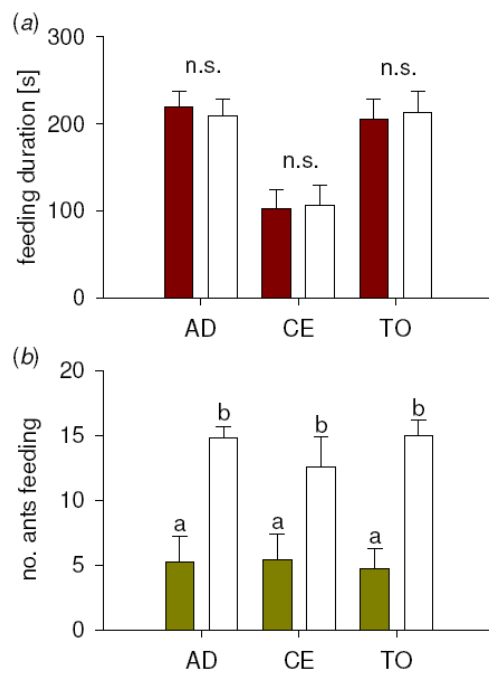


Figure 2. Feeding deterrence test. Oral secretion (coloured bars) was diluted (1:1) with sucrose solution (10% w/v) and offered on cotton wool. Sucrose solution (5% w/v) (white bars) was used as control. (a) *Myrmica rubra*: bars represent time (means \pm s.e.) spent drinking ($n = 15$, Student's t -test), (b) *Solenopsis invicta*: bars represent numbers (means \pm s.e.) of ants feeding ($n = 10$, Kruskal-Wallis ANOVA). Oral secretion was obtained from caterpillars reared on AD = artificial diet, CE = celery or TO = tomato leaves, respectively.

accidental feeding of *M. rubra* one day before the bioassay was carried out. In 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 vs. celery: $p = 0.999$; artificial diet vs. tomato: $p = 0.999$; celery vs. tomato: $p = 0.999$). No significant day

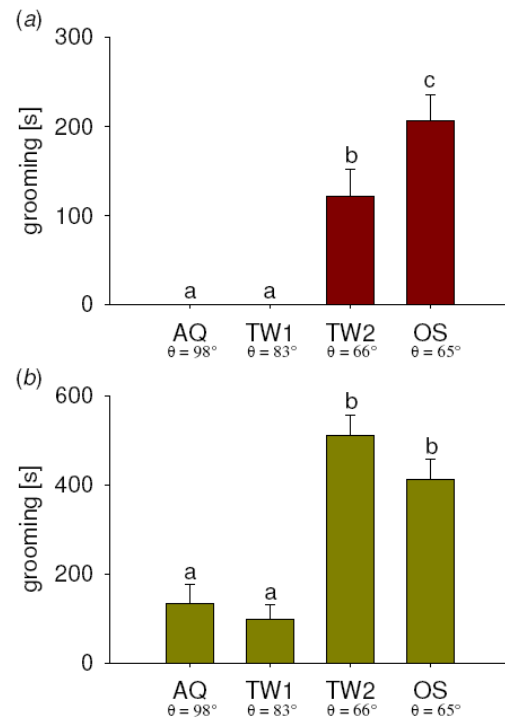


Figure 3. Role of detergency in the defence against ants. (a) *Myrmica rubra* ($n = 15-16$), (b) *Solenopsis invicta* ($n = 15-16$). Ants were treated with a droplet of AQ = water, TW1 = surfactant (low concentration), TW2 = surfactant (high concentration) or OS = oral secretion, respectively. Contact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

effects were apparent as control solutions between all treatments were equally attractive (control_{artificial diet} vs. control_{celery}: $p = 0.999$; control_{artificial diet} vs. control_{tomato}: $p = 0.999$; control_{celery} vs. 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 off 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 ($\theta = 83^\circ$), grooming times of both ant species were not significantly different compared to pure water 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 ($\theta = 66^\circ$), induced significant grooming activity compared to 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 both fluids (HSD test: $p = 0.554$).

(c) *Amphiphilic oral secretion in other species*

All of the tested Lepidoptera, as well as the beetle larvae, had highly amphiphilic OS with similar contact angles: *S. littoralis*: $\theta = 63.8^\circ \pm 0.6$, *S. frugiperda*: $67.5^\circ \pm 0.5$, *H. armigera*: $\theta = 66.2^\circ \pm 0.8$, *L. dispar*: $\theta = 58.4^\circ \pm 0.4$, *P. brassicae*: $52.0^\circ \pm 0.4$, *L. decemlineata*: $\theta = 56.0^\circ \pm 0.7$.

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 were 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 (Simmons & Gurr 2005; Yahara *et al.* 2004) or furanocoumarins in the leaves of celery (Lombaert *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 tasty 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 fifty known host plants were tested and some of them may indeed contain powerful deterrents. However, our results suggest that the role of plant secondary metabolites in the oral secretion 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 on a range of host plants including those of lower toxicity. Plants are highly heterogeneous in their chemical composition with large 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 and Fishbein (2008) showed that within a plant genus not only trade-off between resistance traits and regrowth ability exists but also that phylogenetically older species were more toxic than derived species. Given this variation, herbivores benefit if they do not exclusively rely on re-using 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* were 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 which 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



Figure 4. Wetting of *Myrmica rubra* depends on surfactant concentration. (a) Pure water, (b) Water with low surfactant concentration, (c) Water with high surfactant concentration (contact angle equivalent to *Spodoptera exigua* oral secretion). Contact angle values are given in the lower right corner.

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 an additional impact as in some cases it was observed that the antennae glued together. Thus, proper sensory functioning could be temporarily or permanently impaired.

The use of OS as a defence is probably most effective against single attackers like scouting ants, predatory bugs or spiders. Nevertheless, nearly all caterpillars survived in the bioassays with eight *M. rubra* in the same arena, suggesting that OS can provide good protection against certain ant species. If ants occur in very high numbers, caterpillars can be overwhelmed and eventually die. This was observed when placing caterpillars in the foraging arena close to the nest of *S. invicta* and might be a realistic outcome in the field. *S. invicta* is known to be less efficient in discovering food than other ant species but can compensate by fast recruitment of many nestmates (Calcaterra *et al.* 2008). Caterpillar size in relation to predator size also plays an important role as larger caterpillars produce a lot more OS. *Cotesia marginiventris*, a solitary endoparasitoid of small first and second instar *Spodoptera* larvae, was never found to be seriously affected by the small

amounts of regurgitated OS (personal observation).

The notion that physico-chemical properties of OS are important for defence was further stressed by the ants' grooming responses when treated with droplets of two different dilutions of a non-toxic surfactant. Water with a surface tension comparable to OS ($\theta = 66^\circ$) was as effective as OS in individuals of *S. invicta* and slightly less effective in *M. rubra*. In the latter case, non-surfactant compounds in the OS could have had an additional impact on the ants. In comparison, ants did not respond differently to intermediate levels of surfactant ($\theta = 83^\circ$) than to pure water ($\theta = 98^\circ$) 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, 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 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 cheap alternatives to conventional insecticides in agriculture (Curkovic *et al.* 2007; Oetting & Latimer 1995). Their activity has been attributed to several factors such as drowning pest insects, destructing 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.

ACKNOWLEDGEMENT

Many thanks go to Annett Endler, Caroline Müller and Steffen Pielström for supplying us with ants and to Christa Schaffelner for donating *Lymantria dispar* eggs. We thank Markus Riederer for making lab space and equipment available. Comments by Martin Heil, Marco d'Alessandro, James Blande, Anurag Agrawal, Ted Turlings and three

anonymous reviewers greatly helped to improve this manuscript. Funding was provided by the Deutsche Forschungsgemeinschaft (DFG, SFB 567, TP B9)

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