Physiological adaptations of specialized insects to host plant cardenolides

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To whom it may concern,

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Best wishes,

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To whom it may concern,

I have now read the PhD thesis prepared by Georg Petschenka and checked the English in the following chapters provided to me:

- 1) Target-site sensitivity in a specialized herbivore towards major toxic compounds of its host plant: The Na+K+-ATPase of the Oleander hawk-moth (*Daphnis nerii*) is highly susceptible to cardenolides
- 2) Physiological screening for target-site-insensitivity and immunohistochemical localization of Na+K+-ATPase in cardenolide adapted Lepidoptera
- 3) Functional evidence for an efflux transport mechanism to circumvent neurotoxicity of dietary metabolites in the Oleander hawk-moth (*Daphnis nerii*)
- 4) Bitter pills: evidence for cardenolide deterrence to an invertebrate predator

The language of all chapters is acceptable.

Best wishes,

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Aufführung der Inanspruchnahme fremder Hilfen

Der in Kapitel 2 beschriebene Western Blot wurde von Frau Dr. Julia Offe durchgeführt.

Die in Kapitel 4 vorgestellten Daten wurden von Frau Christiane Bramer erhoben, die statistische Auswertung dieser Studie wurde von Frau Helga Pankoke durchgeführt.

Zusammenfassung

Cardenolide (Herzglykoside; CGs) sind toxische sekundäre Pflanzenstoffe, die spezifisch die Na^+K^+-ATPase hemmen. Dieses im Tierreich allgegenwärtige Enzym erfüllt wichtige physiologische Funktionen und spielt beispielsweise bei der neuronalen Erregungsleitung und der Exkretion eine wichtige Rolle. Cardenolide kommen erratisch in 12 Pflanzenfamilien vor, wobei das reichste Vorkommen innerhalb der Hundsgiftgewächse (Apocynaceae) zu finden ist.

Trotz der hohen Giftigkeit der Herzglykoside, können viele herbivore Insekten diese Toxine nicht nur tolerieren, sondern darüber hinaus sogar sequestrieren und zum Selbstschutz speichern. Ein Paradebeispiel für dieses Phänomen ist der Monarchfalter (*Danaus plexippus*), dessen aposematisch gefärbte Raupen Herzglykoside aus ihren Nahrungspflanzen (*Asclepias*-Arten) aufnehmen und ins Adultstadium transferieren. Auf diese Weise sind die Falter beispielsweise gegen Vögel geschützt. Die Sequestrierung von Cardenoliden kommt jedoch nicht nur bei Schmetterlingen (Danaidae, Arctiidae) vor, sondern ist auch von Käfern (Chrysomelidae, Cerambycidae), Wanzen (Unterfamilie Lygaeinae), einer Blattlausart (*Aphis nerii*) und Heuschrecken (Pyrgomorphidae) bekannt.

Es wurde gezeigt, dass die Na^+K^+-ATPase von *D. plexippus* über eine verringerte Sensitivität gegenüber dem Standard-Herzglykosid Ouabain verfügt. Dieses Phänomen, das als „target site insensitivity“ bezeichnet wird, beruht auf mindestens einer Aminosäurensubstitution an einer für die Bindung von Herzglykosiden relevanten Position des Proteins. Target site insensitivity ist außerdem von *Oncopeltus fasciatus* (Heteroptera: Lygaeidae), *Poekilocerus bufonius* (Caelifera: Pyrgomorphidae), *Chrysochus auratus* und *C. cobaltinus* (Coleoptera: Chrysomelidae) bekannt.

Ein Hauptanliegen der vorliegenden Doktorarbeit war die Klärung der Frage, ob es sich bei target site insensitivity um einen üblichen Weg der Cardenolidresistenz bei Schmetterlingen handelt, die an Herzglykoside angepasst sind. Um diese Frage zu klären, wurde ein photometrischer Test etabliert, mit dessen Hilfe die Herzglykosidsensitivität einer bestimmten Na^+K^+-ATPase unmittelbar überprüft werden kann. Auf diese Weise wurden die Na^+K^+-ATPasen des Oleanderschwärmers (*Daphnis nerii*), der Wüstenheuschrecke (*Schistocerca gregaria*; zu Vergleichszwecken) sowie der herzglykosidadaptierten Bärenspinner *Empyreuma pugione*, *Cycnia oregonensis*, *Euchaetes egle*, *Pygoctenucha terminalis*, *Arctia caja* und des Monarchfalters (*D. plexippus*) untersucht. Diese Untersuchung erbrachte, dass alle getesteten Arten, mit Ausnahme von *D. plexippus*, über Na^+K^+-ATPasen verfügen, die hochempfindlich gegenüber Cardenoliden sind.

Die Untersuchung von Raupenhämolymphe von *E. pugione* erbrachte weiterhin, dass diese Tiere Herzglykosidkonzentrationen in ihrer Hämolymphe aufweisen, die eine vollständige Hemmung der arteigenen Na^+K^+-ATPase *in vitro* verursachen. Um diesen scheinbaren Widerspruch aufzuklären, wurde die Verteilung der Na^+K^+-ATPase in Schmetterlingsraupen immunhistochemisch untersucht. Da sich herausstellte, dass das Enzym weitgehend auf das Nervengewebe beschränkt ist, erwies sich die Kontaktzone zwischen toxischer Hämolymphe und sensitiver Na^+K^+-ATPase innerhalb des Bauchmarks als besonders interessant.

Um herauszufinden, ob die zelluläre Scheide, die das Bauchmark umgibt (Perineurium), als Barriere für Herzglykoside fungiert, wurden Experimente mit radioaktiv markierten Herzglykosiden an isolierten Nervengeweben von *D. nerii* und *Manduca sexta* durchgeführt.

Pflanzen beinhalten gewöhnlich sowohl polare als auch unpolare Cardenolide. Zum Schutz vor polaren CGs würde eine simple Diffusionsbarriere ausreichen, da polare Cardenolide Zellmembranen nicht passiv durchqueren können. Unpolare Herzglykoside hingegen, würden einen aktiven Barrieremechanismus erfordern. Um beide Hypothesen zu überprüfen, wurde ^{3}H-Ouabain als polares und ^{3}H-Digoxin als unpolares CG eingesetzt. Es wurde gezeigt, dass durch Behandlung von *D. nerii*-Nervengeweben mit Urea die Bindung von ^{3}H-Ouabain deutlich erhöht werden kann. Da angenommen wird, dass Urea die perineurale Barriere zerstört, zeigt dieses Experiment, dass das native Perineurium als Diffusionsbarriere für polare Herzglykoside fungiert. Mit der Anwendung der Atmungskettengifte 2,4-dinitrophenol (2,4-DNP) und carbonyl cyanide 3-chlorophenylhydrazon (CCCP) wurde getestet, ob eine energiegetriebene Barriere für das unpolare Herzglykosid Digoxin vorliegt. Beide Inhibitoren erhöhten die Bindung von ^{3}H-Digoxin an die isolierten Nervengewebe signifikant, so dass von einer aktiven Barriere ausgegangen werden kann.

Einer der wichtigsten Efflux-Transporter in der Blut-Hirn-Schranke von Säugern ist P-Glycoprotein (PGP). Verapamil, ein klassischer PGP-Inhibitor, führte ebenfalls zu einer stark erhöhten Bindung von Digoxin, was eine Einbindung eines PGP-artigen Transporters in die Cardenolidbarriere des Schwärmernervengewebes plausibel macht. Zusätzlich wurde die Präsenz eines solchen Carriers immunhistochemisch nachgewiesen.

Darüber hinaus wurde, um die ökologische Relevanz der Cardenolidsequestrierung zu überprüfen, ein Fütterungsexperiment mit einer Radnetzspinne durchgeführt. Hierfür wurden Dummy-Insekten aus Gelatinekapseln verwendet, die mit einer Cardenolidlösung befüllt wurden. Dieses Experiment erbrachte, dass Cardenolide als Deterrentien wirken und Schutz gegenüber Spinnen verleihen können.

Summary

Cardiac glycosides (cardenolides; CGs) are highly toxic secondary plant compounds which bind to and specifically inhibit the ubiquitous animal enzyme Na^+K^+-ATPase. Since this ion pump is involved in essential physiological processes like nervous function or excretion, the hazardousness of cardenolide ingestion is obvious. Cardenolides occur sporadically in 12 plant families and are found most abundantly within the Apocynaceae.

Despite the high toxicity of CGs many herbivorous insects not only tolerate these toxins but even sequester them and use them for their own defence against predators. A prime example for this phenomenon is the monarch butterfly (*Danaus plexippus*) whose aposematically colored caterpillars derive cardenolides from their host-plant (*Asclepias* spp.) and transfer them into the adult stage. As a consequence, the butterfly is protected against avian predators. Sequestration of cardenolides, however, is not restricted to lepidopterans (Danaidae, Arctiidae) but also known to occur in beetles (Chrysomelidae, Cerambycidae), heteropterans (subfamily Lygaeinae), an aphid (*Aphis nerii*) and locusts (Pyrgomorphidae).

Insects which feed on cardenolide-containing plants can be expected to possess physiological mechanisms for tolerating ingested or stored cardenolides. For *D. plexippus* it is known that the Na^+K^+-ATPase of this species possesses a reduced sensitivity to ouabain, a standard cardenolide which is widely used in laboratory studies. This so called target site insensitivity is due to at least one amino acid substitution at a position within the protein which is critical for cardenolide binding. Target site insensitivity towards cardenolides is furthermore known to occur in *Oncopeltus fasciatus* (Heteroptera: Lygaeidae), *Poekilocerus bufonius* (Caelifera: Pyrgomorphidae), *Chrysochus auratus* and *C. cobaltinus* (Coleoptera: Chrysomelidae).

One major objective of the present thesis was to test whether target site insensitivity is a common mode of resistance in cardenolide adapted Lepidoptera. For testing the cardenolide sensitivity of a particular Na^+K^+-ATPase directly, a spectrophotometer assay was established. Using this technique the Na^+K^+-ATPases of the Oleander hawk-moth (*Daphnis nerii*) and, for comparison, the desert locust (*Schistocerca gregaria*) as well as of the cardenolide adapted arctiid moths *Empyreuma pugione*, *Cycnia oregonensis*, *Euchaetes egle*, *Pygoctenucha terminalis, Arctia caja* and the monarch butterfly (*D. plexippus*) were tested on their sensitivity towards ouabain. This study revealed that the Na^+K^+-ATPases of all species tested, with exception of *D. plexippus*, are highly cardenolide sensitive.

Using thin layer chromatography and spectrophotometry it was furthermore shown that caterpillars of *E. pugione* contain cardenolide concentrations within their haemolymph which cause total inhibition of the Na^+K^+-ATPase of this species, *in vitro*. To unravel the paradox that species like *E. pugione* contain high amounts of cardenolides but possess a highly sensitive Na^+K^+-ATPase, the distribution of this enzyme in lepidopteran larvae was investigated with an immunohistochemical approach. Having discovered that the enzyme is largely restricted to the caterpillars' nervous system, the interface between toxic haemolymph and sensitive Na^+K^+-ATPase within the nerve cord was especially interesting.

The lepidopteran nerve cord is surrounded by a cellular layer, the perineurium, which does not express Na^+K^+-ATPase. This strongly suggested that this tissue may function as a barrier to cardenolides, and experiments on isolated nerve cords of *D. nerii* and *Manduca sexta* using radioactively labelled cardenolides were performed.

Plants usually contain polar as well as nonpolar cardenolides. Since polar cardenolides can not permeate cell membranes, access of polar CGs to Na^+K^+-ATPase could be prevented by a simple diffusion barrier. In contrast, nonpolar cardenolides which can passively cross cells might necessitate an active barrier mechanism. Both hypotheses were tested using the polar ^{3}H-ouabain and the relatively nonpolar cardenolide ^{3}H-digoxin. It was shown that treatment of isolated nerve cords of *D. nerii* caterpillars with urea enhanced binding of ^{3}H-ouabain in a time dependent manner. Since urea treatment is believed to disrupt the perineurial barrier, this experiment demonstrates that the native perineurium functions as a diffusion barrier for polar cardenolides. To test for an active barrier to nonpolar cardenolides, isolated nerve cords of *M. sexta* and *D. nerii* were incubated with ^{3}H-digoxin in the presence of the metabolic inhibitors 2,4-dinitrophenol (2,4-DNP) and carbonyl cyanide 3-chlorophenylhydrazone (CCCP). Both inhibitors significantly enhanced ^{3}H-digoxin binding to the nerve cord, suggesting the presence of an active barrier.

In mammals, P-glycoprotein (PGP), which is known to transport digoxin, is one of the most important efflux transporters of the blood brain barrier. Verapamil, a classic PGP inhibitor, was shown to increase digoxin binding to the isolated nerve cord, suggesting the involvement of a PGP homologue in the cardenolide barrier of the hawk-moth nerve cord. The occurrence of such a carrier was furthermore visualized by immunohistochemistry.

Additionally, to investigate the ecological significance of cardenolide-sequestration, a feeding experiment with orb-weaving spiders was performed. For this purpose gelatine capsules filled with a cardenolide solution were used as dummy insects and offered to spiders. This experiment showed that cardenolides act as a deterrent and can confer protection to spider predation.

General Introduction

During feeding herbivorous insects are confronted with a high variety of secondary plant compounds many of which act as toxins and are involved in plant defence. Hence, phytophagous insects need to possess physiological mechanisms which avoid or at least reduce damage caused by the defensive chemicals of their host plants. Moreover, many specialized insects can not only tolerate those substances but also can store them in their body cavity (sequestration; for a recent review see Opitz&Müller 2009) and use them for their own defence against predators. In some instances, sequestered plant toxins even play a role in mating behaviour of insects where they act as precursors for pheromones (e.g. Danaidae; Schneider et al. 1975) or expose morphogenetic potential (Arctiidae; Schneider et al. 1982). Intriguing phenomena like this give an idea of how far-reaching physiological adaptations to particular plant compounds must be.

The present thesis focuses exclusively on cardiac glycosides (CGs) a class of plant toxins that has been intensively studied with regard to plant insect interactions. These substances which are very toxic to humans consist of one or more sugar moieties (often with uncommon sugars whose occurrence is in some cases restricted to cardiac glycosides), and a steroidal skeleton with a lactone group in β position (Fig. 1).

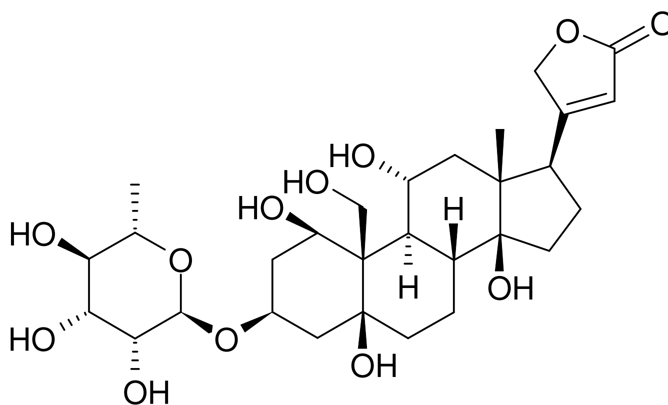

Fig. 1. Ouabain, a cardiac glycoside widely used in biochemical studies

Within cardiac glycosides molecules with a 5-membered mono-unsaturated lactone ring are classified as cardenolides whereas those possessing a 6-membered double-unsaturated lactone ring are called bufadienolides. The steroidal skeleton resembles 10,13-Dimethylsteran. The typical ring juncture (rings A/B: cis; rings B/C: trans; rings C/D: cis), however, leads to a spatial arrangement of the molecule which is decisive for biochemical interactions. Another structural element which is characteristic for

cardenolides is an OH-group at C-14. The high variability of the cardiac glycosides described so far (>500) is caused by an alterable substitution pattern (mainly by oxygen functions). The pharmacokinetic behaviour of CG-molecules is determined by the oxygen bound in the aglycon as well as by the physical properties of the sugars contained in the glycoside (Luckner&Wichtl 2000).

Cardiac glycosides are known from 16 plant families. Their occurrence, however, is erratic (Tab. 1). Besides plants several animals like toads (*Bufo* species), snakes (*Rabdophis*), fire flies (*Photinus*-species) or chrysomelid beetles (*Chrysolina* species) can synthesize CGs as defensive compounds, too (van Oycke et al. 1987; Krenn&Kopp 1998).

Tab. 1. Occurrence of cardiac glycosides in the plant kingdom (Krenn&Kopp 1998; Luckner&Wichtl 2000)

Cardenolide	Ranunculaceae, Moraceae, Brassicaceae, Euphorbiaceae, Fabaceae, Celastraceae, Tiliaceae, Sterculiaceae, Apocynaceae, Scrophulariaceae, Hyacinthaceae, Convallariaceae
Bufadienolide	Ranunculaceae, Crassulaceae, Melianthaceae, Hyacinthaceae

In the European flora, the most prominent source of CGs is Purple Foxglove (*Digitalis purpurea*; Scrophulariaceae) whose ingredients expose positive inotropic action and which has been used against cardiac insufficiency for centuries. Most abundant, however, is the occurrence of CGs within the dogbane family (Apocynaceae; Malcolm 1991). Consequently, almost all chemoecological research dealing with CGs focuses on systems where apocynaceous plants are involved.

The toxic effect of cardiac glycosides is due to the highly specific inhibition of the Na^+K^+-ATPase (Schatzmann 1953) an ubiquitous animal enzyme which is important for nervous function, excretion and energetization of cell membranes. Na^+K^+-ATPase is heterodimeric and consists of a catalytic α-subunit with 10 transmembrane helices and a glycosylated β-subunit. During each reaction cycle three Na^+ are exported from the cell whereas two K^+ are imported to the cell maintaining an electrochemical gradient. This gradient is for example necessary for the Na^+-coupled uptake of glucose and amino acids or the transmembrane transport of ions like Ca^{2+} or H^+. Moreover, the ionic gradient generated by Na^+K^+-ATPase is essential for the maintenance of resting potential and electric activity of muscle and nervous tissue (Lingrel et al. 1990; Jorgensen et al. 2003).

Cardiac glycosides like ouabain, which is widely used in laboratory studies, bind to the Na^+K^+-ATPase α-subunit from the extracellular side where the binding pocket is composed of amino acids from several helices (Qiu et al. 2006). The pharmacological effect of cardiac glycosides in humans is an increased level of intracellular Na^+ leading to

a higher level of intracellular Ca^{2+} which is mediated by Na^{+}/Ca^{2+} exchange. Subsequently, the elevated level of intracellular Ca^{2+} leads to an increase in the strength of heart contraction (Lingrel et al. 1990, and references therein).

Regarding insects, only little data on CG-toxicity is available. Moreover, it is necessary to distinguish between acute and chronic toxicity. With respect to acute toxic effects, however, it is remarkable that non-related (non-adapted) taxa differ greatly in their susceptibility to cardenolides. The LD_{50} of ouabain in locusts (*Schistocerca gregaria*) or cockroaches (*Periplaneta americana*) is as low as 4.4 µg or 0.6 µg per animal, respectively (when injected into the body cavity; Moore&Scudder 1986). In contrast, caterpillars of the tobacco hornworm (*Manduca sexta*) showed no signs of toxic effects at and tolerated an ouabain concentration as high as 10^{-3}M in the haemolymph (Vaughan&Jungreis 1977). Since K^{+} antagonizes ouabain binding to $Na^{+}K^{+}$-ATPase (Glynn 1957) this insensitivity was explained by the high concentration of K^{+} within the lepidopteran haemolymph. The results presented in this thesis, however, suggest that K^{+} plays a minor role at most. Chronic toxic effects of CGs were demonstrated by Karowe&Golston (2006) who fed an artificial diet charged with the cardenolide digitoxin to caterpillars of the generalist *Lymantria dispar*.

A prime example for the defensive use of plant derived cardiac glycosides in insects is the monarch butterfly (*Danaus plexippus*) whose aposematic caterpillars sequester cardenolides from its host plants (e.g. *Asclepias*-species; Parsons 1965) and transfer them into the adult stage. In classic feeding trials these compounds were shown to confer protection to blue jays (Brower et al. 1967). On the whole, sequestration of cardiac glycosides is not restricted to Lepidoptera but also known from heteropterans (Lygaeidae; Duffey&Scudder 1972), an aphid (*Aphis nerii*; Rothschild et al. 1970), locusts (Pyrgomorphidae; von Euw et al. 1967) as well as from chrysomelid beetles (*Chrysochus*; Dobler et al. 1998) and long horn beetles (*Tetraopes*; Duffey&Scudder 1972). Although little details are known there are indications that cardenolide sequestration is an active process (*Danaus*, *Oncopeltus, Chrysochus*) making special adaptations probable (von Nickisch-Rosenegk et al. 1990; Frick&Wink 1995; Detzel&Wink 1995; Dobler 2004).

In comparison to phenomena like sequestration of cardenolides, the question of how insects can tolerate CGs only received comparatively little attention. Here, I addressed this question in detail. The most elegant way to obtain resistance against particular toxins is modification of the target site. This phenomenon, which is called target site insensitivity is known from diverse systems e.g. from carbamate and organophosphate insecticides (Russell et al. 2004). All cardenolide-sequestering insects which have been sufficiently investigated so far also possess target site insensitivity towards cardenolides. The classic example is again *D. plexippus* whose $Na^{+}K^{+}$-ATPase

was shown to possess a reduced *in vitro* sensitivity towards ouabain as early as the 70s (Vaughan&Jungreis 1977). More recently, Na^+K^+-ATPases with reduced ouabain affinity were detected in *Oncopeltus fasciatus* (Lygaeidae; Moore&Scudder 1986), *Poekilocerus bufonius* (Pyrgomorphidae; Al-Robai et al. 1990), *Chrysochus auratus* and *C. cobaltinus* (Chrysomelidae; Labeyrie&Dobler 2004). For *D. plexippus* it was shown that this insensitivity is due to the substitution of at least one amino acid at a position of Na^+K^+-ATPase which is relevant for ouabain binding (Holzinger&Wink 1992). In the leaf beetles *C. auratus* and *C. cobaltinus* the same amino acid substitution as in *D. plexippus* was detected (Labeyrie&Dobler 2004).

One major objective of this thesis was to test whether target site insensitivity is a common phenomenon in cardenolide adapted Lepidoptera and which other strategies might enable these insects to tolerate cardenolides in their body cavity. In a first approach the Na^+K^+-ATPase of the oleander hawk-moth (*Daphnis nerii*) was investigated, which is exposed to high concentrations of cardenolides in its diet yet does not sequester significant amounts of the toxins. In this study, it was also tested whether the presence of cardenolides in the diet alters the ouabain sensitivity of the enzyme (chapter 1). A second study was performed to investigate Na^+K^+-ATPases of cardenolide adapted arctiid species some of which sequester these toxins in high amounts while others apparently do not. In this study I furthermore localized the expression of the target site, Na^+K^+-ATPase, by immunohistochemistry to find out whether compartmentalization might facilitate a safe handling of the toxins (chapter 2). Since the results revealed that the interface between nervous tissue and haemolymph is of special interest for cardenolide resistance, the function of the perineurium (neural sheath) as a cardenolide barrier was addressed in a further study (chapter 3). Lastly, although many studies demonstrate the efficacy of sequestered cardenolides as protection against vertebrates, our knowledge about cardenolides as feeding deterrents to invertebrate predators is insufficient. We therefore designed a feeding experiment using artificial insects which were offered to an orb-weaving spider. The system employed in this study should allow for testing the deterrency of any compound of interest to spiders.

Chapter 1

Target-site sensitivity in a specialized herbivore towards major toxic compounds of its host plant: The Na^+K^+-ATPase of the Oleander hawk-moth (*Daphnis nerii*) is highly susceptible to cardenolides

Abstract

The caterpillars of the oleander hawk-moth, *Daphnis nerii*, (Linnaeus, 1758) (Lepidoptera: Sphingidae) feed primarily on oleander (*Nerium oleander*). This plant is rich in cardenolides which specifically inhibit the Na^+K^+-ATPase. Since some insects feeding on cardenolide plants possess cardenolide-resistant Na^+K^+-ATPases, we tested whether *D. nerii* also possesses this strategy for circumventing cardenolide toxicity. To do so we established a physiological assay which allowed direct measurement of Na^+K^+-ATPase cardenolide sensitivity. Using *Schistocerca gregaria*, as a cardenolide sensitive reference species, we show that *D. nerii* Na^+K^+-ATPase is extremely sensitive to the cardenolide ouabain. Surprisingly, its sensitivity is even higher than that of the cardenolide sensitive generalist, *S. gregaria*. The presence or absence of cardenolides in the diet of *D. nerii* did not influence the enzyme's cardenolide sensitivity, indicating that target-site insensitivity is not inducible in this species. However, despite the sensitivity of their Na^+K^+-ATPase, caterpillars of *D. nerii* quickly recovered from an injection of an excessive amount of ouabain into their haemocoel. We conclude that *D. nerii* possesses adaptations which enable it to feed on a cardenolide rich diet other than those previously described in cardenolide specialised insects, and we discuss other potential resistance mechanisms.

Introduction

Cardenolides and bufadienolides are also referred to as cardiac glycosides (Falbe and Regitz 1995) due to their therapeutic usage in the treatment of heart disease. These compounds occur in 12 different plant families (Luckner and Wichtl 2000), most prominently in the Apocynaceae, e.g. in oleander (*Nerium oleander*), a widely used ornamental plant toxic for humans and other animals (Frohne and Pfänder 2004). Cardenolides, the principal toxic components of *N. oleander*, possess a highly specific mode of action: they bind to and inhibit the Na^+K^+-ATPase (Schatzmann 1953),

an ubiquitous transmembrane enzyme in the cells of all higher eukaryotes that is involved in essential physiological processes, such as nervous function and excretion (Lingrel 1992). Due to their ubiquitous target site, these compounds are expected to cause toxic effects in virtually all animals. Despite the toxic potential of these substances, there are several herbivorous insects of different orders which feed on cardenolide plants.

In this paper we investigated potential Na^+K^+-ATPase-cardenolide resistance in *Daphnis nerii* (Linnaeus, 1758), the oleander hawk moth (Lepidoptera: Sphingidae). The caterpillars of this species use *N. oleander* as their major host plant (Pittaway 1993) and are clearly able to cope with the toxic compounds, small amounts of which are also present in the larval body (Abe and others 1996). However, it remains unknown how the caterpillars avoid intoxication by the cardenolides in their diet.

One possible adaptation to decrease cardenolide toxicity could be the evolution of a cardenolide-resistant Na^+K^+-ATPase similar to that found in the monarch butterfly (*Danaus plexippus*). Monarch larvae feed on toxic apocynacean plants and store high amounts of cardenolides in their body (Brower and others 1982; Parsons 1965). The relative insensitivity of monarch Na^+K^+-ATPase to ouabain, a cardiac glycoside that occurs naturally in the apocynacean genera *Acokanthera* and *Strophanthus* in east Africa (Jäger et al. 1965) and which is widely used as a commercially available inhibitor of cellular Na^+K^+-ATPase activity was first demonstrated by Vaughan & Jungreis (1977) using an enzymological assay. Later analyses showed that this insensitivity was due to an amino acid substitution (Asn122His) in the first extracellular loop of the protein which is involved in ouabain binding (Holzinger and others 1992). Convergently evolved cardenolide insensitive Na^+K^+-ATPases were also detected in *Oncopeltus fasciatus* (Heteroptera: Lygaeidae), *Poekilocerus bufonius* (Caelifera: Acrididae), *Chrysochus auratus* and *C. cobaltinus* (Chrysomelidae) (Al-Robai 1993; Moore and Scudder 1986).

However, not all species specialized on cardenolides, which were investigated so far contained a similar substitution in the first extracellular loop of their Na^+K^+-ATPase protein sequence and this is also true for *D. nerii* (Holzinger and Wink 1996; Mebs and others 2000). Resistance could also be achieved by other alterations of the Na^+K^+-ATPase since also other regions of this protein are involved in the binding of ouabain (Croyle and others 1997; Holzinger and others 1992; Mebs and others 2000; Qiu and others 2005). Other forms of resistance are also hypothetically possible, such as cardenolide impermeable guts or the production of cardenolide degrading enzymes.

The aim of this study was to test whether the oleander specialist *D. nerii* also possesses an insensitive Na^+K^+-ATPase. To test this hypothesis, we performed a physiological assay which allowed us to directly investigate the ouabain sensitivity of the *D. nerii* Na^+K^+-ATPase. Since caterpillars are the developmental stage which is directly

confronted with the cardenolides of the host plant, we used nervous tissue of caterpillars to extract the enzyme.

Additionally, we tested whether the sensitivity of the enzyme was altered by the presence of cardenolides in the diet by comparing the enzyme of *D. nerii* larvae raised on *N. oleander* with the enzyme of *D. nerii* caterpillars raised on *Vinca major* (Apocynaceae), a plant devoid of cardenolides. For comparison, and as a positive control of our method, we also assayed the Na^+K^+-ATPase of *Schistocerca gregaria* (Caelifera, Acrididae), an insect that is known to possess a Na^+K^+-ATPase sensitive towards ouabain (Moore and Scudder 1986). Finally, to test whether *D. nerii* caterpillars can tolerate high amounts of ouabain within the body cavity we injected the larvae with a dose of the toxin sufficient to bring the hemolymph to a level causing total *in vitro* inhibition of the enzyme.

Material & Methods

Na^+K^+-ATPase-Assays

We used *D. nerii* of different genetic backgrounds: some specimens originated from a cross between a strain from Thailand and European individuals while others were derived from different European strains. The caterpillars were raised from eggs at room temperature and ambient light (one replicate) or in a climatic chamber at 27°C and constant light (six replicates) and fed either with fresh leaves of potted *N. oleander* plants or with cuttings of *V. major* collected in surrounding parks and gardens. To extract the Na^+K^+-ATPase, we used four (one replicate) or five (six replicates) fully grown last instar caterpillars. The caterpillars were anesthetized on ice and decapitated. The nerve cord and the brain were dissected on ice, cleaned from adherent tissue and rinsed with deionised water.

The Na^+K^+-ATPase of *S. gregaria* was extracted from dissected brains and thoracic ganglia of nine adult locusts (3 per replicate). The nervous tissues were pooled or singly homogenized on ice in a glass homogenizer with 1 ml deionised water using a motor-driven Teflon pestle (3 min at 800 rpm). Extracts of pooled nervous tissues were diluted with deionised water to 1 ml water per nervous tissue and aliquoted (1 ml) in Eppendorf tubes. Lyophilized extracts were stored at -80°C for a maximum of 9 weeks before use.

Prior to use the lyophilisates were reconstituted with 100 µl deionised water by vortex stirring and sonication in an ultrasonic bath. Pooled extracts were centrifuged at 1000 g at 4° C for 10 min to remove undissolved material. The supernatant was diluted with deionised water to reach a sufficient volume and the protein content was determined

using the Bradford assay (Bradford 1976). Reaction conditions were similar to those described in Moore & Scudder (1986). Three different buffer conditions (I.-III.) were used in order to determine the degree to which the Na^+K^+-ATPase can be inhibited by ouabain and the Na^+K^+-ATPase activity relative to other ATPases: I. 100 mM NaCl, 20 mM KCl, 4 mM $MgCl_2$, 50 mM imidazole (non-inhibited control); II. 100 mM NaCl, 20 mM KCl, 4 mM $MgCl_2$, 50 mM imidazole with 10^{-3}-10^{-8} M ouabain (Fluka) (determination of ouabain sensitivity); III. 100 mM NaCl, 4 mM $MgCl_2$, 50 mM imidazole (determination of the activity of other ATPases: Na^+K^+-ATPase is not active since K^+ is lacking). Reactions were performed in Eppendorf tubes and consisted of the respective buffer (pH 7.4), Tris-ATP (Sigma-Aldrich) with a final concentration of 2.5 mM and ouabain solution or water, respectively. The reaction tubes were preincubated in a water bath at 37°C for several minutes and the reactions started by adding the tissue extract which brought the volume to 500 µl. After 20 min of incubation reactions were stopped by the addition of 250 µl 30% trichloroacetic acid (TCA) and centrifuged at 12000 g for 5 min to precipitate denatured proteins. ATPase-activity was determined by quantifying released phosphate in a microplate reader (Biorad Model 680) at 655 nm using the photometric method described by Taussky & Shorr (1953). The concentration of endogenous phosphate in the samples and the amount of phosphate originating from non-enzymatic hydrolysis of ATP were quantified in control tubes containing buffer, water and either no ATP or no enzyme-solution. Alongside with each series of samples we ran a phosphate calibration curve using a standard series of 0.2-1.2 mM KH_2PO_4 in water mixed 1:2 with 30% TCA as in the reactions. A solution of 30% TCA in water (1:2) was used as a blank for all samples.

For further analysis the enzyme activity was calculated as percent of control after subtracting the activity of other ATPases. To calculate the ouabain doses causing 50 % inhibition (IC_{50}) the averaged curves of each species or treatment were fitted with the solver tool of Microsoft Excel 2003 using a five parameter logistic function.

Ouabain Injections

D. nerii caterpillars were raised on *V. major* at 27°C (13 h/11 h light/dark cycle) to the penultimate instar. Larvae (n = 10) were weighed and injected with 10 µl 10^{-2} M ouabain in 0.9 % NaCl into the posterior dorsal vessel. Control larvae (n = 10) were injected with 0.9 % NaCl only. Afterwards individualized caterpillars were returned to the 27°C chamber and checked for toxic effects.

Results & Discussion

The Na^+K^+-ATPase of the nervous tissue of *D. nerii* caterpillars was highly sensitive to the cardiac glycoside ouabain (Fig. 1). The Na^+K^+-ATPase of *D. nerii* was even more strongly inhibited by ouabain than the Na^+K^+-ATPase of *S. gregaria* that is well known to be sensitive to cardenolides. While the overall activity of the Na^+K^+-ATPase was very similar in both species, the ouabain dose causing 50% inhibition of the enzyme was more than twice as high in *S. gregaria* compared with *D. nerii* (Table 1). Because the pharmacodynamics of all cardiac glycosides is regarded to be uniform (Luckner and Wichtl 2000), we assume that the Na^+K^+-ATPase of *D. nerii* can be inhibited by any cardenolide. Therefore *D. nerii* possesses a target site highly vulnerable towards cardenolides, the major toxic compounds of its host plant.

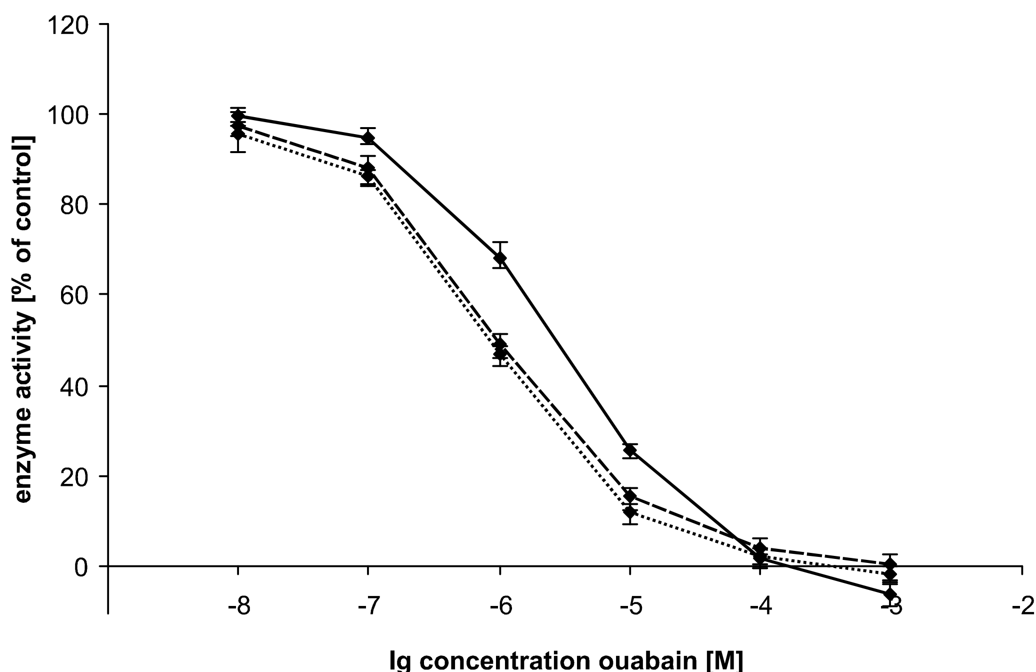

Fig. 1. Inhibition of the Na^+K^+-ATPase of *D. nerii* and *S. gregaria* by ouabain.
- - - = enzyme from caterpillars raised on *V. major*, ········ = enzyme from caterpillars raised on *N. oleander*, —— = enzyme from nervous tissue of *S. gregaria.* Each curve is the average of four (*D. nerii* from *N. oleander*) or three (*D. nerii* from *V. major* and *S. gregaria*) replicates, bars indicate minimum and maximum values. To estimate the contribution of Na^+K^+-ATPase to total P_i-release, KCl was omitted in separate control tubes. The amount of P_i measured under these conditions was subtracted from overall release. The negative values in the *S. gregaria* curve might be due to a small remainder of Na^+K^+-ATPase activity potentially due to contamination with K^+ present in the extract.

There was no noticeable difference in the enzyme's cardenolide sensitivity when comparing caterpillars raised on cardenolide rich oleander leaves (Fig. 1) and caterpillars fed with *V. major* which is devoid of cardenolides and the doses of ouabain causing 50% inhibition are very similar (Table 1). Thus, there is no indication that the occurrence of

cardenolides in the diet alters the cardenolide sensitivity of the Na^+K^+-ATPase (e.g. by the expression of different isoforms of the enzyme).

Tab. 1. *In vitro* enzyme activities and ouabain doses causing 50% inhibition of the Na^+K^+-ATPase (IC_{50}).

	Na^+K^+-ATPase activity [nmol P_i/mg Protein*min]	± SD	IC_{50}
D. nerii from *N. oleander*	121.05	± 18.86	$9,1*10^{-07}$
D. nerii from *V. major*	100.89	± 15.29	$1,04*10^{-06}$
S. gregaria	82.02	± 17.18	$2,81*10^{-06}$

D. nerii was previously reported not to take up cardenolides (Rothschild and others 1970). However, Abe *et al.* (1996) showed that *D. nerii* caterpillars do contain oleander cardenolides in their body. These authors observed different HPLC-profiles when comparing the cardenolides derived from whole larvae with those derived from faeces. Although they did not remove the guts of the caterpillars before extraction, contamination by plant material in the gut likely played a minor role in Abe *et al.*'s (1996) findings because they used caterpillars immediately before pupation where empty guts can be expected. This indicates strongly that at least part of the detected cardenolides originate from the larval body. Based on their results, the authors estimated the amount of bioactive cardenolides to be up to 150-200 µg per larva.

This amount of cardenolides should be sufficient to cause a drastic inhibition of the enzyme, yet *in vivo* the larvae obviously cope with these concentrations. In addition, our injection experiment also showed that *D. nerii* caterpillars can tolerate a high amount of ouabain within the body cavity. Each caterpillar (mean weight ~ 400 mg) received 10 µl 10^{-2} M ouabain solution bringing the hemolymph to a level of ~ 10^{-4} M at minimum, a concentration causing almost total inhibition *in vitro* (Fig. 1). All caterpillars injected with ouabain showed slight signs of a toxic effect: they reacted lethargically when turned on their side and sometimes stayed in that position. While healthy caterpillars adhere tightly to surfaces, these injected caterpillars did not. Caterpillars which had received only saline were much more vital and displayed normal behaviour. At the end, however, all caterpillars recovered and resumed feeding. These findings potentially parallel the results of Vaughan & Jungreis (1977). Although they did not observe toxic effects in *Manduca sexta* caterpillars after the injection of a similar amount of ouabain, the authors showed that the bulk of the toxin was excreted while metabolic degradation could not be detected. The initial toxic effect in the *D. nerii* larvae and the slow recovery might correspond with the excretion of ouabain in these caterpillars.

By contrast to the situation in the monarch, there is no report that *D. nerii* derives chemical protection by the sequestration of cardenolides. Brower *et al.* (1982) reported that the cardenolide content of adult monarchs averaged 616 µg. The cardenolide content of an adult *D. plexippus* is, therefore, at least three times higher than the cardenolide content of a *D. nerii* larva. In addition, the total weight of an adult monarch (~ 0.6 g, own data, n = 6) is only about one tenth of the weight of a *D. nerii* larva (Abe and others 1996: 5.56 g), making the concentration of cardenolides in *D. nerii* caterpillars only about one thirtieth of that observed in *D. plexippus.* Moreover, adynerin, the dominant cardiac glycoside in the larvae detected by Abe *et al.* (1996) is reported not to elicit cardiotonic activity (Imai and others 1965).

The relatively low concentration of cardenolides and the camouflaged habit of the caterpillar (Rothschild 1985), as well as of the moth, suggests a cryptic rather than an aposematic lifestyle. Our finding that the Na^+K^+-ATPase of *D. nerii* is highly sensitive towards ouabain provides indirect evidence that this species possesses mechanisms of resistance other than a modification of the Na^+K^+-ATPase. The relatively low cardenolide content of the larvae suggests that *D. nerii* might absorb only an 'unavoidable' part of dietary cardenolides while excluding the main part of the toxins which pass through the gut. The predominance of adynerin in the caterpillars, a minor compound in the plant (Tschesche and Bohle 1938), may indicate quantitative differences in the absorption of different cardenolides. This might be due to differences in the physical properties of the molecules. Generally, more polar cardenolides should be easier to exclude than less polar ones due to the passive membrane permeation of lipophilic compounds. Mechanisms which prevent absorption of cardenolides are plausible because they are reported to exist even in species not adapted to cardenolides: Tracer feeding experiments demonstrated that neither of the generalists *S. gregaria* and *Periplaneta americana* take up radioactively labelled cardenolides via their guts (Scudder and Meredith 1982) nor does the leaf beetle *Chrysochus asclepiadeus*, a close relative of cardenolide sequestering species that itself is not naturally exposed to cardenolides (Dobler 2004). These observations cannot, however, reveal whether the uptake of cardenolides is prevented or whether they are immediately removed from the hemolymph by an efficient excretion mechanism as observed in *Drosophila melanogaster* (Torrie and others 2004).

The occurrence of cardenolide barriers in guts of generalist insects possibly indicates that cardenolide rich host plants can also be used by non-specialized insects. Such mechanisms might explain how polyphagous insects avoid intoxications by cardenolides in their diets (e.g. *Gymnoscelis rufifasciata* and *Eupithecia* spp. on *Digitalis purpurea*). However, there are also reports of polyphagous lepidopteran species which show toxic effects from the ingestion of cardenolides. Dussourd & Hoyle (2000) show

clearly that caterpillars of several generalistic noctuid moths suffer spasms after the ingestion of cardenolide solutions or latex of *Asclepias* species which contains cardenolides. Furthermore, Karowe & Golston (2006) showed that the cardenolide digitoxin at lower doses deterred the caterpillars of *Lymantria dispar* (Lepidoptera: Lymantriidae) from eating and caused toxic effects at higher doses. These results imply that neither an impermeable gut nor the ability to tolerate cardenolides within the haemocoel are general features of lepidopteran larvae. Basing on our observation that *D. nerii* caterpillars can tolerate excessive amounts of ouabain within their body cavity, we postulate that the target site of cardenolides, the Na^+K^+-ATPase, is insulated from the toxins. Na^+K^+-ATPase is abundant in the nervous tissue of insects (Emery and others 1998). Moreover, immunohistochemical investigations indicate that the Na^+K^+-ATPase in the nervous tissue of caterpillars is expressed disproportionately highly and not detectable elsewhere (G. Petschenka, unpubl. data). For this reasons the perineurium which ensheathes the nervous system could play an important role in protecting the Na^+K^+-ATPase (see Lebovitz 1989). Further experiments focussing on the absorption of cardenolides via the gut, on cardenolide excretion and on the physiological properties of the “blood-brain-barrier” of *D. nerii* caterpillars might enhance our understanding of the multilayer phenomenon of cardenolide resistance.

Chapter 2

Physiological screening for target-site-insensitivity and immunohistochemical localization of Na^+K^+-ATPase in cardenolide adapted Lepidoptera

Abstract

Cardenolides are toxic plant compounds which specifically inhibit the Na^+K^+-ATPase, an animal enzyme which is essential for many physiological processes like the generation of nervous action potentials. Several specialized insects feeding on cardenolide containing plants sequester these toxins for their own defence. Some of these insects were shown to possess Na^+K^+-ATPases with a reduced sensitivity towards cardenolides (target site insensitivity). In the present study we screened five species of arctiids feeding on cardenolide-containing plants for target site insensitivity towards cardenolides using an *in vitro* enzyme assay. All of the arctiid species tested were highly sensitive to the toxins although we detected high amounts of cardenolides in an analysis of the haemolymph of two of these species. In caterpillars of the sequestering arctiid *Empyreuma pugione*, the monarch butterfly (*Danaus plexippus*) and a non-adapted noctuid species (*Autographa gamma*) we localized the target site of cardenolides, the Na^+K^+-ATPase, with an immunohistochemical approach and found that the enzyme is only expressed in the nervous tissue of these species. Western Blot analysis exemplarily performed with *A. gamma* revealed weak additional expression in the labial glands. Summarizing our evidence, we conclude that the expression of a cardenolide sensitive Na^+K^+-ATPase despite the presence of cardenolides in the haemolymph can only be understood if the nervous tissue is specifically protected against the toxins.

Introduction

Cardenolides (cardiac glycosides) are highly toxic secondary plant compounds which specifically inhibit the Na^+K^+-ATPase, an ubiquitous enzyme throughout the animal kingdom. Since this ion pump is essential for physiological processes like nervous function and secondary active transport (Lingrel 1992) ingested cardenolides are

potentially hazardous for all animals. Nevertheless, several insect species have specialized on cardenolide plants as hosts (Malcolm 1991). Especially many plants in the Apocynaceae are rich in cardenolides (Luckner & Wichtl 2000) and serve as hosts to adapted insects (Malcolm 1991).

Insects from at least five orders (Lepidoptera, Coleoptera, Caelifera, Sternorrhyncha, Heteroptera) sequester cardenolides (Parsons 1965; von Euw and others 1967; Rothschild and others 1970; Duffey and Scudder 1972; Rothschild and others 1973; Dobler and others 1998) and derive protection against predators. A prime example for this phenomenon is the well-known monarch butterfly, *Danaus plexippus*, whose aposematic caterpillars ingest cardenolides with their diet (mostly *Asclepias* spp.) and transfer the toxins into the adult stage. As a consequence, the butterfly is protected against avian predators (Brower and others 1967).

In contrast to the well studied phenomenon of cardenolide-sequestration, comparatively less attention has been paid to the underlying mechanisms of cardenolide resistance and the avoidance of autointoxication in specialized insects. Vaughan and Jungreis (1977) first discovered by *in vitro* assays that the Na^+K^+-ATPase of *D. plexippus* is relatively insensitive towards the cardenolide ouabain. Such a target-site-insensitivity could also be detected in physiological assays in a lygaeid bug (Moore and Scudder 1986) and a locust (Al-Robai and others 1990).

In the monarch butterfly it could later be shown (Holzinger & Wink 1992, 1996) that the relative ouabain resistance of the *D. plexippus* Na^+K^+-ATPase is at least partly due to an amino acid substitution (Asn122His) in the first extracellular loop of the enzyme which is critical for ouabain binding. The same resistance conferring substitution was also found in chrysomelid beetles that feed on cardenolide plants and sequester the toxins (Labeyrie and Dobler 2004). However, no such substitution could be detected in further cardenolide sequestering lepidopteran species (Holzinger and Wink 1996; Mebs and others 2000; Mebs and others 2005). All the studies mentioned above were restricted to the first extracellular loop of Na^+K^+-ATPase α-subunit. Yet, studies on vertebrate Na^+K^+-ATPase (Croyle and others 1997; Qiu and others 2003) showed that amino acid residues which may alter ouabain binding are not limited to the first extracellular loop. As Holzinger and Wink (1996) state it therefore can not be ruled out from the molecular studies that the investigated insect species possess cardenolide insensitive Na^+K^+-ATPases, too.

We wanted to know whether arctiid species (Lepidoptera, Arctiidae) which feed on cardenolide plants or actually sequester these toxins also use target site insensitivity to acquire cardenolide resistance. Moreover, all cardenolide sequestering insects which have been physiologically investigated so far were shown to possess Na^+K^+-ATPases relatively insensitive towards ouabain (Vaughan and Jungreis 1977; Moore and Scudder

1986; Al-Robai and others 1990). Hence, we tested whether this "rule" is also true for cardenolide storing arctiids. To overcome the ambiguity on the sequence level we decided to perform direct functional studies of the respective Na^+K^+-ATPases.

The species studied here belong to different tribes of the subfamily Arctiinae (systematics after Jacobson and Weller 2002). We tested three members of the tribe Phaegopterini (*Euchaetes* group), *Euchaetes egle, Cycnia oregonensis* and *Pygoctenucha terminalis* all of which are specialized on cardenolide containing *Asclepias* and *Apocynum* species (Apocynaceae) as caterpillars (Da Costa and others 2004) yet it was previously unknown whether they sequester the toxins. However, the caterpillars have to cope with the toxins since their host plants (*Asclepias* spp. and *Apocynum cannabinum*) are known to contain cardenolides (Malcolm 1991; Niesenbaum and others 2006). Moreover we investigated *Empyreuma pugione*, a species from the tribe Euchromiini. The brightly coloured caterpillars of this moth feed on *Nerium oleander* (Apocynaceae) and have been reported to sequester large amounts of cardenolides (Black 1976) which are also transferred into the adult stage. As an arctiid species without host plant specialization we investigated the highly polyphagous species *Arctia caja* which has been reported to sequester cardenolides from *Digitalis purpurea* (Rothschild 1973). Finally, we reinvestigated the Na^+K^+-ATPase of *D. plexippus* (Nymphalidae) as a positive control.

Since we found that the sodium pump of all investigated species (except of *D. plexippus*) is highly susceptible to ouabain, as a widely used standard cardenolide, we started to look for alternative mechanisms which confer resistance to cardenolides in these organisms.

To determine whether the target site is spatially insulated from the cardenolides present in the caterpillar we analyzed the localization of Na^+K^+-ATPase with an immunohistochemical approach using the monoclonal antibody α5 (target site visualization) and Western blot analysis of tissue extractions. Moreover, we tested whether there are differences in the distribution of Na^+K^+-ATPase within caterpillars which (a) sequester and possess insensitive enzymes (*D. plexippus*), (b) sequester and possess sensitive enzymes (*E. pugione*) or (c) do not sequester cardenolides (*Autographa gamma*, Noctuidae).

To test for the occurrence of sequestered cardenolides in haemolymph of presumably sequestering species we analyzed blood samples of *E. pugione* and *P. terminalis* caterpillars using thin layer chromatography and spectrophotometry.

Material & Methods

Experimental animals

Determination of species

E. pugione (origin: Cuba), *C. oregonensis* (origin: Boulder, Colorado, USA) and *E. egle* (origin: Ithaca, New York, USA) were determined by means of the genitalia following Weller and others (2004) and Da Costa and others (2006). *P. terminalis* (origin: Boulder, Colorado, USA) was determined by D. Bowers (Boulder, Colorado) using a reference museum specimen. *A. caja* was determined after Ebert (1997).

Rearing conditions

Larvae of *E. pugione* were raised on leaves and stems of potted plants of *N. oleander*. Adults and larvae were kept in a climatic chamber at 27°C with 13 h/11 h light/dark cycle. Larvae of *C. oregonensis* and *P. terminalis* were reared on *Apocynum venetum* (both species) or *A. curassavica* (*P. terminalis*), respectively. Caterpillars of *A. caja* (origin Karlsruhe, Germany) were obtained in an intermediate stage and fed with leaves of *Rubus* sp.. Eggs of *D. plexippus* were obtained from a breeder. Caterpillars were reared on leaves of *Asclepias* spp. *(syriaca, curassavica* or *incarnata)*. *D. plexippus* caterpillars used for immunohistochemistry were collected around Ithaca (New York, USA) and raised in the laboratory until fixation. All species mentioned above were kept at 23°C with 16 h/8 h light/dark cycle. Caterpillars of *E. egle* were collected in the field around Ithaca, New York, USA and reared to pupae on *A. syriaca* under ambient conditions. Pupae of *P. terminalis* and *E. egle* were hibernated. After hatching, adults were used directly (*E. pugione*) or frozen and stored at -80°C (remaining species). Caterpillars of *A. gamma* (obtained from wild females caught at different locations in Germany) were reared at room temperature and ambient light on *Taraxacum officinale* and *Rubus* spec. (Immunohistochemistry) or kept on artificial diet (gypsy moth diet, MP Biomedicals) at 26°C with 16 h/8 h light/dark cycle (Western Blot).

Extraction of Na^+K^+-ATPase

Due to the large size of *D. plexippus,* Na^+K^+-ATPase preparations of this species were obtained from crude extracts of dissected nervous tissue. Imagines were thawed on ice. Brain and thoracic ganglia were dissected in cold deionised water, cleaned from adherent tissue and homogenized in 0.5 ml deionised water using a 1 ml all-glass grinder (Wheaton). Nervous tissue from only one individual (or in a single instance two individuals) was used to produce one extract. The tissue extracts were frozen (-80°C),

freeze dried over night and stored at -80°C until analysis. Prior to use, extracts were reconstituted with deionised water by vortex stirring and sonification (10 min, chilled ultrasonic bath). Unsuspended material was removed by centrifugation.

For all other species $Na^{+}K^{+}$-ATPase was partially purified using differential centrifugation. Heads (~18) of living (*E. pugione*) or frozen moths were homogenized in deionised water (200 µl per two heads) in a 1 ml all-glass grinder (Wheaton). Combined homogenates were placed in a chilled ultrasonic bath for 10 min. After that, the extract was centrifuged at 10000 g for 10 min at 4°C. The supernatant was subjected to ultracentrifugation at 80000 g for 30 min at 4°C to sediment membranes (centrifuge: Beckman L-80; rotor: 80 TI). The supernatant was discarded and the microsomal pellet was resuspended in deionised water by vortex stirring and sonification. After reconstitution, the extract was sonificated for further 10 min and assayed directly or stored on ice overnight. If stored, the extract was sonificated for another 10 min before use. Unsuspended material was removed by centrifugation prior to assay.

$Na^{+}K^{+}$-ATPase-Assay

Enzyme preparations were incubated at 37°C in a water bath with different concentrations of ouabain (10^{-3}-10^{-8} M) for 20 min. Assays (500 µl) were performed in 1.5 ml reaction tubes containing enzyme preparation, aqueous ouabain (Fluka and Sigma) or water (control), buffer (pH 7.4) and ATP (ditris-salt; Sigma-Aldrich). Reaction conditions were: 100 mM NaCl, 20 mM KCl, 4 mM $MgCl_2$, 50 mM Imidazol. With every sample, one reaction without ouabain (control), one without enzyme (estimation of non-enzymatic hydrolysis of ATP) and one without ATP (estimation of endogenous P_i) was performed. Reactions were stopped by adding 250 µl 30% TCA and precipitated protein was removed by centrifugation. The amount of phosphate in the supernatant was quantified using the spectrophotometric approach described by Taussky&Shorr (1953) in a microplate reader at 655 nm. 200 µl Aliquots of the samples were stained with 100 µl Taussky-Shorr-Reagent (1% $(NH_4)_6Mo_7O_{24}\cdot4\ H_2O$, 5% $FeSO_4\cdot7\ H_2O$, 1 N H_2SO_4). A phosphate calibration curve with aqueous KH_2PO_4 dilutions (0.2-1.2 mM) mixed 2:1 with 30% TCA was performed with every measurement. $Na^{+}K^{+}$-ATPase activity curves were fitted with a five parameter logistic function using the solver tool from Microsoft Excel (Version 2003) to determine the ouabain concentration causing a 50% inhibition compared to the non-inhibited control (IC_{50}). For all species (except for *C. oregonensis*) the protein content of the extractions was determined following Bradford (1976) with BSA (bovine serum albumin) as a standard.

Target site visualisation

For the immunohistochemical procedure we followed the methods in Patrick and others (2006). Caterpillars of *E. pugione*, *D. plexippus* and *A. gamma* were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4; Yasuhara and others 2000) overnight on ice. After fixation caterpillars were washed three times in PBS, dehydrated in a graded series of ethanol and transferred into absolute isopropanol. Samples were then incubated in isopropanol:xylol (2:1), isopropanol:xylol (1:2) and xylol. Larvae were successively infiltrated with Paraplast Plus. 7-8 µm sections were mounted on slides (Polysine™, Roth), allowed to dry and stored until use. For immunohistochemistry, sections were deparrafinized in Xylol and transferred into water via a series of graded ethanol. After rehydration, sections were permeabilized in PBS with 0.1% Triton X-100 (PBT) for 5 min. Following that, sections were blocked with PBT with 2% BSA for 90 min. For the specific detection of Na^+K^+-ATPase we used the antibody α5 diluted in PBT with 1% BSA to 5 µg/ml. Control sections were treated with PBT/1% BSA only. This monoclonal antibody developed by D. M. Fambrough was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. Following overnight incubation at 4°C in a humid chamber slides were washed three times with PBT/1% BSA/2% NGS. Sections were then incubated for 2 h with the secondary Cy3-labelled antibody (goat anti-mouse, Dianova). After three washings in PBT/1% BSA/2% NGS sections were mounted in mounting medium (ImmunoSelect, Dianova) with DAPI, examined and photographed with a Zeiss AxioCam color camera connected with a Zeiss Axioskop 2.

Western-Blot-Analysis

Last instar caterpillars of *A. gamma* were dissected under ice-cold PBS. Four tissue types (midgut, labial gland, nervous tissue and Malpighian tubules) were separately collected on ice in RIPA buffer (25 mM Tris•HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% Natriumdeoxycholate, 0.1% SDS) with Complete Protease Inhibitor Cocktail (Roche). Tissues were homogenized in the same buffer with an all-glass grinder (Wheaton) and centrifuged for 5 min at 16100 g (4°C). Supernatants were transferred into fresh tubes and the protein content was determined following Bradford (1976). An equivalent of 15 µg of protein of each sample were separated by SDS-PAGE on an 8% acrylamide gel and transferred to a PVDF membrane (Millipore). The blot was blocked, incubated with anti-Na^+K^+-ATPase antibody α5 (1:1000) and goat-anti-mouse secondary antibody conjugated with horseradish peroxidase (Dianova) 1:2500 using the SNAP i.d. system (Millipore).

Signal was detected by chemiluminiscence (Roti-Lumin, Roth) and documented with a Fusion Fx7 (PeqLab).

Preparation and clean-up of haemolymph

Haemolymph of last instar caterpillars of *E. pugione* and *P. terminalis* raised on *N. oleander* or on *A. curassavica*, respectively, was obtained by cutting a larval proleg and collecting a volume of 20-50 µl of extruding haemolymph. Prior to TLC or spectrophotometry samples were extracted by adding 1 ml of methanol vortex stirring and sonification. Then, the sample was centrifuged for 5 min at 12000 g to precipitate proteins. 900 µl of the supernatant were transferred to a fresh tube and 300 µl deionized water were added. Thereafter, the sample was extracted 5 times with 200 µl hexane. Finally, the methanolic extracts were dried in a concentrator and stored at 4℃. For analysis the dried residue was dissolved in 100 µl methanol by vortex stirring and sonification and centrifuged. TLC plates were heat activated for 30 min in an oven (60℃) and extracts were chromatographed twice with ethyl acetate : methanol : water (75:10:7.5; Luckner and Wichtl 2000). For *Empyreuma* each sample was spotted twice on each half of the plate. After chromatography, TLC plates were dried at 60℃ (30 min) and cut into halves. One half was developed with Kedde's reagent for the specific detection of the lactone of the cardenolides while the other half was developed according to Jensen (Luckner and Wichtl 2000) to detect the steroidal part of the cardenolide molecules. Spots which stained positively with both methods for certain represent cardenolides. *Pygoctenucha* extracts from different individuals were run on the same plate. After chromatography, plates were cut into halves and developed after Kedde or Jensen, respectively. For quantification, 20 µl of sample were added to 100 µl 2% 3,5-dinitrobenzoic acid in methanol in the wells of a microplate. After adding 50 µl of 1 M KOH plates were incubated for 5 min and read at 550 nm. A calibration curve was set up using ouabain (0.5-10 mg/ml) as a standard.

Results

All species tested were shown to possess $Na^{+}K^{+}$-ATPase in their nervous system which can be inhibited by the cardenolide ouabain (Fig. 1). As reported previously (Vaughan&Jungreis 1977), the enzyme of *D. plexippus* shows a very low susceptibility to ouabain. In contrast, in the $Na^{+}K^{+}$-ATPase derived from the cardenolide adapted arctiids we detected a pronounced sensitivity to ouabain.

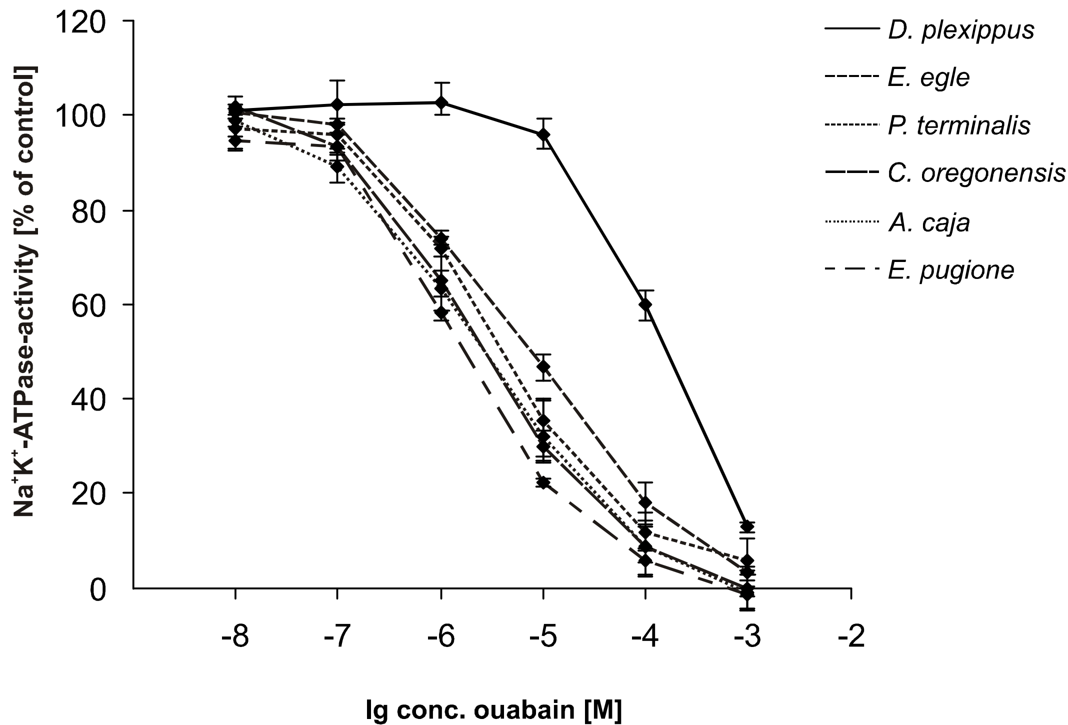

Fig. 1. Ouabain sensitivity of cardenolide adapted arctiids and *D. plexippus*. Each point is the average of four (*D. plexippus*, *C. oregonensis*, *A. caja*, *E.pugione*), three (*P. terminalis*) or two (*E. egle*) determinations (mean ± SD).

Tab. 1: IC_{50}-values of lepidopteran species tested and Na^+K^+-ATPase activities of the respective enzyme extractions

	IC_{50} ouabain [M]	Na^+K^+-ATPase activity [nmol P_i/mg protein*min] ± SD
E. pugione (Arctiidae)	$1{,}84*10^{-6}$	71,72 ± 9,99
C. oregonensis (Arctiidae)	$2{,}75*10^{-6}$	-
A. caja (Arctiidae)	$2{,}74*10^{-6}$	160,86 ± 13,17
P. terminalis (Arctiidae)	$4{,}14*10^{-6}$	517,47 ± 149,69
E. egle (Arctiidae)	$7{,}51*10^{-6}$	246,06 ± 40,17
D. plexippus (Danaidae)	$1{,}54*10^{-4}$	1040,33 ± 436,28

The enzyme activity of the monarch is not affected over three orders of magnitude (10^{-8} to 10^{-5} M) whereas the Na^+K^+-ATPase of the other species already show a strong inhibition at concentrations higher than 10^{-7} M. Comparing the doses at which 50% inhibition was observed (IC_{50}; Tab. 1) the resistant enzyme showed an IC_{50} which is 40fold higher than the averaged IC_{50} values of the other species. In our assays Na^+K^+-ATPase activity (measured as enzymatic ATP-hydrolysis under normal conditions minus ATP-hydrolysis in the absence of K^+) comprised 32-53% of total ATPase activity for the membrane preparations and 79% for the crude preparations obtained from *D. plexippus.* Tab. 1

shows the specific activities of the enzyme extracts tested (except of those for *C. oregonensis* where the protein determination is lacking) under control conditions.

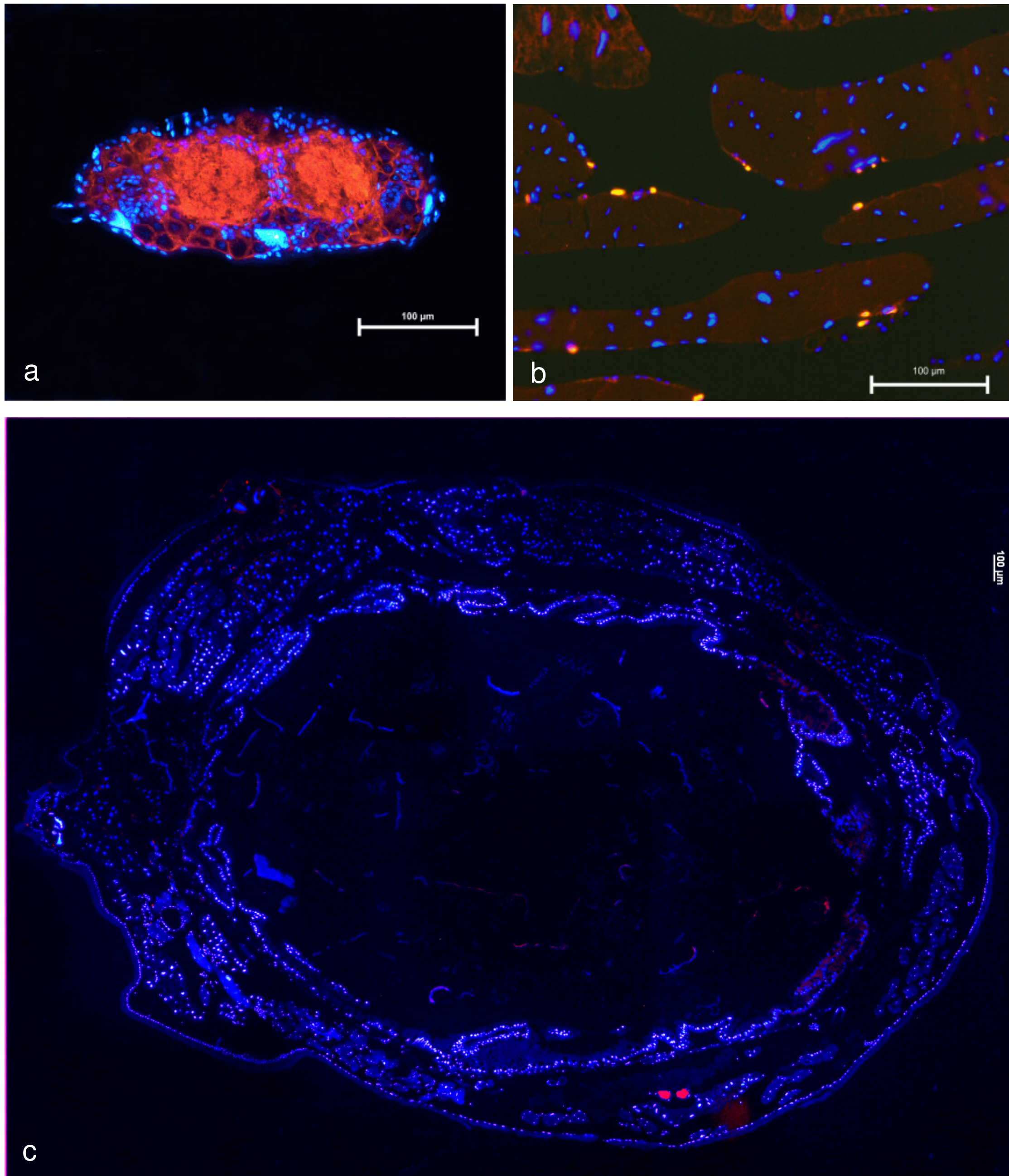

Fig. 2a-c. Target site visualization. Paraffin sections of a) *D. plexippus* ganglion and perineurium, b) *D. plexippus* musculature with innervating nerve fibers, note the absence of surrounding tissue, c) whole cross section through an *E. pugione* caterpillar, note specific label in the nervous tissue only. Orange: $Na^{+}K^{+}$-ATPase, blue: nuclei.

Our immunohistochemical data (Fig. 2a-c) demonstrate that the $Na^{+}K^{+}$-ATPase has an identical distribution in all three species investigated: only the nervous tissue was specifically stained by the monoclonal anti-$Na^{+}K^{+}$-ATPase antibody α5.

In a section through a whole caterpillar of *E. pugione* (Fig. 2c), no other tissue but the cross section of the ventral nerve cord was stained. Accordingly, in *D. plexippus* caterpillars Na^+K^+-ATPase was only revealed in the ganglion itself but not in the surrounding cell layer (Fig. 2a) and only in the innervating cross sectioned nerve fibers but not in the musculature (Fig. 2b). In other tissues like musculature, gut or malpighian tubules where Na^+K^+-ATPase classically occurs in other organisms, no specific signal could be observed.

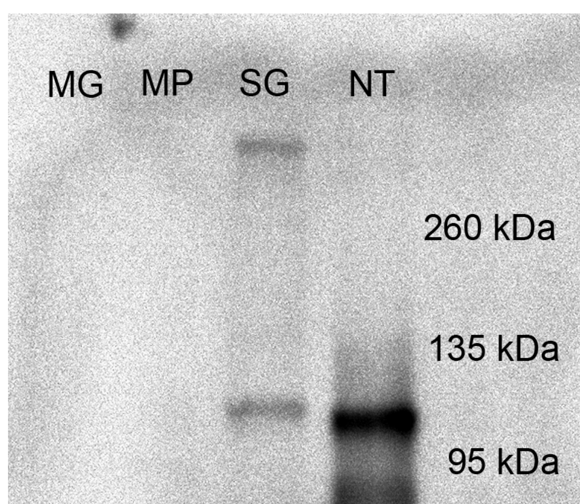

Fig. 3. Western Blot of tissue extractions of *A. gamma*. MG: midgut, MP: Malpighian tubule, SG: labial gland, NT: nervous tissue.

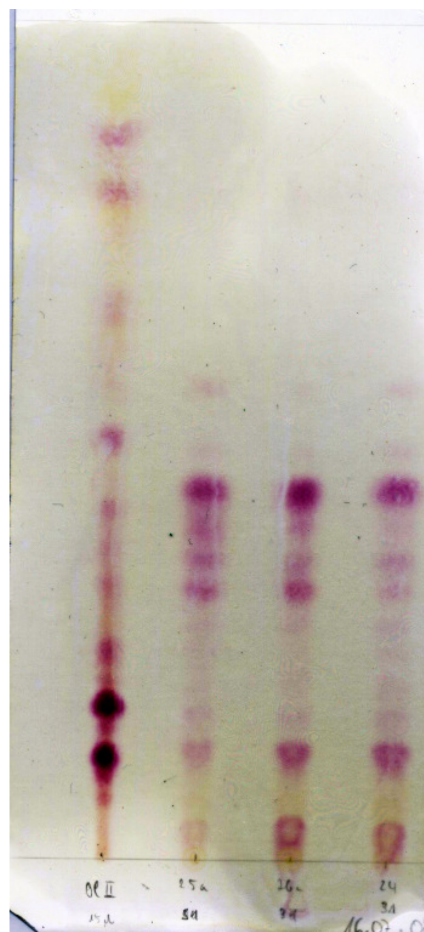

Fig. 4. Thin layer chromatography of haemolymph samples of *E. pugione* caterpillars. Leftmost: extraction from oleander leaves. Remaining three lanes: *E. pugione* haemolymph. Pink spots represent cardenolides.

The western blot analysis supports the pattern revealed by immunohistochemistry: in extractions of midgut and Malpighian tubules of *A. gamma* no Na^+K^+-ATPase was detected, while the nervous tissue provided a strong band at about 115 kDa (Fig. 3). In the extraction of the labial glands, on the other hand, Na^+K^+-ATPase with a slightly higher molecular weight could be detected.

The haemolymph samples from caterpillars of both *E. pugione* and *P. terminalis* were shown to contain cardenolides (Fig. 4). In *E. pugione* a positive signal with both stains was obtained for at least eight spots whereas the Kedde stain alone revealed about 12 spots. In any case, the haemolymph of *E. pugione* contains a wide array of cardenolides with different polarities. However, they only cover the lower 2/3 of the polarity range of cardenolides in *N. oleander* leaves as the direct comparison revealed. The spectrophotometrically determined cardenolide concentration in haemolymph of this species equals to $2.4*10^{-3}$ M ouabain (n=4). In larval haemolymph of *P. terminalis* raised on *A. curassavica* only three very polar cardenolides were detected (data not shown). This number is surprisingly low regarding the report of 20 cardenolides of *A. curassavica* (Malcolm 1989) two of which are less polar than the nonpolar digitoxin. Obviously, P. terminalis caterpillars selectively sequester only single polar cardenolides from A. curassavica or convert nonpolar cardenolides into polar ones.

Discussion

The toxic effect of cardenolides is due to a highly specific target site interaction with extracellular amino acid residues of the $Na^{+}K^{+}$-ATPase. This circumstance enables us to study cardenolide resistance in a detailed manner since we precisely know at which site these toxins act. Resistance to cardenolides, however, must be seen as a complex phenomenon that may be attained by multiple strategies and is influenced by several interacting factors. On the one hand, resistance can be achieved by modification of the target site and most research has focussed on this strategy (Vaughan & Jungreis 1977; Moore & Scudder 1986; Holzinger & Wink 1992, 1996; Mebs and others 2000; Labeyrie & Dobler 2004; Mebs and others 2005). On the other hand, the pharmacokinetics of an ingested compound strongly influence its effects in the body and thus, processes like absorption, distribution in the body, metabolism and excretion play a crucial role. Resistance to cardenolides therefore could also be due to changes of one or several of these processes.

Our data clearly show that within Lepidoptera different strategies of coping with cardenolides have evolved. We already demonstrated the occurrence of a susceptible $Na^{+}K^{+}$-ATPase in the cardenolide specialist *Daphnis nerii* (Sphingidae) in an earlier study (Petschenka&Dobler 2009). This species, however, is not a cardenolide sequesterer in a classical sense and has only comparably small amounts of cardenolides in its body (Abe at al. 1996). Here we show that even species which sequester large amounts of cardenolides may possess $Na^{+}K^{+}$-ATPases which are highly susceptible to these toxins. All five arctiid species investigated here which store or at least can cope with dietary

cardenolides were found to possess sensitive forms of the enzyme. Therefore, target site insensitivity in cardenolide sequestering lepidopterans does not seem to be necessary for coping with cardenolides stored in the body or encountered in the diet.

Sequence data of diverse danaids obtained by Holzinger&Wink (1996) and Mebs and others (2000, 2005) suggest that even species which are closely related to the monarch butterfly miss the resistance conferring histidin residue in position 122 of the enzyme and thus presumably possess cardenolide sensitive forms of $Na^{+}K^{+}$ATPase. This assumption is, however, not unquestionable since other substitutions might still render the enzyme insensitive but if it is true, the insensitive $Na^{+}K^{+}$ATPase of *D. plexippus* might be an exceptional case in cardenolide adapted Lepidoptera.

Our enzyme measurements indicate only slight differences between the five arctiid species which possess ouabain sensitive forms of $Na^{+}K^{+}$ATPase and we do not expect these differences to have ecological implications. The monarch's $Na^{+}K^{+}$ATPase, however, is drastically different and according to the IC_{50} values 40 times less sensitive to ouabain than those of the cardenolide adapted arctiids.

Our curve for *D. plexippus* has a similar characteristic as the one published by Vaughan&Jungreis (1977). However, at high ouabain concentrations of 10^{-4} M and 10^{-3} M our data suggest that the ouabain sensitivity of the enzyme is drastically higher: 90% rest activity vs. 60% rest activity and 70% rest activity vs. 13%, respectively. With regard to the high cardenolide concentration of *D. plexippus* haemolymph of close to 10^{-3} M (Nishio 1980) our findings provide evidence that target site insensitivity alone can not explain the cardenolide resistance of the monarch.

The discrepancy between cardenolide levels in the haemolymph and their effect on the isolated enzyme is even more striking in *E. pugione*: while we measured haemolymph levels of cardenolides of about 10^{-3} M the corresponding ouabain concentration causes total *in vitro* inhibition of the enzyme.

At this point we can not yet decide how cardenolide resistance is achieved in the species tested here. However, given the large quantities of cardenolides in the haemolymph of *E. affinis* and *P. terminalis* (that can be expected to be physiologically active), metabolic detoxification can be excluded as well as enhanced excretion or reduced absorption of cardenolides. All these strategies would counteract an efficient storage in sequestering species.

In insects, $Na^{+}K^{+}$ATPase is generally believed to be mainly expressed in the nervous tissue, yet the enzyme is also known to occur in midgut, muscle, malpighian tubules and rectal pads of some insects (Emery and others 1998). In Lepidoptera, however, $Na^{+}K^{+}$ATPase is reported to be absent in midgut and musculature (Haskell and others 1965; Jungreis and Vaughan 1977; Djamgoz 1986; Fitzgerald and others 1996)

and our data support these findings. Our immunostainings and western blot of representatives of three lepidopteran families, Arctiidae, Nymphalidae, and Noctuidae, only detected Na^+K^+ATPase in the nervous tissue where the expression level is very high and, at a much lower level, in the labial glands. The latter observation fits nicely with the report of Kafatos (1968) who observed a decrease of secretion in adult labial glands after the application of $5*10^{-4}$ M ouabain in saturniid moths. The author interpreted his observation as an indirect effect due to the pronounced flaccidity of ouabain-treated moths since this concentration is much higher than those normally required for direct interference with susceptible active transport. However, we think our finding that lepidopteran labial glands contain Na^+K^+-ATPase makes a direct effect still plausible. The high ouabain concentration necessary to cause an effect on labial gland secretion rate might possibly be explained by rapid excretion from the tissue, a protection mechanism that has been described by Torrie and others (2004) for the Malpighian tubules of *Drosophila*. The size difference between Na^+K^+-ATPase from labial glands compared with Na^+K^+-ATPase from nervous tissue might represent tissue specific isoforms of the α-subunit (see Palladino and others 2003).

The absence of Na^+K^+ATPase in the lepidopteran midgut may be due to a special ion milieu: usually secondary active transport in animal plasma membranes is energized by Na^+K^+ATPase. Yet, the midgut of the tobacco hornworm (*Manduca sexta*) lacks Na^+K^+ATPase and secondary active transport is driven by an ouabain-insensitive potassium pump (Wieczorek and others 1991). This peculiarity is ascribed to the inability of lepidopteran larvae to retain sufficient Na^+ from their diet which is low in Na^+ and rich in K^+. They are thus unable to maintain a haemolymph level of this ion which is appropriate for the use of Na^+K^+ATPase (Klein and others 1996). In this case, basal physiological adaptations caused by the herbivore diet might have facilitated the use of cardenolide bearing host plants by caterpillars.

The question remaining to be addressed is how Na^+K^+-ATPase in the nervous tissue can be protected against high cardenolide levels. The lepidopteran nervous tissue as the main site of Na^+K^+-ATPase expression is surrounded by a cellular layer (perineurium) and an acellular neural lamella (Pichon and others 1972). The perineurium itself does apparently not express Na^+K^+-ATPase. A role of this tissue in the protection of nervous Na^+K^+-ATPase against cardenolides is highly suggestive and was already proposed by Vaughan&Jungreis (1977). Our chromatographic data demonstrate that there are significant amounts of polar and less polar cardenolides in the haemolymph of sequestering species which render a barrier necessary. Rubin and others (1983) demonstrated that ouabain is not able to reach the Na^+K^+-ATPase in the intact nerve cord of *M. sexta* thus suggesting the perineurium to be a diffusion barrier for polar

cardenolides. Based on this situation we assume that in lepidopterans the target site is spatially insulated from the toxins in the haemolymph. Concerning nonpolar cardenolides active exclusion mechanisms might be involved as suggested for nicotine in the nerve cord of *Manduca sexta* (Murray and others 1994).

The high level of haemolymph K^+, a well known antagonist to cardenolide toxicity on Na^+K^+-ATPase (Glynn 1957), was suggested to mediate relative *in vivo* insensitivity in lepidopterans by Vaughan&Jungreis (1977). However, to maintain normal electrical activity the nerve cord extracellular space in *M. sexta* requires low K^+ and high Na^+ concentrations whereas the Na^+:K^+ ratio in the haemolymph is 25.4:25.1 mM (Pichon and others 1972) and the perineurium is considered to restrict the paracellular pathway of such ions. Therefore, for nervous Na^+K^+-ATPase a high K^+ concentration is probably not the basis for insensitivity towards cardenolides. It might play a role at the endings of nerve fibers where, as our immunohistochemical data suggest, the perineurium is absent or at the Na^+K^+-ATPase in the labial glands.

Target-site-insensitivity towards cardenolides as detected in the monarch butterfly, lygaeid bugs and leaf beetles (Vaughan&Jungreis 1977; Moore&Scudder 1986; Labeyrie&Dobler 2004) suggests that this mechanism might be the silver bullet for cardenolide resistance. In lepidopterans, however, where Na^+K^+-ATPase is almost exclusively restricted to the nervous tissue, target-site-insensitivity might not be necessary if the enzyme is well protected by the perineurium. It is plausible that this protective barrier may be an ancestral condition since nervous function needs specific ionic conditions and protection from a variety of compounds. Our ongoing physiological investigations focussing on the active and passive processes at the lepidopteran perineurium will potentially elucidate the role of this tissue as a barrier for cardenolides.

Chapter 3

Functional evidence for an efflux transport mechanism to circumvent neurotoxicity of dietary metabolites in the Oleander hawk-moth (*Daphnis nerii*)

Abstract

Cardenolides are plant toxins which specifically inhibit the physiologically important Na^+K^+-ATPase. Hence, herbivorous insects feeding on cardenolide-containing plants need to protect their Na^+K^+-ATPase from these toxins. The most obvious adaptation to circumvent cardenolide toxicity is target site insensitivity as observed in the monarch butterfly (*Danaus plexippus*). However, many other lepidopteran species which are exposed to dietary cardenolides lack this adaptation. We here focussed on the caterpillars of *Daphnis nerii*, the oleander hawk-moth, which ingest large amounts of cardenolides while feeding on oleander leaves and use the non-cardenolide species *Manduca sexta*, the tobacco hornworm, as a comparison. Both species possess Na^+K^+-ATPases which are highly sensitive to the cardenolide ouabain, yet, caterpillars of both species were shown to be able to tolerate large amounts of cardenolides in their body cavity. Using ^{3}H-ouabain as a polar cardenolide and ^{3}H-digoxin as a nonpolar cardenolide we tested for the presence of a cardenolide barrier at the caterpillars' nerve cord which might be preventing the access of cardenolides to Na^+K^+-ATPase. By radioactive binding assays with isolated nerve cords we demonstrated that the perineurium, the tissue surrounding the nervous tissue, functions as a diffusion barrier for the polar cardenolide ouabain in *D. nerii*. For the nonpolar digoxin, we found that an active barrier that can be inhibited by the metabolic inhibitors CCCP or 2,4-DNP limits the access of cardenolides to the nerve cord. The strong effect obtained by the well-known P-glycoprotein (PGP) inhibitor verapamil suggests that a PGP-like efflux transporter is involved in the active cardenolide-barrier. Immunohistochemistry with the anti-PGP antibody C219 resulted in a specific labelling of the perineurium of *D. nerii* ganglia. In contrast, the target site Na^+K^+-ATPase is only expressed within ganglia as was corroborated for *M. sexta* with the monoclonal antibody α5. Our data suggest that the lepidopteran perineurium serves as a diffusion barrier for polar cardenolides and provides an active barrier for nonpolar cardenolides. Our findings

may explain the high *in vivo* resistance to cardenolides observed in lepidopteran larvae which do not possess insensitive Na^+K^+-ATPases.

Introduction

Over the course of evolution plants have evolved a vast diversity of secondary plant compounds many of which act as chemical weapons against herbivores. In return, herbivores developed strategies to overcome plant defences. As can be expected, mechanisms of resistance in insects are numerous and include detoxification of toxins by enzymes, excretion, exclusion (gut barriers) and target site insensitivity (Scudder&Meredith 1982; Després et al. 2007).

In our current research, we focus on the resistance to one specific class of plant toxins, cardenolides or cardiac glycosides (CGs). These toxins are specific inhibitors of the Na^+K^+-ATPase, an ubiquitous animal enzyme which is essential for physiological processes (Schatzmann 1953; Lingrel&Kuntzweiler 1994). The occurrence of cardenolides is erratic and scattered over 12 plant families (Luckner&Wichtl 2000). Herbivorous insects like the monarch butterfly (*Danaus plexippus*) which feed on cardenolide plants often sequester these compounds and thus derive protection against predators (Brower et al. 1967). The relative cardenolide resistance of the *D. plexippus* Na^+K^+-ATPase (Vaughan&Jungreis 1977) is a prime example for target site insensitivity which is also found in other cardenolide specialists (Moore and Scudder, 1986; Al-Robai et al., 1990; Labeyrie and Dobler, 2004). In earlier studies, however, we found that lepidopterans which are adapted to cardenolides do not necessarily possess cardenolide insensitive Na^+K^+-ATPases (Petschenka and Dobler, 2009; Petschenka et al. in prep.). Moreover, within cardenolide adapted Lepidoptera the monarch seems actually to be an exceptional case (Holzinger and Wink, 1996; Mebs et al., 2000; Mebs et al., 2005; Petschenka et al. in prep.).

Expression of Na^+K^+-ATPase is apparently restricted to the nervous tissue in Lepidoptera with small amounts present in the labial glands. Since we know about the occurrence of dietary cardenolides in the haemolymph of caterpillars the interface between insect blood and nervous tissue is of special interest (Petschenka et al., in prep.).

The ventral nerve cord of insects is, like in other organisms, surrounded by the perineurium, a tissue maintaining ionic conditions appropriate for the electrical activity of the nervous tissue which can be different from the composition of the haemolymph (Pichon et al., 1972). Additionally, this tissue is believed to function as a blood brain barrier for toxic plant compounds present in the herbivores' haemolymph. In the tobacco hornworm (*Manduca sexta*) for example it has been suggested to protect the nervous

tissue from dietary nicotine (Murray et al. 1994). However, so far there is only limited functional evidence for such a protective function. In this study, we wanted to test whether the perineurium can function as a barrier to haemolymph cardenolides and therefore potentially contribute to the resistance to these toxins.

We used the oleander hawk moth (*Daphnis nerii*, Lepidoptera, Sphingidae; Fig. 1) in our experiments, a cardenolide specialist which feeds primarily on oleander (*Nerium oleander*), a plant rich in these toxins (eg. Abe et al. 1996). Due to the large size of the caterpillars physiological experiments can be performed easily. To compare the situation found in *Daphnis nerii* to other spinghid moths not specialized on cardenolide plants, we included *M. sexta* in our study and carried out some more detailed studies with this species (due to the better availability compared to *D. nerii*).

Fig.1: The Oleander hawk-moth (*D. nerii*). The major host plant of the caterpillars of this species is *N. oleander* whose high toxicity is based on cardenolides. Foto by T. Kleinteich.

D. nerii caterpillars possess only comparatively small amounts of cardenolides in their body (~150-200 µg at maximum; Abe et al. 1996). However, regarding the high susceptibility of the Na^+K^+-ATPase of this species (Petschenka&Dobler 2009) even minute amounts of these compounds in the haemolymph could be fatal. Since oleander and other cardenolide plants contain cardenolides of a wide polarity range we speculated that the perineurium, on one hand, forms a diffusion barrier for polar cardenolides as it has already been shown for *M. sexta* by Rubin et al. (1983). On the other hand, nonpolar cardenolides

which are able to enter cells by diffusion might require an active barrier mechanism. Both hypotheses are tested here by physiological experiments. Since we found evidence for an active efflux system in the exclusion of cardenolides from the nervous tissue, we used different inhibitors to narrow down the type of transporter constituting the barrier.

In the mammalian brain one of the most important efflux transporters is the P-glycoprotein (PGP) efflux system (de Boer et al. 2003). This 170-kDa membrane bound protein is an ABC-transporter with a wide array of substrates which is responsible for the phenomenon of multidrug resistance in cancer cells. PGP extrudes xenobiotic compounds which diffuse into cells under consumption of ATP. In *M. sexta*, the perineurium of larval nerve cords was shown to be P-glycoprotein immunopositive and the involvement of this carrier in nicotine resistance was suggested (Murray et al. 1994). We here tested, by the application of quinidine and verapamil as well known PGP inhibitors whether a PGP-like transporter is involved in the physiological blood-brain-barrier of the hawk-moth nerve cord. The fact that the cardiac glycoside digoxin is a well known P-glycoprotein substrate suggested a role for this protein in the active cardenolide barrier.

To visualize the occurrence of a PGP-like transporter in the blood-brain-barrier of *D. nerii* we applied a monoclonal anti-PGP antibody on frozen sections of caterpillar nerve cords. Furthermore, we visualized the target site of cardenolides in the hawk-moth nerve cord with an anti-Na^+K^+-ATPase antibody.

Combining our results we suggest that the perineurium as a blood-brain-barrier enables caterpillars to tolerate polar as well as nonpolar cardenolides within their body cavity.

Material and Methods

Radiochemicals and inhibitors

^{3}H-ouabain (12 Ci/mmol; dissolved in 9:1 ethanol:toluene or 30 Ci/mmol; dissolved in ethanol) was purchased from Amersham and Perkin Elmer. ^{3}H-Digoxin was purchased from Perkin Elmer (40 Ci/mmol; dissolved in ethanol). 2,4-dinitrophenol (2,4-DNP; Fluka), carbonyl cyanide 3-chlorophenylhydrazone (CCCP; Sigma), verapamil hydrochloride (Sigma) and quinidine (Sigma) were used as 0.05 M stock solutions in ethanol. In our binding experiments we used ^{3}H-cardenolide concentrations of 0.35 and 0.7 µM, respectively. We decided to use such low amounts because Rubin et al. (1983) observed non-specific binding of ^{3}H-ouabain to native nerve cords of *Manduca* at concentrations above 10 µM.

Diffusion barrier

To test for diffusion barriers to polar cardenolides, we followed the experimental design of Rubin et al. described for *M. sexta* (1983). Caterpillars of *Daphnis nerii* (European origin) were raised on *Vinca major* at 23°C (16 h/8 h light/dark cycle). Prior to dissection last instar caterpillars were chilled on ice and decapitated. Ventral nerve cords were surgically removed, placed in cold incubation buffer (125 mM NaCl, 5 mM $MgCl_2$, 0.5 % bovine serum albumin (BSA) and 12.5 mM imidazole, pH 7.3), cleaned from adherent tissue and trimmed to a chain of 8 ganglia plus intervening connectives (abdominal ganglia plus metathoracic ganglion; Johnston&Levine 1996). For each of three replicates a series of six caterpillars was used. One nerve cord of a series was used as a control and was kept in incubation buffer at room temperature for the duration of the urea treatment. The additional five nerve cords were immersed in 3 M urea in incubation buffer for 5, 10, 12, 15 or 20 min, respectively. After urea treatment, the cords were washed twice with incubation buffer for at least 5 min each. Following urea treatment, the cords (including the control cord) were individually incubated in 100 µl incubation buffer with 0.7 µM ^{3}H-ouabain for 1 h at 37°C. After incubation, cords were washed in an excess volume of 10 mM imidazole (pH 7.3) for 30 min on ice. Each cord was then transferred to 200 µl 0.2 M NaOH/1 % SDS and digested overnight. To 150 µl of this extract 3 ml liquid scintillation cocktail (Ultima Gold) were added and radioactivity was determined (Wallac 1409 liquid scintillation counter, easy count mode). The remainder of each sample was stored at -20°C for later protein determination. Protein content of the samples was measured with the BCA assay (Thermo Scientific) using BSA as a standard.

Active barrier

M. sexta: Eggs of *M. sexta* were kindly supplied by Dr. Markus Huß (University of Osnabrück). Caterpillars were reared on gypsy moth diet (MP Biomedicals) supplemented with streptomycin, chloramphenicol, methyl benzoate and formalin at 26°C and 16 h:8 h light:dark cycle. Only last instar caterpillars before the wandering stage were used in the experiments.

D. nerii: Caterpillars of *D. nerii* (origin Thailand) were raised from eggs at 27°C at 13 h:11 h light:dark cycle). Hatched caterpillars were initially fed with *Vinca major* since the soft leaves of this plant are easier to consume for the neonate caterpillars. Small larvae (second instar) were transferred to *N. oleander* and raised to the last instar on this plant.

Caterpillars of both species were chilled on ice, decapitated and nerve cords were dissected and placed in ice-cold *Manduca* saline: 5.0 mM K_2HPO_4, 10.0 mM $MgCl_2$, 1.0 mM $CaCl_2$, 10.0 mM NaCl, 10.0 mM KOH, 7.4 mM L-proline, 7.7 mM tripotassium citrate,

2.8 mM disodium succinate, 2.0 mM glucose, 175.0 mM sucrose, 5.6 mM malic acid, 10.0 mM HEPES, pH 6.7 (Woods&Chamberlin 1999). Again, the posterior eight ganglia were used. Until incubation, tissues were kept on ice in *Manduca* saline. To test the hypothesis that the nerve cord of *M. sexta* and *D. nerii* possesses an energy driven barrier which prevents CGs from reaching the Na^+K^+-ATPase, the metabolic inhibitors 2,4-DNP and CCCP were applied. To test whether a PGP-like transporter is involved in this barrier, we used verapamil and quinidine (pre-dissolved in ethanol) which are well known competitive PGP-inhibitors (Horio et al. 1991). Control tubes received the same amount of ethanol. The concentration of ^{3}H-digoxin in the assay was 0.35 µM (ethanol concentration 3.36 %). Each nerve cord was incubated in a volume of 100 µl *Manduca* saline at 37°C. After 30 min tubes were placed on ice, the radioactive solution was removed, replaced by 1 ml of cold 10 mM imidazole (pH 7.3) and mixed by vortexing. The washing buffer was replaced once, the tubes were inverted and kept on ice for 30 min. The short washing step was performed to remove adherent radioactive solution while the long washing step was performed to remove unbound ^{3}H-digoxin (see Rubin et al. 1983). In an additional experiment (data not shown) we found that nearly all adhering radioactivity is removed from the tissue after the 30 min washing step. After washing, the samples were digested and radioactivity counted as described above. Since the dissected nervous tissues are very uniform samples, the radioactivity measured in this experiment and the experiment below is expressed as dpm (disintegrations per minute)/nerve cord and not referred to the protein content.

Statistical analysis

If necessary, data were squared or log-transformed to achieve homogeneity of variances (Levene's test) and approximately normal distributions (Shapiro-Wilk). Data were analyzed by ANOVA using a randomized block design with the experiment as blocking factor. Post hoc comparisons are based on Tukey's HSD test. All statistical tests were performed with SPSS (IBM).

Comparative permeability for ouabain and digoxin

This experiment was performed to demonstrate the different permeability of the perineurium of *D. nerii* caterpillars for ouabain and digoxin. As incubation buffer, physiological saline (PBS: 137 mM, NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4; Yasuhara et al. 2000) was used, otherwise the assay followed the procedures described above. Control nerve cords were incubated in buffer with 0.7 µM ^{3}H-digoxin or ^{3}H-ouabain only. In parallel, nerve cords were incubated with the labelled compounds plus CCCP (1 mM). The CCCP was added to disrupt the physiological barrier

for digoxin to get an estimate of the amount of digoxin reaching the target site by diffusion. To compare between experiments using different substrates, the data of the radioactive measurements were corrected for the particular specific activity.

Immunohistochemistry

PGP-like transporter: Nerve cords of chilled *D. nerii* caterpillars (last instar) were dissected and immersed in PBS. Then, tissues were fixed for 1 h at room temperature in Lana's fixative (15% picric acid, 4% paraformaldehyde (PFA) in 0.5 M sodium phosphate buffer, pH 7; Murray et al. 1994). After fixation, tissues were washed three times for 10 min each in PBS and successively cryoprotected in 5, 10 and 15% sucrose in PBS for 1 h each. Following cryoprotection tissues were embedded in OCT compound, frozen in isopentane in liquid nitrogen and stored at -80°C until sectioning. 16 µm sections were cut on a Leica CM 1950 cryostat and allowed to dry at room temperature. Slides were stored at -80°C until use. The anti-PGP antibody C-219 (Abcam; dissolved in PBS) was applied at a concentration of 10 µg/ml. In the control sections, the primary antibody was omitted. The primary antibody was detected with the NOVADetect Detection System (Dianova) employing a biotinylated secondary antibody (goat anti-mouse) and horseradish peroxidase-labelled streptavidin. 3,3'-diaminobenzidine was used as a chromogenic substrate. Stained sections were shortly washed with deionised water, transferred into 80% ethanol (via 60% ethanol) and mounted in Euparal. Sections were investigated under a Zeiss Axioskop 2 and photographed with a Zeiss AxioCam color camera.

Na^+K^+-ATPase: to visualize the target site of cardenolides within the hawk-moth nerve cord we used the monoclonal anti-Na^+K^+-ATPase antibody α5 developed by D. M. Fambrough (Developmental Studies Hybridoma Bank, University of Iowa). For sample processing and incubation, we followed the protocol described by Patrick et al. (2006). Nerve cords of last instar *M. sexta* caterpillars were dissected and fixed in 4% PFA in PBS (pH 7.1) for 1 h at room temperature. After washing in PBS, nerve cords were dehydrated in a graded series of ethanol, transferred into isopropanol and successively transferred into xylol (isopropanol:xylol = 7:3; xylol:isopropanol = 3:7; xylol). Following Paraplast Plus embedding 8 µm paraffin sections were cut. Sections were deparaffinised with xylol, rehydrated in a graded series of alcohol (isopropanol; 96, 90, 80, 70, 50 % ethanol) and transferred into water. After rehydration, slides were permeabilised with PBT (PBS with 0.1 % Triton X-100) for 5 min, blocked with 2% BSA in PBT for 90 min and incubated with the primary antibody α5 (5 µg/ml in PBT with 1% BSA) overnight at 4°C. Control slides were incubated with PBT/1% BSA only. The next day, slides were washed with PBT/1% BSA with 2% NGS (normal goat serum) to remove unbound antibody and incubated with

the secondary antibody (Cy3 labelled goat anti-mouse antibody; Dianova) at a dilution of 1:400 in PBT/1% BSA/2% NGS for 2 h at room temperature. Finally, slides were washed with PBT/1% BSA/2% NGS and mounted in a medium containing DAPI (ImmunoSelect, Dianova). Slides were investigated under a Zeiss Axioskop 2 and photographed with a Zeiss AxioCam color camera.

Results

Diffusion barrier

Protection from polar haemolymph cardenolides can be realized by a simple diffusion barrier which blocks the paracellular pathway. Treatment with urea is believed to disrupt the perineurial barrier and was shown to greatly enhance ouabain diffusion into the nerve cord of *M. sexta* (Rubin et al. 1983). In our experiment (Fig. 2) with *D. nerii* caterpillars we found a corresponding situation: Up to 10 min incubation in 3 M urea ouabain binding to the isolated *D. nerii* nerve cord linearly increases with time. After 10 min of incubation the curve reaches a plateau possibly indicating complete permeabilisation of the diffusion barrier or saturation of the ouabain binding sites by the applied amount of ouabain.

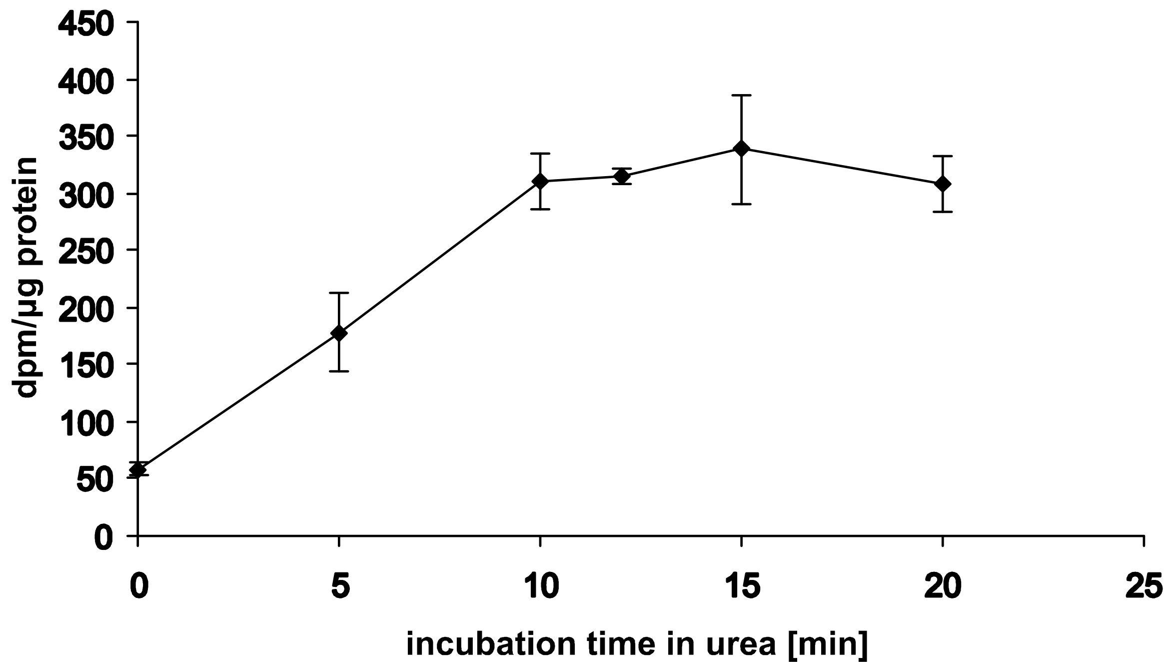

Fig.2: Disruption of the perineurial barrier with urea in isolated nerve cords of *D. nerii* (n=3; mean ± SD).

Active barrier

Digoxin was commonly assumed to be absorbed passively via the gut (Caldwell et al. 1969) but, more recently, it was suggested that also active processes are involved in digoxin uptake (Yao&Chiou 2006). However, since it is lipophilic digoxin can be expected to permeate cell membranes. Therefore, we assume non-polar cardenolides to necessitate more sophisticated mechanisms of exclusion than polar ones.

The application of the metabolic inhibitors (ionophores) 2,4-DNP and CCCP on the isolated nerve cord of *M. sexta* significantly enhanced binding of ^{3}H-digoxin (Fig. 3a) 1.7 fold and 2.3 fold, respectively. For *D. nerii* we tested only the more effective agent CCCP (Fig. 4).

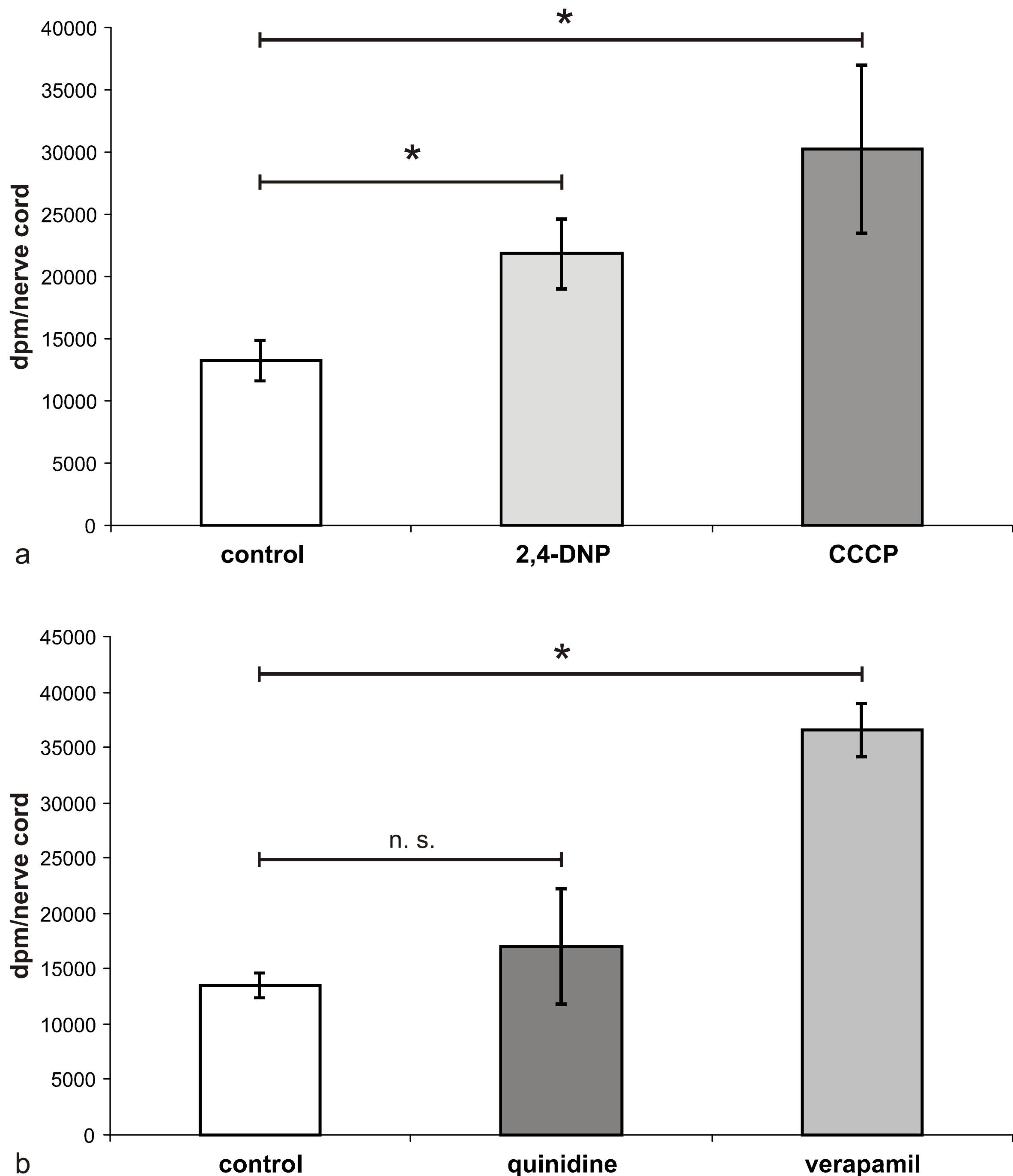

Fig.3: Influence of inhibitors on ^{3}H-digoxin binding in isolated nerve cords of *M. sexta.* a) Metabolic inhibitors 2,4-DNP and CCCP. b) PGP inhibitors quinidine and verapamil (n=5; mean ± SD). Asterisks above bars indicate significant differences.

The picture was similar to the one obtained from *M. sexta*, the increase of ^{3}H-digoxin binding, however, was much stronger than in *M. sexta* (4.5 fold compared to 2.3 fold).

To assess the involvement of a PGP-like transporter in the physiological barrier the classical PGP inhibitors verapamil and quinidine were tested for their effect on digoxin binding to the nerve cord. In *M. sexta* verapamil enhanced ^{3}H-digoxin binding to the nerve cord 2.7 fold. Quinidine also produced an effect (Fig. 3b) which was, however, not

significant. In *D. nerii*, where only verapamil was applied, a similar result was obtained. Interestingly, as with CCCP, this effect was also stronger than in *M. sexta* (3.4 fold compared to 2.7 fold).

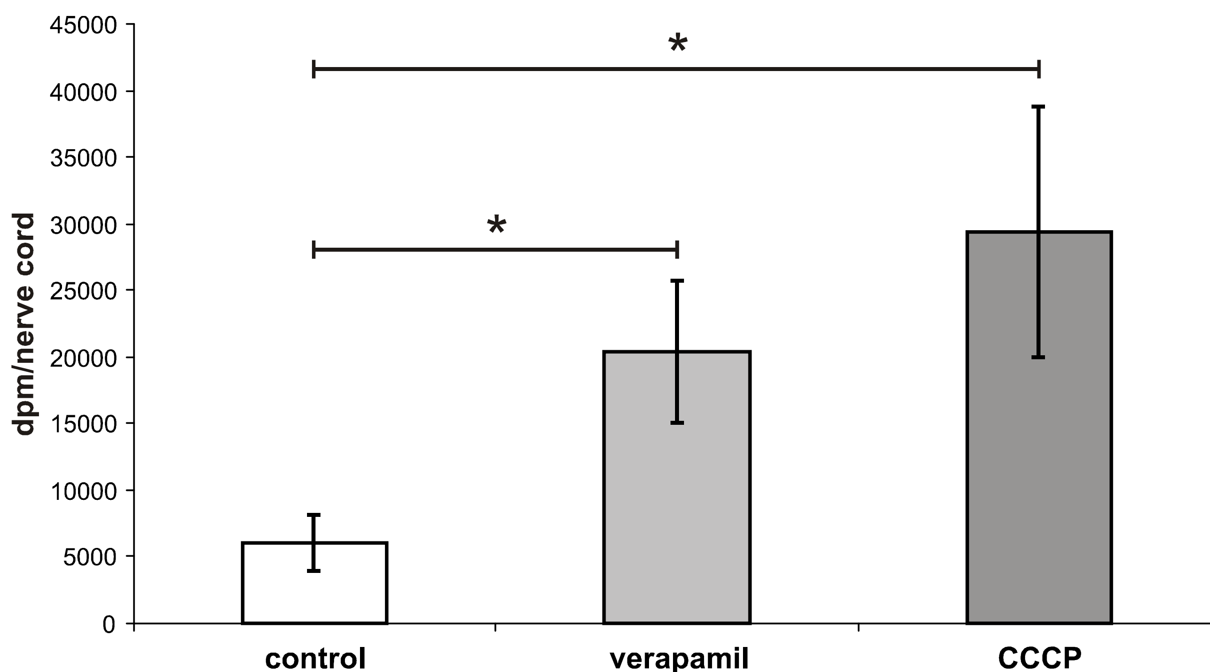

Fig.4: Influence of the metabolic inhibitor CCCP and the PGP inhibitor verapamil on ^{3}H-digoxin binding in isolated nerve cords of *D. nerii* (n=6; mean ± SD). Asterisks above bars indicate significant differences.

Comparative permeability

Digoxin binding to the *D. nerii* nerve cord is about 15 times higher than ouabain binding when the CCCP treated tissues are compared (Fig. 5). Although there is no energy supply by glucose due to the use of PBS instead of *Manduca* saline, the physiological barrier is still active and the amount of bound digoxin is much higher in the CCCP treatment compared to the control. Among the tissues which were incubated in ouabain no difference between CCCP treatment and control was observed. This experiment, however, provides only a rough estimation since we can not be sure that the physiological barrier is completely abolished by the action of CCCP. Moreover, we did not determine nonspecific binding and therefore comparisons are only valuable when the nonspecific binding of ouabain and digoxin is comparable.

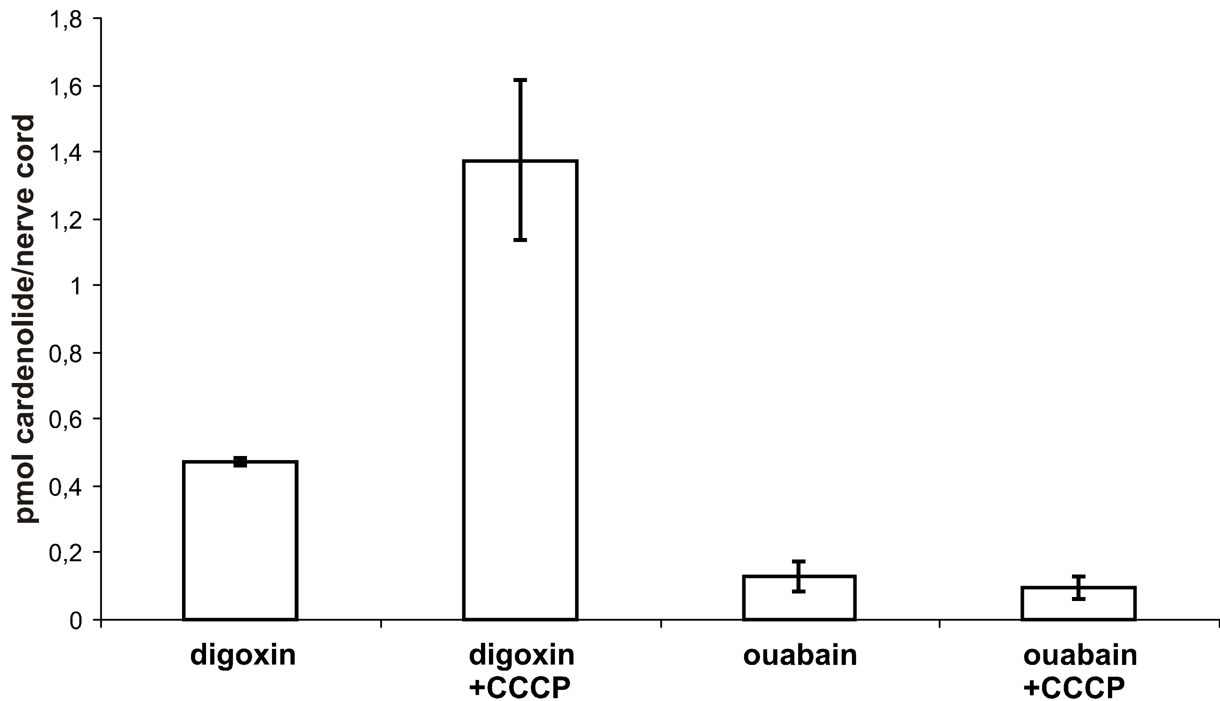

Fig.5: Comparative permeability of ouabain and digoxin in the nerve cord of *D. nerii* (n=3; mean ± SD).

Immunohistochemical detection of a PGP-like transporter

The anti-P-glycoprotein antibody C219 is the most widely used antibody for the immunodetection of PGP. It was raised against membrane preparations of a hamster and a human cell line and binds to a conserved epitope of the protein (van den Elsen et al. 1999). We applied this antibody to cryosections of ganglia from *D. nerii* caterpillars and found specific staining (brown precipitate) only in the periphery of the respective ganglion (Fig. 6).

Immunohistochemical detection of Na^+K^+-ATPase

Application of the monoclonal anti-Na^+K^+-ATPase antibody α5 revealed a strong signal in larval nerve cords of *M. sexta* ganglia (Fig. 7). The occurrence of Na^+K^+-ATPase is apparently restricted to the neurons within the ganglion and no specific signal could be observed in the perineurium.

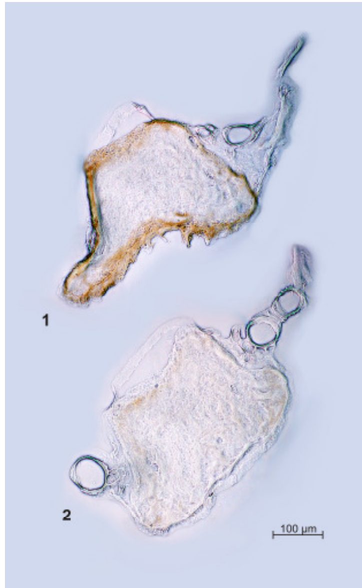

Fig.6: Frozen section of a *D. nerii* ganglion treated with the anti-PGP antibody C219. Specific labelling (brown precipitation) can be seen in the periphery of the ganglion. 1: treatment; 2: control (primary antibody omitted). Scale bar: 100 µm.

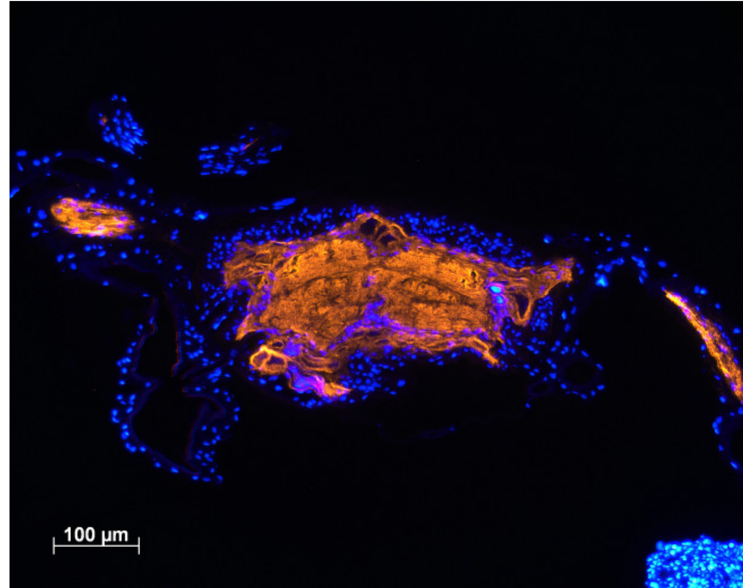

Fig.7: Paraffin section of a ganglion of a *M. sexta* caterpillar. Orange: specific label of $Na^{+}K^{+}$-ATPase. Blue: nuclei stained with DAPI. Scale bar: 100 µm.

Discussion

The physiological investigation of plant insect interactions is a complex matter. To unravel the mechanisms which potentially render herbivorous insects resistant to toxic secondary plant compounds we here transferred knowledge from more extensively investigated vertebrate systems to insect plant science. In our study we focussed on *D. nerii* and *M. sexta*, two closely related species that differ in their host plants and the secondary compounds they are exposed to. Whereas *M. sexta* is naturally not exposed to dietary cardenolides, *D. nerii* is an oleander specialist and encounters high amounts of cardenolides of a wide polarity range in its natural diet, oleander. This species is not a classical cardenolide-sequesterer (like for example the monarch butterfly, *D. plexippus*) because its caterpillars possess only relatively low amounts of these toxins in their body (Abe et al. 1996). This could be achieved by a relative impermeability of the gut membrane to cardenolides as has been observed in generalist insects like *Schistocerca* and *Periplaneta* (Scudder&Meredith 1982). Such impermeability is not surprising in the

case of polar cardenolides that most likely are unable to passively cross the gut membrane, yet in these species the guts are even impermeable for the highly nonpolar cardenolide digitoxin. Since the Na^+K^+-ATPase of *D. nerii* is, however, highly susceptible to cardenolides (Petschenka& Dobler 2009) even the low amounts penetrating into the haemolymph make mechanisms necessary which mediate cardenolide resistance.

An earlier study on cardenolide adapted caterpillars revealed that Na^+K^+-ATPase is largely restricted to the caterpillars' nervous tissue (Petschenka et al., in prep.). On the other hand, we found that *D. nerii* caterpillars can tolerate excess amounts of ouabain if they are injected into the larvae's body cavity (Petschenka & Dobler 2009). We therefore postulated a mechanism which prevents cardenolides from reaching the Na^+K^+-ATPase within the larvae's nervous system and thus focussed here on the interface between cardenolide-containing haemolymph and the ventral nerve cord.

Our data show that ouabain gains access to the Na^+K^+-ATPase when the nerve cord of *D. nerii* has been treated with urea. Since urea is believed to disrupt the perineurium (Rubin et al. 1983) our results provide evidence that the native perineurium is not permeable for ouabain and we can assume that this applies to other relatively polar cardenolides, too. Therefore, the target site of cardenolides, the Na^+K^+-ATPase, is shielded from polar cardenolides dissolved in the haemolymph. In *M. sexta*, the existence of a diffusion barrier for ouabain in the nerve cord was already known. In an attempt to visualize Na^+K^+-ATPase in the nervous tissue of *M. sexta* Rubin et al. (1983) observed that ouabain can not penetrate the native nerve cord. After treatment of the nerve cord with urea ouabain gained access to the Na^+K^+-ATPase. We here observed a similar time dependent disruption of the diffusion barrier in *D. nerii* caterpillars to the one published by Rubin et al. (1983). The diffusion barrier is probably constituted by the cells of the perineurium which form tight junctions (Pichon et al. 1972) impeding the paracellular pathway for diffusing compounds. This barrier is most likely not selective for cardenolides but represents a diffusion barrier for any polar compound. The ionic composition of the haemolymph would not be suitable for nervous function and thus the perineurium is assumed to be responsible for the maintenance of the necessary ion concentrations in the nerve cord's extracellular space (Pichon et al. 1972). Therefore, the tightness to ouabain of the perineurium of *D. nerii* is likely not a specialization. On the other hand, however, the LD_{50} of injected ouabain for *Schistocerca* and *Periplaneta* is as low as 4.4 µg and 0.6 µg per individual, respectively (Moore&Scudder 1986). This might mean that the perineuria of *Schistocerca* and *Periplaneta* are not as tight to ouabain as the one of the hawk-moth. However, the Na^+K^+-ATPase in these insects may not be restricted to the nervous tissue but also occurs in other tissues, e.g. in the gut and the malpighian tubules (Anstee&Bell 1975; Tolman&Steele 1976; Peacock 1977).

To test for the existence of an active barrier for nonpolar cardenolides in the hawk moth perineurium we used the relatively nonpolar cardenolide digoxin which is known to passively permeate cells. By the application of the ionophores CCCP and 2,4-DNP we aimed at blocking the respiratory chain in our test tissue and thus to interrupt the supply of ATP. Consistent with the idea of an active barrier that protects the $Na^{+}K^{+}$-ATPase there is a higher binding of digoxin to the nerve cords when the metabolic inhibitors CCCP or 2,4-DNP were applied. This fits with the hypothesis of an active efflux mechanism: when the energy supply is depleted digoxin can no longer be actively removed from the cells and reaches its target site.

Carriers of the PGP family are strong candidates to mediate the observed effect: in the mammalian brain they constitute the most important part of the blood brain barrier by extruding infiltrating compounds under consumption of ATP (de Boer et al. 2003). Furthermore, Mayer et. al (1996) demonstrated that PGP is responsible for excluding digoxin from the brain of wild type mice.

To test whether a PGP-like transporter is involved in the energy driven digoxin barrier we incubated nerve cords of *M. sexta* and *D. nerii* with two of the most widely used PGP inhibitors, quinidine and verapamil. These compounds are known to elevate the plasma level of digoxin in humans when coadministered with this drug and this phenomenon is primarily attributed to the inhibition of PGP (Mayer et al. 1996, and references therein).

Both in *D. nerii* and in *M. sexta* the application of verapamil increased the amount of digoxin bound to the nervous tissue. These observations suggest that the efflux barrier for digoxin is mediated by a PGP-like transporter. When comparing the data of the two hawk moth species it is conspicuous that digoxin binding under control conditions is about twice as high in *M. sexta* as in *D. nerii*. At the moment, however, it is difficult to judge whether this difference is due to a quantitative or qualitative difference in the perineurial barrier of both species.

The presence of a PGP-like protein is furthermore indicated by our immunohistochemical data which revealed specific binding of the anti-PGP antibody C219 in the periphery of *D. nerii* ganglia. The presence of this protein in the nervous system of *M. sexta* was already demonstrated by Murray et al. (1994) though on the whole, the data on the occurrence of PGP-like proteins in insects is limited. PGP is encoded by the mdr gene family and at least three mdr genes are present in the *Drosophila* genome (Tapadia&Lakhotia 2005 and references therein).

Regarding plant insect interactions, our knowledge about the involvement of PGP is insufficient though the excretion of nicotine in Malpighian tubules of *Manduca* caterpillars (Madrell&Gardiner 1976) was suggested to be based on a PGP homologue (Gaertner et

al. 1998). Especially the wide substrate range suggests a potential key role of this transporter in the resistance of herbivorous insects towards toxic secondary plant compounds. PGP might, in addition, no only be part of the blood brain barrier but also be responsible for rendering insect guts impermeable for plant toxins. PGP could in theory enable generalist species to cope with a wide array of diverse toxic secondary plant compounds.

We can not expect, however, that this mechanism is the only mode of cardenolide resistance. It is known that cardenolides are metabolically modified within the insect body. If regions of the molecule are affected which mediate biochemical interactions, metabolism also results in detoxification. In addition, excretion by the Malpighian tubules can be expected to reduce haemolymph levels of cardenolides. The situation is furthermore complicated by the fact that digoxin is not only substrate to PGP but also to organic anion transporting polypeptide (OATP) and potentially even additional carriers (Yao&Chiou 2006). Further physiological studies using model organisms should address this complexity.

Chapter 4

Bitter pills: evidence for cardenolide deterrence to an invertebrate predator

Abstract

Sequestration of toxic plant compounds by herbivorous insects as a defence against predators has been observed in many tritrophic systems. In this study, we focused on the potential benefit of sequestered cardenolides, potent toxins that block the $Na^{+}K^{+}$-ATPase, for the cardenolide-storing arctiid moth *Empyreuma pugione* against the orb-weaving spider *Nephila senegalensis*. Using three types of assays (live moths, ouabain-loaded non-toxic flies, and toxin-laden gelatine capsules) we demonstrate that cardenolides elicit significant rejection behaviour. Based on our results we conclude that cardenolides are potent protective agents which confer protection against spiders.

Introduction

Sequestration of toxic plant compounds by herbivorous insects has been described in many instances and it is widely accepted that this sequestration provides protection against predators. Several classes of sequestered plant metabolites such as pyrrolizidine alkaloids, cardenolides (cardiac glycosides; CGs) and iridoid glycosides have been intensively investigated (reviewed in Opitz&Müller 2009). Brower's classic demonstration of a blue jay vomiting after consumption of a cardenolide-sequestering monarch butterfly (*Danaus plexippus*, Danaidae; Brower and others 1968) is a prime example of protection conferred by stored plant toxins. The removal of pyrrolizidine alkaloid-laden *Utetheisa pulchellus* (Arctiidae) from their webs by *Nephila* spiders provides another example of protection obtained by storing allelochemicals (Eisner 1982).

In this paper we focus on the protective effects of cardenolide sequestration whose defensive value against vertebrate predators has been demonstrated several times. Cardenolide sequestration by monarch caterpillars have been shown to protect adult

monarchs from both birds (Parsons 1965; Brower and others 1967) and mice (Glendinning and others 1990). Likewise, arctiid species in several genera (e.g. *Cycnia*, *Syntomeida* and *Empyreuma*) which feed on cardenolide-containing plants are known to sequester these toxins (Rothschild and others 1973; Black 1976; Nishio 1980) and this sequestration is assumed to be a highly effective deterrent in the bat *Eptesicus fuscus* (Hristov and Conner 2005). Moreover, Evans et al. (1986) found that cardenolide sequestration significantly improves protection of the hemipteran *Caenocoris nerii* against quails.

To our knowledge, there are only two experimental studies addressing the protective effect of sequestered cardenolides to invertebrate predators. Berenbaum and Miliczky (1984) demonstrated that Chinese mantids (*Tenodera ardifolia sinensis*) vomit after consuming cardenolide sequestering milkweed bugs (*Oncopeltus fasciatus*). The authors raised the prey organisms either on sunflower seeds (devoid of cardenolides) or on cardenolides-containing seeds of *Asclepias syriaca*. Mantids feeding on *O. fasciatus* individuals which had access to cardenolides showed clear signs of rejection: they hurled the milkweed bugs away, regurgitated orange fluid and groomed their heads and forelegs. Sunflower-raised milkweed bugs, however, were accepted and eaten by naïve mantids. This experiment demonstrated that mantids reject milkweed bugs only when they had access to *Asclepias* seeds. Since seeds of *Asclepias syriaca* contain cardenolides (Vaughan 1979), it is highly plausible that the defence of the bugs is based on sequestered cardenolides. The excellent study of Malcolm (1989) is, to our knowledge, the only study to date which demonstrates an adverse effect of cardenolides to a spider. In this case, cardenolides-sequestering aphids (*Aphis nerii*) caused *Zygiella x-notata* spiders to build poor quality webs and to be less effective at catching prey.

The published evidence reporting predators' negative responses to cardenolide-sequestering insects typically leave some uncertainties due to the high complexity of the investigated interactions. Since insects often possess multiple chemical defences, it can be difficult to determine with certainty that a predator is deterred by plant derived cardenolides, other secondary plant compounds, or by substances synthesized *de novo* by the insect. Here, we set out to definitively show the importance of cardenolide sequestration *per se* for predator deterrence. As a test system, we employed the spider *Nephila senegalensis.* Spiders of the genus *Nephila* are known to discriminate against chemically protected prey and unpalatable prey is rejected or even released unharmed from their webs (Vasconcellos-Neto and Lewinsohn 1984; Orr and others 1996).

Our study was divided into three levels of decreasing complexity and increasing mechanistic control. First, we transferred imagos of *Empyreuma pugione*, a moth which is known to sequester high amounts of cardenolides (Black 1976) into webs of *Nephila* and

observed the behaviour of the spider. Secondly, as a system of intermediate complexity, we injected a defined amount of ouabain, as a well known reference cardenolide, into flies (*Calliphora vicina*) and offered untreated flies as a palatable control. Thirdly, we developed an "artificial insect" assay using meat broth-filled gelatine capsules which allowed us to test the influence of the chemical directly without any confusion introduced by parameters which are not controllable. Altogether, our data demonstrated that the observed rejection of the cardenolide sequestering insects can be explained by the adverse effect induced by loading a palatable prey with cardenolides.

Material & Methods

Animals

E. pugione offspring of a wild population from Cuba were reared in our laboratory for several generations at 27°C (13h light/11h dark). Caterpillars were fed with shoots of potted *Nerium oleander* plants. The presence of cardenolides in individuals of our *Empyreuma*-strain was confirmed by thin layer chromatography (G. Petschenka, unpubl. data). *N. senegalensis* were kept in the laboratory at about 25-27°C, fed with *C. vicina* and sprayed with water every second day. All spiders used were of the second generation raised in captivity, the parental generation was collected in Namibia in 2006. Spiders were allowed to construct their webs in frames of acrylic glass (60x60 cm), with a rear panel and an open front. Due to the extreme sexual size dimorphism of *Nephila* (Kuntner and others 2008), only females (adult or subadult) were used for the experiments. *C. vicina* were obtained as pupae from a pet shop and allowed to hatch. Flies were routinely fed with a dietary supplement before use. If the spiders failed to recognize a prey artificially flipped into their web we used a vibrating device made from an electric toothbrush to elicit predation behaviour in the spider.

Experimental design

1. *Empyreuma*-Assay: 12 imagos of *E. pugione* (6 ♀♀, 6 ♂♂) were anesthetized with CO_2 and weighed. We used 12 spiders which were starved for three days prior to the experiment. The moths were flipped into the webs and the behaviour of the spiders was recorded. For each trial, we noted the time until the spider cut the prey out of the web ("retention time") and recorded the residual weight of the prey. For each moth excised from the web we recorded whether the body (thorax) was unharmed or injured by the spider's attack.

2. Charged-Fly-Assay: Flies (*C. vicina*) were anesthetized with CO_2 and weighed. Subsequently, they were injected with 10^{-2} M aqueous ouabain (Fluka) solution (CG-flies) or water (control flies), respectively, using a tuberculin syringe. Each fly was treated with an amount of ouabain sufficient to bring the ouabain concentration of the individual fly to $3.3*10^{-3}$ M (based on the fly's weight) a dose similar to amounts detected in *Syntomeida epilais* a cardenolide sequestering arctiid species phylogenetically related to *Empyreuma* (von Nickisch-Rosenegk et al. 1990; Weller et al. 1999). Control flies received an equivalent amount of water. Volumes injected ranged from 16 to 27 µl per fly. Fluids were injected ventrally via the prothorax into the abdomen. After injection, flies were weighed a second time and their body length was determined. All spiders used (10) were naïve with regard to cardenolides and starved for five days before the experiment started. Flies were flipped into the webs. The assay was divided into three steps: First, every spider received a control fly. Second, after the control fly was consumed and cut out from the web a CG-fly was offered. Third, after excision of CG-flies spiders received a further control fly. This three step procedure was chosen to test for learning effects in the spiders. For all flies the retention time in the web (time span between attack of the spider and excision) as well as weight and length after excision were determined.

3. Capsule-Assay: This series of experiments was conducted to reduce the predator-prey-toxin-system to maximal simplicity. All 12 spiders used were naïve with regard to cardenolides and starved for five days before the experiment began. The method which we developed for this study allowed a direct test of the deterrence potential of any chemical compound to *Nephila*-spiders. To create "dummy"-insects, we used gelatine capsules (1.43 cm x 0.52 cm) filled with a liquid. This system resembles insects in possessing a hard integument and a fluid filling. To prevent a weight loss of the capsule due to soaking of the gelatine followed by evaporation of liquid, a problem that we encountered in preliminary tests, the inner wall of the capsules was treated with pharmaceutical lard (*Adeps suillus*) to create a hydrophobic surface preventing the fluid from soaking the gelatine wall. For this purpose, the fat was melted at 50°C, then allowed to cool down to 23°C, and, using a fine brush it was distributed evenly on the inner surfaces of both halves of a capsule. The two halves were then immediately joined together. After about two hours at ambient temperature the fat was hardened and the capsules were filled with 200 µl of the test solution consisting of 1% meat extract (Fluka), 5% starch (to enhance the viscosity of the fluid) and $5*10^{-3}$ M ouabain or water (controls), respectively. Each filled capsule was weighed before being flipped into a spider's web. Predation behaviour of the spider was elicited as described above. Retention time in the web and weight after excision were determined for each capsule. Six additional control capsules were exposed to ambient conditions (27°C) for one hour and subsequently

weighed to get an estimate of the weight loss by evaporation over time. However, the weight loss was negligible (2.6%) and not considered any further.

Statistical analysis

All data were tested for homoscedasticity (Levene's test) and normality (Shapiro-Wilk). If necessary, data were log-transformed to achieve homogeneity of variances and approximate normal distributions. To improve clarity, primary data are illustrated as % values in Figure 1a and 3a. The Charged-Fly-Assays were analyzed by paired t-tests. For the capsule-assay, groups were compared using the t-test for equal variances. All statistical tests were performed with the statistical software JMP 7.1 (SAS).

Results

Predation behaviour of *N. senegalensis*

After introducing prey (moths, flies or capsules) into the webs, spiders attempted to grasp the prey or tested for the presence of prey by plucking the web. At first contact, spiders bit the prey and enwrapped it with silk. After enwrapping, prey was translocated to the feeding site. Spiders always selected positions above the hub of the web. After several minutes of resting in the hub, spiders began to test the prey. During this period the spiders tested different sites of the prey. For all types of cardenolides-containing prey (*Empyreuma*, fly, or capsule) the spiders showed a similar behaviour: they bit into the prey several times, each bite being followed by wincing.

Empyreuma-Assay

Of the 12 moths fed to the spiders, 7 were cut from the web without lesions. In the remaining 5 individuals, the thorax was injured by the spider. Of all moths, however, only 2 survived. The time until the moths were removed from the web ranged from 90 to 205 minutes (145 minutes on average).

Charged-Fly-Assay

Our experiments with *N. senegalensis* and palatable flies injected with ouabain showed unambiguously that the cardenolide elicited rejection behaviour in the spiders. Body

length of ouabain injected flies was barely reduced after excision from the spider webs. Control flies, on the contrary, were completely consumed by the spiders and only undigestible remains were discarded from the web (Table 1, Fig.1a).

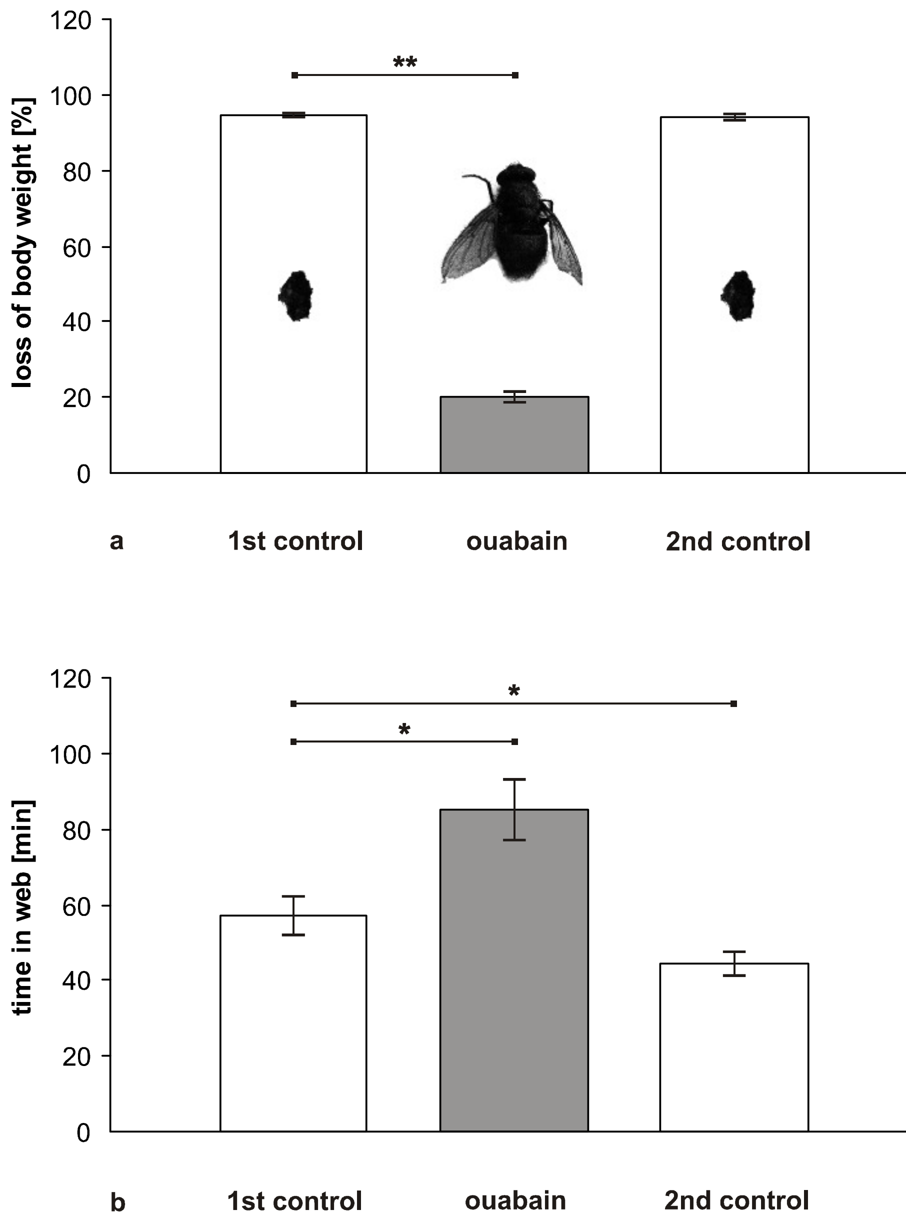

Fig. 1. Reaction of *N. senegalensis* to injected flies. Each spider was presented first with a control fly (water-injected) followed by an ouabain injected fly and a second control fly. a) % reduction of body weight after the spider's attack, b) retention time of the flies in the spider webs. Asterisks above columns indicate significant differences (n=10; *: p≤0.05; **: p≤0.01). The inserted photographs illustrate the typical appearance of control or ouabain injected flies after spider attack.

The weight loss of the flies provides a similar picture: the spiders consumed 74.5 % less of the ouabain injected flies compared to the control (paired t-test, $t_{1,9}$ = 17.086, p<0.001; Fig. 1a). Flies treated with ouabain remained significantly longer in the webs than the controls (paired t-test, $t_{1,9}$ = -2.564, p<0.030; Fig. 1b). The spiders' reaction to the water treated flies presented last did not indicate that the spiders learned to avoid the flies after

having been presented with an ouabain injected individual: the second water injected fly at the end of the experiment was eaten as readily as the first one and the weight loss did not differ from the first control (paired t-test, $t_{1,9} = 0.344$, $p>0.739$). However, the retention time in the web was significantly shorter than the values observed for the first control flies (paired t-test, $t_{1,9} = 2.879$, $p<0.018$; Fig. 1b).

Tab. 1. Loss of body length in injected flies after attack by *N. senegalensis*. Experimental design as in Fig. 1.

loss of body-length [mm]	**control 1**	**ouabain-injected**	**control 2**
average	6,6	0,2	6,3
SD	0,97	0,42	0,48

Capsule-Assay

Capsules were attacked by the spiders and those capsules containing just meat broth were readily emptied and lost 66.6 % of their original weight (Fig. 2).

Fig. 2. *N. senegalensis* attacking a gelatine capsule filled with meat broth. Spiders penetrated the capsule's wall with the chelicerae and fed on the fluid filling.

The weight loss of the ouabain capsules as a measure of consumption, on the other hand, was 62.6 % lower and rather negligible (Fig. 3a; t-test, $t_{2,10} = -11.729$, $p < 0.0001$). Moreover, the retention time of the CG-capsules in the spider webs differed significantly between the groups and was significantly shorter than the retention time of the control capsules (t-test, $t_{2, 10} = -11.729$, $p < 0.0001$; Fig. 3b). This result differs from the results of

the charged-fly test: the retention time of the flies treated with ouabain was significantly longer than the retention time of the water-injected and palatable flies.

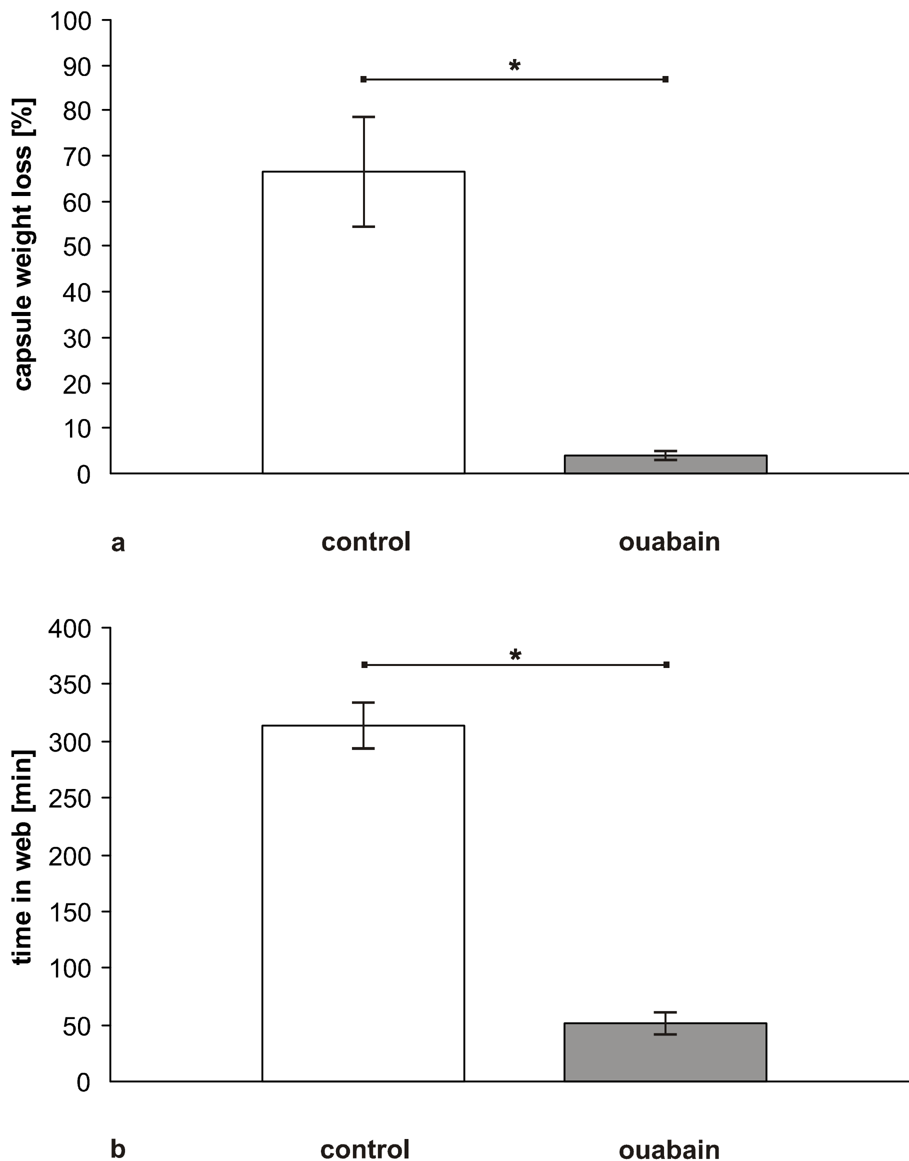

Fig. 3. Reaction of *N. senegalensis* to gelatine capsules filled with meat broth only or with meat broth containing $5*10^{-3}$ M ouabain (n=6 in both groups). a) % weight loss, b) retention time in the spider webs (**: $p \leq 0.01$).

Discussion

Our results clearly demonstrated that the cardenolide ouabain elicits rejection of prey by *N. senegalensis*. This finding potentially has important ecological significance since cardenolide sequestration or de novo synthesis of these compounds is a widespread phenomenon in insects of different orders and families: Lepidoptera - Danaidae (Parsons 1965), Arctiidae (Rothschild and others 1973); Coleoptera - Chrysomelidae (Pasteels and

Daloze 1977; Dobler and others 1998), Cerambycidae (Duffey and Scudder 1972); Caelifera - Pyrgomorphidae (von Euw and others 1967); Heteroptera - Lygaeidae (Duffey and Scudder 1972); Sternorrhyncha - Aphididae (Rothschild and others 1970).

We think that especially our capsule assay provides some advantages over trials involving whole sequestering animals due to its reduced complexity. Using whole insects, the effect obtained is not clearly attributable to one particular secondary plant compound. Vasconcellos-Neto and Lewinsohn (1984) reported that field collected *Danaus gillipus,* which are known to store cardenolides (Cohen 1985), were always (n=6) rejected or released by *N. clavipes*. This reaction might be due to the presence of cardenolides. Yet, since *D. gilippus,* like other danaids, is also a pharmacophagous PA sequesterer (Dussourd and others 1989), this observation could also be due to stored PAs, which are well known to elicit releasing behaviour in *N. clavipes* (Eisner 1982).

To our knowledge, the only other study providing direct evidence for cardenolide mediated rejection or toxicity to an invertebrate predator is a study by Malcolm (1989): The author fed spiders (*Zygiella x-notata*) with the cardenolide sequestering aphid *Aphis nerii* and reported that the structure of the webs was disrupted as it is the case when psychoactive drugs are fed to spiders. Moreover he fed the spiders with a solution of the fox-glove cardenolide digitoxin and showed that the spiders reacted in the same way. This finding provides direct proof that this abnormal and disadvantageous behaviour is elicited by the applied cardenolide. Malcolm (1989) did not find this effect when feeding the spiders with ouabain. Unlike digitoxin, ouabain is a polar cardenolide and might possibly not be absorbed by the gut of spiders (Malcolm 1991) and thus not induce a toxic effect. Based on our data, however, ouabain obviously can stimulate receptors of the spider and elicit an adverse response. Moreover, ouabain was reported to elicit receptor activity in insects when applied to maxillary sensilla styloconica of caterpillars of *Pieris brassicae* (van Loon and Schoonhoven 1999).

Comparing the results of the charged-fly-assay and the capsule-assay there is a conspicuous difference: The ouabain infiltrated flies remained significantly longer in the webs than the palatable controls whereas the unpalatable capsules where removed much faster than the palatable ones. Since ouabain is the basis for unpalatability in both cases the reason for this behavioural difference has to be searched in the carrier. Flies as a complex system surely possess chemical characteristics which are absent in the capsules. These chemical properties might act as positive stimuli which elicit a positive response by the spider. Under these circumstances there might be interference between the positive stimuli of the carrier (fly) and the negative stimuli of the ouabain. This assumption might be an explanation for the longer retention time of the flies charged with ouabain. Moreover, the distribution of the ouabain within the flies is probably uneven. If

so, whilst testing ouabain infiltrated and ouabain free body parts the spider might experience a mixture of positive and negative stimuli which possibly prolongs the testing process. In summary, there is no doubt that ouabain mediates a highly negative response in *N. senegalensis*.

7 of the 12 *Empyreuma* tested, were rejected by the spiders and this observation suggests the unpalatability of *E. pugione* to *N. senegalensis*. However, the observation that only 2 of them survived the attack requires an explanation regarding the protective value of cardenolides. Vasconcellos-Neto & Lewinsohn (1984) divide the responses of *N. clavipes* to butterflies into three distinct categories: 'eaten', 'rejected' or 'released'. Butterflies that ended up being released were not even bitten and resumed normal flight before hitting the ground. The *Empyreuma* in our study have to be classified as 'rejected'. The survival of two individuals rather seems to be a recovery from rejection than a classical release as described by Vasconcellos-Neto & Lewinsohn (1984) since the removal took a very long time (90-205 min) and the *Empyreuma* were bitten. Mere rejection of a prey organism, however, is only advantageous when the spider associates the bad experience with this species during future encounters. Berenbaum & Miliczky (1984) report that the mantids which had bad experience with cardenolide laden milkweed bugs did not attack *O. fasciatus* anymore. If *Nephila* possesses a comparable learning capacity prey organisms like *Empyreuma* would derive a clear advantage from the storage of cardenolides. The data obtained from our Charged-Fly-Assay, however, do not indicate learning capacity since the control fly offered last was consumed as readily as was the first control fly. Potentially, the flies lack certain cues which are normally present in chemically protected prey and facilitate learning (aposematic colour, warning odour etc.). This assumption is in agreement with the report of Malcolm (1989) who found a learning effect in spiders fed with aposematic oleander aphids (*A. nerii*).

Possibly the fact that the spiders were starved in our study (*Empyreuma*-Assay: 3 days; Charged-Fly and Capsule-Assay: 5 days) also contributes to the behaviour observed here. Gelperin (1968) reports that mantids prey on, taste and discard milkweed bugs on first encounter. If sufficiently starved, however, they will prey on and eat the hemipterans even without signs of toxic effects. The same could apply to starved *N. senegalensis* in our experiments and therefore potentially contribute to the fact that the *Empyreuma* in our study were 'rejected' and not 'released'.

Altogether, our results clearly show, that ouabain mediates a negative response in the spiders tested. This supports that insects derive protection against vertebrates and invertebrates from sequestered cardenolides and thus that cardenolide sequestration is advantageous. The comparison of our results with literature reports suggests that factors like starvation or experience might modify the invertebrate predator's reaction on

chemically protected prey. Studies elucidating the influence of these factors might help to get a better understanding of the interaction of invertebrate predators and cardenolide storing prey. Since cardenolides sequestered by insects are qualitatively different future investigations should also focus on structure-activity relationships and potential ecological implications.

General Discussion and Outlook

Herbivorous insects are confronted with a high variety of toxic plant compounds which they ingest during the feeding process. Generally, plant toxins can exert either a specific or a non-specific toxic effect. One major weapon acting against many classes of toxic allelochemicals is the biotransformation of such substances, a process which is mainly realized by three enzyme super-families: cytochrome P450 monooxygenases, glutathione S-transferases and carboxylesterases (Després et al. 2007).

Besides the majority of toxins which act in a non-specific manner, there are numerous compounds which act specifically. Prominent examples comprise pyrethroids which affect sodium channels (Vijverberg et al. 1982; Kakko et al. 2000), nicotine which acts on cholinergic receptors (Klaassen 2008), and cardiac glycosides (CGs) which bind to and inhibit the ubiquitous $Na^{+}K^{+}$-ATPase (Schatzmann 1953).

Focussing on cardiac glycosides, it is known that these toxins are substrates to cytochrome P450 monooxygenases (Marty&Krieger 1984 and references therein; Kirby et al. 2006) and modification of regions of the molecule which are relevant for specific binding to $Na^{+}K^{+}$-ATPase might lead to detoxification. Moreover, the results of Black (1976) indicate CGs to be substrates for insect glycosidases. Marty&Krieger (1984) speculated that ketone reductases could metabolize the cardenolide uscharidin in *D. plexippus* larvae into two more polar cardenolides.

Regarding cardenolide toxicity in insects, however, it is important to differentiate between species which sequester and those which do not sequester CGs. For species which do not store CGs it should be favorable to deactivate the toxins. Insects, on the other hand, which derive protection by the sequestration of CGs should contain the toxins in an active form. *D. plexippus* was shown to possess comparatively low levels of monooxygenase activity (Marty et al. 1982), an observation fitting into this scheme.

Cardenolides are bitter tasting and induce emesis in vertebrates. In mammals, emesis is induced by excitation of a chemoreceptor trigger zone in the medulla (Hoffman and Bigger 1980 as cited in Malcolm 1991). Since sequestered cardenolides do elicit emesis in predators they are still physiologically active. It is, however, not clear whether the emetic property means that sequestered cardenolides still act as $Na^{+}K^{+}$-ATPase inhibitors. Moreover, regarding the evolution of cardenolide-sequestration as an anti-predator defence, we cannot state whether stored cardenolides need to keep their potential to inhibit $Na^{+}K^{+}$-ATPase or whether it is sufficient they keep their deterrent potency, e.g. their bitter taste to vertebrates. Furthermore, we do not know whether both features are structurally related and whether sequestered cardenolides still possess the

features which mediate $Na^{+}K^{+}$-ATPase inhibition. Several insect species were shown to contain sequestered cardenolides in an unchanged form (Black 1976; Seiber et al. 1980, Dobler et al. 1998). To determine whether sequestered cardenolides retain their inhibitory potency, I developed a new method (based on the inhibition of $Na^{+}K^{+}$-ATPase from locust rectum) to quantify cardenolide levels of lepidopteran haemolymph. This method detects only those cardenolides which still have an inhibitory effect on $Na^{+}K^{+}$-ATPase. The preliminary data obtained with this method showed that haemolymph cardenolides from caterpillars of *Empyreuma pugione* and *Danaus plexippus* still cause *in vitro* inhibition of $Na^{+}K^{+}$-ATPase. The amount of active cardenolides equals to $1.2*10^{-3}$ M ouabain (n=2) in *D. plexippus* and $5.3*10^{-4}$ M (n=6) in *E. pugione* caterpillars, respectively. Thus, we can assume sequestered cardenolides to retain their inhibitory (toxic) potential.

The presence of active cardenolides in the body cavity of sequestering species necessitates tolerance mechanisms other than enzymatic degradation. The most elegant mechanism to avoid intoxication is the modification of the target site, the $Na^{+}K^{+}$-ATPase. This so called target site insensitivity is known to occur in *D. plexippus* (Vaughan&Jungreis 1977), leaf beetles of the genus *Chrysochus* (Labeyrie&Dobler 2004) and a locust, *Poekilocerus bufonius* (Al-Robai et al. 1990). Moreover the lygaeid bug *Oncopeltus fasciatus* is known to possess an ouabain-insensitive $Na^{+}K^{+}$-ATPase (Moore&Scudder 1986) and our preliminary data indicate that target site insensitivity towards cardenolides is a common feature within the hemipteran subfamily Lygaeinae. However, the investigations of the present thesis showed that within Lepidoptera even species which contain high amounts of cardenolides, like *E. pugione,* possess $Na^{+}K^{+}$-ATPases highly sensitive to cardenolides. Hence, such species must possess alternative mechanisms of cardenolide resistance.

The evidence as to how sequestering lepidopterans avoid cardenolide intoxication is either still missing or leaves many open questions. Even species like *D. plexippus,* which possess $Na^{+}K^{+}$-ATPases relatively insensitive to cardenolides, can be expected to possess adaptations apart from target site insensitivity. The data presented here show that the $Na^{+}K^{+}$-ATPase of this species *in vitro* is inhibited by 88% at cardenolide concentrations which occur naturally in *D. plexippus* haemolymph (Nishio 1980). This observation clearly reveals the necessity of additional protective mechanisms. Metabolism of cardenolides was demonstrated in *D. plexippus*, *Syntomeida epilais* (Arctiidae) and *E. pugione* (Black 1976; Seiber et al. 1980). However, as already discussed, stored cardenolides are still active and loss of toxicity might not be favourable for sequesterers. An observation which is important in this context is the accumulation of cardenolides in the integument as found in *D. plexippus* (Brower&Glazier 1975; Frick&Wink 1995). The haemolymph is known to be an especially important compartment regarding cardenolide

toxicity in lepidopterans (see chapters 1-3), but appears to be only a transient compartment for sequestered cardenolides in monarch larvae. Adult *D. plexippus* whose larvae had been fed with ouabain were shown to possess no ouabain in the haemolymph but relatively high amounts in the integument and wings. Besides the argument that the integument is the first compartment a predator is faced with (see Brower & Glazier 1975; Nishio 1980) accumulation of cardenolides in the integument could mean a reduction of the haemolymph level. Therefore, elimination of haemolymph cardenolides would reduce exposure of nervous Na^+K^+-ATPase to cardenolides. In contrast, Black (1976) found that the wings of *S. epilais* were the site with the lowest concentration of cardenolides. The haemolymph, however, was the richest compartment in this species. Black ascribed this circumstance to the reflex bleeding behaviour of *S. epilais* adults.

In species which do not efficiently sequester cardenolides, the gut is a first barrier to ingested cardenolides. Only few studies addressed the permeability of the insect midgut to cardenolides. In agreement with vertebrate systems, mainly polar cardenolides like ouabain can be expected not to cross the gut passively. Conversely, nonpolar cardenolides can diffuse through cell membranes due to their physicochemical properties (see Wright 1960). Interestingly, midguts of the generalists *Schistocerca gregaria* and *Periplaneta americana* were found to be completely impermeable even to the non-polar cardenolide digitoxin (Scudder&Meredith 1982). Such a barrier, preventing diffusible compounds from entering the body cavity, must be active since digitoxin is expected to cross membranes passively (enteral resorption 90-100%, Luckner&Wichtl 2000). Moreover, *S. gregaria* and *P. americana* probably do not encounter cardenolides in their natural diet, suggesting that the mechanism which renders the midgut impermeable to cardenolides is non-specific and most likely ancestral. Since our data on the lepidopteran perineurium (chapter 3) indicate that an insect homologue of P-glycoprotein (PGP) transports the cardenolide digoxin, a role of such a transporter in the midgut barrier of insects is highly suggestive. This assumption is furthermore plausible since the midgut of caterpillars of *Manduca sexta* (Murray et al. 1996) and *Heliothis virescens* (Lanning et al. 1996) were found to be immunopositive to this transporter. Another putative mechanism which could confer tolerance to dietary cardenolides is the peritrophic enevelope. Barbehenn (1999) reports that digitoxin has only low permeability through the peritrophic envelope, a phenomenon which is due to the association of lipophilic and amphiphilic allelochemicals with lipid aggregates.

In cardenolide specialists like the oleander hawk-moth, which ingest large amounts of cardenolides but possess only low amounts in their haemolymph, an impermeable gut might be a mechanism of resistance. Otherwise, the low cardenolide content of the body cavity might be explained with efficient excretion via the Malpighian tubules. Transport of

ouabain by Malpighian tubules is reported from *O. fasciatus* (Heteroptera; Meredith et al. 1984), *Zonocerus variegatus* (Caelifera; Rafaeli-Bernstein&Mordue 1978) and *Drosophila melanogaster* (Diptera; Torrie et al. 2004). Moreover, Vaughan&Jungreis (1977) found that *M. sexta* larvae excrete 87% of injected radiolabelled ouabain within 48 hours, suggesting that lepidopteran Malpighian tubules can excrete ouabain, too. The widespread occurrence of ouabain excretion suggests that insect Malpighian tubules generally can excrete this compound. Possibly, the interplay of an impermeable gut and cardenolide excretion assures exclusion of cardenolides from the body cavity.

Cardenolide-sequestration, *per se,* is an arbitrary definition depending on the concentrations detected in the insects' tissues and even species like the oleander hawk-moth, which do not efficiently store cardenolides, were shown to possess physiologically relevant amounts of these compounds (Abe et al. 1996; Petschenka&Dobler 2009). It may be expected that mechanisms which protect against internal cardenolides are derived from an ancestor. Our data suggest the occurrence of a digoxin carrier in the cardenolide specialist *Daphnis nerii,* as well as in *M. sexta* which is not adapted to cardenolides. The ability of the PGP homologue in the perineurium of *M. sexta* to transport digoxin suggests that this feature is ancestral. Hence, PGP homologues in the blood brain barrier of lepidopterans might generally be able to transport digoxin. It is, therefore, likely that the same mechanism of resistance which underlies cardenolide resistance in non-sequesterers like *D. nerii* forms the basis for insensitivity in sequestering species which possess sensitive $Na^{+}K^{+}$-ATPases. It is known that substrate specificity of human P-glycoprotein is altered by mutations (Ishikawa et al. 2004) and future studies should unravel whether PGPs of specialized insects show higher affinities to toxins of their respective host plants than to others.

The factors which are involved in the behaviour of cardenolides in insects are illustrated in Fig. 1

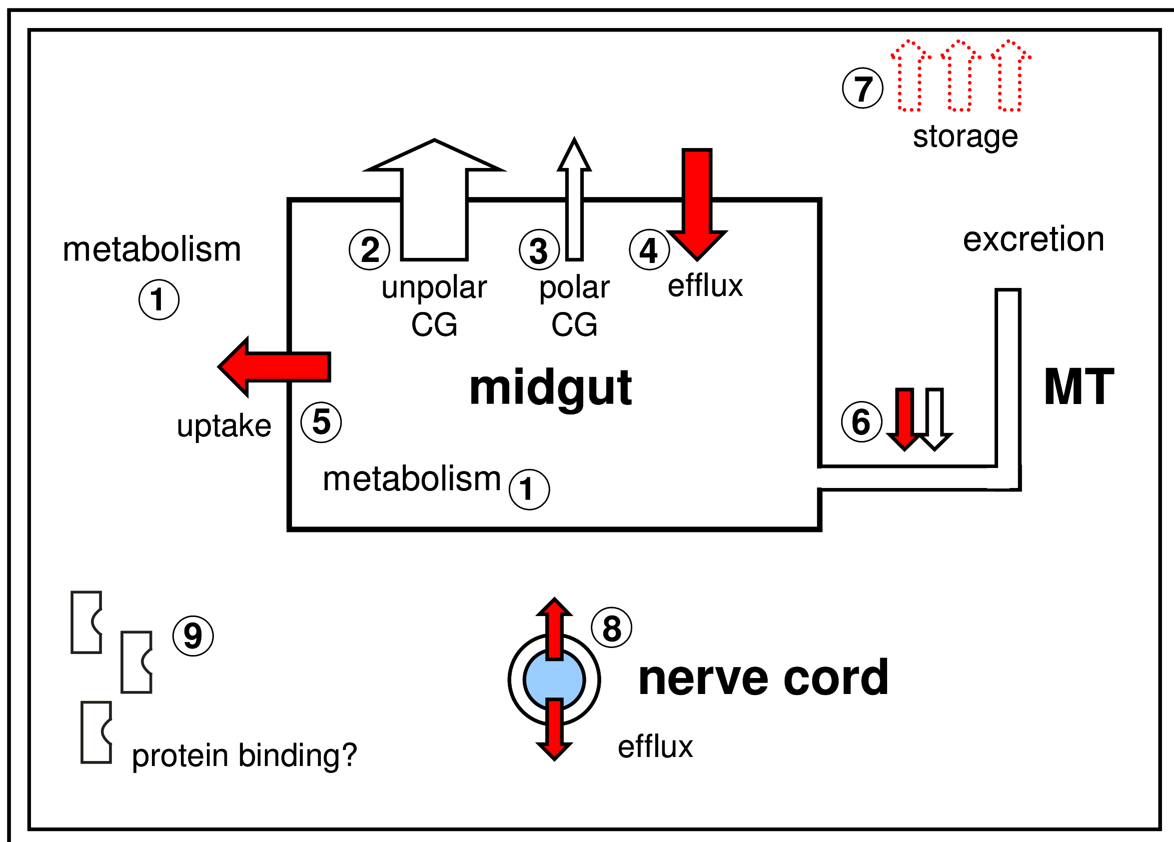

Fig. 1. Compilation of the possible fate of cardenolides in insects after various sources. Schematic section through a hypothetic insect. Active processes: red, passive processes: white; dotted red line: potentially active process. MT=Malpighian tubules. (1) Ingested cardenolides may be metabolized during their gut passage and also within the body cavity (Abe et al. 1996; Seiber et al. 1980). (2) Unpolar CGs can passively cross the gut membrane whereas polar CGs can not cross the gut by diffusion or can cross it only to a smaller extent (3). Cardenolides which entered gut cells might be removed by efflux carriers like PGP (4) whose occurrence in the lepidopteran midgut is suggested by Murray (1996) and Lanning et al. (1996). Studies of sequestering species like *D. plexippus*, *S. epilais* and *O. fasciatus* propose active carrier-mediated transport of cardenolides (5) via the gut membrane (von Nickisch-Rosenegk et al. 1990, Detzel&Wink 1995, Frick&Wink 1995). Reduction or regulation of haemolymph cardenolides could be achieved by excretion via the Malpighian tubules (6). Excretion of ouabain can occur either actively or passively (Meredith et al. 1984; Rafaeli-Bernstein&Mordue 1978; Torrie et al. 2004). (7) Sequestration of cardenolides into the integument (*D. plexippus*; Brower&Glazier 1975) or into the extradorsal space as seen in lygaeid bugs (Scudder et al. 1986) might reduce haemolymph concentrations. (8) Access of cardenolides to nervous Na^+K^+-ATPase (light blue) is prevented by the perineurium which constitutes a diffusion barrier for polar cardenolides and which contains efflux carrier extruding non-polar cardenolides. (9) Hypothetically, a further possibility to reduce toxicity of stored haemolymph cardenolides would be binding to proteins, yet so far there is no evidence for such a process.

According to our findings (chapter 3) storage of polar cardenolides should be less costly than storage of non-polar cardenolides which necessitate an active cardenolide barrier in the perineurium. Interestingly, sequestering species metabolize cardenolides to more polar derivatives as described for *S. epilais*, *E. pugione*, *D. plexippus* and *O. fasciatus* (Black 1976; Seiber et al. 1980; Martin et al. 1992). Malcolm (1991) presumes that in *D. plexippus* higher polarity cardenolides are less easily mobilized in the haemolymph and are easier to store. In the light of our findings, polar cardenolides might also be favoured

because their storage is not costly. Moreover, universal detoxifying enzymes like cytochrome P450 monooxygenases use lipophilic substrates (Timbrell 2002). Polarization could therefore prevent cardenolides from being metabolized by cytochrome P450 monooxygenases. However, care has to been taken to avoid circular reasoning, since cytochrome P450 monooxygenases convert non-polar compounds into polar ones.

The defensive value of sequestered cardenolides is undoubted. As detailed in chapter 4 cardenolides deter vertebrate as well as invertebrate predators from feeding on cardenolide-sequesterers. Regarding the presentation of sequestered cardenolides to attacking predators different strategies have evolved. The accumulation of cardenolides in the wings of monarch butterflies is thought to provide an avian predator with the possibility to detect unpalatability by pinching a portion of the wing and to release the butterfly alive. In comparison, cardenolide-sequestering lygaeid bugs like *O. fasciatus* or *Lygaeus kalmii* emit cardenolide-containing droplets by integument rupture (Scudder&Meredith 1982; Scudder et al. 1986) thus confronting the attacking predator with a solution of cardenolides. The fluid emitted by these heteropterans stems from a compartment called the dorsolateral space, a specialized layer of the epidermis where sequestered cardenolides get enriched (Scudder et al. 1986). Similarly, arctiids like *S. epilais* or *E. pugione* show reflex bleeding and emit droplets of cardenolide-laden, bitter-tasting (personal observation) haemolymph (Fig. 2). This haemolymph is aposematically colored and should allow for cardenolide detection by a predator without hurting the prey. A further mode of cardenolide presentation is found in leaf beetles of the genus *Chrysochus*: here, sequestered cardenolides are concentrated in specialized glands. Upon disturbance, these beetles emit droplets of cardenolide-rich glandular secretion (Dobler et al. 1998).

Fig. 2. *E. pugione*. Upon disturbance the moth releases droplets of toxic haemolymph behind the head and also at the leg bases.

In *E. pugione* (Fig. 3a) not only the caterpillars (Fig. 3b) are aposematically orange with white spots but also the eggs are very large and yellow (Fig. 3c). We extracted eggs of *E. pugione* using thin-layer-chromatography and found them to be rich in cardenolides (unpublished results). It is therefore likely that the conspicuousness of *E. pugione* eggs is correlated with an efficient protection caused by cardenolides. Interestingly, the closely related species *S. epilais* produces egg batches of comparatively small, white eggs.

Fig. 3. a: *E. pugione*. b: caterpillar of *E. pugione* on oleander. The caterpillars have large white spots contrasting with the orange ground color. c: eggs of the same species.

Cardenolide-insect interactions provide a useful model to study plant-insect-interactions. The large number of cardenolide-sequestering species from at least five insect orders offers multiple opportunities for comparisons. Moreover, the occurrence of cardenolide-sequestration in species-rich taxa (e.g. Danaidae, Arctiidae, Lygaeinae, Chrysomelidae, Pyrgomorphidae) allows for the investigation of the evolutionary biology of cardenolide-sequestration and cardenolide-resistance. Cardenolides, *per se*, are very attractive due to the profound pharmacological analysis which they have received. The same applies to their target site, the $Na^{+}K^{+}$-ATPase. Due to the ubiquitous occurrence of this animal enzyme and its physiological importance, a vast amount of knowledge is available. Decades of scientific research on cardenolides and their biochemical action provide us

with a wide array of molecular biological tools. The commercial availability of antibodies against digoxin, $Na^{+}K^{+}$-ATPase, P-glycoprotein, several purified cardiac glycosides, fluorescently and radioactively labelled cardenolides and even more tools enables us to study cardenolide-insect interactions in a detailed manner extraordinary within chemoecology.

All these possibilities await their application to cardenolide-insect research and with this thesis I have tried to do first steps in this direction. Apart from the application of the sophisticated methods available, comparative approaches with phylogenetic background are desirable. Moreover, it is necessary to enhance the specificity of the systems under investigation. For example, the $Na^{+}K^{+}$-ATPase of *D. plexippus* has so far exclusively been tested for its sensitivity to ouabain. This cardenolide, however, does not occur in the monarch's host-plant. Furthermore, the host-plants contain several structurally diverse cardenolides. In a comparative approach we plan to apply an array of different cardenolides on *D. plexippus*-$Na^{+}K^{+}$-ATPase. Preliminary data have already shown that the enzyme of this species is much more sensitive to digitoxin than to ouabain.

Despite a wealth of descriptive data on the occurrence of cardenolide sequestration in insects, up to now most of the physiology involved is not understood. Using modern techniques on a comparative basis, cardenolide-insect interactions are a promising and fascinating field for future plant-insect research.

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