Chemoecology 15:261–267 (2005)
0937–7409/05/040261–7

DOI 10.1007/s00049-005-0321-9

CHEMOECOLOGY

Sequestration of phorbol esters by aposematic larvae of *Hyles euphorbiae* (Lepidoptera: Sphingidae)?

Anna K. Hundsdoerfer[1,2], Jeannette Ndaya Tshibangu[1], Bernhard Wetterauer[1] and Michael Wink[1]

[1]Institute of Pharmacy and Molecular Biotechnology, Heidelberg University, Im Neuenheimer Feld 364, D-69120 Heidelberg, Germany
[2]current address: Zoological Museum, Natural History State Collections Dresden, Königsbrücker Landstr. 159, D-01109 Dresden, Germany

Summary. The larvae of the hawkmoth species *Hyles euphorbiae* have a conspicuous aposematic colouration and show gregarious behaviour. It has thus been suggested that they sequester phorbol esters from their food plants which include different species of the genus *Euphorbia* (Euphorbiaceae) for chemical protection against predators. To test this hypothesis in more detail, we fed larvae an artificial diet with three doses of 12-tetradecanoyl-phorbol-13-acetate (TPA), then examined the faeces and the larval tissues, such as integument, haemolymph and gut of the caterpillars for the presence of TPA. In order to determine the ability of the larvae to detoxify phorbol esters, other larvae were directly injected with a TPA solution and analysed in the same manner. Our study indicates that the larvae of *Hyles euphorbiae* do not sequester phorbol esters. Upon oral application TPA was not found in the larval integument or the haemolymph. Instead, it was mostly metabolised (about 70-90 %). Nevertheless, about 10-30 % were retained and recovered in the faeces. The larvae were also able to metabolise and thus detoxify the phorbol ester when TPA was injected directly into the body. These hawkmoth caterpillars are relatively large and have a gut full of plant material, which they regurgitate into the direction of the predator when attacked in nature. Since phorbol esters are very potent toxins and irritants, we postulate that the gut content (and especially the plant slurry disgorged as regurgitant from the anterior gut) alone could be aversive for a potential predator, even if some metabolism has taken place. Thus, although *H. euphorbiae* caterpillars do not actively sequester phorbol esters, their aposematic colouration appears to be based on chemical defence through phorbol esters retained in the gut.

Key words. phorbol ester – TPA – sequestration – detoxification – metabolism

Taxa. *Hyles euphorbiae*, Macroglossinae, Sphingidae, Lepidoptera, Insecta

Introduction

Sequestration of plant toxins in herbivores is often correlated with aposematic colouration and gregarious behaviour (Bowers 1993). Aposematic colours may warn predators that an individual or species is chemically defended and unpalatable. *Hyles euphorbiae* (Linnaeus) (Lepidoptera: Sphingidae) is a large hawkmoth species distributed in Central/Southern Europe and Western Asia (details in Hundsdoerfer *et al*., 2005). The larvae have aposematic colouration and show gregarious behaviour and were thus suggested to sequester phorbol esters that are characteristic secondary metabolites of their host plants, several different species of the genus *Euphorbia* (Euphorbiaceae). The genus *Euphorbia* (Euphorbiaceae) is chemically defined by the occurrence of a large number of polyfunctional diterpenoids with the tigliane (phorbol), ingenane and daphnane skeletons (Evans & Soper 1978). Most of them are skin irritants and toxic, in addition, many of them are skin tumour promoters. Non-irritant polyfunctional macrocyclic diterpenoids with the lathyrane and jatrophane skeletons have also been isolated from *Euphorbia* species.

The main cellular target of phorbol esters is the protein kinase C (PKC) (Castagna 1987; Blumberg 1988) and it is likely that a part of the toxic properties of phorbol esters is directly connected with this biochemical activity. TPA (12-tetradecanoyl-phorbol-13-acetate; also called phorbol 12-myristate 13-acetate, PMA) is the most abundant biologically active phorbol-12,13-diester of croton oil (*Croton tiglium*, Euphorbiaceae) and also the most irritant and co-carcinogenic compound (Hecker, 1968a, b) of this oil. Highly toxic to frogs and acting as tumour promoter of mouse skin (Hecker 1971), TPA is widely used as a standard phorbol ester in biochemical experiments.

A TPA isomer occurs in *Euphorbia cyparissias* (Ott and Hecker, 1981), the most common food plant of *Hyles euphorbiae* in Central Europe. However, *E. cyparissias* has been observed to be too toxic for strains of *H. euphorbiae* from Southern Europe (H. Harbich, personal communication). Instead, these caterpillars thrive on other spurge species such as *E. characias*, *E. paralias*, *E. myrsinites* or *E. segetalis*. However, these species also cause severe irritation of skin and eyes during collection (personal observations and refer to the following citations). The diterpene esters of the latter three species have been analysed (Evans and Kinghorn, 1974; Öksüz *et al*. 1995; Ferreira *et al*. 1998; Jakupovic *et al*. 1998a, b). *E. myrsinites* contains diterpene esters from the ingenol- and myrsinol-type (Evans & Kinghorn 1974; Öksüz *et al*. 1995), but none of the phorbol-type, i.e., the tigliane derivatives such as TPA. In order to enable a clear

Correspondence to: Anna K. Hundsdoerfer, e-mail: anna.hundsdoerder@snsd.smwk. sachsen.de and Michael Wink, e-mail: wink@uni-hd.de

Table 1 Recipe for the artificial diet as described in Harbich (1994). It was used for the experiment of the oral application of TPA to *Hyles euphorbiae* larvae in this study. The leaf powder consisted of ground dry leaves and stems of *Euphorbia myrsinites* in this case

Ingredient	Amount	Action
water	100 ml	bring to a boil
Agar-agar leaf powder	2.5 g 2.5 g	add slowly to boiling water under constant stirring, leave to boil for another 10 s
wheat germ brewer's yeast ascorbic acid	7.5 g 1.5 g 0.4 g	stir into the mixture after it cooled down to 70 °C

identification of the experimentally administered TPA in the larval tissues, *E. myrsinites* was thus chosen as food plant for the caterpillars in our experiments.

The diterpene esters from *E. paralias* have been shown to exhibit molluscicidal and anti-feedant properties (Abdelgaleil *et al*. 2002) and those of *Jatropha curcas* (Euphorbiaceae) to exhibit insecticidal activities against several species of e.g. moths, butterflies, aphids, bugs, bettles, flies and cockroaches (Wink *et al*. 1997). There are only a few other herbivores that feed on *Euphorbia* species in Central Europe (Manojlovic and Keresi, 1997; personal observations) indicating the effectiveness of phorbol esters for chemical defence against herbivores.

Marsh *et al*. (1984) have performed some experiments to test the possible sequestration of phorbol esters by *H. euphorbiae*. They did, however, not discern the larval body tissues and the larval gut that contains a large amount of plant material, possibly with remains of diterpenes. However, they claim to have also found small amounts of ingol diterpene esters in the pupae and adults. In order to deepen the research about the question whether *H. euphorbiae* larvae are able to sequester dietary phorbol esters as acquired defence compounds against predators, we fed an artificial diet with three doses of TPA to larvae of *H. euphorbiae*, then examined the faeces, and the larval tissues such as integument, haemolymph and gut of the caterpillars for the presence of TPA. In order to examine the ability of the larvae to detoxify phorbol esters, a TPA solution was directly injected into other larvae and that were analysed in the same manner.

Material and Methods

For the purposes of this experiment caterpillars of *Hyles euphorbiae* from Spain were reared on shoots of *Euphorbia myrsinites* (Euphorbiaceae) in the laboratory. They represented the progeny of one pair of adult moths that were collected as caterpillars in Spain (2003) and reared to pupation in captivity. Only these sibling larvae were used for the experiments. The larvae for the sequestration experiments were fed an artificial diet (Table 1) after moulting from the fourth to the fifth and last instar. This diet was specially developed for hawkmoths (Harbich, 1994) and includes a small amount of dried food plant (*E. myrsinites* in this case). The larvae used in the detoxification experiments were allowed to continue to feed *ad libitum* on *E. myrsinites* throughout the experiment. The weight of each larva was monitored when it was fully fed (to improve comparison) and the faeces collected regularly and completely; they were stored in glass containers at –80° C until extraction. The HPLC chromatograms of an extract of the foodplant, *E. myrsinites*, were compared to those of the larval tissues with the same methods, to check for possible sequestration of diterpene esters before the beginning of the experiment.

Sequestration experiments

Eight larvae of similar size were fed with artificial diet, whereby the three control larvae were given a diet without TPA *ad libitum* throughout the experiment. During a time interval of 4.5 h the five experimental larvae were fed 2-3 cubes of 1g diet containing 0.5-1.2 mg TPA (Fluka) each. Each larva was fed a total of 2 mg TPA. The TPA was introduced into the diet by injecting and mixing a TPA solution (concentration 200 µg TPA/µl solvent DMSO: MeOH, 4:1, v/v) into the soft cube just before it polymerised fully. The control larvae were fed several cubes of diet mixed with the solvent only to test whether a difference in acceptance to the cubes without solvent could be detected. Since this was not the case, the control larvae were only given cubes without any treatment.

After 24 h the eight larvae were cooled to 4 °C and dissected into haemolymph, integument and gut. These tissues were also stored in glass containers at –80 °C until extraction. Almost all animals had tolerated the experimental treatment, except for one larva (one of the three control larvae). It appeared to develop more slowly than the other seven and upon dissection parasites were found in the integumental fat body. This larva was excluded from the evaluation.

Detoxification experiments

Ten larvae of similar size feeding on *E. myrsinites* were chosen for the experiment. Five of them were injected with 1.5 mg TPA in solution each (solvent DMSO:MeOH, 4:1, v/v; concentration 200 µg/µl) in three doses of 0.5 mg within 4 h. The other five larvae were injected with the solvent only as a control.

After 48 h the ten larvae were also cooled to 4 °C and dissected into haemolymphe, integument and gut, and again, these tissues were stored in glass containers at –80 °C until extraction.

Extraction

A specific volume of methanol was added to each tissue (1.0 ml to each integument, 0.5 ml to each gut and haemolymph, respectively) and a varying amount of methanol was added to the faeces, depending on the weight and volume of the individual sample. The mixture was thereafter homogenised with an Ultra-Turrax (Ika-Werk; Janke & Kunkel). For the extraction of very small amounts of faeces, they were cut up with scissors in methanol. To obtain a clean raw extract the container was firstly centrifuged for 15 min. at about 1,880 × g (room temperature). In cases when the supernatant had not become transparent, it was centrifuged again at about 8,800 × g for 10 min. The clear supernatant was mixed with acetonitrile (ACN) 1:1, causing a precipitate to be formed, that was removed by centrifugation for at least 30 min. at nearly 15,000 × g (4 °C). When necessary, the former procedure (adding ACN and removing the precipitate) was repeated and thereafter the supernatants were stored at –80 °C until analysis.

HPLC Analyses

HPLC analyses were carried out using an Eurochrom 2000 HPLC system equipped with a HPLC interface box (Knauer), two Knauer pumps (HPLC pump 64), a Knauer injection system, a Knauer UV detector (Variable Wavelength Monitor) set at 238 nm. The HPLC solvents were degassed online through a ERC 3215a and mixed in a dynamic mixing chamber (Knauer). The extracts were analysed on a LiChrospher RP18 column, 5 µm, 3 i.d. × 125 mm with a 3 i.d. × 30 mm pre-column (Merck). Elution was performed at a flow rate of 1 ml/min, using a solvent gradient consisting of a linear increase from 60 % to 100 % of methanol (solvent B) in a water background (solvent A) over periods of 20 min, followed by 100 % solvent B for 5 min. The column was equilibrated in 60 % methanol.

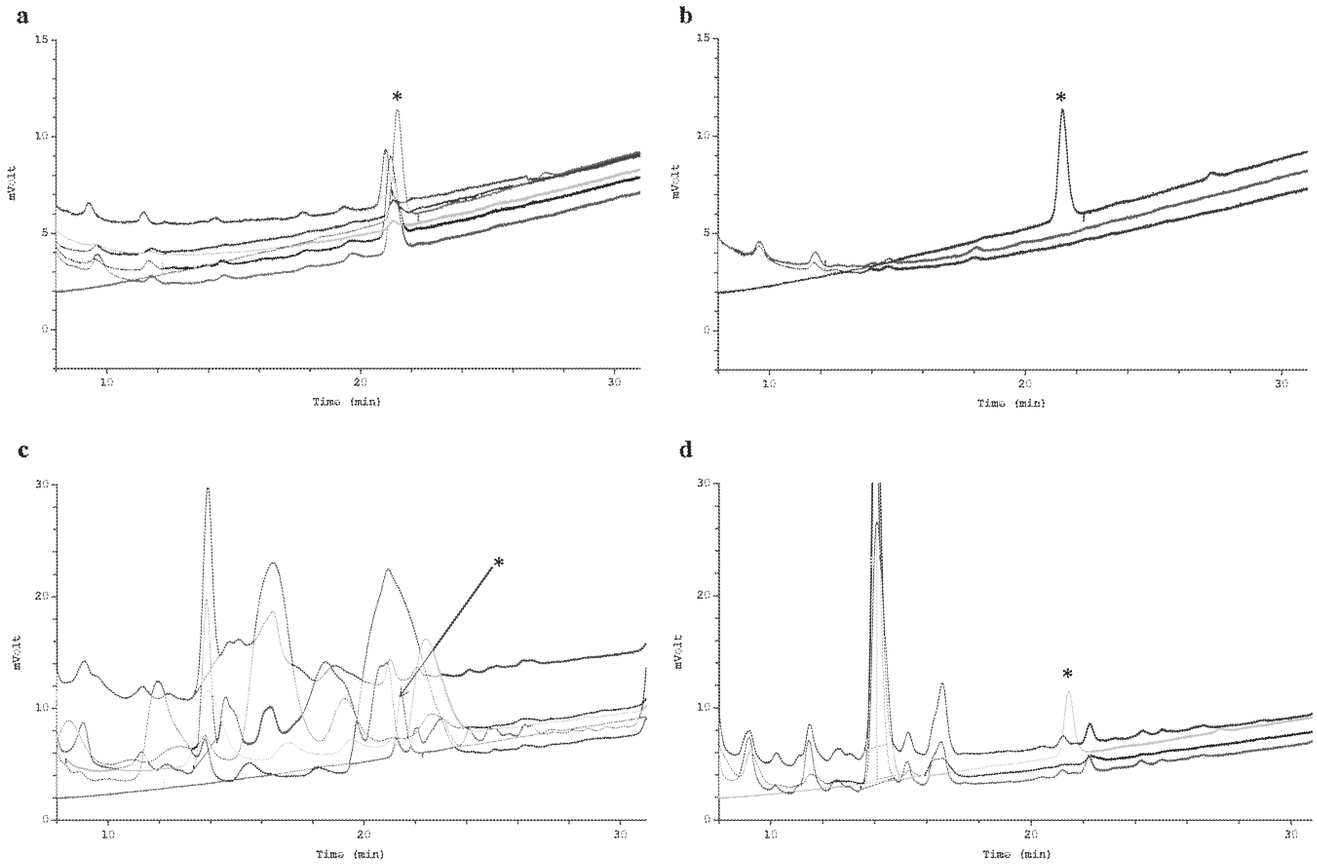

Fig. 1 The HPLC profiles of the larval faeces extracts (collected after 20 h) of the five individual larvae **a.** fed with TPA in the sequestration experiment, as compared to **b.** the faeces extracts of the two control larvae; and **c.** injected with TPA in the detoxification experiment, also **d.** as compared to the faeces extracts of the three control larvae. * TPA standard peak, Rf = 21.5

The extracts of the faeces from the detoxification experiment indicated that there were many substances near the retention time of TPA that were not separated well enough through this procedure, so for these samples the method had to be adapted to a linear gradient of 75 % solvent B to 95 % in 30 min, followed by linear increase to 100 % solvent B in 1 min. and isocratic 100 % solvent B for 4 min.

Mass Spectroscopy Analyses

TPA was identified by co-chromatography of authentic TPA. In order to make sure that the HPLC peaks really represented TPA, they were analysed by ESI-MS-MS.

Putative TPA peaks were collected and subjected to mass spectroscopy. We added 1 % formic acid to the samples, which were dissolved in methanol with a small percentage of water (depending on the HPLC gradient used, see above). The mixtures were subjected to Electro-Spray Ionisation (ESI) in the mass spectrometer VG Quattro II (Micromass). The conditions were: capillary: 3.01 kV, HV lens: 0.48 kV, cone: 33 V, RF lens: 0.7 V, source temperature: 80 °C; MS1: ion energy: 1.6 V, MS2: ion energy: 4.1 V, multiplier: 650 V.

Recovery Analysis

To determine the effectiveness of the extraction procedure, integument tissues of three detoxification control larvae as well as three portions of faeces of two sequestration control larvae were mixed with 200 µg TPA each and extracted and analysed by means of the method described above. Pure TPA was used as an external standard for quantification. The six extractions resulted in a mean recovery value of 81 % ± 0.11.

Results

The HPLC chromatograms of the food plant, *E. myrsinites*, contained about five very small peaks that should represent its diterpene esters. The larval tissues did not contain these peaks.

Sequestration experiment

After a few bites into the TPA treated cubes of the artificial diet, the larvae stopped eating for several minutes. Thereafter, it seemed as though they ate the TPA cubes more avidly than the control larvae ate the untreated ones. Cubes with a TPA concentration of up to 1.2 mg/g diet were consumed very rapidly and soon the maximal dose of 2 mg per larva was reached (the fastest larva ingested 2 mg TPA in two cubes within about 2 h). All larvae excreted normally, irrespective of whether they were fed TPA or not.

Since larvae were reared on *E. myrsinites* they did not contain any endogenous phorbol esters related to TPA. The HPLC profiles of the extracts from animals that had received TPA orally revealed that the integument and haemolymph

Table 2 The amount of TPA (µg) recovered in the faeces of *Hyles euphorbiae* larvae after oral application of 2 mg (in 2-3 doses). The times are to be understood as hours after the last dose. The percentage of TPA recovered from the 2 mg application (% recovered) is used to calculate the percentage of unmetabolised TPA in the faeces (% unmetabol.) under consideration of the recovery rate of 81 %

	Time								
Larva No.	2 h	4 h	5,5 h	16 h	20 h	24 h	Total	% recovered	% unmetabol.
1	0.0	8.7	95.0	122.3	0.0	0.0	225.9	11.3	13.9
2	0.0	4.3	137.4	150.4	0.0	0.0	292.0	14.6	18.0
5	0.0	9.4	1.9	217.2	0.0	0.2	228.7	11.4	14.1
6	10.3	0.0	117.5	73.5	0.0	0.1	201.4	10.1	12.4
7	119.0	93.5	95.7	153.0	19.3	0.0	480.4	24.0	29.7

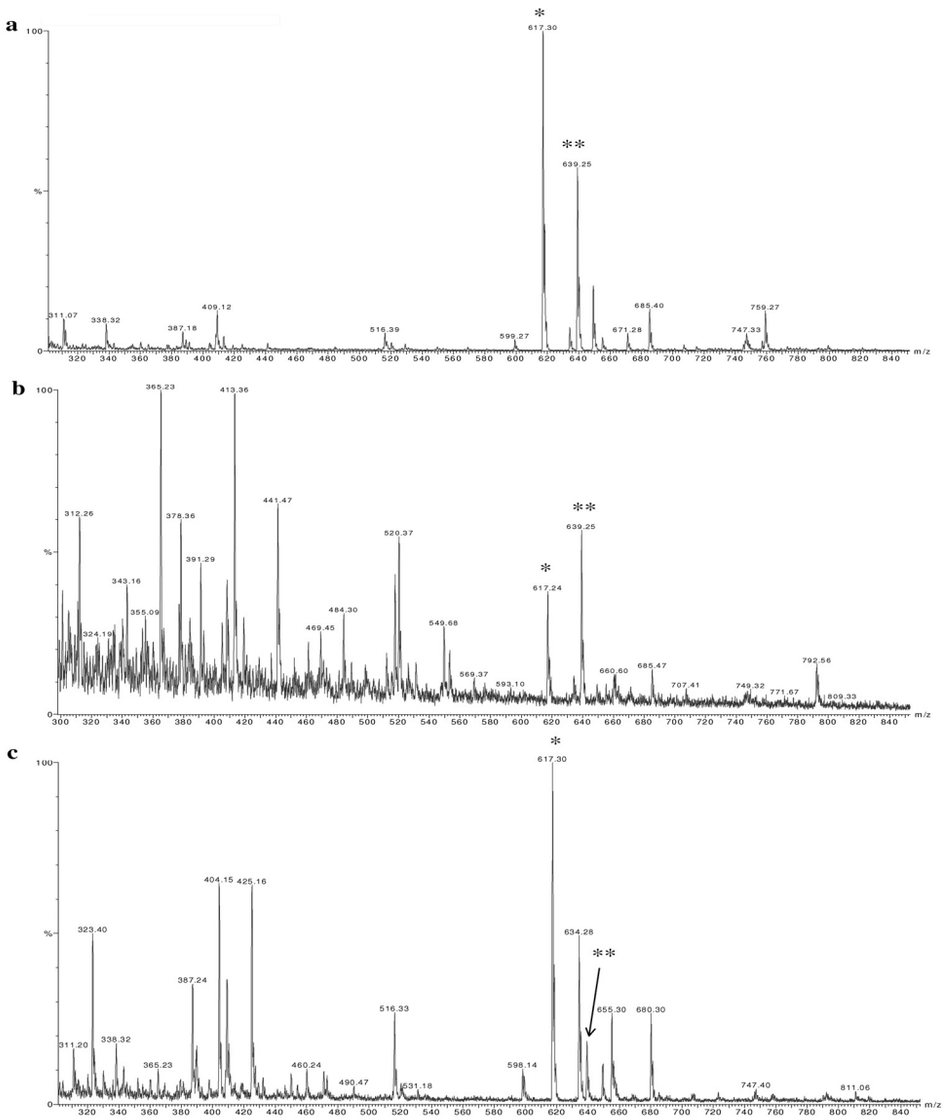

Fig. 2 Identification of TPA by LC-MS. Mass spectra of **a.** TPA standard; **b.** putative TPA peak of the extract of faeces collected after 16.5 h from a larva fed with TPA in artificial diet; **c.** the putative TPA peak of the integument extract of a larva injected with TPA. * $[M+H]^+$ TPA at m/z 617.3 Da (calcd. 617.40 Da), ** $[M+Na]^+$ TPA at m/z 639.3 Da

tissues were without TPA (data not shown). The analyses of the gut tissues indicated a very small peak near the standard TPA peak in the extracts of both the experimental and control larvae. However, nearly all extracts of the faeces collected displayed distinct peaks at the retention time of TPA (e.g. Fig. 1a; Table 2) in TPA fed larvae. In the extracts of the faeces collected only 2 h after the last dose of artificial diet with TPA, these peaks were also visible, although only very small in most larvae (data not shown). The faeces of the control larvae did not show any distinct peaks, irrespective of how many hours they were collected after the begin of the experiment (e.g. Fig. 1b). The mass spectrum of the putative TPA peak from the extract of faeces collected after 16.5 h (from one larva fed with TPA in artificial diet) produced molecular ion peaks of TPA $[M+H]^+$ and $[M+Na]^+$ (Fig. 2b). Considering the dose applied, about 10-30 % of TPA was recovered from the faeces (Table 2). Since no TPA was found in the integument of the haemolymph, indicating that TPA is not stored, metabolism of phorbol esters appears to take place to an important amount. With the methods applied here we did not detect the metabolites.

Detoxification experiments

When the caterpillars were injected with 1.5 mg TPA (equivalent to 0.47 ± 0.09 g/kg body weight), they stopped feeding and became motionless soon after the full dose had been applied. However, control larvae, injected with the solvent only, showed no signs of stress or abnormal behaviour. Accordingly, the TPA-treated caterpillars did not gain weight or develop in a similar fashion as the control larvae. However, after about 45 h the treated larvae had apparently recovered; they resumed feeding and excreted normally.

The HPLC profiles of the extracts from the integument of the TPA injected larvae displayed small, but distinct single peaks at the retention time of TPA, whereas the ones from the control larvae did not (data not shown). Mass spectra obtained from the putative TPA peaks from the integument extracts (of all individuals separately) showed the presence of TPA in all cases (Fig. 2c). The haemolymph and gut extracts showed very small peaks near the TPA peak (data not shown), as already observed in the gut tissue extracts of the experimental and control larvae of the sequestration experiment and described above. We suggest that these peaks do not represent TPA. Examples of the HPLC profiles of the faeces extracts are presented in Fig. 1c, d. The faeces from the five TPA injected larvae showed many substances not present in that of the control larvae; but a distinct TPA peak was not found suggesting that the new peaks are detoxification products. All experimental larvae appeared to display an individual pattern of metabolites: none of the additional peaks were present in all the extracts from the faeces of individual larvae (Fig. 1c).

Discussion

Our study indicates that the larvae of *Hyles euphorbiae* do not sequester phorbol esters to any significant degree. This result corresponds with that of other studies of hawkmoth caterpillars (family Sphingidae) that demonstrated that they mostly feed on toxic plants without sequestering the toxins for their own defence (e.g. Wink and Theile, 2002). We doubt the claim of Abe *et al*. (1996) to have found *Daphnis nerii* to sequester cardenolides from *Nerium oleander*. In their study they did not analyse the larval gut separately from the larval body. The claim of Marsh *et al*. (1984) to have found ingol diterpene esters in larvae, pupae and adults of *H. euphorbiae* has to be assessed with the same critique, to not have differentiated between larval tissues and the plant material in the larval gut. Furthermore, they used Thin Layer Chromatography (TLC) for the analyses, which is not as precise as HPLC. In preliminary tests we had also attempted to use TLC, but this method proved to be too insensitive for the small amounts of diterpenes we extracted. Nevertheless it would still be worth an experiment, to feed larvae of *H. euphorbiae* with artificial diet continuously containing high concentrations of TPA and then extracting the pupae and adults.

In the case of *H. euphorbiae*, TPA may be diffused or transported from the gut into the haemolymph and the metabolising tissues when the larvae orally consume the substance, but it does not appear to be deposited or stored in the integument. Instead, they apparently fully metabolise most of the phorbol ester, while retaining a small percentage unmetabolised in the faeces for excretion. Nevertheless, we could not detect TPA metabolites in the faeces (or any other tissue) after oral application by means of this method.

In addition, our injection experiments clearly show that the larvae were able to metabolise and thus detoxify phorbol esters (Fig. 1c). We could not detect TPA after injection but several metabolites that differed between individuals. A high detoxification capacity for dietary secondary metabolites has also been detected in other Sphingid larvae that obtained toxins either orally or via injection (Wink and Theile 2002).

How to explain the aposematic colouration of *H. euphorbiae* larvae? Our experiments unambiguously show that larvae of *H. euphorbiae* do not sequester phorbol esters for chemical defence unlike the situation in the aposematic bug *Pachycoris klugii* (Heteroptera: Scutelleridae), that store phorbol esters in their integuments for their own chemical defence (Wink *et al*. 2000). Our results imply that the larval body (without the gut) is unprotected and palatable, we thus expect the pupae, the adult moths and the eggs to be unprotected and palatable since no phorbol esters are stored in the larva and thus can not be transferred to the pupa. Nevertheless, these hawkmoth caterpillars are relatively big animals and have a large gut full of plant material containing phorbol esters. For a potential predator, an adverse effect would be likely if it would devour larvae entirely. In addition, larvae of *H. euphorbiae* regurgitate into the direction of a predator when attacked in nature, as well as under laboratory conditions.

We demonstrated that about 10-30 % of the phorbol ester TPA passes through the gut without much metabolism. The LD_{50} of TPA for frogs is very low (0.0002 g/g body weight; summarized in Hecker, 1971) demonstrating the acute toxicity of this phorbol ester. More importantly, the irritant properties of TPA (and other phorbol esters) are extremely strong (0.05 µg/ear of a mouse in a standard procedure; summarized in Hecker, 1971). We thus postulate that if 10-30 % of the phorbol ester can pass through the entire gut unmetabolised, the freshly homogenised plant slurry, constituting the regurgitant, still contains sufficient

amounts of the toxic/irritant principles to serve as chemical protection. In a natural attack situation, the regurgitant probably comes into contact with the predator more readily than the haemolymph (Müller *et al*. 2003), provided that the caterpillar has a short reaction time, which is readily indicated to be the case in *H. euphorbiae*. In the genus *Pieris* (Lepidoptera: Pieridae) a similar situation was reported: *P. brassicae* caterpillars are aposematic and gregarious, but they do not sequester host plant glucosinolates. Instead, they also regurgitate and the regurgitant (but not the haemolymph) produced an aversive effect on potential predator ants (Müller *et al*. 2003). Therefore, although we have not performed experiments to corroborate our hypothesis, we postulate that phorbol esters which are an efficient plant defence of *Euphorbia* species against generalist herbivores, can be used by the caterpillars with the specially adapted behaviour of regurgitation for their own defence against predators without the necessity of sequestration. Thus, we hypothesise *H. euphorbiae* is chemically as well protected as its food plant.

We expect that this defence behaviour, including chemical protection, is mainly directed against large predators, such as vertebrates. These have been shown to be mostly susceptible towards the toxic, irritant or tumour promoting action of phorbol esters (e.g. mice and frogs in Evans and Soper, 1978 as well as humans in Ferreira *et al*. 1998) and thus to avoid contact with them (e.g. sheep, cattle and rats in Kronberg *et al*. 1995). Thus, although *H. euphorbiae* caterpillars do not sequester phorbol esters, we postulate their aposematic colouration to be based on their chemical defence mechanisms against vertebrates.

Another question concerns the potential tolerance of *H. euphorbiae* larvae to phorbol esters themselves, i.e. how they avoid autointoxication. In other specialised insects that store toxic secondary metabolites tolerance has been achieved through target site modification: for an example the binding site for cardiac glycosides has been changed in the monarch butterfly (*Danaus plexippus*) by a single point mutation, so that these compounds no longer bind to the Na^+, K^+ -ATPase (Holzinger and Wink, 1996). In the case of *H. euphorbiae* and phorbol esters, however, the target site is the protein kinase C (PKC), which is ubiquitous in mammalian tissues (Smith and Meldrum, 1992) and is also expected to be ubiquitous in insect tissues due to its central role in many essential biochemical pathways (Alberts *et al*. 1994). The protein is soluble in subcellular compartments in its inactive form and bound to cellular membranes in its active form (Wolf *et al*. 1985). Phorbol esters are actively metabolised and detoxified, thus they are not expected to reach a high number of target sites: if consumed orally, the phorbol esters are probably absorbed in such low concentrations that they can be fully detoxified immediately. We thus assume that the autointoxication is prevented by a very active detoxification system.

At the beginning of the sequestration experiment, the TPA in the diet did appear to have an aversive effect on the caterpillars (refer to results). This was overcome after a few minutes after which the diet with TPA appeared to be consumed more avidly than the one without. Glendinning (2002) described mechanisms for overriding the aversive response of unpalatable, non-toxic food. These can be pre-ingestive (gustatory or olfactory) or post-ingestive (systemic) responses, whereby both are based on changes in signalling pathways. It appears that the TPA-naïve caterpillars (the food plant powder used for the diet was from *E. myrsinites*, which was especially chosen because it does not contain phorbol esters that are structurally similar to TPA) had to activate a specific mechanism before being able to consume TPA without being harmed. We postulate the mechanism to act on the taste sensillae and the metabolism tissues because their ability for active detoxification was found to be the decisive step in avoiding autointoxication (as discussed above). Therefore, the first mechanism for overcoming the aversive response of the larvae to the TPA in the diet is therefore expected to be systemic, not only gustatory or olfactory. The latter would only act on the sensitivity of the taste sensillae. In accordance with this, the response to overcome the firstly deterrent effect of TPA took several minutes, whereas a taste- or smell-mediated one would take less than 6 s (observed in the hawkmoth *Manduca sexta*; Glendinning, 2002). We therefore postulate that the systemic response to overcome the aversion against TPA in the diet of *H. euphorbiae* is coupled to the onset of the activation or acceleration of the metabolism and detoxification mechanisms.

When injected directly into the larvae, the TPA does reach its PKC target sites and accordingly the caterpillars appear to be poisoned (described above). The caterpillars apparently recuperate by firstly storing the toxic compound in an inert compartment, i.e., the integument. They then appear to have individual-specific metabolism and detoxification mechanisms to ultimately excrete the toxin and its metabolites (Fig. 1c). This resistance against the lethal toxicity of phorbol esters is extraordinary since these are quite powerful toxins: the insecticidal effect on *M. sexta* (Sphingidae) was produced at phorbol ester concentrations between 1 and 10 µg/100 mg diet (Wink *et al*. 1997). *H. euphorbiae* caterpillars, however, greedily ate a total of 2 mg TPA (in one case in 2 g diet, i.e., an average concentration of 100 µg TPA/100 mg diet) within 4 hours, as pointed out by our study, and were injected with 1.5 mg TPA without dying. They thus tolerated at least a 10-100 times higher dose in the diet and presumably an even higher magnitude of the comparable dose of direct internal application.

Wink and Theile (2002) have demonstrated that *H. euphorbiae* is not generally more tolerant to plant toxins than *M. sexta*: when injected with the alkaloid nicotine (from tobacco), the caterpillars suffered strongly and although they were still alive after 72 h, they were in bad physiological condition. In this case, *M. sexta* tolerated the highest amount of nicotine of all Lepidoptera studied, an ability which is not surprising because it naturally feeds on tobacco. Therefore, although sphingids usually do not sequester the toxins of their food plant (with the possible exception of *Daphnis nerii*, Abe *et al*. 1996), they nevertheless appear to have developed efficient detoxification mechanisms for the specific toxins they encounter in their diet.

The caterpillars injected with TPA in our experiment were TPA-naïve since they fed on *E. myrsinites*, which does not contain phorbol esters (it contains ingenol- and myrsinol-type diterpene esters, see Introduction). An interesting experiment would be to inject TPA into caterpillars that have already once recuperated from a sufficiently high first TPA dose to check whether the detoxification mechanism had been fully activated to protect them from becoming poisoned a second time. In addition, caterpillars

that had been injected with extracts of *E. cyparissias* could be injected with TPA to examine whether the structural isomer of TPA contained in this plant (Ott and Hecker, 1981) can also activate the detoxification mechanism.

Conclusion

Our study indicates that the larvae of *Hyles euphorbiae* do not sequester phorbol esters. The larvae metabolised and thus detoxified the phorbol ester when TPA was injected directly into the body. After oral consumption TPA was metabolised to about 70-90 %, whereby the remaining TPA was retained in the faeces and excreted. These hawkmoth caterpillars have a large gut full of plant material, which they regurgitate into the direction of the predator when attacked in nature. We postulate that yet unmetabolised phorbol esters are still present in the gut (especially in the food plant slurry of the anterior part, which is used as regurgitant) in sufficient amounts to act toxic and irritant upon predators. Thus, although *H. euphorbiae* caterpillars do not sequester phorbol esters, their aposematic colouration could be based on chemical defence mediated by phorbol esters transiently present in their gut.

Acknowledgements

We would like to thank Katja Sesterhenn, Maren Schneider, Bianca Menrath and Stephanie Hainbuch for laboratory support. Thanks also to Pablo Ibieta for helpful discussions about extraction procedures and analytical protocols. We sincerely thank Carla de Beer (London) for her corrections of the English language.

References

Abdelgaleil SAM, El-Aswad A, Nakatani M (2002) Molluscicidal and anti-feedant activities of diterpenes from *Euphorbia paralias* L. Pest Manag Sci 58: 479–482

Abe F, Yamauchi T, Minato K (1996) Presence of cardenolids and ursolic acid from oleander leaves in larvae and frass of *Daphnis nerii*. Phytochemistry 42: 45–49

Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD (1994) Molecular biology of the cell. New York and London: Garland Publishing

Blumberg PM (1988) Protein kinase C as the receptor for the phorbol ester tumor promoters: sixth Rhoads memorial award lecture. Cancer Res 48: 1–8

Bowers MD (1993) Aposematic caterpillars: life-styles of the warnigly colored and unpalatable. *In* Stamp NE, Casey TM (eds) Caterpillars: Ecological and evolutionary constraints on foraging. New York: Chapman and Hall

Castagna M (1987) Phorbol esters as signal transducers and tumor promoters. Biol Cell 59: 3–13

Evans FJ, Kinghorn AD (1974) A new ingenol type diterpene from the irritant fractions of *Euphorbia myrsinites* and *Euphorbia biglandulosa*. Phytochemistry 13: 2324–2325

Evans FJ, Soper CJ (1978) The tigliane, daphnane and ingenane diterpenes, their chemistry, distribution and biological activities. A review. Lloydia 41: 193–233

Ferreira M-JU, Madureira AM, Ascenso JR (1998) A tetracyclic diterpene and triterpenes from *Euphorbia segetalis*. Phytochemistry 49: 179–183

Glendinning JI (2002) How do herbivorous insects cope with noxious secondary plant compounds in their diet? Entomol Exp Appl 104: 15–25

Harbich H (1994) Erfahrungen bei der Aufzucht von Sphingidenraupen mit einem Kombinationsfutter (Lepidoptera: Sphingidae). Entomol Z 104: 112–117

Hecker E (1968a) Cocarcenogeneic principles from the seed oil of *Croton tiglium* and from other Euphorbiaceae. Cancer Res 28: 2338–2349

Hecker E (1968b) Cocarcenogenic substances from Euphorbiaceae. Planta Med Suppl: 24–45

Hecker E (1971) Isolation and characterization of the cocarcenogenic principles from croton oil. Pp 439–484 *in* Busch H (eds) Methods in cancer research, vol. 6. New York and London: Academic Press

Holzinger F, Wink M (1996) Mediation of cardiac glycoside insensitivity in the monarch (*Danaus plexippus*): Role of an amino acid subsitution in the ouabain binding site of Na+, K+ -ATPase. J Chem Ecol 22: 1921–1937

Hundsdoerfer AK, Kitching IJ & Wink M (2005) The phylogeny of the *Hyles-euphorbiae*-complex (Lepidoptera: Sphingidae): molecular evidence from sequence data and ISSR-PCR fingerprints. Org Divers Evol 5: 173–198

Jakupovic J, Morgenstern T, Marco JA, Berendsohn W (1998a) Diterpenes from *Euphorbia paralias*. Phytochemistry 47: 1611–1619

Jakupovic J, Jeske F, Morgenstern T, Tsichritzis F, Marco JA, Berendsohn W (1998b) Diterpenes from *Euphorbia segetalis*. Phytochemistry 47: 1583–1600

Kronberg SL, Lynch WC, Cheney CD, Walker JW (1995) Potential aversive compounds in leafy spurge for ruminants and rats. J Chem Ecol 21: 1387–1399

Manojlovic B, Keresi T (1997) Previous studies of phytophagous insects for biological control of plants from the genus *Euphorbia* L. (Euphorbiales: Euphorbiacea J. St. Hill.). Zast Bilja 48: 23–48

Marsh N, Rothschild M, Evans F (1984) A new look at Lepidoptera toxins. Symp R Entomol Soc London 11: 135–139

Müller C, Agerbirk N, Olsen KE (2003) Lack of sequestration of host plant glucosinolates in *Pieris rapae* and *P. brassicae*. Chemoecology 13: 47–54

Ott HH, Hecker E (1981) Highly irritant ingenane type diterpene esters from *Euphorbia cyparissias* L. Experientia 37: 88–91

Öksüz S, Gürek F, Gil RR, Pengsuparp T, Pezzuto JM, Cordell GA (1995) Four diterpene esters from *Euphorbia myrsinites*. Phytochemistry 38: 1457–1462

Pittaway AR (2004) Sphingidae of the western Palaearctic. http://tpittaway.tripod.com/sphinx/list.htm

Smith SE, Meldrum BS (1992) The protein kinase C activators, phorbol 12-myristate 13-acetate and phorbol 12, 13-dibutyrate, are convulsant in the pico-nanomolar range in mice. Eur J Pharm 213: 133–135

Wink M, Koschmieder C, Sauerwein M, Sporer F (1997) Phorbol esters of *Jatropha curcas* - Biological activities and potential applications. *In* Gübitz GM, Mittelbach M, Trabi M (eds) Biofuel and industrial products from *Jatropha curcas.* Graz: Dbv-Verlag Univ

Wink M, Grimm C, Koschmieder C, Sporer F, Bergeot O (2000) Sequestration of phorbolesters by the aposematically coloured bug *Pachycoris klugii* (Heteroptera: Scutelleridae) feeding on *Jatropha curcas* (Euphorbiaceae). Chemoecology 10: 179–184

Wink M, Theile V (2002) Alkaloid tolerance in *Manduca sextra* and phylogenetically related sphingids (Lepidoptera: Sphingidae). Chemoecology 12: 29–46

Wolf M, Cuatrecasas P, Sahyoun N (1985) Interaction of protein kinase C with membranes is regulated by Ca^{2+}, phorbol esters, and ATP. J Biol Chem 260: 15718–15722

Received 8 March 2005; accepted 1 September 2005.