Evolution of adaptations to cardiac glycosides in the hemipteran subfamily Lygaeinae

Dissertation

Zur Erlangung der Würde des Doktors der

Naturwissenschaften des Fachbereichs Biologie, der Fakultät für Mathematik,

Informatik und Naturwissenschaften,

der Universität Hamburg

vorgelegt von

Christiane Bramer

aus Lübben

Hamburg, 2014

Genehmigt vom Fachbereich Biologie
der Fakultät für Mathematik, Informatik und Naturwissenschaften
an der Universität Hamburg
auf Antrag von Frau Professor Dr. S. DOBLER
Weiterer Gutachter der Dissertation:
Professor Dr. J. GANZHORN
Tag der Disputation: 16. Mai 2014

Professor Dr. C. Lohr
Vorsitzender des
Fach-Promotionsausschusses Biologie

“Milkweed bugs (Lygaeinae) are the butterflies of the bug world – black on red associated with poison may deter predators, but it attracts scientists!” Jeffrey R. Aldrich

Table of contents

Eidesstattliche Versicherung und Aufführung der Inanspruchnahme fremder Hilfen

Der in Kapitel 1 durchgeführte Enzym - Assay wurde von Herrn Dr. Georg Petschenka durchgeführt, die DNA - Sequenzanalysen der Na/K-ATPase dieser Studie wurde von Frau Vera Wagschal durchgeführt.

Die in Kapitel 2 durchgeführte MALDI-TOF-MS Analyse wurde am Department für Organische Chemie an der Universität Hamburg erstellt.

Die in Kapitel 4 vorgestellten Daten wurden von Herrn Christian Schweitzer erhoben.

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertationsschrift selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Hamburg, den 14.03.2014

General Introduction

In evolutionary terms, the so-called 'arms race' between plants and insects has given rise to highly specialized chemical interactions. Noxious phytochemicals that are repulsive, unpalatable or poisonous are one of the primary defence mechanisms of plants against insect herbivores. Such plant secondary compounds usually display detrimental effects on non-adapted insect herbivores and consequently restrict their host range *via* feeding or oviposition choices (Awmack & Leather, 2002). Therefore, adapted herbivores in several insect orders have evolved different ways to overcome these plant defence barriers (Vaughan & Jungreis, 1977; Schoonhoven *et al*., 2005; Dobler *et al*., 2011). Including mechanisms of detoxification (Scott & Wen, 2001; Li *et al*., 2002,2007; Després *et al*., 2007), possession of impermeable guts (Scudder & Meredith, 1982b; Petschenka *et al*., 2013) or the avoidance of noxious plant parts (Dussourd & Eisner, 1987, Després *et al.,* 2007) are most efficient and widespread adaptations to exploit a high variety of chemicals encountered in their food. Moreover, some specialized insects not only avoid poisoning by the toxin, further they acquire and store them in various tissues, glands or compartments (sequestration) where they act as defensive compounds for insect's own benefits (Opitz & Müller; 2009). These naturally occurring substances have either beneficial or toxic effects, depending on dosage or biological activity.

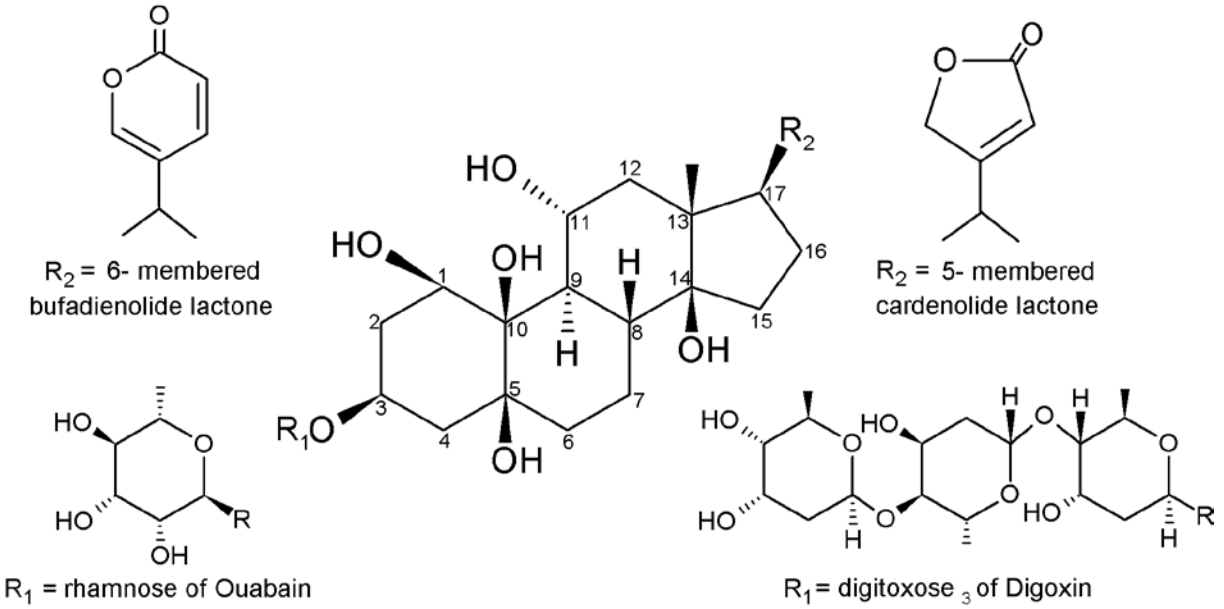

Fig. 1. Chemical structure of cardiac glycosides. A steroid backbone (here representing ouabain) of four fused rings is connected at C17 to a 5-membered mono-unsaturated lactone ring in the case of cardenolides (R2) or to a 6-membered double-unsaturated lactone ring in bufadienolides. Cardenolides occur as free genins or with sugar moieties glycosidically bound at C3 (R1). Rhamnose attached at C3 yields ouabain (g-strophantin), three molecules digitoxose$_3$ yield digoxin.

All cardiac glycosides (CGs) share as common features the basic steroid skeleton (aglycone or genin) with a lactone ring in *β* position at C17 and are characterized by a 14*β*-hydroxyl group and sugar moieties (glycoside) attached through an OH of carbon 3*β* (Fig. 1). A wide variety of sugar molecules are known to occur in natural CGs but only a few such as glucose, rhamnose and fructose are widespread among plants. Representative examples of common CGs in biochemical research are illustrated in Figure 1: digoxin with three molecules of digitoxose linked to the aglycone and ouabain which has a single rhamnose molecule. The typical AB cis and AB trans conformation is a feature of medically important cardenolides in the plant families Apocynaceae. Depending on the structure of the aglycon, the CGs can be divided into two groups: cardenolides and bufadienolides. Cardenolides have a 5-membered mono-unsaturated γ-lactone ring, at the C17 position whereas bufadienolides possess a 6-membered double-unsaturated δ-lactone ring (Fig. 1).

With a high diversity of chemical forms (>500), these secondary metabolites are erratically distributed among 14 angiosperm families that include both cardenolide- and bufadienolide-containing species (Tab. 1)(Malcolm, 1991; Luckner & Wichtl, 2000).

Tab. 1. Plant families that include cardiac glycoside (cardenolide or bufadienolide) containing species. Cardenolides have been recorded from 62 genera of 10 plant families (Malcolm, 1991; Luckner & Wichtl, 2000; Hänsel & Sticker, 2007). Bufadienolides are found in 11 genera and 6 families (Krenn & Kopp, 1998; Steyn & van Heerden, 1998). The family names have been adapted to the current taxonomic classification by the KEW world checklists of selected Plant Families (as of October 18th 2013).

Cardenolides

Plant Families	Genera
Ranunculaceae	*Adonis*
Moraceae	*Antiaris, Antiaropsis, Castilla, Dorstenia, Maquira, Naucleopsis, Ogcodeia, Streblus*
Brassicaceae	*Acachmena, Cheiranthus, Conringia, Erysium, Hesperis Syrenia*
Euphorbiaceae	*Mallotus*
Fabaceae	*Coronilla, Securigera*
Celastraceae	*Elaeodendron, Euonymus, Lophopetalum*
Malvaceae	*Corchorus, Mansonia*
Apocynaceae	*Acokanthera, Adenium, Anodendron, Apocynum, Asclepias, Aspidoglossum, Beaumontia, Calotropis, Carissa, Cerbera, Cryptolepis, Cryptostegia, Glossostelma, Gomphocarpus, Gongronema, Marsdenia, Melodinus, Menabea, Nerium, Pachycarpus, Pentopetia, Periploca, Pergularia, Plumeria, Roupellina, Strophantus, Tanghinia, Thevetia, Trachycalymna, Urechites, Vallaris, Xysmalobium*
Plantaginaceae	*Digitalis, Isoplexis, Penstemon*
Asparagaceae	*Convallaria, Polygonatum, Rohdea, Ornithogalum*

Bufadienolides

Plant Families	Genera
Crassulaceae	*Bryophyllum, Cotyledon, Kalanchoe, Tylecodon*,
Asparagaceae	*Drimia*,
Iridaceae	*Homeria, Moraea*
Melianthaceae	*Bersama, Melianthus*
Ranunculaceae	*Helleborus,*
Santalaceae	*Thesium*

Bufadienolides have been recordet from only 11 genera of 6 plant families but have been proved in only few animal families. In the animal kingdom, these substances are most wiespread in toads such as Bufonidae but also occur in snakes, fireflies, and other insects (Krenn & Kopp, 1998; Steyn & van Heerden, 1998).

Cardenolides on the other hand have a larger distribution among a total of 62 genera in 10 plant families (Malcolm, 1991; Luckner & Wichtl, 2000; Hänsel & Sticker, 2007) which have been found in a wide range of habitats among the world and particularly in tropical and temperate regions (Agrawal *et al.* 2012). The most prominent occurrence of cardenolides is reported from the dogbane family Apocynaceae (including the former Asclepiadaceae) which produce cardenolides as an abundant toxic principle (Agrawal *et al.* 2012). In general, cardinolides are an important class of naturally occurring drugs whose actions include both beneficial and toxic effects on the heart in vertebrates and insects as neurotoxins (Scholz & Schmitz, 1984; Langford & Boor, 1996; Malcolm, 1991; Wink, 2009). As highly specifically acting substances, CGs are potent and highly selective inhibitores of the ubiquitous membrane-bound enzyme Na/K-ATPase, which is responsible for the establishment and maintenance of the osmotic balance of cells (Hansen, 1984). This enzyme transports 3 Na^+ ions in exchange for 2 K^+ ions against the electrochemical gradients existing across the plasma membrane by utilizing ATP as driving force and is therefore also called the sodium pump. It exists as a heterodimer and is composed primarely of a large catalytic α-subunit and a smaller glycosylated β-subunit (Lingrel *et al.,* 1990). The α-subunit consists of 10-transmembrane (M1 to M10) domains and five extracellular loops. Several amino acids in the first, second and third extracellular loop form a highly conserved cardenolide-binding pocket (the target site) that mediates sensitivity to inhibition by cardiac glycosides (Laursen *et al.,* 2013).

Insects in at least five different orders show adaptations to this class of compounds, which become ingested during the feeding process and further they use cardenolide-rich plants as primary hosts (Després *et al.,* 2007; Opitz & Müller, 2009; Dobler *et al*., 2011). Well documented examples of insects which sequester these dietary compounds are especially known from Lepidoptera: Danaidae (Parsons, 1965; Nishida, 2002), Coleoptera: Chrysomelidae (Dobler *et al*., 1998), Heteroptera: Lygaeidae (Von Euw *et al*., 1971; Duffey & Scudder, 1972; Scudder & Duffey, 1972; Moore & Scudder 1985), Orthoptera: Pyrgomorphidae (von Euw *et al.,* 1967) and Homoptera: Aphididae (Rothschild *et al*., 1970).

These bitter tasting and emesis provoking plant compounds are powerful drugs because the toxins can be used as an extremely effective defence to realize their impact on the next trophic level (Malcolm, 1991; Rowell-Rahier & Pasteels, 1992). The best-known example for the defensive use of cardenolides is given by Brower's classic demonstration of

a blue jay eating a toxic monarch butterfly and subsequently vomiting up its prey (Brower, 1969).

Similar to the monarch butterfly (*Danaus plexippus* (Linnaeus, 1758)) pioneering studies have shown that the large milkweed bug, *Oncopeltus fasciatus* (Dallas, 1852) is also able to store high concentrations of bitter-tasting and toxic chemicals from their host plants (Duffey & Scudder, 1972; Scudder and Duffey, 1972; Duffey and Scudder, 1974; Isman *et al*., 1977; Scudder *et al.,* 1986). As a common adaptation, both species possess Na/K-ATPases with has a strongly lowered binding affinity towards cardenolides caused by an altered form of the target site. This phenomenon called target site insensitivity is at least partially due to an amino acid substitution of asparagine for histidine at position 122 in the first extracellular loop of the Na/K-ATPase (Holziger *et al*., 1992; Holziger & Wink, 1996, Dobler *et al.,* 2012; Zhen *et al.,* 2012). Target site insensitivity in general is a rarely observed resistance mechanism in insects and could also be shown in the grasshopper *Poekilocerus bufonius* (Al-Robai *et al.,* 1990) and in *Chrysochus* leaf beetles (Labeyrie & Dobler, 2004). Within the Lygaeinae, in addition to *O. fasciatus* also the small milkweed bug (*Lygaeus kalmii* Stål, 1874) encounters dietary cardenolides in its host plants and is known to possess a altered Na/K-ATPase which confer a lowered binding affinity to cardenolides (Zhen *et al*., 2012).

Some of the larger species of Lygaeinae are called “milkweed bugs” which reflects their worldwide common association with plants of the milkweed family Apocynaceae which is known to comprise many plant genera containing cardenolides (Scudder & Duffey, 1972; Agrawal *et al.*, 2012). Lygaeinae are the most diverse lygaeid subfamily with 57 genera and 640 species (Slater & O’Donnell, 1995) and mainly characterized by a bright coloration in red, orange and yellow combined with a black pattern. While the bug family Lygaeidae are cryptic coloored and are generally ground dwelling seed feeders, specimens within the Lygaeinae are mostly found on the reproductive parts of plants where they are predominantly seed feeders of conspiciously placed plant seed pods.

Scudder & Duffey (1972) published a list of host plants of selected lygaeid species, which demonstrates that the majority of Lygaeinae including *O. fasciatus* and *L. kalmii* seem to be closely associated with apocynaceous plants. This plant family, occurring in temperate and subtropical regions is known to be a rich source of cardenolides (Tab. 1) (Burrows & Tyrl, 2013). Species such as *O. fasciatus* and *L. kalmii* which can be found on *Apsclepias* species, *Cosmopleurus fulvipes* (Dallas, 1852) on *Calotropis* or *Caenocoris nerii* (Germar, 1847) which is found and feeds almost exclusively on *Nerium oleander,* an apocynaceous shrub (Fig. 3), in fact appear to use apocynaceous plants throughout their life cycle (von Euw *et al*., 1971; Feir & Suen, 1971).

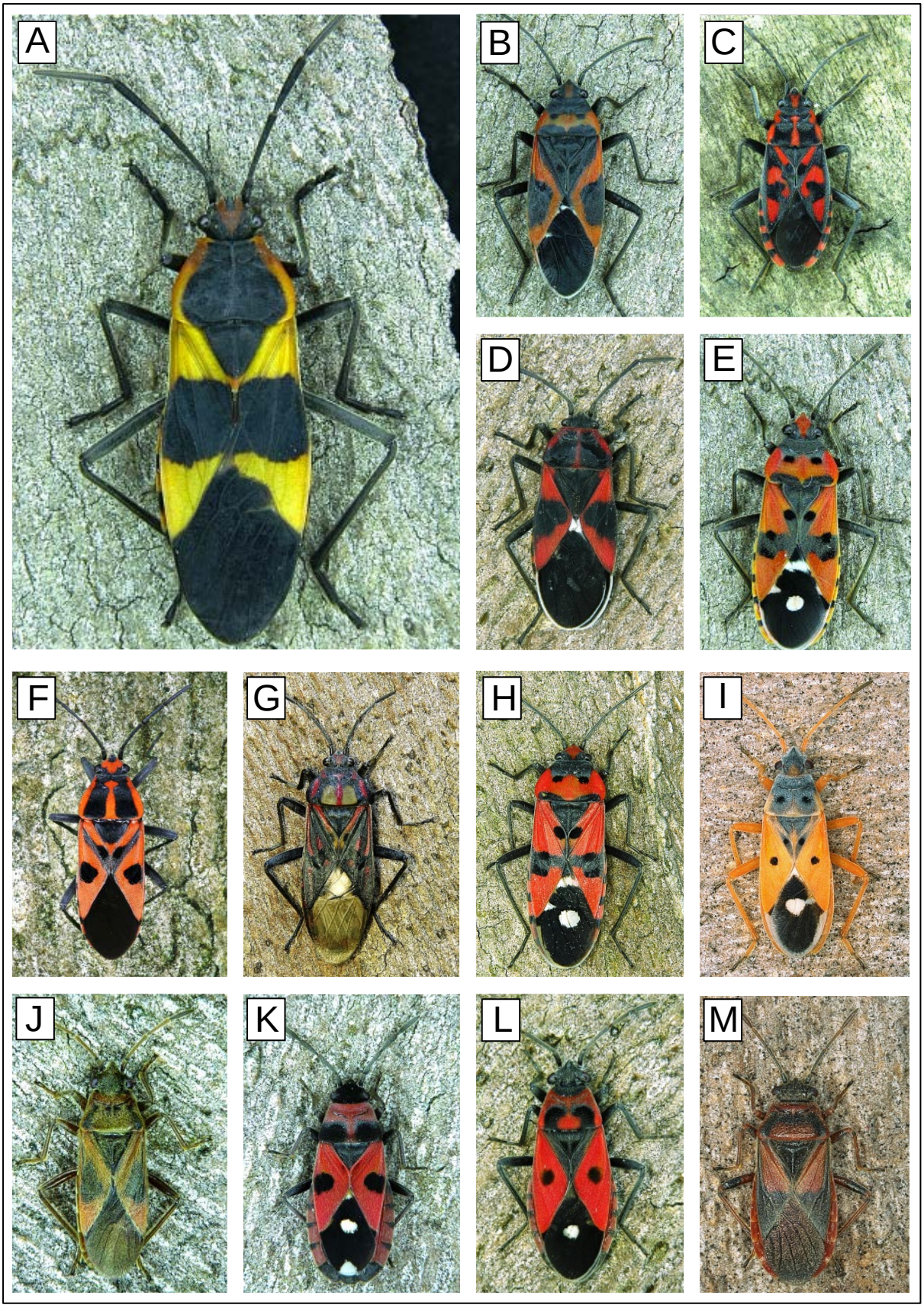

Figure 2..13 typically colored Lygaeinae representing 9 genera of this subfamily; (A) *Oncopeltus fasciatus* (B) *Lygaeus kalmii* (C) *Spilostethus saxatilis* (D) *Tropidothorax leucopterus* (E) *Lygaeus equestris* (F) *Spilostethus hospes* (G) *Spilostethus pandurus* (H) *Lygaeus simulans* (I) *Cosmopleurus fulvipes* (J) *Arocatus longiceps* (K) *Horvathiolus superbus* (L) *Melanocoryphus albomaculatus* (M) *Arocatus melanocephalus*

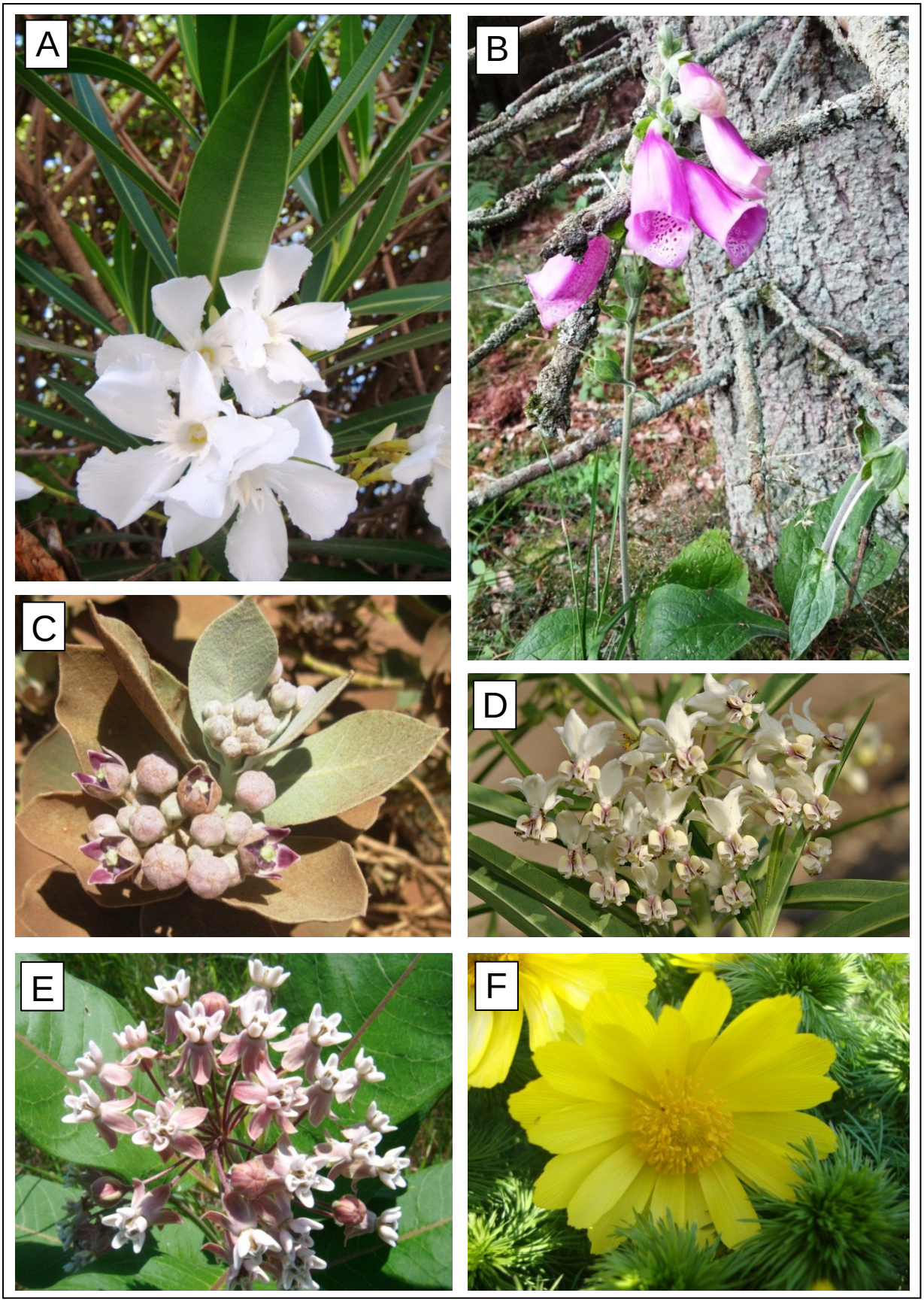

Figure 3. Common cardenolide-rich plant species used as host plants by several species of lygaeinae. (A) *Nerium oleander*; Apocynaceae, (B) *Digitalis purpurea*; Plantaginaceae, (C) *Calotropis procera*; Apocynaceae, (D) *Gomphocarpus physocarpus*; Apocynaceae, (E) *Asclepias syriaca*; Apocynaceae, (F) *Adonis vernalis*; Ranunculaceae

It is conspicuous however, that a few species within the subfamily use host-plants which belong to distantly related families of the Apocynaceae but do contain cardenolides (Fig. 2) (Winkler & Wichtl, 1985; Wichtl & Junior, 1977; Burrows & Tyrl, 2013). Examples include *Lygaeus equestris* (Linnaeus, 1758) on *Adonis vernalis* (Ranunculaceae) (Solbreck & Kugelberg, 1972; Deckert, 2007) or *Horvathiolus superbus* (Pollich 1781) on *Digitalis* (Plantaginaceae) (Aukema *et al*., 2005; Wachmann *et al.* 2007). Whereas the majority of the Lygaeinae is specialized to feed on few closely related plant species belonging to a single plant family only a few species use plants of further families however species similar in one or more properties such as biochemistry. Such prevailing host specificity on cardenolide containing plants (illustrated in Tab.1.and Fig.3.) suggest that the Lygaeinae probably expanded their host range by following similar plant secondary chemistry.

Ehrlich and Raven's essay (1964) on coevolution proposed that plant species that evolve a novelty, purchased as defence against herbivory and radiate into diverse species share the similar chemistry. In parallel, insects developed counteradaptations to this highly effective chemical defence and therefore they are able to use a range of the same chemically distinctive plants (Futuyma & Agrawal, 2009). This proposal has stimulated work on the physiological, genetic and ecological mechanisms of insect-plant interactions, focused especially on the role of plant secondary chemistry. According to this idea here, we studied the evolution of resistance to host plant toxins and their use as defense chemicals which are likely to form coadapted strategies in the hemipteran subfamily Lygaeinae using a comparative approach combining systematic, genetic, physiological and morphological methods.

Aposematically colored bugs of the Lygaeinae are not only adapted to feed on previously mentioned host plants, further several species have been shown to sequester or contain (dried museum specimens) cardenolides (Scudder & Duffey, 1972) in their bodies. Such uniform host specificity and the presence of an altered Na/K-ATPase published already for two lygaeid species appears to be likely that target site insensitivity is an apomorphic feature of the subfamily Lygaeinae. Therefore, the major objective of this thesis was to elucidate the evolutionary history of adaptations to cardenolides which enable species of the hemipteran subfamily Lygaeinae handling and utilization of host plant toxins. Based on an established molecular phylogeny of this subfamily, here we interpret using a comparative approach the occurrence and the efficiency of adaptations among the different genera focused on molecular, physiological, biochemical, morphological and ecological aspects.

One aim of this study was by focusing on selected species, to explore the sequestration process of cardenolides in terms of the quantity and polarity range and to determine the phylogenetic origin of this ability (Chapter 1).

It has been well documented, that *O. fasciatus* sequesters cardenolides at any stage of theirs life history (Scudder & Duffey, 1972; Duffey *et al*., 1978; Scudder *et al*., 1986) and tolerates effectively large amounts of cardenolides from a variety of its *Asclepias* host plants (Malcolm, 1991). Undoubtedly, this bug, altogether, can store about 317 µg cardenolides which is up to 6.5 times more than found in monarch butterflies (Duffey & Scudder, 1972; Malcolm, 1991). However, it was shown that individual cardenolides are selectively sequestered in *O. fasciatus*. Therefore, in a comparative approach I analyzed the uptake (including accumulation) of nine Lygaeinae using two purified cardenolides, which represent a polarity range of these toxic compounds present in the natural died of the insects.

The presence of cardenolides in body parts of sequestering species necessitates mechanisms to prevent intoxication. One possible mechanism as mentioned previously consists in a modification of the target site in the Na/K-ATPase that has a strongly lowered binding affinity towards cardenolides. How sequestering Lygaeinae avoid cardenolide intoxication is demonstrated by our data from a second approach (in Chapter 1) in which we tested the inhibition of Na/K-ATPase activity under ouabain stress. Further, I investigated whether target site insensitivity as a common adaptation, evolved at the basis of this hemipteran group (Chapter 1). Moreover, my study emphasizes the close association with apocynaceous hosts and reveals that this is the original one whereas the use of other plants as cardenolide sources is a derived trait.

It is interesting to note that individual cardenolides are selectively sequestered in *O. fasciatus*, whereas intermediate and more polar toxins were highly concentrated in the insect which disagree with the cardenolide profile exactly as it occurs in its host plants (Duffey & Scudder, 1974; Isman *et al.,* 1977; Scudder *et al.,* 1986). Further experiments suggest a selective permeability of the gut, whereby the uptake of nonpolar cardenolides than to polar is favored (Yoder *et al*., 1976; Scudder & Meredith, 1982b). In *O. fasciatus* there was found to be a high concentration of polar- and an absence of apolar cardenolides in general (Duffey & Scudder, 1974; Yoder *et al.,* 1976; Duffey *et al.,* 1978; Moore & Scudder, 1985). The very polar compounds detected represent products arising from metabolic alteration in the species. The ability to metabolically alter cardenolides has only been shown for *O. fasciatus* after ingestion of the non-polar digitoxin, which was changed to at least two polar products, yet polar ouabain appeared to be unaltered by the insect (Duffey & Scudder, 1974; Scudder & Merredith, 1982b).

I was therefore interested in investigation how Lygaeianae in particular *O. faciatus* handle different polar cardenolides (Chapter 2). It was of interest to examine the metabolism

process in *O. fasciatus* in detail and to compare this feature with several species of the cardenolide adapted subfamily. Furthermore, I tried to elucidate the mechanism and to take conclusions by which certain lygaeinae are able to handle glycosides because toxicity has been correlated with their polarity.

In order to utilize sequestered CGs as chemical deterrence to predators, species have to store the sequestered amounts in the haemolymph, cuticle, specialized tissues or glands. *O. fasciatus* accumulates considerable quantities (1000 times more concentrated than in the body) of cardenolides in specialized areas which are called as dorsolateral space (Scudder & Meredith, 1982a; Scudder *et al.,* 1986). This storage compartment is a specialized layer of the epidermis where sequestered cardenolides get enriched (Scudder *et al.* 1986). It could demonstrate that this structure is located primarily in the lateral parts of the integument in the mesothorax and metathorax as well in the sterna II to VII of the abdomen (Scudder & Duffey, 1972). When the bug is squeezed, stored cardenolides will be expressed as discrete droplets of fluid issue at precise points along the dorsolateral margins of the thorax and abdomen.

Primarily the study in Chapter 3 was undertaken to clarify the presence and the distribution of a glycoside storage compartment in several Lygaeinae. By mapping the observed morphological features on a recent phylogeny of the Lygaeinae we here report that the adaptation for storage and release of plant compounds evolved in a stepwise manner. Moreover, the study represents a detailed description of the adult cardenolide storage compartement in the thoracic region of the lygaeid species *O. fasciatus.*

The repellent droplets, released out of the storage compartment are located on aposematic areas where they can be immediately sighted by a predator (Scudder & Duffey, 1972; Scudder *et al*., 1986). Cardenolides generally have a strong deterrent effect as antipredatory defences in vertebrates as has been demonstrated for blue jays (*Cyanocitta cristata*), quails (*Coturnix coturnix*), mice and bats (Brower *et al.,* 1967; Brower, 1969; Evans *et al.,* 1986; Glendinning, 1990; Hristov & Conner, 2005). To our knowledge, there are only few experimental studies addressing the protective effect of sequestered cardenolides on arthropod predation (Levey, 1983; Berenbaum & Miliczky, 1984; Malcolm, 1989; Petschenka *et al*., 2011).

The study in Chapter 4 was performed to investigate to what extent predators are affected by their prey's food. To investigate whether *O. fasciatus* gained improved protection by feeding on the toxic host plant vs. the nontoxic alternative we used naive orb-weaving *Nephila senegalensis* (Araneae: Nephilidae) as predators.

Chapter 1

Evolution of resistance traits: How Lygaeinae (Heteroptera, Lygaeidae) cope with toxic host plant cardenolides

Abstract

Insect host-plant utilization and the way how these herbivores cope with a stunning array of toxic secondary plant compounds is one of the best studied species interactions. Sequestration of unpalatable or toxic plant substances is a widespread strategy to enhance the insect's own protection against predation. However, this strategy has to rely on the insect's insensitivity to the toxins. The large milkweed bug *Oncopeltus fasciatus* (Heteroptera; Lygaeinae) is a well known example for being adapted to the defensive traits of its milkweed host plants (Apocynaceae). One of the most conspicuous adaptations consists in a Na/K-ATPase which has a reduced binding affinity for cardenolides (target site insensitivity). Besides *O. fasciatus* nearly all members of the subfamily Lygaeinae are associated with apocynaceous plants, and hence the black and red warning coloration occurring in this taxon also appears to hint to the use of cardenolides for defensive purposes. In this study we investigate the resistance traits the Lygaeinae evolved as adaptations to host plant cardenolides. Furthermore, in a comparative approach across the subfamily we examined the uptake of cardenolides by feeding assays with two pure compounds. As phylogenetic backbone we constructed a molecular phylogeny which enabled us to determine the origin of adaptations. Molecular investigations and physiological studies indicate that target site insensitivity towards cardenolides is a common feature in all Lygaeinae. Given that all tested species but the European *Arocatus*, which do not encounter cardenolides in their host plants, are able to sequester cardenoldies, we assume that this trait is a basal feature in the subfamily, too.

Introduction

Insect resistance to host plant toxins is a central issue in studying the macroevolution of plant-herbivore interactions and can only be satisfactorily understood by the integration of macroevolutionary pattern with evidence from functional, genetic and ecological approaches (Futuyama & Agrawal, 2009). The use of distantly related host plants through chemically similar properties may is a result of common adaptations to the defence plant chemistry in the hemipteran subfamily Lygaeinae. Therefore, a phylogenetic reconstruction of ancestral adaptations and resistance traits, using a comparative approach combining systematic, genetic and physiological methods may reveal the evolution of coadapted strategies in the Lygaeinae.

The best investigated representative of the Lygaeinae is the large milkweed bug (*Oncopeltus fasciatus*) which is specialized on host plants in the genus *Asclepias* (Apocynaceae) that contain moderate to high concentrations of toxic cardenolides (cardiac glycosides, CGs) (Agrawal *et al.,* 2012). These bitter tasting and emetic plant compounds act in vertebrates and insects as neurotoxins (Malcolm, 1991; Wink, 2009). As highly specifically acting substances, CGs binds to the Na/K-ATPase and inhibits its action. Like the monarch butterfly (*Danaus plexippus*) (Vaughan & Jungreis, 1977; Holzinger & Wink, 1996), *O. fasciatus* possesses a modified form of the Na/K-ATPase showing reduced sensitivity to cardenolides (target site insensitivity, Moore & Scudder; 1986). Moreover, *O. fasciatus* sequesters cardenolides from *Asclepias* seeds and stores the toxins for its own defense against predators (Scudder & Duffery, 1972; Scudder *et al.,* 1986). Both adaptations are likely to be involved in interactions across three trophic levels as they allow *O. fasciatus* a) to use *Asclepias* seeds as a food source and b) to store these toxins in the body and use them as a defense against predators (Berenbaum & Miliczky, 1984; c. f. Chapter 4).

The phenomenon of cardenolide sequestration, per se, involves the uptake, transfer to, and concentration of cardenolides in the storage compartments where the toxins are preserved to be used as antipredator defence. Physiological studies on permeability of insect guts suggest that polar cardenolides require an energy dependent transport and presumably intestinal carriers in the gut epithelium which allow polar cardenolides to enter the haemolymph (Nickisch-Rosenegk *et al.,* 1990; Frick & Wink, 1995). Conversely, apolar cardenolides can be expected to cross the gut passively due to their physiological properties (Wright, 1960). Cardenolides taken up from the gut are accumulated in a modified integument, a specialized layer of the epidermis where sequestered cardenolides get enriched (Scudder & Meredith, 1982a; c.f. Chapter 3).

Approximately 80 % of the Lygaeinae from at least five continents use plants of the milkweed family Apocynaceae as primary hosts (Scudder & Duffey, 1972). Whereas the

majority is specialized to feed on few closely related plant species belonging to a single plant family, only a few species of the aposematically colored bugs utilize further families but with the same chemically properties. Moreover, several species have been shown to sequester or contain (in dried museum specimens) cardenolides (Scudder & Duffey, 1972). Gene sequence analyses suggested that in addition to *O. fasciatus*, the small milkweed bug (*Lygaeus kalmii*) also possesses a Na/K-ATPase with a reduced sensitivity to cardenolides (Dobler *et al.,* 2012; Zhen *et al.* 2012). This, together with the typical black and red aposematic coloration, render it likely that the use of sequestered cardenolides as defensive compounds and the possession of target site insensitivity of Na/K-ATPase are basal features of this hemipteran group. In a comparative approach we tested 10 species of the Lygaeinae for the coadapted traits target site insensitivity and cardenolide sequestration. The parallel construction of a molecular phylogeny of 20 Lygaeinae and 4 outgroup species (2 lygaeids, one species each of Oxycarenidae and Pyrrhocoridae) enabled us to address the following questions: 1) is the ability to tolerate and to sequester cardenolides a basal feature of the Lygaeinae, 2) if basal, are target site insensitivity and cardenolide sequestration then maintained in species which no longer have to cope with cardenolides, and 3) is the secondary use of non-apocynaceous cardenolide plants like *Adonis vernalis* (Ranunculaceae), *Digitalis purpurea* (Plantaginaceae) or *Urginea maritima* (Asparagaceae) likely due to a preadaptation to cardenolides?

To approach these questions we performed a comparative analysis of sequestration of two radioactively labeled cardenolides, which represent a polarity range of toxic compounds present in the natural diet of the insects. Through *in vitro* spectrophotometric assays we tested nervous tissues of seven Lygaeinae and one outgroup species for target site insensitivity against the cardenolide ouabain.

Mutations conferring resistance to cardenolides were shown only in a few studies (Holzinger *et al.,* 1992; Labeyrie & Dobler, 2004; Dobler *et al.,* 2012; Zhen *et al.,* 2012; Petschenka *et al.,* 2013) demonstrating that due to specific amino acid substitutions several insect species are resistant to ouabain. In the present study, we also sequenced Na/K-ATPase genes of 11 species of Lygaeinae (plus 2 outgroups) and analyzed the resulting amino acid sequences for critical substitutions to understand the molecular basis of resistance.

Our integrative analysis of two probably coadapted traits in a phylogenetic framework affords reconstructing the evolution of adaptations to cardenolides in the Lygaeinae.

Material and Methods

Specimens for sequestration assays and in vitro analysis of Na/K-ATPase

Adults of several species of the Lygaeinae were obtained both from the field and from laboratory cultures. As outgroups we used species of the families Phyrrhocoridae, Berytidae and Lygaeidae (Ischnorhynchinae). For feeding assays the species were reared in the laboratory from the egg stage on a diet of husked sunflower seeds (*Helianthus annuus* L.) and water. Colonies were kept under artificial light at a 16 h/8 h light/dark cycle at 26°C (*C. fulvipes, H. superbus, O. fasciatus, S.pandurus*) or 30°C (*L. equestris, L. kalmii, L. simulans*).

All further species including *Arocatus longiceps*, *A. melanocephalus, Kleidocerys resedae* (Lygaeidae)*, Metatropis rufescens* (Berytidae), and *Pyrrhocoris apterus* (Pyrrhocoridae) were field collected individuals. *A. longiceps* (Berlin, Germany) and *A. melanocephalus* (Kallinchen, Germany) were maintained on sunflower seeds and seeds of *Platanus* (2 days) under ambient conditions (*A. l.*) or at 26°C (*A. m.*, 16 h/8 h light/dark cycle). *K. resedae*, *M. rufescens,* and *P. apterus* (Hamburg/Hamburg/Straupitz, Germany) were maintained on sunflower seeds (26°C, 16 h/8 h light/dark cycle) (2-3 days).

Tropidothorax leucopterus (Grießheim, Germany) was reared on sunflower seeds and cut branches of *Vincetoxicum hirundinaria* (26°C, 16 h/8 h light/dark cycle).

Sequestration Assay

To assess the ability to sequester cardenolides, we fed 9 species of the Lygaeinae of 6 genera (see Tab.1.) and the three outgroup species (*K. resedae*, Lygaeidae; *M. rufescens*, Berytidae; and *P. apterus*, Pyrrhocoridae) with radioactively (^{3}H) labeled cardenolides.

As plants typically produce several cardenolides with a wide polarity range we used the polar [^{3}H]-ouabain and the relatively apolar cardenolide [^{3}H]-digoxin (both Perkin Elmer LAS GmbH, Rodgau, Germany). Both cardenolides which probably do not occur in the natural host plants of Lygaeinae were used due to their commercial availability. Individuals were immobilized with a lasso made of dental floss (see insert in Fig. 2) and their proboscis was manually introduced into a droplet (2 µl) of 5% sucrose solution containing 5 µM ^{3}H-cardenolide dissolved in ethanol on parafilm (final concentration of ethanol 17.7%). After feeding, specimens were kept for 10 days at 26°C and supplied with water and sunflower seeds ad libitum to allow for gut clearance of cardenolides. The incubation period of 10 days was chosen as we observed that keeping treated individuals for only 3 days before analysis

produced less reliable results (see supplemental figure 1 and discussion) which might be due to incomplete gut clearance of cardenolides. After 10 days, individuals were frozen in liquid nitrogen and homogenized with a pestle (glass or stainless steel). To evaluate stored cardenolides, samples were extracted with 1 ml methanol (3 x) by vortex stirring. After centrifugation, an aliquot (200 µl) of the pooled supernatants was added to 3 ml scintillation cocktail (Ultima Gold, Perkin Elmer) to quantify radioactivity (amount of [^{3}H]-cardenolide taken up) with a liquid scintillation counter (Wallac 1409). In addition, the residual radioactivity on the Parafilm used as feeding support and the radioactivity of the drinking solution (2 µl aliquots) were determined to calculate the percentage of radioactivity actually stored by the hemipteran specimens. To do so, the residual radioactivity (in disintegrations per minute (dpm)) on the parafilm support was subtracted from the initial total radioactivity of the drinking solution (= total amount ingested by the hemipteran specimen). Dpm values obtained by extraction of the specimens were then divided by these values to calculate the percentage of cardenolides stored after 10 days. All feeding experiments (each species was tested for ouabain and digoxin, separately) were repeated 3 to 13 times (see Fig. 2 for number of replications) using individual specimens.

DNA sequences for molecular systematics

Our taxonomic sampling included twenty species of *Lygaeinae* selected to represent the presumed phylogenetic breadth within this subfamily. Some species were represented by more than one individual (indicated by Roman numerals). In addition, DNA was sequenced from *K. reseda* and *Belonochilus numenius,* species belonging to closely related taxa (Ischnorhynchinae and Orsillinae) and *P. apterus* and *Oxycarenus lavaterae* (Pyrrhocoridae and Oxycarenidae) as representatives of distant relatives (Henry, 1997) (Tab.1). The target sequences were 1714bp from the 3' half of the mitochondrial genes for *cytochrome oxidase subunit* I and II (COI/II) including the intermittent tRNA leucine gene ($tRNA^{Leu}$) and 507bp of the large nuclear ribosomal subunit (28S rDNA). DNA extraction was performed using either the Qiagen DNeasy Tissue kit or a DNA extraction system for dry museum material described by Gilbert *et al.,* (2007). The target sequences were amplified by standard polymerase chain reaction (PCR) protocols. To generate homologous sequences for the 28S rDNA fragment, we used the primers described by Muraji & Tachikawa, (2000). Amplification of the target gene region COI/II was achieved by amplifying two or three smaller overlapping fragments using primers previously reported by Maus *et al.* (2001) and Weller *et al.,* (2004). Complementary strands of a single individual were edited and aligned using Sequencer 4.6 (Gene Codes Corporation, Ann Arbor, MI).

Table 1. List of species used in the different treatments. The coloured names represent the species used as outgroup, members of the Lygaeidae (green), Oxycanedidae (orange), Pyrrhocoridae (blue).

Species	Phylogeny	Sequestration	Assays	Sequencen
Kleidocerys resedae	X	X	X	X
Belonochilus numenius	X			
Oxycarenus lavaterae	X			
Arocatus longiceps I	X			
Arocatus longiceps II	X	X	X	X
Arocatus longiceps III	X			
Arocatus aenescens	X			
Arocatus melanocephalus I	X	X		X
Caenocoris nerii	X			X
Cosmopleurus fulvipes	X	X		X
Graptostethus izzardi	X			
Graptostethus servus	X			
Haemobaphus concinnus	X			
Horvatiolus superbus	X		X	X
Lygaeus equestris I	X			
Lygaeus equestris II	X	X	X	X
Lygaeus kalmii I	X			
Lygaeus kalmii II	X	X	X	X
Lygaeus simulans I	X			
Lygaeus simulans II	X	X		
Melanocoryphus albomaculatus	X			X
Metatropis rufescens		X		
Oncopeltus fasciatus I	X			X
Oncopeltus fasciatus II	X	X	X	
Spilostethus hospes	X			
Spilostethus macilentus	X			
Spilostethus saxatilis I	X			
Spilostethus saxatilis II	X			
Spilostethus pandurus I	X	X		
Spilostethus pandurus II	X		X	
Spilostethus pandurus III	X			X
Tropidothorax leucopterus I	X	X		
Tropidothorax leucopterus II	X		X	X
Tropidothorax leucopterus III	X			
Pyrrhocoris apterus	X	X		X

Phylogenetic analyses

Phylogenetic reconstructions were carried out using maximum likelihood (ML) analysis and Bayesian inference. Prior to likelihood analyses, best-fit models of nucleotide substitution were selected with likelihood ratio tests as implemented in Modeltest v3.7 (Posada & Crandall, 1998). Models of evolution and parameters were estimated for each gene position separately. ML analyses was performed with TreeFinder (Jobb, 2008) using the model of

sequence evolution (GTR+G) for all partitions. Tree searches were started from five trees derived by a random walk of 10 nearest neighbour interchange (NNI) steps around a center tree (neighbour joining tree) generated by PAUP. The robustness of the ML tree was evaluated by bootstrap analyses with 1000 replicates using the same program.

Bayesian analysis was conducted with MrBayes v3.0b4 (Huelsenbeck & Ronquist, 2001), fitting a GTR+I+G model to each of the four data partitions. Two independent runs were carried out with four parallel Markov Chain Monte Carlo (MCMC) chains of 1 million generations and trees sampled every 200 generations.

The topologies derived from ML searches were evaluated under the fourfold GTR+G model with parameters estimated from each tree and compared by the approximately unbiased (AU) test (Shimodaira, 2002) as implemented in Treefinder using 50000 RELL bootstrap replicates.

In vitro assay of Na/K-ATPase

To test for the occurrence of target site insensitivity to cardenolides we assayed Na/K-ATPase of 7 Lygaeinae and one lygaeid outgroup (Tab.1) *in vitro*. Na/K-ATPase assays were performed as described in Petschenka *et al.* (2013). Briefly, brains and thoracic ganglia of hemipteran specimens (killed and stored at -80°C) were dissected under deionized water, pooled (see supplemental Table. 2 for numbers of individuals used), and homogenized in deionized water (500 µl) using an all glass grinder (Wheaton). Extracts were frozen at -80°C, lyophilized and stored frozen until use. Prior to assay, lyophilisates were reconstituted by adding 1000 µl water, vortex stirring and incubation for 10 min in a chilled ultrasonic bath. Undissolved residues were removed by centrifugation at 5,000 x g (3 min). Cardenolide sensitivity of Na/K-ATPase was determined by quantification of inorganic phosphate released from ATP by Na/K-ATPase at different concentrations of ouabain (a water soluble cardenolide widely used in biochemical studies) over a period of 20 min at 37°C. To test for linearity of P_i release over the period of incubation we measured a time course of P_i release (reactions stopped after 0, 5, 10, 15, and 20 min) using a Na/K-ATPase preparation of *O. fasciatus* at all reaction conditions and found that P_i release was always linear (see Petschenka *et al.,* 2013). Linearity under all conditions ensures that ouabain inhibition curves are not biased by non-linear Pi release over time under different incubation regimes.

Sequencing of Na/K-ATPase genes

For amino acid substitution screening we analyzed the nervouse tissues of 11 different species of the Lygaeinae (Tab.1) and two outgroup species (*K. resedae,* Lygaeidae and *P. apterus,* Pyrrhocoridae). The nervous tissue was dissected in RNA later (Qiagen, Hilden, Germany) or water and homogenized. Sequencing of Na/K-ATPase genes was performed as described in Petschenka *et al.* (2013). Sequence fragments were assembled with Sequencer 4.6 (Gene Codes., Ann Arbor, MI) and compared with sequences deposited in GenBank using the BLAST algorithm.

Results

Phylogeny of Lygaeinae

Analysis of the combined dataset

The final 2221 bp alignment consisted of 1714 bp of the mitochondrial COI/II and $tRNA^{Leu}$ genes and of 507 bp of the nuclear 28S gene obtained for 35 individuals. The unconstrained maximum likelihood tree generated with a GTR+G model fitted to each gene partition (Fig. 1) and the tree derived from a Bayesian analysis did not differ, except for minimal conflicts in well supported clades with ML bootstrap values >50% and posterior probabilities (PP) >0.50.

Phylogenetic relationships

In all analyses, the subfamily Lygaeinae was recovered as a monophyletic group. Of its eleven genera, all individuals of the same species from different populations cluster together and are supported by high bootstrap values. The genera *Arocatus*, *Graptostethus* and *Spiostethus* with the inclusion of *Haemobaphus concinnus* are represented by several species and are recovered as monophyletic. Overall, three clusters including *Arocatus* + *Caenocoris*, *Graptostethus* + *Tropidothorax* and *Horvathiolus* + *Melanocoryphus* are recovered as monophyletic, too. In all analyses the subfamily Lygaeinae is split into two well supported sister groups. The smaller one consists of a monophyletic genus *Arocatus (A. aenescens, A. rusticus, A. longiceps* and *A. melanocephalus)* with *Caenocoris nerii* at the bottom of this clade. In the second major group, *Cosmopleurus fulvipes* appears with strong

support as the most basal lineage and sister group to all remaining Lygaeinae O. fasciatus is recovered as sister taxon of the monophyletic cluster *Horvathiolus* + *Melanocoryphus*. Their sister group relationship on the other hand, is less certain since in some reconstructions *Oncopeltus* + *Tropidothorax* form a monophyletic group while *Graptostethus* was recovered as sister group to *Horvathiolus* + *Melanocoryphus*. Of the two genera represented by most species, *Spilosthetus* and *Lygaeus*, all individuals cluster closely together, yet in both cases the genera are paraphyletic with respect to other species. In *Spilostethus, H. concinnus* is included and placed in between a basal *S. macilentus* and the remaining species. In the genus *Lygaeus* (*L. equestris* + *L. simulans*) are strongly supported as monophyletic by our data but the new world species *L. kalmii* is located on a branch basal to *Lygaeus* and *Spilostethus* and does not seem to belong to the same genus.

Host plant records

For a total of 20 specimens of the subfamily *Lygaeinae* positive host-plant information is available and included in Figure 1. The phylogenetic reconstruction indicated that the majority (80 %) of the analyzed *Lygaeinae* are associated with Apocynaceae (green) or use apocynaceous plants as at least one potential host. However, a few species of different genera feed on non apocynaceous host plants, too. Species with deviating host plants from other plant families are the following: *L. equestris* is adapted also to *Adonis vernalis* (Ranunculaceae), *H. superbus* as well as *M. albomaculatus* feed often on *Digitalis purpurea* (Plantaginaceae), *A. longiceps* is adapted to and feeds almost exclusively on *Platanus* spec. (Platanaceae) while *A. melanocephalus* is specialized on *Ulmus* spec. (Ulmaceae). Nearly all mentioned plant families contain cardenolides, except for *Platanaceae* and *Ulmaceae.* In general, our analyses did not examine whether these species are truly monophagous or use more than one plant species throughout their live cycle. Rather, we try to demonstrate that the use of apocynaceous plants seems to be a general phenomenon in the subfamily with the European *Arocatus* representing the only known exceptions.

Remarkably several species, i.e. *T. leucopterus*, *M. albomaculatus*, *S. saxatilis, L. simulans* and *L. equestris* in addition to the plant species mentioned above, are also closely associated with *Vincetoxicum hirundinaria* (Wachmann *et al.,* 2007), a host devoid of CGs (Dobler *et al.,* 1998). The first species in particular cannot survive without *V. hirundinaria* because it depends on this plant for growth and development.

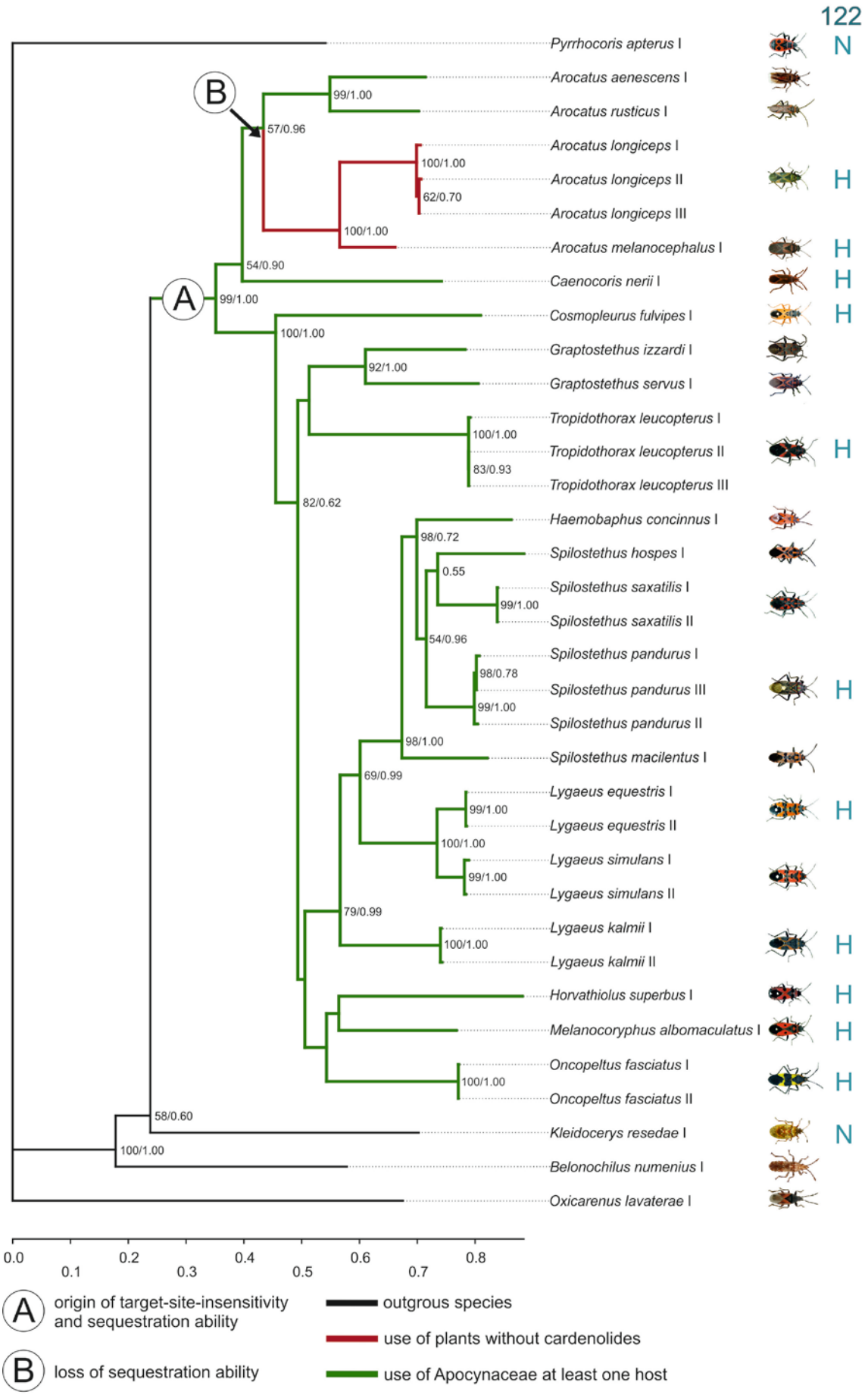
122
Pyrrhocoris apterus I
N
Arocatus aenescens I
Arocatus rusticus I
Arocatus longiceps I
Arocatus longiceps II
H
Arocatus longiceps III
Arocatus melanocephalus I
H
Caenocoris nerii I
H
Cosmopleurus fulvipes I
H
Graptostethus izzardi I
Graptostethus servus I
Tropidothorax leucopterus I
Tropidothorax leucopterus II
H
Tropidothorax leucopterus III
Haemobaphus concinnus I
Spilostethus hospes I
Spilostethus saxatilis I
Spilostethus saxatilis II
Spilostethus pandurus I
Spilostethus pandurus III
H
Spilostethus pandurus II
Spilostethus macilentus I
Lygaeus equestris I
H
Lygaeus equestris II
Lygaeus simulans I
Lygaeus simulans II
Lygaeus kalmii I
H
Lygaeus kalmii II
Horvathiolus superbus I
H
Melanocoryphus albomaculatus I
H
Oncopeltus fasciatus I
H
Oncopeltus fasciatus II
Kleidocerys resedae I
N
Belonochilus numenius I
Oxicarenus lavaterae I
99/1.00
57/0.96
100/1.00
62/0.70
100/1.00
54/0.90
99/1.00
100/1.00
92/1.00
100/1.00
83/0.93
82/0.62
98/0.72
0.55
99/1.00
54/0.96
98/0.78
99/1.00
98/1.00
69/0.99
99/1.00
100/1.00
99/1.00
79/0.99
100/1.00
100/1.00
58/0.60
100/1.00
A
B
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
A origin of target-site-insensitivity and sequestration ability
B loss of sequestration ability
outgrous species
use of plants without cardenolides
use of Apocynaceae at least one host

Figure 1. Maximum likelihood tree of the subfamily Lygaeinae based on the combined dataset of COI, COII, $tRNA^{Leu}$ and 28S genes. Values above branches indicate ML bootstrap support values (>50%) in 1,000 replicates. The black branches represent two lygaeid species from subfamilies other than the Lygaeinae (Ischnorhynchinae, Orsillinae) and *P. apterus* (Pyrrhocoridae) as well as *O. lavaterae* (Oxicarenidae) which were used as outgroups. The green branches indicate that the respective species use at least one apocynaceous plant as host whereas the red branches refer to a use of non apocynaceous plants as hosts. Capital letter A marks the evolutionary origin of target site insensitivity and cardenolide sequestration. Capital letter B displays the loss of the ability to sequester cardenolides. Evidence for sequestration was determined here or obtained from the literature. The single letter behind the bug pictures indicates the amino acids at positions 122 for each tested species (Histidine: H; Asparagine: N). Bug photographs illustrate representative species.

Amino acid substitutions in the Na/K-ATPase gene

Our current genetic screen of the Na/K-ATPase α subunit of 11 Lygaeinae and *P. apterus* (Pyrrhocoridae) as well as *K. resedae* (Ischnorhynchinae) as outgroup can only reveal with certainty the identity of the amino acid at position 122 of the protein. This position is well supported as decisive for cardenolide binding as has been previously shown for the monarch butterfly *D. plexippus* and other insects (Holziger & Wink, 1996; Labeyrie & Dobler, 2004; Dobler *et al.,* 2012; Zhen *et al.,* 2012; Dalla *et al.,* 2013). All members of the subfamily Lygaeinae investigated here possess an amino acid substitution of asparagine for histidine at position 122 (N122H) in the first extracellular loop of the Na/K-ATPase which is essential for ouabain binding. Both outgroup species however, had the conserved asparagine residue representing the original condition. Mapping the character states on the phylogeny of the Lygaeinae yields a uniform pattern of a resistance conferring substitution even in species which are not confronted with cardenolides due to a CG free diet.

Cardenolide sequestration in the Lygaeinae

The percentage of cardenolides stored by hemipteran specimens from orally delivered compounds (ouabain and digoxin) was determined based on the radioactivity recovered 10 days after ingestion of 2 µl 5 µM [^{3}H]-Ouabain (1.46 ng) or [^{3}H]-Digoxin (1.56 ng) (Figure 2). Seven out of nine Lygaeinae tested stored relatively high portions of ingested cardenolides. *A. longiceps* and *A. melanocephalus* contained the smallest amounts of both cardenolides (less than 6 % of the initial amount taken up orally). The ratios for digoxin (blue bars) in all other species ranged from 81.1 % to 93 %. Ouabain ratios in these species ranged from 51.3 % to 94.6 % with the exception of *T. leucopterus* which showed an exceptionally low value of 9.5 %, only. In two of the three outgroup species less than 2.1% of both compounds were recovered whereas *P. apterus*, the most distantly related outgroup species, still had 37 % of

the initially imbibed ouabain and 5.6 % digoxin. These results suggest that throughout the sequestering Lygaeinae (all species without *Arocatus*) digoxin as apolar cardenolide is favored over ouabain.

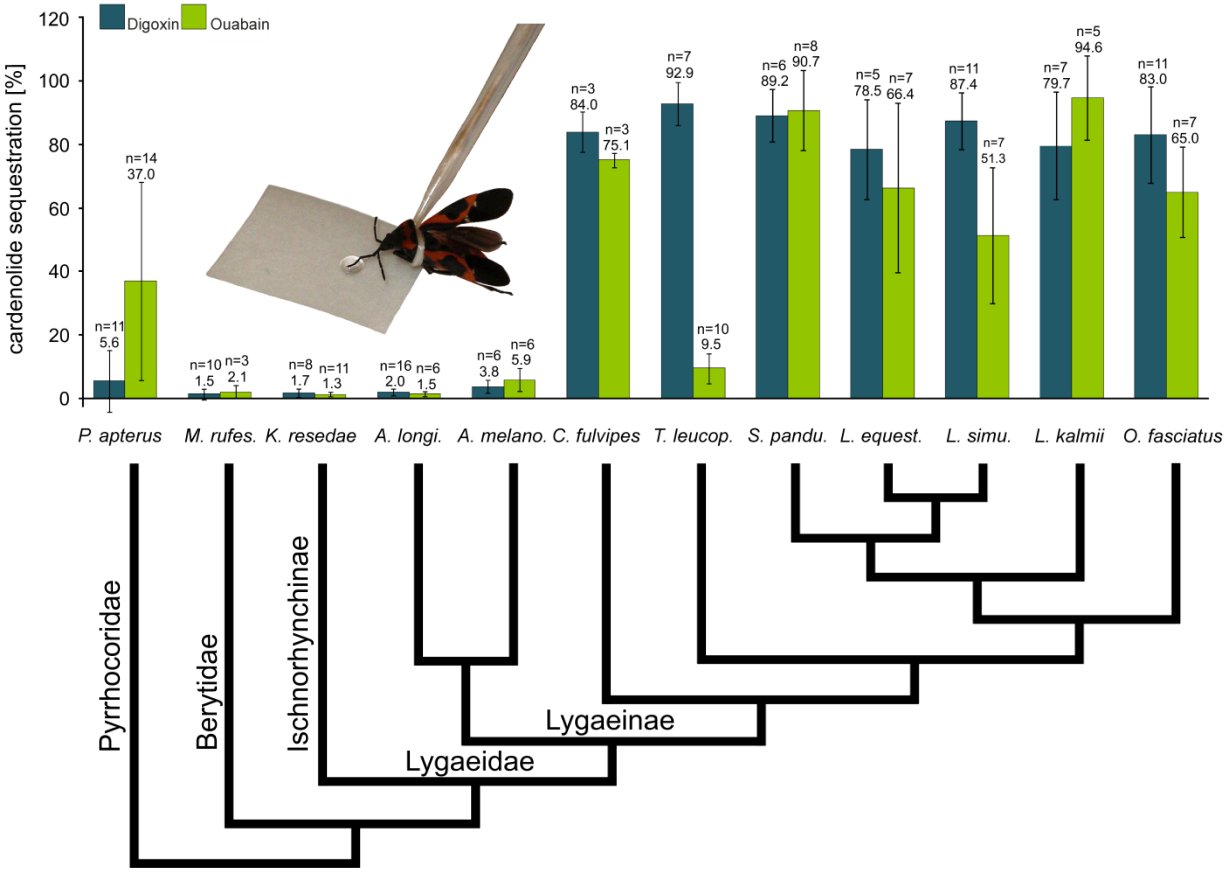

Figure 2. ^{3}H-cardenolide- content in 9 Lygaeinae and 3 outgroup species (Pyrrhoccoridae, Berytidae, Ischnorhynchinae 10 days after oral application. Bars indicate the proportion of stored cardenolides (digoxin = blue, ouabain = green). The total amount ingested was set to 100%. The picture of a feeding *L. kalmii* illustrates the method used to force the bug to drink the test solutions.

Ouabain resistance of the Na/K-ATPase in vitro

All 7 Lygaeine tested here (Tab. 1, Tab. 2) showed a very unique pattern of *in vitro* inhibition of Na/K-ATPase by ouabain (Fig. 3) that does not reveal differences between species. Lygaeinae Na/K-ATPase is almost not affected over three orders of magnitude of ouabain concentration (10^{-8} to 10^{-5} M) and still had 50 % activity at ouabain concentrations of less than 10^{-3} M. Specifically, we found that the Lygaeinae possess an extremely insensitive Na/K-ATPase with an average of IC_{80} of $2.46*10^{-4}$M. Instead of the usually indicated half minimal inhibitory concentration (IC_{50}) we used the IC_{80} because the enzymes of the Lygaeinae are not sensitive enough to determine the exact 50% inhibition concentration. The

individual IC_{80} values of all individuals are presented in Tab.2. Our analysis furthermore revealed a dramatic difference regarding sensitivity to ouabain in the outgroup species *K. resedae* (Ischnorhynchinae) compared to the Lygaeinae. The enzyme of *K. resedae* is highly sensitive and showed with an IC_{80} of $2.06*10^{-7}$M already an inhibition at ouabain concentrations as low as 10^{-7} M. Together with the Na/K-ATPase sequence data we conclude that target site insensitivity to cardenolides probably is a unique feature of all Lygaeinae.

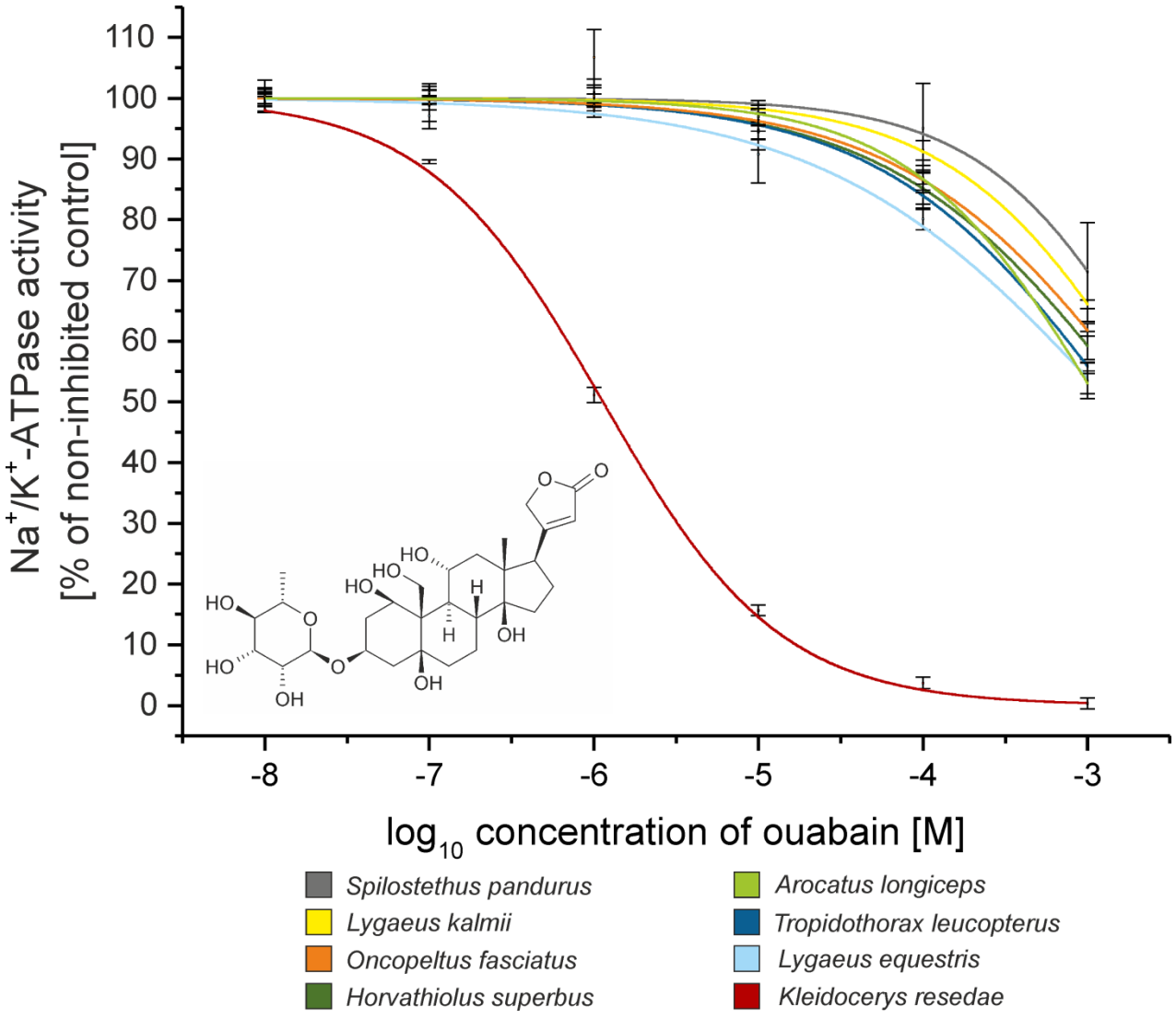

Figure 3. In vitro sensitivity of the Lygaeinae Na/K-ATPases to ouabain. Each curve illustrates the inhibition of the enzyme over a magnitude of six different ouabain concentrations (10^{-8} to 10^{-3} M). The single data points show the average of the replicates per species which are alos represented by different colors. For comparison the inhibition curve of *D. plexippus* Na/K-ATPase is included (data taken from Petschenka *et al*. 2013).

Table 2. Ouabain sensitivity of Na/K-ATPases of the investigated species. Number of replicates and mean IC_{80} values of ouabain are given. IC values were calculated as the mean values of all replicates per species.

hemipteran species	IC_{80} value of Na/K-ATPase for ouabain	Nr. of replicates	Nr. of individual used per replicate
Arocatus longiceps	$1.89*10^{-4}$ M	3	19-23
Horvathiolus superbus	$1.82*10^{-4}$ M	3	16
Lygaeus equestris	$8.68*10^{-5}$ M	3	8
Lygaeus kalmii	$3.70*10^{-4}$ M	3	10-15
Kleidocerys resedae	$2.06*10^{-7}$ M	3	20
Oncopeltus fasciatus	$2.19*10^{-4}$ M	3	6
Spilostethus pandurus	$5.58*10^{-4}$ M	3	5
Tropidothorax leucopterus	$1.54*10^{-4}$ M	3	7-9

Discussion

Our functional, genetic and ecological approach allows for reconstructing the macroevolutionary history of adaptations to toxic cardenolides in the hemipteran subfamily Lygaeinae. The data presented here show that cardenolide sequestration is restricted to the Lygaeinae and does not occur in the outgroup species tested. Seven of the nine Lygaeinae are obviously able to store the orally ingested cardenolides ouabain and digoxin in their body (Fig. 2). Given the uniform distribution of this trait across our molecular phylogeny we assume that sequestration of cardenolides is a basal feature of the Lygaeinae. Our finding supports the universality of this trait suggested by Scudder & Duffey (1972) who detected cardenolides in dried museum specimens of > 20 lygaeine genera.

Nevertheless, two species of Lygaeinae also proved to be unable to sequester cardenolides. The absence of sequestration in the European *Arocatus* species *A. longiceps* and *A. melanocephalus* correlates with the life-history of these two species which are inhabiting and feeding on plants (*Platanus*: Platanaceae, or *Alnus*: Betulaceae, and *Ulmus*: Ulmaceae, respectively) not known to produce cardenolides. Feeding on non-apocynaceous hosts is most likely not the ancestral state in *Arocatus* as representatives of the genus in other geographic regions (e.g. Africa and Australia) are known to use CG plants as hosts (*A. aenescens*, A. *chiasmus*, A. *continctus*, A. *montanus* and A. *rusticus* feed on *Araujia*, *Asclepias*, *Gomphocarpus*, *Nerium*, and *Parsonsia* (Cassis & Gross, 2002) or were even shown to possess cardenolides (*A. rusticus*) (Scudder & Duffey, 1972). In our phylogeny, *A. longiceps* and *A. melanocephalus* are revealed as sister taxa nested in a monophyletic genus *Arocatus* which moreover is recovered as sister group to *C. nerii*, a species known to sequester cardenolides (Von Euw et al., 1971). We hence assume that the ability to sequester cardenolides was lost in the two European species. This finding may indicate that

physiological adaptations which are necessary to sequester cardenolides are costly and are therefore reduced when not needed.

Arocatus which moreover is recovered as sister group to *C. nerii*, a species known to sequester cardenolides (Von Euw et al., 1971). We hence assume that the ability to sequester cardenolides was lost in the two European species. This finding may indicate that physiological adaptations which are necessary to sequester cardenolides are costly and are therefore reduced when not needed.

The lower, but unequivocal presence of ouabain (digoxin 5.6 %, only) in the outgroup species *P. apterus* (Fig. 2) is not necessarily in disagreement with a monophyletic origin of sequestration in the Lygaeinae as *P. apterus* also is an aposematic species and might derive toxins from its host plants as well. Moreover, the outgroup species do not possess cardenolide resistant Na/K-ATPases (see sequencing and physiological data) which might be a prerequisite for cardenolide sequestration.

Testing the resistance traits which prevent self-intoxication in the sequestering Lygaeinae, we found that uninhibited Na/K-ATPase activity under ouabain stress is a common feature in the subfamily Lygaeinae. Our *in vitro* investigations of Lygaeinae Na/K-ATPase of brain and thoracic ganglia showed a relative uniform pattern of strong insensitivity towards oubain (Fig. 3). All seven species investigated here possess the same cardenolide insensitive form of Na/K-ATPase i.e. a much less ouabain-sensitive Na/K-ATPase (IC_{80} = $2.46*10^{-4}$M) than the outgroup species *Kleidoceris resedae* (IC_{80} = $2.06*10^{-7}$M) and a slightly higher insensitivity than the monarch butterfly (IC_{80} = $2.32*10^{-5}$M) (Petschenka *et al*., 2013). The characteristics of Na/K-ATPase inhibition by ouabain described here, resembles the one described by Moore & Scudder (1986). In our *in vitro* investigations we focused on nervous Na/K-ATPase, only, as the nervous tissue is a rich source of Na/K-ATPase facilitating *in vitro* assays. Moreover, the nervous system was shown to be the main site of Na/K-ATPase expression in other insect orders (Lepidoptera: Petschenka *et al.* 2012) rendering the nervous system most relevant for toxicological interpretation.

The presence of cardenolide resistant Na/K-ATPases in all Lygaeinae tested suggests that target site insensitivity of Na/K-ATPase, like the ability to sequester cardenolides, also is a basal feature of the Lygaeinae. Both species, *A. longiceps* and *A. melanocephalus,* which are not exposed to dietary cardenolides and do not store the toxins, still possessed a modified, cardenolide insensitive Na/K-ATPase.

Responsible for a uniform insensitivity in the Lygaeinae are amino acid substitutions at positions of the enzyme which are known to be involved in binding of ouabain (Fig.1). Our preliminary molecular investigations of the Na/K-ATPase of several Lygaeinae demonstrated that an amino acid substitution of asparagine for histidine at position 122 (N122H) is present in the first extracellular loop of the Na/K-ATPase of all species and is at least partly

responsible for insensitivity in the *in vitro* enzyme assays (Dobler *et al.*, 2012; Dalla *et al.,* 2013). Further amino acid substitutions at positions which are known to be involved in ouabain binding of Na/K-ATPase in the Lygaeinae lead to a further increased insensitivity (Dobler *et al*., 2012, Dalla *et al*., 2013). Previous studies could show that up to eight different substitutions may be responsible for the lower cardenolide binding characteristics in CG adapted *O. fasciatus* and *L. kalmii* (Dobler *et al.*, 2012; Zhen *et al.,* 2012). Whether all of these substitution arepresent in all the Lygaeinae investigated here could not yet be unequivocally clarified. The possession of several Na/K-ATPase gene copies in the Lygaeinae (Zhen *et al.,* 2012; Dobler *et al*., 2012; Dobler *et al.,* unpublished data) renders this investigation difficult. Evidence from genetic modifications of the *Drosophila* Na/K-ATPase show that accumulation of substitutions at positions forming the ouabain binding pocket in mammals (Croyle *et al.*, 1997; Qiu *et al*., 2005; Yatime *et al.,* 2013) lead to increased insensitivity of the enzyme (Dobler *et al.,* 2012; Dalla *et al.,* 2013). Two combined substitutions, either Q111T-N122H as observed in *O. fasciatus* or Q111V-N122H as present in *D. plexippus* have been shown to reduce the inhibition by ouabain strongly, whereas the enzyme occurring in *K. resedae* represents the typical sensitive form. As the enzyme of Lygaeinae however, is far more resistant to ouabain *in vitro* than the enzyme of the monarch butterfly this increased resistance is likely due to at least a third substitution at an additional position. Enzyme assays performed with the single mutation T797A discovered in *L. kalmii* showed a 250-fold increased resistance (Dalla *et al.,* 2013) while a combination of four substitutions (Q111T, N122H, F786N, T797A) introduced into the Drosophila Na/K-ATPase gene leads to enzyme characteristics closely similar to the Na/K-ATPase inhibition curves observed here (Dalla & Dobler, unpubl. data). Therefore, it is likely that due to combinations of at least four potentially important substitutions at different positions the strongly increased resistance in the Lygaeinae can be explained.

The worldwide host association of Lygaeinae with plants of the milkweed family Apocynaceae, suggests a very old relationship between these two taxa. Remarkably, several Lygaeinae use plant species as hosts which belong to non-related families but are known to produce cardenolides as well. Examples include *L. equestris* which is well known to feed on *Adonis vernalis,* a cardenolide producing Ranunculaceae (Junior & Wichtl, 1980; Winkler & Wichtl, 1986; Deckert, 2007), or *H. superbus* which often is associated with *Digitalis purpurea* a Plantaginaceae (Péricart, 1998; Wachmann *et al*., 2007, Petschenka pers. obs.). Furthermore, *S. pandurus* uses *Urginea maritima* as a host (Vivas, 2012), an Asparagaceae known to contain bufadienolides which are structurally related to cardenolides and have the pharmacodynamical activity. Most likely these Lygaeinae also sequester cardenolides from these sources which has been shown for *L. equestris* (Petschenka, 2010, unpublished data) collected from *A. vernalis*. Species like *Tropidothorax leucopterus*, *Melanocoryphus*

albomaculatus, *Spilostethus saxatilis, Lygaeus simulans* and *Horvathiolus superbus,* in addition to the plant species mentioned above, are also closely associated with *Vincetoxicum hirundinaria* (Wachmann *et al.,* 2007). The current use of plants from non-apocynaceaous families most likely represents host shifts which were facilitated by the preadaptation of the Lygaeinae to CGs. As they, like many other species of the Lygaeinae, can use a variety of plants as nutritional resources the availability of a certain class of chemicals may predict host plant associations more strongly than the supply of nutrition. Given that Lygaeinae are the most species rich lineage within the Lygaeidae (sensu Henry, 1997) with more than 500 species compared to the sister groups Orsilinae (250 species) and Ischnorhynchinae (75 species), the adaptations to dietary CGs may represent a key innovation of this group. In general, it is assumed that species that are specialized in their food utilization are often more diverse than taxa including more generalist feeders because the rate of evolution is thought to be higher among specialists (Whitlock, 96).

Taken together, results from three comparative approaches revealed that sequestration and target site mutation as resistance traits in the Lygaeinae are ancestral adaptations and have apparently originated at the basis of the subfamily. Even species, who do not normally encounter dietary cardenolides and do not store the toxins, still possess a modified insensitive Na/K-ATPase. These results lead us to conclude that target site insensitivity and sequestration of cardenolides are basal and plesiomorphic characters of the Lygaeinae which derive from an originally host plant use.

Chapter 2

Metabolic alteration of cardiac glycosides in Lygaeinae: detoxification or optimized uptake?

Abstract

Several Lygaeinae (Heteroptera) are morphologically and physiologically adapted to sequester cardenolides for their own protection against predation. When fed on *Asclepias* seeds, a high concentration of polar cardenolides was found in *Oncopetus fasciatus,* despite a wide polarity range of cardenolides was available. These hints to a selective sequestration or a metabolic alteration process of cardenolides. Previous studies could show that different polar metabolites arise through change of apolar cardenolides, however, little is known about the details of the metabolic processes. We therefore performed a comparative study in which 10 different species of Lygaeinae plus one outgroup were tested for metabolic alteration of two purified [3H]-labeled cardenolides: the apolar digoxin and the polar ouabain by using different treatments. Both treatments yielded always the same metabolites. Ouabain was always recovered unchanged whereas digoxin alteration seems to follow a uniform mechanism which is, at least in part, associated with the selective accumulation of cardenolides in the glycoside storage compartment. Further, we elucidated the metabolic alteration of digoxin in *O. fasciatus* over a period of 170 h and identified the structure of the storable metabolite. As MALDI analysis indicates this metabolite arises by at least one amination step. Metabolic alteration of apolar cardenolides is a general phenomenon in the Lygaeinae which may have evolved as an adaptation to handling and accumulating cardenolides derived from their host plants.

Introduction

Specializing on plants with toxic secondary compounds requires in herbivores traits to overcome these chemical defences. These counter-mechanisms include for instance sequestration (and compartmentalization) of toxic compounds used for own defenses or the metabolic modification of the molecules to avoid specific binding to targets. Whereas the former mechanism requires an active form of toxins, the latter mechanism represents a detoxification mechanism which might lead to excretion of substances via the Malpighian tubules (Després *et al.,* 2007). Biotransformation is one of the major weapons against many classes of toxic allelochemicals to resist intoxication. Specialized Lygaeinae (Heteroptera) for instance possess a wide spectrum of adaptations to cope with toxic host plant cardenolides with sequestration being one of them. Cardenolides in general bind to and inhibit the ubiquitous Na/K-ATPase responsible for maintaining cellular potentials (Lingrel *et al*., 1990; Jorgensen *et al*., 2003; Horisberger, 2004). To cope with these toxic compounds Lygaeinae possess amino acid substitutions in the target site of the Na/K-ATPase which lower the binding affinity for crdenolides (Dobler *et al.* 2012, Zhen *et al.* 2012 and Chapter 1).

The phenomenon of cardenolide sequestration, per se, involves the uptake, transfer, and concentration of cardenolides in the storage compartments. Physiological studies on permeability of insect guts suggest that polar cardenolides such as ouabain require an energy dependent transport and presumed intestinal carrier in the gut epithelium which allow polar cardenolides to enter the haemolymph. Conversely, apolar cardenolides can be expected to cross the gut passively due to their physiochemical properties (Wright, 1960). Further, there are two opposing mechanisms in uptake and accumulation of cardenolides in the species. The uptake of apolar cardenolides occurred far more rapidly (77 % in 30 min) than the uptake of polar ones (3 % in 30 min.) (Yoder *et al*., 1976; Duffey *et al.,* 1978; Scudder & Meredith, 1982b; Detzel & Wink, 1995). In the reverse situation, the results of Duffey *et al.,* (1978) showed that the uptake of polar cardenolides from the haemolymph into the glycoside storage compartments is faster than the transfer of metabolized apolar cardenolides. Nevertheless, the mechanisms of sequestration, transport and accumulation of cardenolides in the haemolypmph or the integument are not known in detail.

Interestingly, in *Oncopeltus fasciatus* a high concentration of polar- and an absence of apolar cardenolides was detected (in several studies) (Duffey & Scudder, 1974; Yoder *et al.,* 1976; Duffey *et al.,* 1978; Moore & Scudder, 1985; Scudder *et al.,* 1986). Next to the sequestration and target-site mutation, Lygaeinae also possess the ability of metabolic alteration of cardenolides. They apparently use biotransformation not for detoxification but rather to transform cardenolides into storable forms. Studies on accumulation and distribution of cardenolides in *O. faciatus* (Duffey & Scudder, 1974; Moore & Scudder, 1985) illustrate

that the ratios of cardenolide concentration differ among several insect tissues. Whereas wings, gut and haemolymph are characterized by low cardenolide content, the glycoside storage compartment represents the greatest storage capacity of the body. In vivo and in vitro evidence using indicator cardenolides which cover a wide polarity range of natural occurring plant compounds, indicated a metabolic alteration of apolar cardenolides (Duffey & Scudder, 1974; Scudder & Meredith, 1982b). However, these reports did not address the metabolic mechanisms in detail.

Thus, it was of interest to examine the metabolic process in *O. fasciatus* in detail and to compare it among several species of this cardenolide adapted subfamily. Using thin-layer-chromatography the cardenolide profiles after ingestion or injection of polar ouabain or apolar digoxin were determined in these species. Finally, this study was undertaken to identify the cardenolide derivative present in the defensive secretion stored in the glycoside compartment. This may elucidate the mechanism by which certain Lygaeinae are able to handle glycosides because their toxicity has been correlated with polarity. We elucidated the metabolite structure to obtain information on enzymes required for apolar cardenolide metabolism in the Lygaeinae and their occurrence in the insect body tissues. This ultimately allows to deduce the role of metabolism in the ability to cope with large amounts of cardenolides.

Material and Method

Insect handling

Adult Lygaeinae were obtained both from the field and from laboratory cultures. The species *Oncopeltus fasciatus*, *Horvathiolus superbus*, *Lygaeus equestris*, *L. simulans*, *L. kalmii, Spilostethus pandurus* and *Cosmopleurus fulvipes* were reared in the laboratory from the egg stage and raised on husked sunflower seeds (*Helianthus annuus* L.) and water which was available from cotton wicks in plastic tubes. All species were reared in a climatic chamber at 16 h/8 h light/dark at 26°C (*C. fulvipes, H. superbus, O. fasciatus, S.pandurus*) or 30°C (*L. equestris, L. kalmii, L. simulans*). *Tropidothorax leucopterus* (from Grießheim, Germany) was reared on sunflower seeds and cut branches of *Vincetoxicum hirundinaria* and was also kept at 26°C, 16 h/8 h light/dark cycle. For *Arocatus melanocephalus* (from Kallinchen, Germany), *A. longiceps* (from Berlin, Germany) and *Kleidocerys resedae* (from Kamburg, Germany) field collected individuals were used.

Treatment of species for different analyses

A) Metabolite profile 72 h after injection

For this treatment six species (*A. melanocephalus, O. fasciatus, T. leucopterus, L. simulans, L. equestris, C. fulvipes*) were used and each of them were injected with a polar or an apolar cardenolide. A solution containing [3H]-ouabain (polar) or [3H]-digoxin (apolar) (Perkin Elmer LAS GmbH, Rodgau, Germany) and 1.125 % NaCl (1:4) in water was laterocranial injected between fifth and sixth abdominal segment by a fine capillary syringe (Hamilton 701 NR; ga22S/51mm/pst3; ROTH GmbH+Co). The specific activity of radiolabelled cardenolides was 6 Ci/mmol for [3H]-ouabain and 7.08 Ci/mmol for [3H]-digoxin. Before injection, species were cooled for 5 min at -20°C to immobilize them in order to prevent injury or puncture of the gut during injection. After injection species were kept for 72 h on water and sunflower seeds (as previously described).

A) Ingestion of cardenolide solutions by forced drinking

The Lygaeinae and K. resedae used in this experiment were forced to drink cardenolide solutions (composed of 5 µM cardenolide in water with 17.7 % ethanol and 5 % sucrose) by fixing them with a lasso of dental floss. The proboscis was manually introduces into a 2 µl droplet containing cardenolide on parafilm. After feeding, the species were again kept for 72 h on water and sunflower seeds.

B) *Metabolite profile over a period of 170 h*

Before starting the experiments fifth instar larvae were separated from adults to ensure that all individuals had approximately the same age. In this treatment 75 adults of the large milkweed bug, *O. fasciatus* were injected with 2 µl of the nonpolar digoxin as described above to determine metabolite profiles over a period of 170 hours. Species were kept as described above over a period of 170 hours. Within this period three individuals each (two females, one male) were frozen separately at 25 different times.

C) Accumulation of different polar cardenolides in the glycoside storage compartment

Six adult *O. fasciatus* each were injected as previously described with [3H]-ouabain or the apolar [3H]-digoxin diluted in 1.125 % NaCl and kept on water and sunflower seeds for 72 h.

Detection of metabolites of cardenolides in the examined Lygaeinae

All species of treatment A, B and C given drinking solution or treated by injection were frozen after the end of the treatment. Individuals of each species were separately frozen in liquid nitrogen, homogenized with a glass pestle and extracted with 1 ml 100 % methanol. The sample was shaken for one hour and then centrifuge at 8000 rpm. Supernatant was transferred and methanol evaporated overnight. Dried residual sample was dissolved in 50 µl methanol. Species of treatment D were squeezed through slight pressure at specific points of the body surface whereby the release of defence fluid out of the storage compartment was initiated as previously described by Duffey & Scudder (1974). Crude methanol extracts and the fluid of defence droplets were separated by thin-layer chromatography (TLC) (pre-coated TLC sheets with silica gel 60, ALUGRAM® SIL G) and analyzed using a multichannel radioactivity detector (Rita-32a, Raytest). The plates were developed in filter-paper-lined, saturated chambers with ethyl acetate-methanol-water (30:4:3). As control for cardenolides or cardenolide derivates we performed a second TLC by using kedde reagent (1:1 mixture of 5% 3,5-dinitrobenzoic acid in MeOH and 2 N KOH) for the specific detection of the lactone of the cardenolides.

Structure analysis by MALDI-TOF- MS

O. fasciatus individuals were injected with a solution of 3 µl digoxin (10^{-6} M dissolved in ethanol and water 1:3) and kept on sunflower seeds impregnated with digoxin by applying the compound directly as a solution in chloroform-methanol (2:1). After six days the defence fluid was collected from the insects as described above and dissolved in water. To check for presence of metabolite in the defence fluid, TLC separation was performed as control. The sampled fluid (2 µl) was spotted on a TLC sheet and developed as described previously. MALDI-TOF (Matrix-associated laser desorption ionization-time of flight mass spectrometry) analyses were performed on an ultrafleXtreme instrument (BrukerDaltonics, Bremen, Germany) equipped with a smartbeam-II laser with a repetition rate of 1 kHz. The matrix (α-cyano-4-hydroxycinnamic acid (4-HCCA) was dissolved in 2:1 water:acetonitrile with 0.1 % trifluoroacetatic acid (TFA). The matrix was shaken and centrifuged for 1 to 2 min at 14 000 rpm. The clear liquid was decanted for use. Digoxin (Sigma) was used as standard for instrument calibration and as internal mass standard. The calibration standard (1 mg/mL) was prepared in 0.1 % TFA in water. The defence fluid sample was analyzed using a 10:1 (µl each, matrix/sample) mixture. Samples were spotted on a MALDI target plate and allowed to air dry. The spots were measured within the FlexControl software (version 3.3). The spectra

were processed using FlexAnalysis software (version 3.3). The MALDI-TOF MS analyses were performed at the Department of Organic Chemistry, University of Hamburg.

Results

Cardenolide alterations in the Lygaeinae

Body samples of all six Lygaeinae 72 hours after injection with [3H]-labelled ouabain showed no alteration by the insects, since the radio TLC analysis revealed only a single cardenolide identical in R_f value (R_f = 0.06-0.07; ouabain, R_f = 0.07) with the reference ouabain (Fig.1 A1-E1). The same result (R_f = 0.07) was obtained 72 hours after imbibing [3H]-labelled ouabain. All nine tested species had unchanged ouabain in the body fluid samples. Nevertheless, the amount of ouabain recovered from the body samples of *Arocatus* species was less than 20 % of the amount ingested or injected previously.

In contrast, after injection or ingestion of [3H]-labelled digoxin the cardenolide was converted into metabolites. In a total of six species (*L. equestris*, L. simulans, *O. fasciatus, T. leucopterus*, *C. fulvipes*) digoxin was altered to at least two metabolites. Three separate fractions could be recovered by TLC (Fig. 2 A2-E2): fraction I representing a very polar cardenolide detected at R_f = 0.02-0.03, fraction II chromatographed in an intermediate position at R_f = 0.10-0.13, and fraction III with R_f = 0.50 corresponded to unchanged digoxin. In species of the genera *Arocatus* (*A. melanocephalus*, *A. longiceps*) the analysis showed only two cardenolides including unchanged digoxin. The metabolite corresponded with an R_f = 0.02 to fraction I of the other species. In additional analyses up to 144 hours after ingestion of digoxin no second less polar metabolite appeared which was formed out of the unstable polar product (fraction I). During this time the cardenolide concentration in the body sample decreased linearly up to a detection limit after 130 hours. Chromatography of the body sample in *H. superbus* indicated that even after 72h the radioactive label introduced with digoxin occurred in a single peak coincident with the fraction II. Analyzing the metabolite profile in K. resedae, there was no evidence for cardenolides in the body fluid after 72 hours.

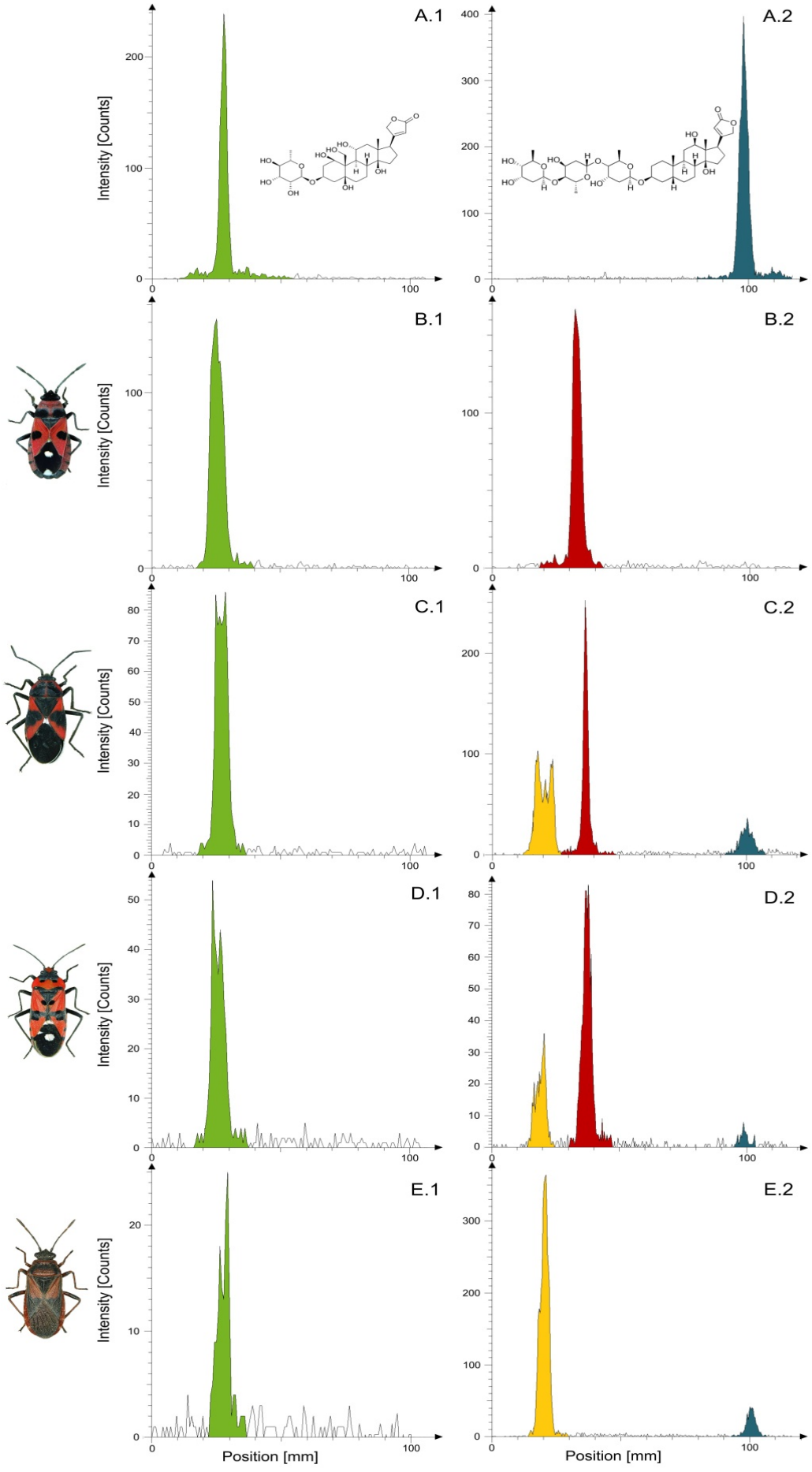
A.1
A.2
B.1
B.2
C.1
C.2
D.1
D.2
E.1
E.2
Intensity [Counts]
Position [mm]

Figure.1. A diagram representing the radioactive analysis of chromatograms of the body sample 72 h after ingestion of digoxin or ouabain in different species of Lygaeinae. Abscissa represents distance along the TLC plate: origin at 15 mm and solvent front at 180 mm. Ordinate shows the intensity in counts. A1) show the position of ouabain standard with an R_f = 0.07 and A2) show the standard of digoxin with R_f = 0.50. B1) represents the position of ouabain after 72h of *H. superbus*, C1 of *T. leucopterus*, D1 of *L. equestris* and E1 of *A. melanocephalus*. B2 shows the metabolized digoxin after 72h in *H. superbus*, C2 of *T. leucopterus*, D2 of *L. equestris* and E2 of *A. melanocephalus*.; The colours of the peaks represents the different compounds: Ouabain (green), Digoxin (blue), first metabolite (yellow) and second metabolite (red).

Profile of digoxin metabolites in O. fasciatus

Chromatography of the body-samples showed that in adults of *O. fasciatus* injected with [3H]-labelled digoxin the cardenolide was converted into at least two metabolites (fraction I and II) in the insect compared to the unchanged standard (Fig.1 A-E). Immediately (30 min) after injection of digoxin 70 % of this apolar cardenolide was transformed into a very polar metabolite (fraction I) (R_f = 0.03; cf. digoxin, R_f =0.50) (Fig.2). Already after one hour a second less polar metabolite (fraction II) (R_f = 0.12) appeared which was formed out of the unstable polar product (first metabolite). The total amount of unchanged digoxin 12 hours after injection was found to be 10 % of the total digoxin available. Metabolite amounts, however, were found to be 90 % after 16 hours summing both fraction I and II. Over a period of 66 hours most of the intermediate product (80%) was modified into a final stable product (15%) and only a low amount of unchanged digoxin (5%) could be found. Finally after 163 hours digoxin and the first polar metabolite had completely vanished. Among the three individuals analyzed no deviations in this time course could be detected.

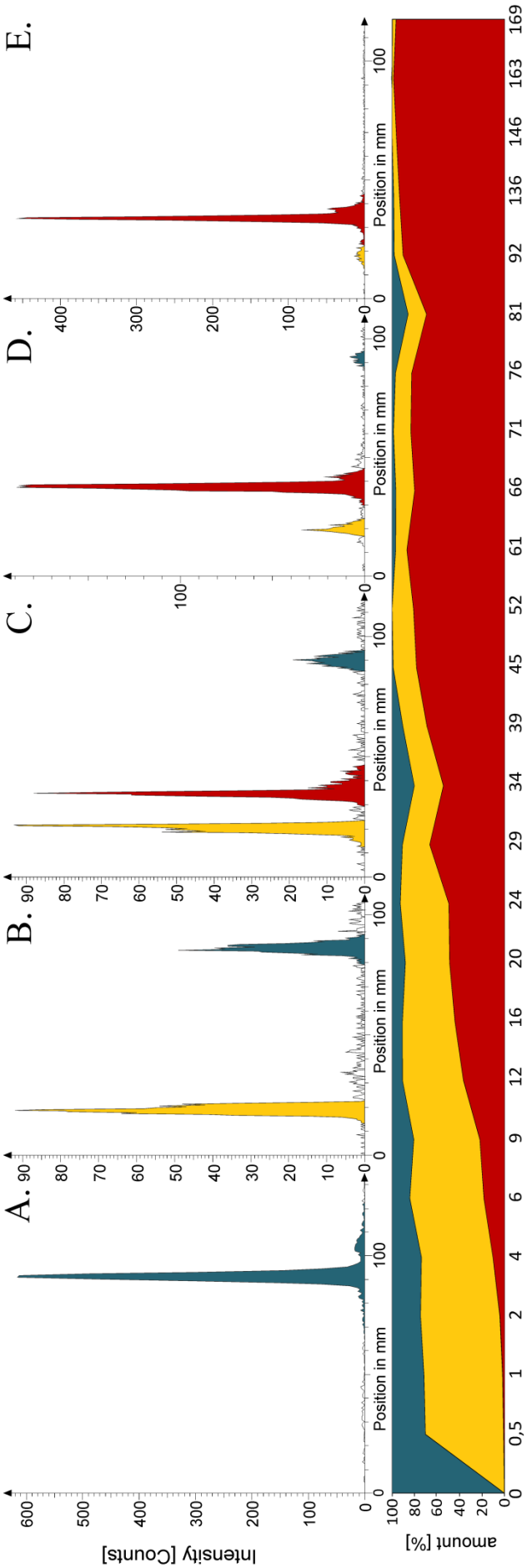

Figure. 2. Metabolite profile in *Oncopeltus fasciatus* over 170 hours after injection of digoxin. Abscissa represents distance along the TLC plate, origin at 15 mm and solvent front at 180 mm. Ordinate shows the intensity in counts. A. At time 0 100% digoxin (blue) was injected. B. After 30 min 70 % of the injected digoxin was transformed into a very polar metabolite (yellow). C. After 12 hours most of the digoxin (blue) disappeared and a second less polar metabolite (red) appeared that was formed out of the unstable polar product (yellow). D. After 66 h most of the intermediate product (yellow) was modified into the second final product (red). E. After 163 h digoxin (blue) and the first polar metabolite (yellow) had completely vanished

Uptake of cardenolides into the storage compartments in O. fasciatus

Chromatograms of TLC separation indicated that the uptake of polar ouabain from the haemolymph into the storage space within 72 hours after injection was faster than the uptake of metabolized digoxin. Fluid of the storage compartments contained 50-70 % ouabain whereas only 1-3 % of digoxin was taken up after 72 h. However, in the defence fluid only the second polar metabolite (fraction II with R_f = 0.12) could be detected.

Structure analysis of metabolite fraction II in the defence fluid of O. fasciatus

The defence fluid released from the storage compartments after the insect was treated with digoxin was analyzed by MALDI- TOF-MS. A typical MALDI-TOF mass spectrum of the cardenolide digoxin showed a single intense peak at *m/z* 803.415 [+Na^+] corresponding to the mass of digoxin with an additional sodium ion (Fig. 3). The measurement of the fluid sample yielded several peaks of different masses (Fig. 4). Based on the mass of digoxin we speculate that the intense peak at *m/z* 820.420 [+Na^+] might be the metabolite of fraction *II* identified by TLC separation of the defence fluid in addition to a sodium ion. This mass coincides almost perfectly with the structure of the metabolite illustrated in Figure 5. We assume that through amination an NH_3 is introduced into the genin of digoxin at the C-20 of the 5 membered lactone followed by a hydrogenation which entails the resolution of the double bond. Mass spectra analyses and structure analyses identified two more metabolites. Both mass spectra of *m/z* 782.489 with a protonated molecular ion and *m/z* 804.449 with an added sodium ion are expected to be derivates of the metabolite of fraction //. The masses [*m/z* 782.489 + H^+] and [*m/z* 804.449 + Na^+] agree with the assumed structures shown in Figure 5. Through amination of NH_3 at different positions of the genin of digoxin it is possible that molecules of the same chemical formula could be present as derivates.

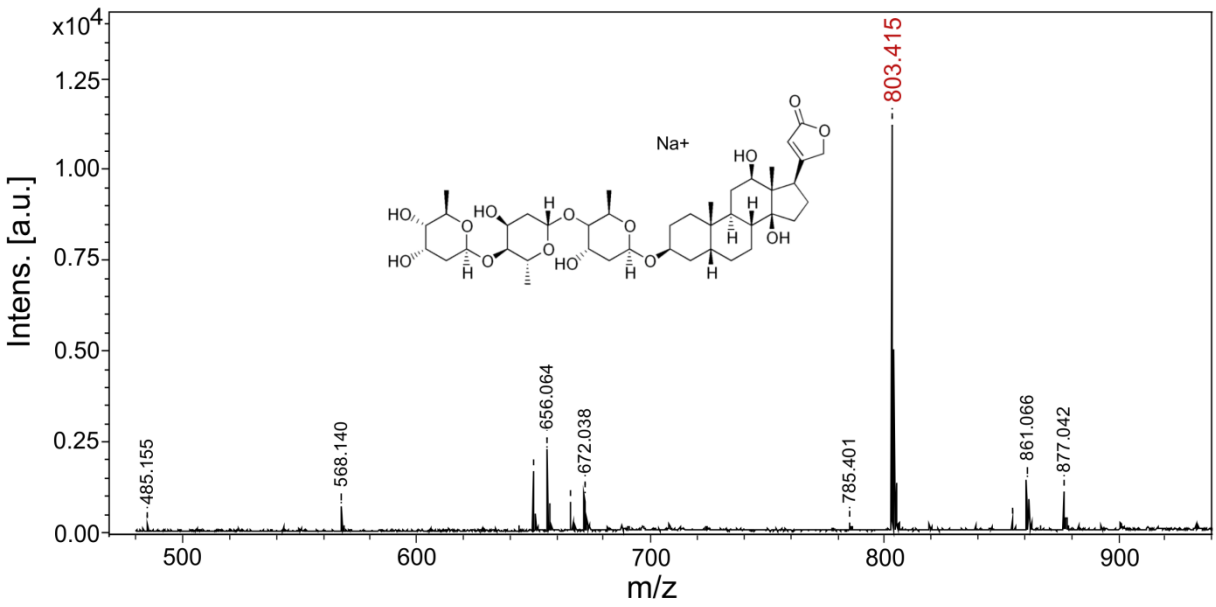

Figure 3. MALDI-TOF mass spectrometric analysis with the cardenolide digoxin used as standard showing an intense fragmentation of *m/z* 803.415, corresponding to the mass of ionized digoxin

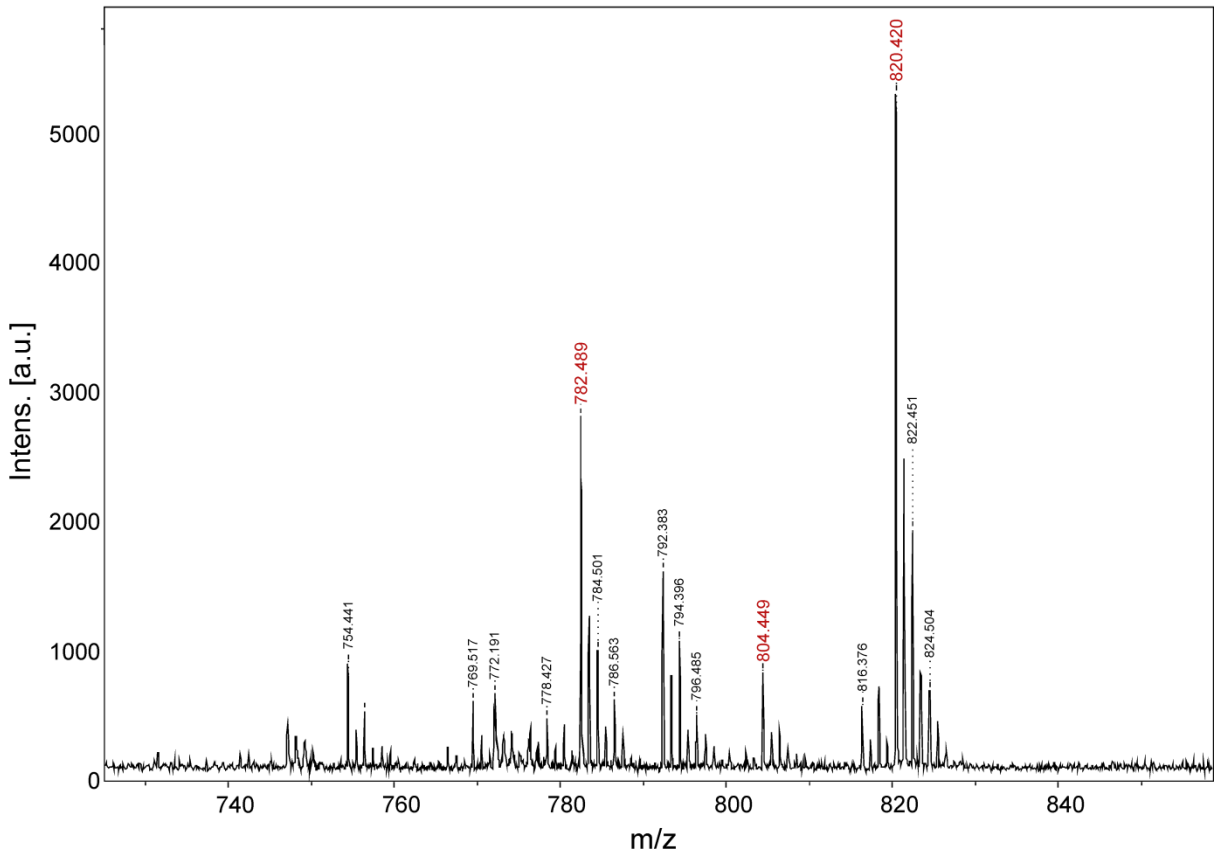

Figure 4. MALDI-TOF mass spectrum of defence fluid of *O. fasciatus* showing an intense peak of *m/z* 820.420, that is expected to be the metabolite identified as fraction // in thin-layer-chromatography, the two other marked spectra with *m/z* 782.489 and *m/z* 804.449 are expected to correspond to the mass of two metabolic derivatives

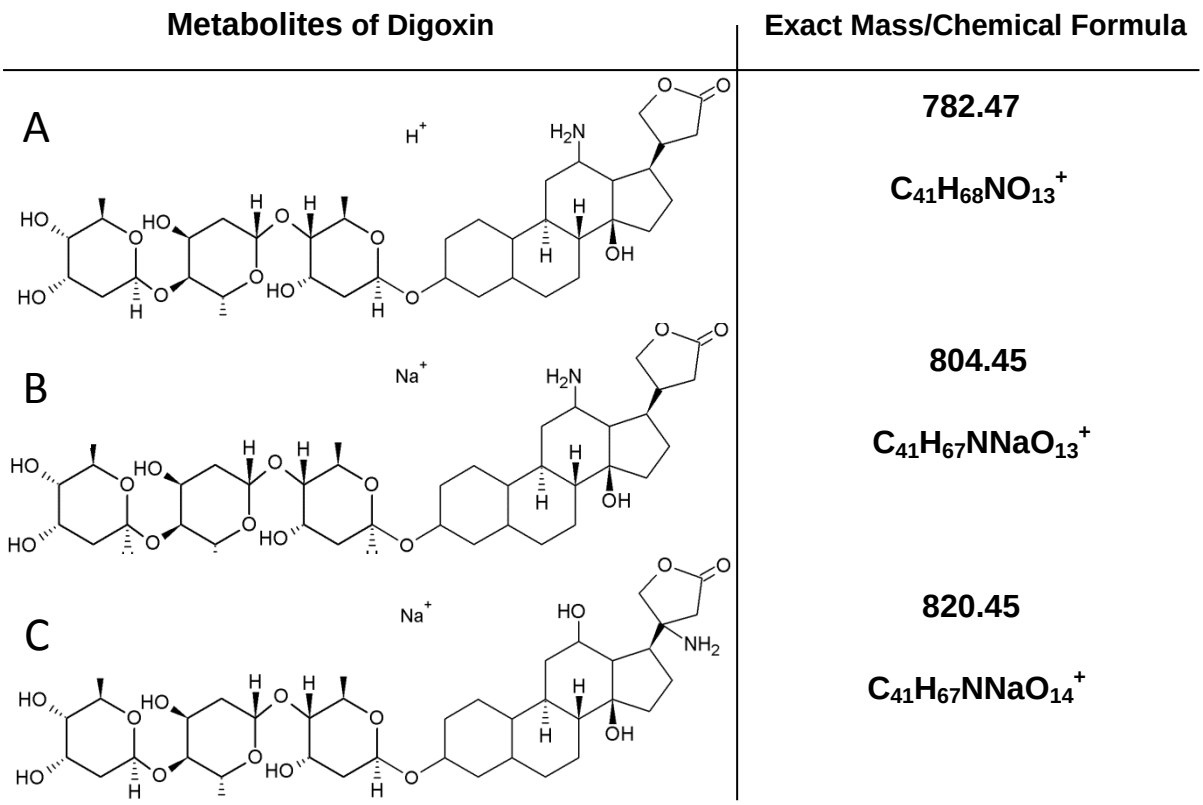

Figure 5. Structure and the according mass of metabolites of digoxin, the metabolite structure of C represents the expected metabolite found in fraction *II* of body fluid samples of Lygaeinae, A and B are expected to be derivates of metabolite shown in C.

Diskussion

This study was undertaken in order to clarify certain aspects of the metabolic alteration of cardenolides in the Lygaeinae and to elucidate the mechanisms by which these species are able to handle these toxic compounds. Similar to previous studies (Scudder & Meredith, 1982; Duffey & Scudder, 1974) ouabain does not appear to be metabolized in any of the examined Lygaeinae. Labelled digoxin, however, was recovered from body samples as three separate fractions which included two metabolites of digoxin as well as unchanged digoxin. Detailed determination of the metabolite profile in *O. fasciatus* over a period of 170 hours uncovered the metabolic transformation of apolar digoxin with regard to quantity and polarity of the metabolites. Previous studies using the more apolar cardenolide digitoxin had already reported the presence of two rather polar metabolites in *O. fasciatus* 24 hours after ingestion (Scudder & Meredith, 1982b; Duffey & Scudder, 1974), yet did not uncover the interdependence and the temporal appearance of the individual fractions. The fact that digoxin was converted first to a more polar metabolite than ouabain but afterwards transformed into a slightly more apolar cardenolide point to two enzyme catalyzed reactions involved in digoxin metabolism. By using TLC and kedde reaction we confirmed the presence

of a lactone in the metabolite representing fraction / and //, which primarily provide evidence of a cardenolide or a cardenolide derivate.

The comparative studies testing digoxin uptake in several species of Lygaeinae produced the same results and mostly uncovered the occurrence of two metabolites with the same R_f values as found in *O. fasciatus*. Only in body extracts of *H. superbus* a single metabolite corresponding to fraction // was detected which suggests a faster enzymatic transformation than in other sequestering Lygaeinae. Testing the outgroup species *K. resedae*, we found not cardenolides in body fluids after 72 hours. These findings suggest that both cardenolides have not been taken up by the gut which in turn is an indication for the possession of an impermeable gut.

Nevertheless, it is important to differentiate between cardenolide sequestering species and those who do not sequester but take up cardenolides. In non-sequestering Lygaeinae, i.e. *A. longiceps* and *A. melanocephalus,* we could only detect the first metabolite (fraction *I*) in the extractions regardless of time. This metabolite disappeared rapidly, most likely by excretion via the Malpigian tubules, which are suggested to be relevant for the excretion of toxic plant compounds in general (Nation, 2001). It is tempting to speculate, that the rapid excretion of metabolized cardenolides originated as an enzymatic detoxification mechanism whereas the further transformation to the second metabolite represents an adaptation to accumulation and storage of cardenolides. Since apolar cardenolides are suspect to be more emetic than their polar counterparts (Blum, 1981) it appears plausible that the transformation evolved to prevent selfintoxication by minimizing the mobility and penetration ability of diffusible cadenolides in body tissues. In accordance with this Malcolm (1991) presumed that polar cardenolides are easier to store.

Enzymes required for digoxin metabolism seem to be widespread in body tissues of the Lygaeinae. The results presented here show that transformation of digoxin takes place at least in the haemolymph or in associated tissues (e.g. the fat body), whereas Scudder & Meredith (1982b) demonstrated a similar transformation already in the gut lumen. Since non-sequestering species are able to produce digoxin metabolites (only fraction /), it might be that involved enzymes are not specific for biotransformation of CGs, but rather they are engaged in diverse biochemical functions such as detoxification of xenobiotics. Such detoxification enzymes can sometimes be restricted to specific organs or tissues or vary according to insect developmental stages and sex (Després *et al.,* 2007). In contrast, the second metabolic transformation of CGs occurs only in sequestering species and appears to be lost in the non-sequestering *Arocatus* species.

In insects which do not efficiently sequester cardenolides, the gut is a first barrier for ingested cardenolides at least for polar compounds (Scudder & Meredith, 1982b; Wink & Schneider, 1990). Only few studies addressed the permeability of the gut to cardenolides.

Physiological studies on permeability of *O. fasciatus* guts showed that the uptake of apolar cardenolides occurred far more rapidly (77% in 30 min) than the uptake of polar ones (3% in 30 min) which require an energy dependent transport by presumed intestinal carriers in the gut epithelium which allow these polar cardenolides to enter the haemolymph (Yoder *et al*., 1976; Scudder & Meredith, 1982b; Detzel & Wink, 1995). The uptake of differing polar carddenolides in the storage compartment however, showed that polar cardenolide will be favoured. Digoxin derivates were concentrated in the defence fluid just above the detection limit 72 h after *O. fasciatus* had been injected with digoxin solution. A very small percentage (1-3 %) of the radioactively labeled metabolite (assigned to fraction //) could be found in the fluid released out of the storage compartments. In the reverse situation, we found that the uptake of polar CGs from the haemolymph into the storage space was about 20 times faster (50-70% in 72h). These results suggest that gut uptake is unspecific and allows a broad spectrum of cardenolides to enter the haemolymph whereas the passage into the storage compartments seems to underlie a selective process. The preferential and far more rapid uptake of originally polar CGs (e.g. ouabain) into the storage compartment, suggests either that a selective carrier occurs in Lygaeinae or that selective transport of transformed cardenolides from the place of metabolic alteration to the storage compartment is time-consuming. In the former case, it might be plausible to assume that an ABC transporter acts to carry CGs into the storage compartments as described for Chrysomelina larvae by Strauss *et al.* (2013). Nevertheless, the sequestration of cardenolides is the result of the presence of several barriers with various degrees of selectivity.

Sequestration of polar carenolides in general requires energetic costs for carriers and possible transport mechanisms (from gut lumen to storage compartments) whereas in the case of a unchanged apolar cardenolide the uptake would be less costly and there for more advantages. Nevertheless, use all tested Lygaeinae the costly enzymatically metabolic conversion to transform digoxin in derivates. The advantage of such a mechanism remains unexplained and requires further examinations.

As a first hypothesis derived from the MALDI-TOF MS analysis we are able to suggest an addition of NH_3 followed by a hydrogenation at the lactone ring of digoxin. We assume that the substitution happens at C-20 of the genin which results in a resolution of the double bond. This hypothesis is supported by the exact molecular mass which would arisen through amination of the digoxin and through the presence of predicted derivates of this metabolite, which possess NH_3 at various sites of the genin. A point to be clarified is, whether the saturated lactone still can react to develop the violet colour as evidence for this amination hypothesis.Moreover, it is necessary to perform further structure analyses to resolve the mechanism which will provide further evidence for the details of the uptake, transportation and concentration of cardenolides. Further, the relative importance of the metabolism of

apolar cardenolides versus the apparent preference for the accumulation of the originally polar cardenolides is not comprehensible up to know. To uncover the advantages of an energy dependent metabolic process, complete elucidation of the associated enzymes required for transformation is still needed.

Chapter 3

Stepwise evolution of storage compartments for defensive toxins in the Lygaeinae (Heteroptera: Lygaeidae)

Abstract

Although most species of the heteropteran subfamily Lygaeinae are known to accumulate cardiac poisonous chemicals, in the case, cardenolides, from their host plants, little is known about the morphological adaptations to these traits. Only the large milkweed bug *Oncopeltus fasciatus* a representative of this group has previously been shown to store large amounts of these plant toxins in a modified integument called the dorsolateral space. As additional morphological adaptation special weak areas of the cuticle exist on thorax and abdomen which rupture under pressure to release the cardenolide-rich droplets as a feeding deterrent against predators. The present study reexamines this thoracic storage compartment of *O. fasciatus* in detail and presents a comparative analysis of the integument as storage area for defensive compounds in several species of Lygaeinae. By mapping the observed morphological features on a recent phylogeny of the Lygaeinae we here report that the adaptation for storage and release of plant compounds evolved in a stepwise manner.

Introduction

Heteropteran species are well-known as stink bugs because they possess metathoracic scent glands (MTGs) excreting allelochemicals of an offensive odor (Remold, 1963; Staddon, 1979; Staddon & Daroogheh, 1981; Aldrich, 1988) which can be an effective anti-predator defense (Remold, 1962; Schaefer, 1972; Staddon, 1979). Most representatives of the hemipteran subfamily Lygaeinae, however, are known to have reduced MTGs (Schaefer, 1972; Scudder & Duffey, 1972), yet the chance of surviving a predator attack is increased. This could be shown through predation assays in which species hold on toxin plants have been tested directly against sunflower reared Lygaeinae (Sillen-Tullberg *et al.*, 1982; Sillen-Tullberg, 1985; Evens *et al.,* 1986; Chapter 4). As alternative defense system Lygaeinae bugs acquire toxic and emetic cardenolides (cardiac glycosides, CGs) by sequestration from their food plants. The quality of being distasteful and poisonous is frequently combined with a bright coloration of red or orange and black warning colors (Guilford, 1990).

The large milkweed bug *Oncopeltus fasciatus* (DALLAS, 1852) is one of these brightly patterned Lygaeinae which utilize sequestered CGs as chemical deterrents and for this accumulates considerable quantities of these toxic compounds in specialized areas which are called dorsolateral space (Scudder & Duffey, 1972; Scudder & Meredith, 1982a). These structures have been shown primarily in the lateral parts of the integument in the meso- and metathorax as well as in the sterna II to VII of the abdomen (Scudder & Meredith, 1982a). In Heteroptera most representatives possess a duplication of the integument in the lateral parts of the meso- and metathorax (Taylor, 1918) which will be called epimeral duplicature in the following which is divided ventral in the supracoxal lobe and dorsal in the posterior epimeral lobe. An investigation of these regions showed that the cuticle of the metathorax is extended dorsally forming a pipe-like structure which releases the droplets through a slit between two overlapping cuticular flanges. In addition the integument also has several discrete weak points which can disrupt to release chemicals if the adult bug is squeezed. On release, these repellent droplets are located on brightly colored areas where they can be immediately spotted by a predator. Thus, concentration of cardenolides in the storage compartments and release of a toxic CG containing fluid along the thorax and abdomen is probably a more effective anti-predator strategy than the use of MTG chemicals. However, the release mechanism of the defensive fluid is only known for *O. fasciatus.* According to the assumption of Scudder and Duffey (1972) these structural specializations seen in the abdomen of *O. fasciatus* created for cardenolide accumulation are also present in other Lygaeinae but are not a universal characteristic of this subfamily. Further examinations of the sequestering Lygaeinae, *Lygaeus kalmii* (SCHILLING, 1829) supposed to have found a two layered integument very similar to that in the large milkweed bug (Scudder & Meredith, 1982a). However, the exact 3-dimensional extensions of the dorsolateral space are not known and comparative analyses of other species of Lygaeinae are also lacking.

Therefore, one aim of the present study was the detailed description of the glycoside storage compartments (“dorsolateral space”) in the thoracic region of *O. fasciatus* using histological sections and micro-computer tomography (µ-CT). Further ten additional representatives of Lygaeinae and one closely related outgroup species were examined and the findings compared with the storage compartment observed in *Oncopeltus*. The major goals were to reconstruct the basic patterns of this specific defense mechanism in Lygaeinae and to uncover the evolution of the morphological adaptations to the storage and release of toxic CGs. This comparative approach revealed that a modification of a thickened double layered integument is responsible for the origin of a uniform defense system combined with the stepwise evolution of a toxin releasing system as anti-predator defense.

Material & Methods

List of examined taxa

Adult specimens of 10 species of Lygaeinae from 8 genera and 1 closely related species of the Ischnorhynchinae were examined. Species which were studied by serial cross sections include ***Kleidocerys resedae*** (PANZER), 1797 (Germany: Hamburg, Martin-Luther-King-Platz), ***Arocatus melanocephalus*** (FABRICIUS, 1798) (Germany: Zossen, Kallinchen), ***Arocatus aenescens*** STÅL, 1874 (Australia: Queensland, old forestry camp, Bulburin FR), ***Caenocoris nerii*** (GERMAR, 1847) (Spain: La Gomera, between San Sebastiàn and Chejelipes), ***Cosmopleurus fulvipes*** (DALLAS, 1852) (Marokko: Field near Chegaga), ***Horvathiolus superbus*** (POLLICH, 1781) (Germany: Rheinland, Bad Unkel (Stux)), ***Lygaeus equestris*** (LINNAEUS, 1758) (Germany: Frankfurt Oder, Mallnow), ***Oncopeltus fasciatus*** (USA: New York State, Ithaca), ***Spilostethus pandurus*** (SCOPOLI, 1763) (Namibia: Brakwater), ***Tropidothorax leucopterus*** (GOEZE, 1778) (Germany: Grießheim, Rheinwiesen). The species we investigated by SR-µCT were ***Arocatus longiceps*** STÅL, 1872 (Germany: Berlin, Cental Station) and ***O. fasciatus,*** in addition the latter species was also examined with the SEM.

Methods

Histology

Specimens were dehydrated in an ethanol series and embedded in Araldite. Cross sections of 1 µm thickness were produced using a Reichert-Jung Ultracut E microtome and glass knifes. Sections were manually transferred to adhesion microscope slides (Histobond®+) and stained with a mixture of toluidine blue O (MERCK) and pyronin G (SERVA) 4:1. The serial sections were digitalised using a semiautomatic slide scanner composed of a Leica DM 5000 microscope and equipped with MetaMorph software. The digitalised sections were finished with Adobe Photoshop CS6 (colour correction, unsharp masking) and aligned using Visage Imaging™ Amira® 5.4 software.

Synchrotron Radiation based Micro-Computer Tomography (SR-µCT)

The SR-µCT scans were performed at Beamline BW2 of the German Electron Synchrotron Facility (DESY, Hamburg; project number I-20070032) using a stable low photon energy beam (8kV) and absorption contrast (see Beckmann et al. [2006, 2008], Friedrich et al. [2008]). The specimens were dehydrated in an ethanol series, critical point dried (EmiTech K850 Critical Point Dryer; Ashford, Kent, UK) and mounted with superglue on a metallic holder. The samples were scanned in 180° rotation, resulting in an image stack with a physical resolution of 4 µm (voxelsize: 1.98 µm). The high density resolution of the SR-µCT-data allowed to discriminate different types of tissue (e.g., skeleton, muscles).

3-dimensional reconstructions

Based on both µCT-image stacks and histological sections the extension of the cardenolide (or cardiac glycoside) storage compartment (CSC) of several species was reconstructed three-dimensionally using Visage Imaging Amira 5.4 software. Due to the thin delimitation of the CSC the structures had to be manually outlined with the “brush tool” using a Wacom Cintiq 21UX interactive pen display. The obtained segmented image stacks were used for the automatic creation of surface objects in Amira software.

Scanning Electron Microscopy (SEM)

For the examination of external skeleton structures the body of a specimen (with legs dissected) was cleaned by ultrasonic bath and afterwards dehydrated in ascending concentrations of ethanol. The specimen was fixed in formaldehyde-acetic acid- ethanol (FAE), critical point dried (EmiTech K850 Critical Point Dryer, Ashford Kent) and glued to a fine pin with nail polish. The sample was then coated with platinium (Polaron SC7650 Spulter Coater) on both sides and examined in LEO 1525 SEM.

Results

Thoracic glycoside storage compartments of *O. fasciatus*

The glycoside storage compartment of adult *O. fasciatus* is an enormous, complexly branched space underlying the cuticle of wide regions of the thorax. This compartment originally described as dorsolateral space (Scudder & Meredith, 1982a) is now called as glycoside storage compartment (GSC) due to new results concerning the actually locations storing cardiac glycosides. Moreover, the new name designates the type of compounds known to be accumulated in this compartment described in the recent study.

The glycoside compartment shows main concentrations in the posterolateral parts, i.e. the epimeral regions (Fig.1.A-C). The compartment is located between the cuticle and the epidermis, which is underlain by a thick basal lamina (Fig.2.I). The comparison of three specimens revealed no distinct intraspecific variability. The location of the glycoside containing spaces is identical. However, minor variation in size of some parts was observable.

Three main compartments can be distinguished which are illustrated as pictures in Fig.1. A-C and further are represented ba a supplementary 3D video. The anteriormost compartment is located in the prothorax (Fig.1.A-C, represented in yellow). It is composed of a circum-cervical ring just behind the head. This structure called as anterior protergal lobe (apl) is a marginal evagination of the integument enclosing the neck around the prothorax (structure by Govid & Dandy, 1970). Ventrally paired lobes widely spread in the prosternal region to the edge of the coxa. The posterior half of the lateral prothoracic skeleton is completely underlain by an extensive compartment. It is dorsally connected with paired, lap-like extensions situated in the pronotum. A ventral interconnection with the precoxal room is not developed. The prothoracic storage room is not connected to the following segmental compartments.

The main part of the second, mesothoracic compartment fills the epimeral area. It extends into almost every corner of the epimeron (em). The epimeron is folded in the central region forming flattened cuticular duplicatures. Its ventral flange, the supracoxal lobe (scl), covers the coxal joint, whereas the dorsal lobe masks the intersegmental membrane and the spiracle. In cross section the lobes appear double layered with a narrow space between both sheets of cuticle. The single connection to the body cavity is directly above the coxal joint (Fig. 2.I). Almost the whole space in the cuticular lobes is occupied by extensions of the main mesothoracic compartment. This compartment also expands along the dorsal pleural margin on the anepisternum and enters the pleural wing process (pwp). A thin channel (vcb_1) runs ventral along the anterior rim of episternal (es) and sternal sclerites and interconnects the spaces of both body halves. A second, posterior bridge (vcb_2) is formed directly in front of the mesocoxae. This channel is about twice time thicker as the anterior one. Two anteriorly directed lobes are developed close to the mid line. A distinct dorsal interconnection of the main compartments is not present. A dorsal clasp-like storage space is located below the posterior rim of the mesoscutum but is completely delimited from the main compartments of the mesothorax.

The third compartment, located in the mesothorax, is similar structured as its mesothoracic counterpart. Both storage spaces are connected by a short bypass from the posterolateral corner of the mesothorax with the anterodorsal metathoracic edge.

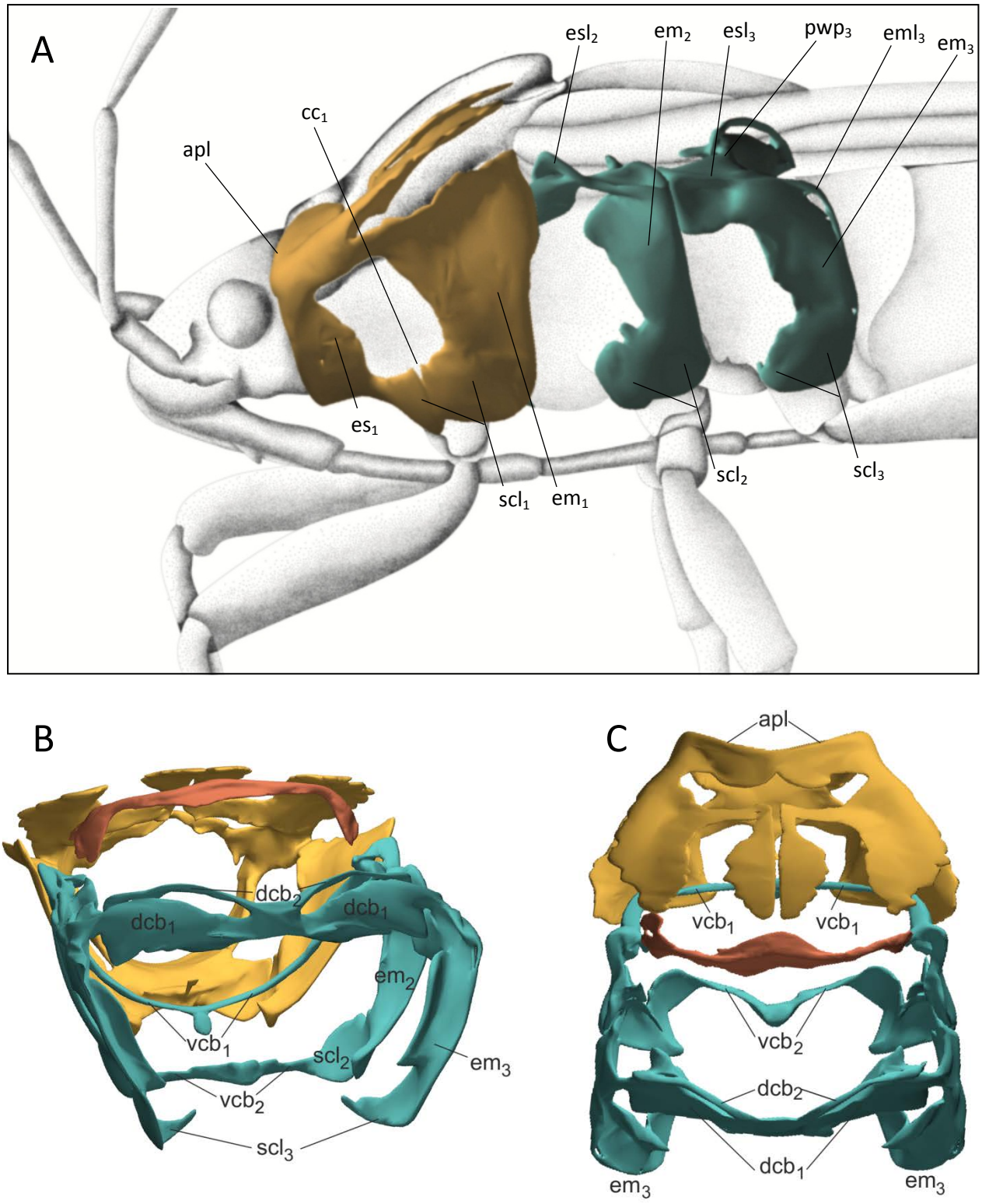

Figure 1. A-C) Thoracic glycoside storage compartments of *O. fasciatus*, 3D-reconstructed based on SRµCT-data. A, drawing of external features combined with reconstruction of the three biggest storage compartments in lateral view. B, posterolateral view. C, dorsal view. Distinct, unconnected compartments are differently colored: yellow - prothoracic compartment, blue - meso- and metathoracic compartments,red - dorsal mesothoracic clasp. anterior protergal lobe (apa), coxal cleft (cc), dorsal connecting bridge 1 (dcb_1), dorsal connecting bridge 2 (dcb_2), epimeron (em), episternum (es), pleural wing process (pwp), supracocal lobe (scl), ventral connecting bridge 1 (vcb_1), ventral connecting bridge 2 (vcb_2),

The lateral main elements of the metathoracic compartment differ only slightly from the condition present in the mesothorax. Only the anepisternal part is wider distributed. Distinct differences are observable in the interconnecting channels: neither an anterior nor a posterior ventral connection is present but well-developed dorsal bridges (dcb_1 + dcb_2) fill large areas under the metanotum, immediately behind the mesophragma (Fig. 1 B + C). The larger one (dcb_1) of these two connecting bridges has its origin in the pleural wing process and runs from both sides along the mesophragma. The second conjunction (dcb_2) arises from the larger connecting bridge medially behind the wing base and runs along the postscutellum as a narrowly centered deviating bridgeand merges with the parallel running storage area (dcb_1) at the end of the mesophragma.

Comparison of thoracic glycoside storage compartments in the Lygaeinae

By comparing the cross-sections respectively the SR-µCT scans of 10 different species of Lygaeinae, we determined the extent of the glycoside storage compartment and can show that this feature follows a uniform basic pattern. In general, it appears that all available regions of the integument are used for cardenolide storage. The storage compartment in further Lygaeinae shows the same construction and revealed no distinct variability as described in *O. fasciatus.* Further, all investigated species show the same intersegmental connections: a thin bridge (vcb_1) anterior, and a bigger one posterior (vcb_2) situated, which ventral interconnect the space of the body halves. Equally present are both dorsal bridges (dcb_1 and dcb_2) which run along under the metanotum. Examinations could not show that further arias as described in *O. fasciatus* will be used to accumulate cardenolides.

The differentiating feature of the glycoside storage compartment within the Lygaeinae is the structure of the lateral thoracic storage area. The main storage region as previously shown (Fig.1 A-C) represents the epimeron of meso- and metathorax. In this region is developed a special structure called as epimeral duplicature (ed) which arises through evagination of the dorsal and ventral integument of the epimeron (see Fig.2 A-I). The dorsal part of the epimeral duplicature forms the structure called epimeral lobe (eml) whereas the ventral part builds the supraxocal lobe (scl). The cavity among the epimeral duplicature represents the main part of the storage compartment. The glycoside storage compartment of the epimeral duplicature in *O. fasciatus* is represented by a well and large developed space with a cuticle thickness of 17.08 µm (Fig. 2I).

In comparison I could demonstrate that *K. resedae* representing the subfamily Ischnorhynchinae exhibit no storage capacity due to a missing glycoside storage compartment (Fig. 2.A). However, it has been shown that *K. resedae* exhibit a strong cuticle

which takes the whole region for the integument as storage space in *O. fasciatus*. Species of the genera *Arocatus* and *Caenocoris* although have a compartment to store cardenolides however, the capacity of storing substances is limited. The cuticular sides of the integuments are still connected in some areas, which probably cause better stabilization but restrict the space for fluid filling (Fig.2. B-D). Further the cuticle of the integument of those small bugs is quite thick in contrast to the other genera of this lygaeid subfamily. Comparing the cuticle thickness of the species we found that the cuticle of the genera *Arocatus* and *Caenocoris* is 1.5 times thicker relative to the cross-section of the body.

In the genera *Cosmopleurus, Horvathiolus* and *Lygaeus* I could observe stabiliments, too, which cross the double layered integument in the basal region, yet do not appear to reduce the storage capacity of the compartment (Fig. 2.E,F,G). The space between the cuticle and basal lamina in the epimeral duplicature reaches the maximum extension in the species of the genera *Horvathiolus, Lygaeus, Spilostethus* and *Tropidothorax* as well as in *O. fasciatus* (Fig.2.F-I). Since the basal lamina borders the storage space proximal directly to the body cavity, therefore species have the ability to extend the storage compartment continuously into the body as shown in Figure 2. (F,G,I).

Abbreviations

apl	anterior protergal lobe
as	angled slit
at	adipose tissue
bs	basal lamina
c	cuticle
dcb	dorsal connecting bridge
drs	dorsal releasing sites
ed	epimeral duplicature
em	epimeron
emg	epimeral wing groove
eml	epimeral lobe
es	episternum
esg	episternal wing groove
esl	episternal lobe
f	flange
gc	glycoside compartment
m	midgut
ms	median suture
pbp	predetermined breaking point
prs	posterior releasing sites
pwp	pleural wing process
scl	supracoxal lobe
t	trachea
vcb	ventral connecting bridge

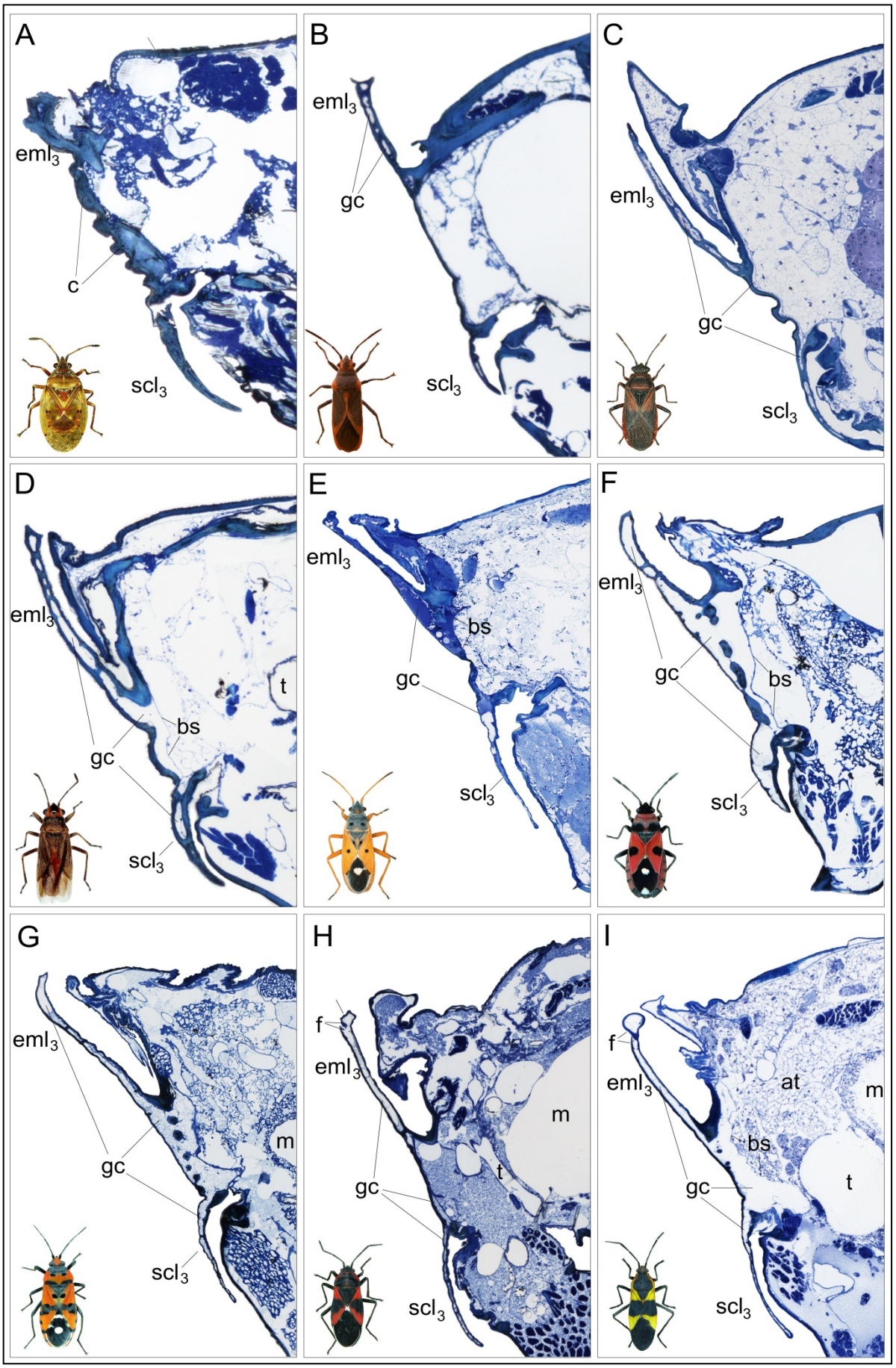
A
eml3
c
scl3
B
eml3
gc
scl3
C
eml3
gc
scl3
D
eml3
t
bs
gc
scl3
E
eml3
bs
gc
scl3
F
eml3
gc
bs
scl3
G
eml3
m
gc
scl3
H
f
eml3
m
t
gc
scl3
I
f
eml3
at
m
bs
t
gc
scl3

Figure 2. Lateral part of histological cross sections through the metathoracic cuticular duplicature showing the conditions of the glycoside storage compartment and drop releasing systems in eight Lygaeinae and one Ischnorhynchinae. Note the differences in cuticule thickness and size of intercuticular space. A) *Kleidocerys ressedae* B) *Caenocoris nerii* C) *Arocatus melanocephalus* D) *Arocatus aenescens* E) *Cosmopleurus fulvipes* F) *Horvathiolus superbus* G) *Lygaeus equestris* H) *Tropidothorax leucopterus* I) *Oncopeltus fasciatus.*; adipose tissue (at), basal lamina (bs), cuticle (c), epimeral groove (emg), flange (f), glycoside compartment (gc), metepimeral lobe (eml_3), midgut (m), supracoxal lobe (scl), trachea (t),

Thoracic release sites of the Lygaeinae

By mechanical pressure on certain points of the surfaces of the adult *O. fasciatus* discrete droplets of distasteful fluid (space fluid) are secreted (Fig.3 A+B). Without exception, the integument of all segments of thorax and abdomen possesses an external modification of the cuticle creating a weak point which can easily disrupt. The cuticle of the specific areas along the dorsolateral margins of the body is somewhat thinner and breaks off at a predetermined breaking point (pbp). Only in the area of the metathorax a second segmental orifice is situated. In this region the cuticle forms a slit by two overlapping flanges for releasing droplets without disrupting the cuticle (Fig.2.I, Fig.6.5 and Fig.5).The angled slit is situated inferior of an epidermal lobe at the posterior margin of the metathoracic epimeron (Fig.4.6). This region of secretion releasing we designated as posterior releasing side (prs).

By comparing twelve different species of the group we ascertained that there are enormous differences in the characteristic of the releasing sites of the Lygaeinae. Like *O. fasciatus, T. leucopterus* species also have both morphologically modified opening systems: the predominant breaking point (pbp) situated distally on a lobe at the dorsal edge of the metepisternum which we called dorsal releasing side (drs) and a slit formed by two overlapping flanges at the dorsal margin of the metepimeron (prs) (Fig.2.H) very similar to that in *O. fasciatus.* Gentle pressure on the thorax causes the release of droplets out of the epimeral slit whereas strong pressure leads further to an opening of the breaking points (pbs) in the metepisternum. An examination of six more species of Lygaeinae (*H. superbus, S. pandurus, S. saxatilis, L. equestris, L. simulans, C. fulvipes*) could establish that strong pressure of the thorax leads to the release of liquid only in the area of the metathoracic dorsal releasing site. Histological sections clearly demonstrated that in *H. superbus, S. pandurus, L. equestris* and *C. fulvipes* there is also a braking point of the metathoracic dorsal releasing side. The breaking point occurs as a distally situated slit in the cuticle of the epimeral lobe (Fig.4.6). This orifice is caused by a very thin region in the cuticle (Fig. 4.2) and is most likely proximally closed by an underlying cell membrane. An analysis of the region of the posterior releasing site (prs) in *S. pandurus* suggests that the cuticle of the integument of the metepimeral lobe (eml_3) is extremely thin but a slit formed by two cuticular flanges is missing (Fig.4.3).

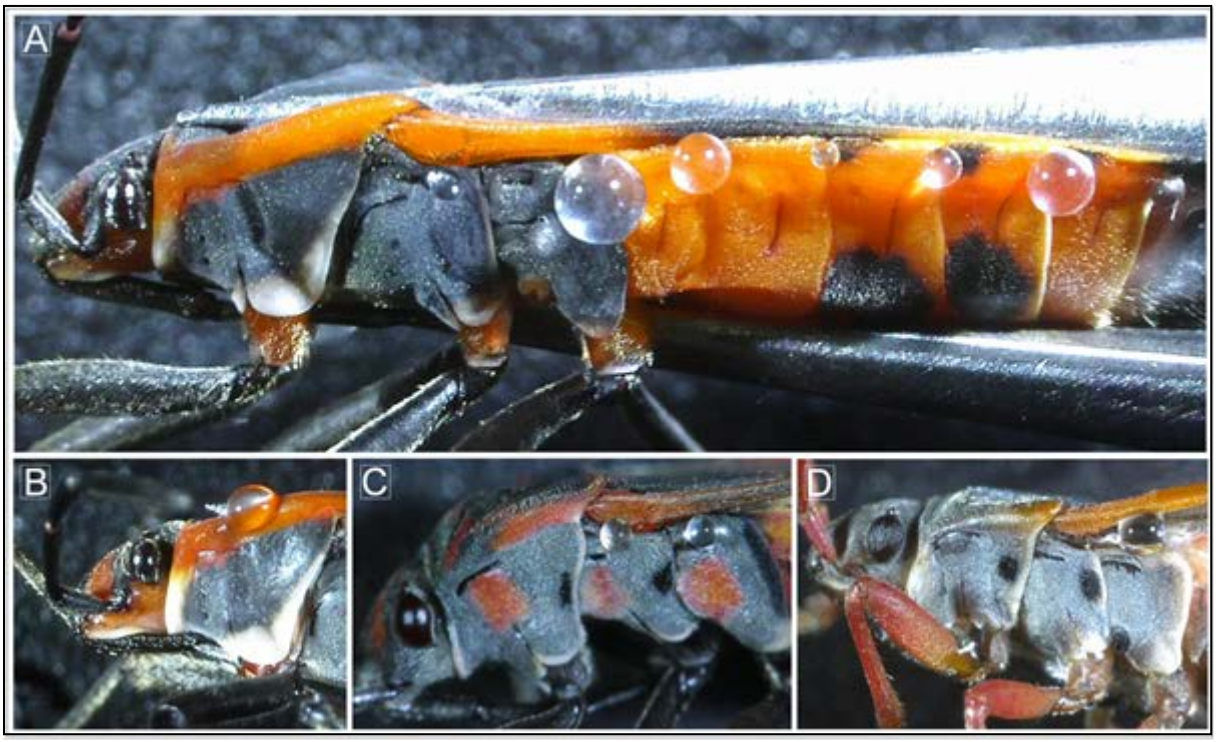

Figure 3. Lateral view of two different species of Lygaeinae showing releasing sites of glycoside compartments of thorax and abdomen. A). *O. fasciatus* excretes defence fluid of the storage compartment by seven segments through mechanical pressure. B). *O. fasciatus* ♀ releasing a droplet in the region indicated by the arrow of the prothorax. C). *S. pandurus* releasing a droplet of the pbp of the es2 and es3. D). The release of a droplet of distasteful glycoside-rich fluid from a tergo pleural weak point of the metathorax of *C. fulvipes*.

The region of the eml_3 shows an additional cuticular modification (Fig.4.4) as determined in the esl_3 described for predominant breaking points. Despite strong pressure on the eml_3 we couldn't observe release of glycoside-rich fluid out of this region in *S. pandurus.*

As a common feature between both opening systems (predominant breaking point and the releasing system) we could observe that the slit of the eml_3 as well as the preaking point of the esl_3 are located exactly in the region inferior to the end of the epimeral wing grooves which serves as fixture for the edge of the wing. A further group species, *C. nerii, A. aenescense, A. rusticus, A. longiceps and A. melanocehalus,* exhibited neither a slit as mechanical opening nor thinly constructed weak points for predominant breaking points (pbp) (Fig.4.1). Observations showed that pressure exerted on these species does not result in the release of fluid.

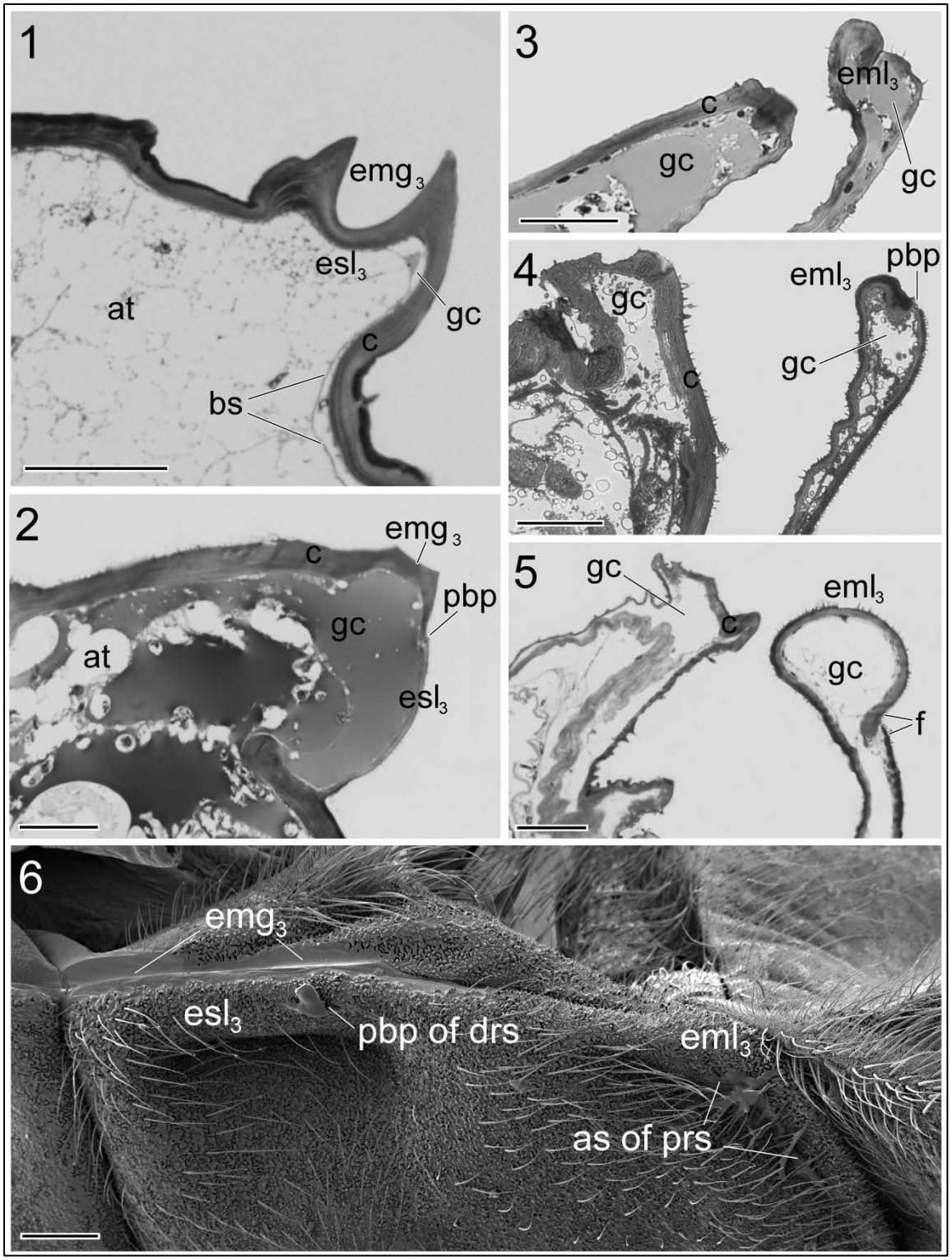

Figure 4. Representation of all variants of the glycoside releasing site in the metathorax of the Lygaeinae. 1,2) Dorsolateral view of the thoracic transverse section through metepisternal lobe (esl_3) of 1 *A. melanocephalus* without dorsal releasing sides (drs) and 2 *C. fulvipes* with a predetermined braking point (pbp) of the drs. 3,4,5) Dorsolateral view of the thoracic transverse section through metepimeral lobe (eml_3) of 3. *C. fulvipes* with no posterior releasing site (prs), 4 *S. pandurus* showing a pbp of the prs and 5 *O. fasciatus* with an angled slit (as) of the prs formed by two overlapping flanges (f). 6) SEM of the dorsal part of the metathorax of *O. fasciatus* (lateral view); representing anteriorly the esl_3 with the distally situated predetermined breaking point (pbp) of the drs and posterior the eml_3 with the inferior situated (as) of the prs (Scale bars 1-5 50 µm, scale bar 6 100 µm); glycoside compartment (gc), basal lamina (bs), cuticle (c), adipose tissue (at), epimeral groove (emg)

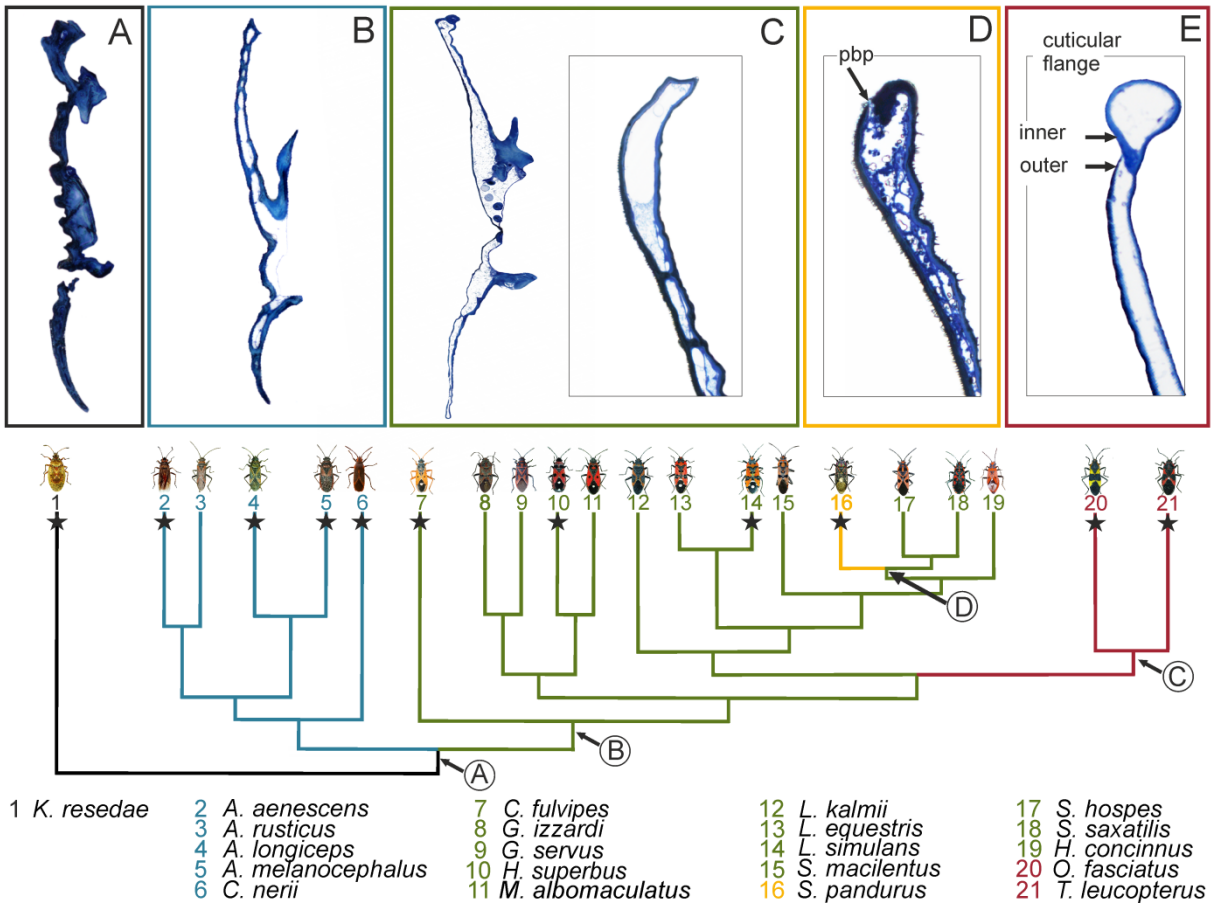

Figure 5. Stepwise evolution of a storage- and releasing system for poisonous cardiac glycosides in the subfamily Lygaeinae. The top pictures display the three different forms of the storage compartment (A-C) and the three forms the metepimeral lobe (C-E) found in the subfamily. The phylogenetic tree based on combined data of COI, COII and 28S genes comprises ten genera of the Lygaeinae and *Kleidocerys resedae* as outgroup species. The coloration of the branches indicates the species clusters with the same morphological adaptations. Circled letters show the origins of new features: A) the origin of a storage compartment, B) the origin of a predominant breaking point (pbp) at the episternal lobe ($esl_{3)}$, C) the origin of a mechanical opening system at the epimeral lobe of the metathorax (eml_3), D) origin of a predominant breaking point (pbp) at the metepimeral lobe (eml_3). The black stars represent the species which were examined either by SR-µCT or by histological section series.

Discussion

The integument of insects not only acts as water barrier to prevent tissue desiccation but also is very often involved in defense strategies against predators, parasites and disease agents (Boucias & Pendland, 1998, Neville, 1975). As a physical barrier a strong exoskeleton offers an efficient protection since it is first contacted by an attacking predator. Nevertheless, a thinner cuticle can be advantageous in the insect's defense strategy as well. In some cases, mechanical defense mechanisms disappeared in order to focus prospectively on chemical deterrence. One impressive demonstrations of such a case represents the defense system of the subfamily Lygaeinae, shown in the present study.

The epimeral duplicature in Heteroptera such as in *K. resedae* generally consist of a very strong cuticle to protect specimens as mechanical boundary (Larsén, 1945), whereas in the Lygaeinae the cuticle thickness is reduced to get space for cardiac glycoside storage by dietary sequestration from plants. As we have been able to show, the glycoside storage compartment in *O. fasciatus* as well as in all examined species of Lygaeinae underlies vast areas of thoracic regions of the external integument. Since nearly all available areas of integument are used for glycoside storage, it seems almost impossible for predators to overcome the chemical defense barrier.

The glycoside rich compartment arises as a result of extensive vacuolation of an unusual epidermal cell layer (Scudder & Meredith, 1982a) which in turn enables the insect to fill all areas of integument underlying the vacuolated tissue. Vacuolated epidermal cell layer are examined only in a few insects including *Schistocerca gregaria* which used storage vacuoles of a maturation pheromone (Neville, 1975). In popular leaf beetle (*Chrysomela populi*), sequestered phenolglucoside are deposited in a reservoir of defensive glands which include vesicles of defensive compounds (Strauss *et al.,* 2013). With respect to the occurrence of a similar structure, it might be possible that a mechanism as described in Strauss *et al.,* (2013) equally filled vesicles of the secretory epidermal cells in the lygaeinae which via exocytosis are emitted in the glycoside storage compartment.

The basal lamina underlying the compartment serves as border between haemolymh and glycoside fluid. The separate storage of glycoside fluid may enable the species to decrease the concentration of glycosides in the haemolymph which despite an existing insensitivity to the toxins might cause poisoning. Differences in the expansion of the storage compartment in lateral and dorsal regions between species may depend on several factors, including the type of diet, the age and the sex of the examined individuals. Because we also know that the amount of glycosides in the females is higher than in male species and that adults acquire about one-tenth of the cardenolides over a weak if they are only for a short time on a glycoside diet as they are normally on glycosides over live time cycle.

Connecting bridges found between bilateral parts as well as different segments are expected to work as pressure balance or as supply to refill depleted compartments. Mechanical pressure resulting from the capture by a predator probably causes release of glycoside-rich fluid from specialized cuticular structures. As already described by Scudder and Meredith (1982a), the releasing sites in *O. fasciatus* can be found on the dorsolateral margin of thorax and abdomen. However, this study shows that the prothoracic segment also possesses a weak point which can rupture to release glycoside fluid. Since Scudder & Duffey (1972) could not find any abdominal releasing sites they described a dichotomy in the subfamily which divides bugs in two groups with regard to their glycoside use. In contrast, we could show that all Lygaeinae use glycosides in the same manner as a special defense

strategy in which separate body parts are used as toxin reservoirs. The main difference among the species could be observed when comparing the releasing sites.

The release of droplets can be compared with “reflex bleeding” of insects which represents the release of haemolymph at a predefined spot. In according species the haemolymph contains remarkable amounts of defensive substances which are used in protection as well. As defensive action, at least five insect orders including mainly coleoptera perform reflex bleeding where insects present haemolymph, through integumental rupture (Wallace & Blum, 1971; Blum & Sannasi, 1974). Additional representatives are known from Plecoptera, Homoptera (Cercopidae), Lepidoptera and Hymenoptera (Symphyta) (Peck, 2000; Boevé, & Schaffner, 2003; Capinera, 2008). However, in these cases the toxic substances are dissolved in the haemolymph whereas in the Lygaeinae only specialized and secluded compartments release concentrated cardiac glycoside fluid.

Mapping the observed morphological differences (glycoside compartment and releasing sites) on the phylogeny of the Lygaeinae it becomes unambiguously clear that a stepwise adaptation to cardiac glycoside accumulation took place. The original condition represented by the outgroup species *K. resedae* features a strong integument possibly as a mechanical defense strategy (Fig 5.A). Within the Lygaeinae we can discriminate between four different characteristics that evolved for glycoside storage and release. The presence of glycoside compartments caused by reduction of cuticle thickness represents a synapomorphy of the whole subfamily. As most basally situated clade and sister taxon to all other Lygaeinae, the genera *Arocatus* and *Caenocoris* still show the original condition of this stepwise evolution. The adaptation for glycoside storage implies a thinner cuticle which allows the storage of fluid in few cavities, yet no fluid exit points are present in the too thick cuticle. This basal morphological transformation may not confer any advantage for the individual in an encounter with a predator because distastefulness only gets noticed when the bug is sacrificed. Scudder & Duffey (1972) pointed out that the effectiveness of glycosides does not depend on secretion onto the surface as demonstrated by *Danaus plexippus*. However, we believe that protection in the monarch butterfly is caused by the storage of glycosides in wing scales which represents a similar way of external presentation. In contrast to the most basal clade, species of the sister group including the other genera of Lygaeinae possess an integument with a thinner cuticle and a larger storage compartment relative to a cross-section of the body. At the base of this clade, structures for releasing droplets out of the glycoside compartment must first have originated. By comparing cuticles of lygaeid genera we detected an external modification of the cuticle (predetermined breaking point, pbp) in the dorsal metepisternal lobe (esl_3) which evolved first as simplest and most basal opening system (at B in Fig. 4). As synapomorphic feature of at least *O. fasciatus* and *T. leucopterus* (at C in Fig. 4) a mechanical opening system which by gentle

pressure opens two cuticular flanges to release droplets out of the compartment represents the next step in the adaptation to cardiac glycoside containing plants. The development of such a special opening mechanism may ensure a targeted release of fluid and reduce the damage of tissue by rupturing. All these adaptations reflect a stepwise approximation to an elaborate protection system in the Lygaeinae, the more so as experiments with predators showed that the presentation of distasteful fluid on the outer parts of the body enabled the survival of the tested bugs without any ill effects.

The mechanical opening system, described above, was found only in the genera *Oncopeltus* and *Tropidothorax,* two closely related species. The phylogenetic analysis of the COI/II and 28S sequence data (see Chapter 1) left open whether *Oncopeltus* is sister taxon to a monophyletic *Horvathiolus* and *Melanocoryphus* while *Tropidothorax* is distatntly placed as sister taxon to *Graptostethus* or wether *Oncopeltus* and *Tropidothorax* are sister taxa (as described in Fig. 5). The first tree had the higher likelihood, yet in an AU test (Shimodaira & Hasegawa, 1999), a statistical test that compared the sitewise likelihood of both trees, the alternative tree with *Oncopeltus* and *Tropidothorax* as sister tax did not provide a worse fit to the data. This latter phylogenetic hypothesis agree with a monophyletic origin of the complicated opening system in these two taxa.

While *Oncopeltus* as sister taxon to *Horvathiolus* and *Melanocoryphus* and *Tropidothorax* as sister taxon to *Graptostethus* might be the best tree we decide by considering our morphological results that the alternative tree with *Oncopeltus* and *Tropidothorax* as sister groups with a common ancestor don't explain the data worse. A remarkable outcome of our analysis is that a convergent evolution of the releasing site at the posterior metepimeral lobe was found in *S. pandurus*. This species does not possess a similar mechanical opening system as found in *Oncopeltus* and *Tropidothorax* but a predetermined breaking point [as discovered in the region of the episternal lobe is located on the dorsal epimeral lobe. In closely-related species like *L. equestris* none of the opening systems could be found, yet seems to be a thin cuticle in the region of the exit point in *L. equestris* similar to that shown in *S. pandurus*. We could observe that at the posterior end of the metepimeral wing groove the distal edge straightens and the cuticle gets thinner. The observation of the µCT- and histological section series of *O. fasciatus* and *T. leucopterus* confirm our assumption that the wing groove in the epimeron become less deep and forms the inner flange of the mechanical opening system. Furthermore in this context it seems not surprising that the predominant braking point at the episternal lobe is also located posterior of the episternal wing groove. These results point out that a convergent evolution of the opening systems in *O. fasciatus* and *T. leucopterus*, on one hand, and *S. pandurus,* on the other, is entirely possible since the opening areas developed out of the same structures.

Taken together, the data presented here argue for a model of a stepwise evolution in which the defense strategy of toxin release became more and more sophisticated. Obviously the storage of plant sequestered cardiac glycosides in the integument of the Lygaeinae is a result of a reduction of cuticle thickness which caused a certain loss of mechanical protection but also a gain of effective chemical defense.

Chapter 4

Deterrent effect of cardenolides: effects of diet on defence of *Oncopeltus fasciatus* against the golden orb web spider *Nephila senegalensis*

Abstract

Larvae and adults of the large milkweed bug *Oncopeltus fasciatus* (Heteroptera: Lygaeidae) are specialized to feed almost exclusively on milkweed species of the genus *Asclepias*, which contain high concentrations of toxic cardenolides (cardiac glycosides). Such toxic compounds are known to act as deterrents against predators. In this study, we investigated to what extent predators are affected by their prey's food. *O. fasciatus* were reared either on *Asclepias incarnata* seeds as a natural diet or on sunflower seeds (cardenolide-free). To investigate whether *O. fasciatus* gained improved protection by feeding on the toxic host plant vs. the nontoxic alternative we used naive orb-weaving *Nephila senegalensis* (Araneae: Nephilidae) as predators. The predation trials suggested that the cardenolide fed bugs were significantly less likely to be killed than the bugs reared on sunflower seeds. Yet, when testing for avoidance learning in the spiders no visible aversion could be detected after a negative experience with a toxic prey.

Introduction

Many insects exhibit various strategies of defence against insectivorous animals. One common way to avoid predation besides mechanical defenses and camouflage, is the use of toxic chemicals combined with aposematic colorations (Bowers, 1993; Endler & Mappes, 2004; Nishida *et al*., 2002; Lindstedt *et al.*, 2011). In addition, these defences can occur in one or multiple complex modalities which for instance imply combining of color patterns, smell, and taste (Moore *et al.,* 1990; Marples *et al.,* 1994; Marples & Roper, 1996).

Conspicuous or bright colorations as warning signals quite simply point out unprofitability for predators that discriminate among the various preys mainly on the basis of visual signs. This rests on the assumption that visually oriented predators learn to avoid distasteful prey more quickly when they have conspicuous color patterns than when they are

cryptic (Gittleman & Harvey, 1980; Gambarele-Stille & Tullberg, 1999; Speed, 1993, 2000; Ripii *et al.,* 2001; Ham *et al.,* 2006; Exnarova *et al.,* 2006, 2008). The distastefulness of insects may either stem from noxious or emetic substances which are acquired from their host plants (sequestration) or which are synthesized by the insect *de novo* (Opitz & Müller, 2009).

Several species of the hemipteran subfamily Lygaeinae are known to tolerate and take up deterrent plant chemicals, the most famous example being the large milkweed bug (*Oncopeltus fasciatus*). Both the nymphs and adults of this brightly black and orange colored species obtain a variety of potentially toxic, plant produced cardenolides from *Asclepias* seeds whereas species reared on sunflower seeds lacked these chemicals (Duffey & Scudder, 1972; Moore & Scudder, 1985; Scudder *et al.,* 1986). In addition, *O. fasciatus* possess specific morphological adaptations for cardenolide uptake and accumulation. When the bugs are squeezed by a predator, the stored bitter-tasting cardenolides are extruded as discrete droplets of fluid at defined points along the dorsolateral margins of the thorax and abdomen. The presence of deterrent chemicals on the surface of *Asclepias*-fed *Oncopeltus* can influence the tendency of predators to release this bugs either immediately upon contact with the bitter tasting fluid or through visualization because repellent droplets are located on brightly colored areas where they can be immediately spotted by a predator (Scudder & Duffey, 1972; Scudder *et al*., 1986).

Cardenolides generally have a strong deterrent effect as antipredatory defences in vertebrates as has been demonstrated for blue jays (*Cyanocitta cristata*), quails (*Coturnix coturnix*), mice and bats (Brower *et al.,* 1967; Brower, 1969; Evans *et al.,* 1986; Glendinning, 1990; Hristov & Conner, 2005). Through a bitter taste and emesis provoking stimuli it can be expected that cardenolides have general a repellent effect. To our knowledge, there are only few experimental studies addressing the protective effect of sequestered cardenolides on arthropod predation (Levey, 1983; Berenbaum & Miliczky, 1984; Malcolm, 1989; Petschenka *et al*., 2011). The experiment performed with Chinese mantids (*Tenoders ardifolia snensis*) demonstrated that mantids after rejection of a milkweed bug only when it had access to Asclepias seeds have learned to reject aposematic bugs after encountering an unpalatable conspecific. Field observations suggested that orb weaving spiders (Araneae) are able to detect toxic Lygaeinae before biting into the prey and cut them out. Since the visual sense plays a very minor role for orb weaving spiders, the aposematic color patterns of the bugs can not serve as a warning signal. Moore *et al.,* (1990) pointed out that many species relying on toxins for defence also provide some odours as repellent signals. In this case might olfactory senses explain the rejection of the aposematic bug by the blind spider.

Petschenka *et al*., (2011) illustrated that ouabain, (a cardenolide commonly used in physiological research) *per se* deters potential enemies from otherwise suitable prey items,

yet this cardenolide does not occur naturally in host plant species of *O. fasciatus*. We therefore conducted predation assays with the golden orb web spider *Nephila senegalensis* (Walckenaer) and tested the effect of a natural cardenolide composition obtained from the host plant *Asclepias incarnata*. Furthermore, we investigated whether the spiders learn avoidance of unpalatable prey by signals conveyed non-visually. Feeding on toxic host plant species would thus confer a further advantage.

Material and methods

Plant and insect material

The milkweed seeds (*Asclepias incarnata* L.) for feeding-assays were received from the botanical garden, in Hamburg. Adult male and female of the large milkweed bug *O. fasciatus,* taken from a laboratory culture were kept under artificial light (16:8 h light-dark cycle) at 26°C. The insects were reared from the egg stage either on a diet of husked sunflower seeds (*Heliothus annuus* L.) and water, or on milkweed seeds (*A. incarnata*). Specimens reared on sunflower seeds were used as controls since this food source does not contain cardenolides. Specimens on *A. incarnata* seeds derived Cardenolides from these host plant during larval feeding. All used bugs were descendants derived from a number of couples obtained from the Aquazoo, Düsseldorf. All *N. senegalensis* spiders used in this study were of the first generation raised in captivity, the parental generation had been collected near Kapstadt, South Africa in 2011. Due to the extreme sexual size dimorphism of *Nephila* only females were used for the experiments. Spiders were placed in frames of acrylic glass (60 cm x 60 cm), with a rear panel and an open front and maintained on a diet of *Calliphora* flies. Animals were kept under artificial light (12:12 h light-dark cycle) at 23-27°C and sprayed with water every second day.

Predator-Assay

All 13 spiders used were naïve with regard to cardenolides and starved for two days before beginning the test series because a hungry predator could be more willing to try aposematic prey. The experiments consisted of two feeding trials in which consecutively two different prey items were presented. For each trial a cardenolide rich and a cardenolide lacking *O. fasciatus* were offered to the spiders in different order. The bugs were flipped into the webs and the behavior of the spiders was recorded. Further, for each trial we noted the time until the spider cut the prey out of the web (“time in web”) and as well as the residual weight of the

prey. In the first trial, six spiders received a cardenolide rich *Oncopeltus* first. After consumption or rejection of the prey an cardenolide rich *Oncopeltus* was hurled into the web. In the second trial seven additional spiders were tested and were presented first with a cardenolide rich *Oncopeltus* and afterwards with a cardenolide lacking one. We used these two trials to check that the spiders behavior is not affected by the sequence (if the cardenolide rich bug offered the spider first or second). We measured time in the web from the attack of the spider to the excision of the insect and determined the weight before and after excision.

Statistical analysis

The retention time in the web and the loss of weight after attack were separately analyzed for both trials. All statistical comparisons used the Wilcoxen signed-rank test, which explicitly incorporates paired data. Differences were accepted as statistically significant in all analyses if $p < 0.05$. To improve clarity, primary data on weight loss are illustrated as % values. Statistical analysis was carried out with JMP 7.1 (SAS).

Results

Attacking behaviour of *N. senegalensis*

After introducing the prey into the web, the spiders responded in both trials at once to the bug's presence by vibrating the radii with their forelegs (plucking). (Fig. 1 A). A swift attack after recognition of the prey was accompanied by directly grasping it with the fangs (Fig.1 B and C). With few exceptions, naïve *N. senegalensis* released the cardenolide rich prey (species reared on *Asclepias*) immediately upon contact (Fig.1 D). To do so, the spiders either cut some of the threads around the prey or pulled the bug out by using their pedipalps. In a single case, after completing a short test bite, the *Nephila* even backed away and immediately begun to clean itself by wiping the fangs. The spider was instantly repelled and acted confused and disoriented. After a while, the spider undertook numerous bites while rotating the bug with the forelegs. This prey was released after a period of 56 minutes and died from the bites, whereas for all other bugs in this trial the presence in the web lasted only about one minute and the prey survived.

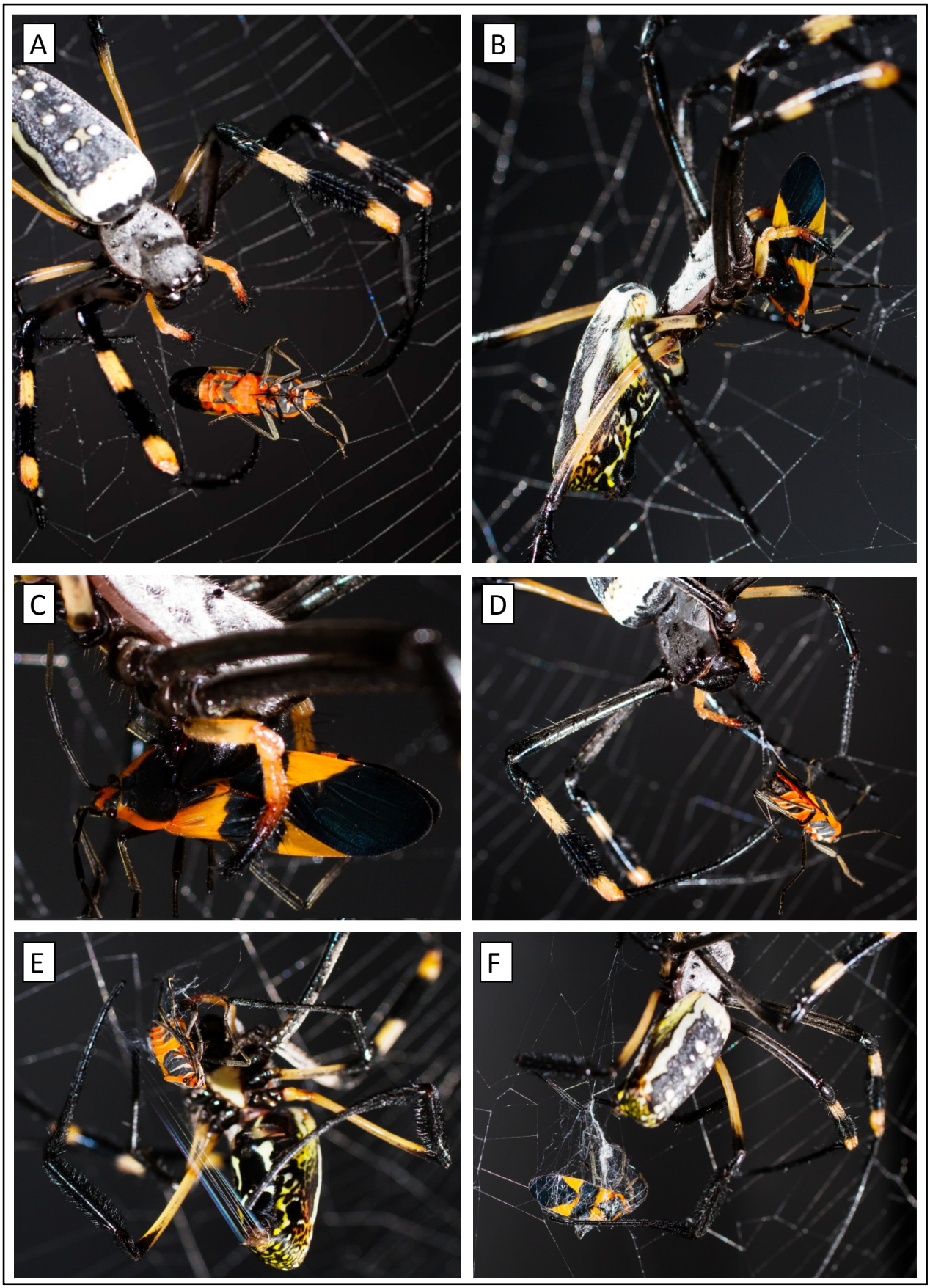

Figure 1. Predation on *Oncopeltus fasciatus* reared (D) solely on *Asclepias incarnata* seeds or (E, F) exclusively on sunflower seeds (*Helianthus annuus*). (A) The spider is approaching the bug, and then grasps it immediately to inflict its bites (B, C). (D) *Nephila* releases the prey from the web by removing the silk enclosing the bug. (E) The spider spins loops of silk around the prey before translocating the completely enveloped bug to its feeding site (F).

If the bugs were reared on sunflower seeds the *Nephila* spiders killed the bugs after the first test bite by injection of poisonous saliva. After the bug was wrapped in silk by a few loops, the spider brought the prey to the center of the orb and started feeding (Fig. E and F). During feeding, which lasted 1.4-2.6 h, the spider twisted the bug and reduced the size to a small chunk of dry remnants. All 13 bugs that we offered in both trials were treated similarly.

Effect of food plant

This experiment using natural predators was conducted to test whether the insects containing cardnolides have a deterrent effect for a higher survival. Our data suggest that food plant species strongly affected the palatability of the prey for *Nephila* spiders and had significant effects on the survival of the bugs. The *Asclepias* diet clearly ensured a higher survival rate of *Oncopeltus* than when reared on nontoxic seeds (*H. annuus*). The weight loss of the prey as a measure of consumption was significantly higher for *O. fasciatus* reared on sunflower seeds than for those raised on *A. incarnata* seeds (Wilcoxon signed-rank test: Figure 2A $p = 0.0156$ and Figure 2C $p = 0.0078$). The spiders consumed 94.6 % less of the bugs reared on *Asclepias* compared to the control in treatment one and about 88.6 % less in treatment two. In addition, we observed that of 13 *Asclepias* reared bugs that we offered the various *Nephila* individuals, 12 survived. These results demonstrate that the cardenolide rich bugs repelled the *Nephila* spiders significantly (Fig. 1 D).

An analysis of the time in the web also showed significant differences between individuals fed on the nontoxic plant or the toxic hosts. Compared to the control, *O. fasciatus* reared on cardenolide containing *Asclepias* remained significantly shorter in the web (Wilcoxon signed-rank test: Figure 2B $p = 0.0156$ and Figure 2D $p = 0.0078$). While nontoxic *O. fasciatus* were kept for 118 minutes in web by the spider, cardenolide rich *O. fasciatus* in contrast stayed only 5.5 minutes in web. That's on average, 95.3 % time difference between both experiments. In addition, the time that the bugs were kept in the net did not differ significantly between both trials for bugs reared on the same diet. Only in one experiment an *Asclepias* reared bug was held 97.5 % longer in the web and 87.6 % more of it was consumed than of all other individuals treated the same way.

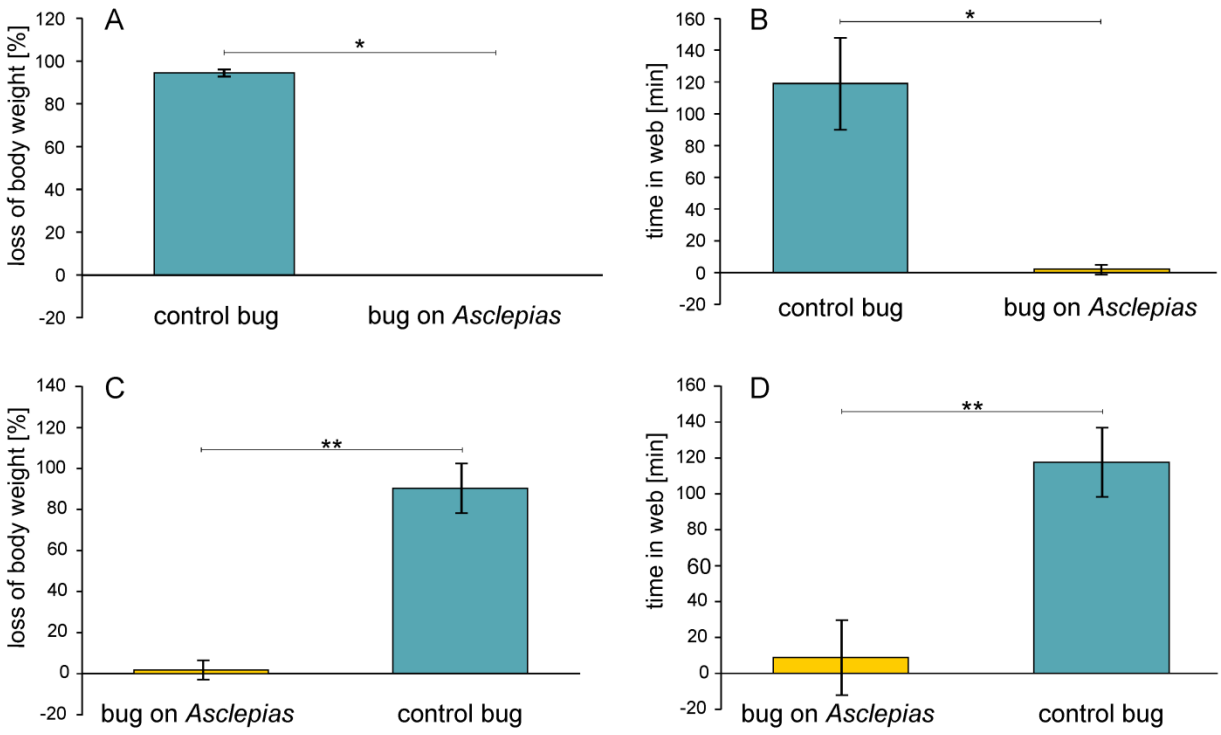

Figure 2. Reaction of *N. senegalensis* of two different types of prey. (A,B): Each spider (n=6) was tested first with an *Oncopeltus* kept on sunflower seeds followed by an *Oncopeltus* reared on *Asclepias incarnata* seeds. (C,D): 7 spiders were offered first a bug reared on *Asclepias* and immediately a control bug. A and C % reduction of body weight after spider attack, B and D time *Oncopeltus* spend in the web. Asterisks above columns indicate significant differences in Wilcoxon signed-rank test (*:$p \leq 0.05$; **: $p \leq 0.01$). Bars represent mean ±SD.

Learning trial for prey avoidance

The *Nephila* spiders usually directly approached the prey. In all experiments the spiders responded quickly to bugs introduced into the web and generally attacked the palatable *O. fasciatus* in spite of negative experiences with previous prey. Immediately (within 3 minutes) after the first encounter and removal from the web of an unpalatable *Oncopeltus*, another bug was offered. None of the spiders refused to attack the second (palatable) bug or touched it with more caution. As shown in Figure 2, comparing the first and the second trial revealed no difference in loss of body weight of sunflower reared bugs. The negative experience with *O. fasciatus* reared on *Asclepias* thus did not reduce the tendency on *Nephila* to attack *Oncopeltus* again. In addition, comparing the time in the web of palatable *O. fasciatus* in both trials (Figure 2) does not reveal any effects of learned avoidance in attacking this prey.

Discussion

During evolution insects have developed an impressive set of mechanisms to defend themselves against natural enemies. Exploitation of chemical defences acquired through feeding on toxic host plants might be the most widespread strategy for the insect`s own benefit against predation. This study focuses on the potential benefits of cardenolide sequestration from *Asclepias* host plants against an invertebrate predator (*N. senegalensis*). We can show that the large milkweed bug *O. fasciatus* had a significantly lower mortality then their sunflower-reared conspecifics. In the single case where a bug reared on *Asclepias* seeds was killed, the spider undertook numerous test bites which may have been caused by increasing hunger. Altogether, these results demonstrate that cardenolide sequestration is advantageous and ensures the survival of the single individual.

Nevertheless, different predators vary in their sensitivity to the distastefulness of a given insect. Generally, it appears that cardenolide containing Lygaeinae have a strong deterrent effect on one of their main vertebrate predators, the insectivorous birds (Evans *et al*., 1986; Sillen-Tullberg *et al.* 1982; Sillen-Tullberg, 1985; Tullberg *et al.,* 2000) whereas in case of mice as possible predators experimental data are lacking. However, could be shown that cradenolide sequestration by monarch caterpillars causes protection of the adults from mice (Glendinning, 1990). By their bitter taste and emetic effect cardenolides are expected to induce a general negative reaction in vertebrate predators. Yet, there is only little experimental evidence to suggest that cardenolides are effective against invertebrates (Berenbaum & Miliczky, 1984; Malcolm, 1989; Petschenka *et al.,* 2011).

In some instances, the deterrent effect of cardenolides differs between predators possibly due to differences in taste perception. Berenbaum & Miliczky (1984) demonstrated that cardenolides in *O. fasciatus* act as deterrents against Chinese mantids (*Tenoders ardifolia snensis*), whereas Levey (1983) suggested that mantids did not distinguish between cardenolide containing prey and prey lacking these toxins. On the other hand, it could be shown that lygaeid species such as *Spilostethus pandurus* or *Lygaeus kalmii* were refused by predatory bugs or ants (Levey, 1983; Jones, 1934). Malcolm (1989), however, demonstrated that feeding on cardenolide containing *Aphis nerii* caused the orb-web spider *Zygiella x-notata* (Clerck) to build disrupted webs, yet they did not reject the toxic prey. Part of the difference in the behavior of these predators may also depend on the concentration of unpalatable compounds sequestered from the host plants species. Cardenolide concentrations in *Asclepias* in particular show extremely large variability between plant species and among plant parts which can be responsible for differences in acceptability for predators (Malcolm, 1991). Therefore, Petschenka *et al*. (2011) used *Nephila* spiders to test

directly for the influence of ouabain against invertebrate predators without disturbing factors. In this study, in addition, we provide evidence that cardenolides sequestered from a natural host plant species, the swamp milkweed *A. incarnata* which has a low constitutive cardenolide concentration (Roeske *et al.*, 1976; Malcolm, 1991) still cause rejection by *Nephila* spiders.

An observation, which could be important in this context, is the accumulation of crdenolides in the integument as found in all lygaeinae bugs. A fluid filled compartment called the glycoside storage compartment arises through a specialized layer of the epidermis where sequestered cardenolides get enriched. In some cases it is the mechanical action of the predator itself, which causes the release of the defence substances. Through mechanical pressure on certain surfaces of the adult bugs, single discrete droplets of distasteful fluid are secreted. Although a direct release of cardenolide fluid couldn't be observed it seems possible that the spider after tasting bitter repellents released the bug immediately upon contact without visible injury. Nevertheless, it is known that rejection of distasteful or otherwise obnoxious prey only occurs after a *Nephila* spider has bitten it (Robinson & Robinson, 1973). Nephilidae generally thought to have a primitive prey capture strategy and usually attack all types of prey by biting (Robinson & Myrik, 1971; Eberhard, 1982; Higgins, 2008), whereas the prey-catching behavior of other orb-weaving spiders differs: They do not immediately kill the captured prey but rather they quickly wrap prey in silk for immobilization ("wrap attack" strategy) (Olive, 1980). Observations showed that the first bite of *Nephila* upon contact is a short-duration bite made to test the prey, whereas a second long bite will inject the venom and kill a palatable prey. Obviously, *Nephila* spiders possess receptors which will be stimulated by bitter tasting cardenolides and induce a negative response. Therefore, it seems advantageous for Lygaeinae to be able to release distasteful fluid to outer parts of the body to reduce the damage of an unavoidable bite. Despite of a test bite performed by the spider, a survival rate of nearly 100 % illustrates the protective function of sequestered cardenolides for the single prey. If bugs are attacked by a vertebrate predator a quail respectively, the survival of the prey is much lower and the supposed chemical defence only secures the bug from being eaten, not from being killed (Evans *et al.*, 1986). Therefore, it is advantageous that chemical defenses frequently co-occur with sounds or odours but often with bright colorations, to advertise their unpalatability or toxicity that encouraged predators to learn to avoid distasteful prey.

Avoidance learning is a widespread phenomenon which occurs in vertebrates as well as in invertebrates (Berenbaum & Milictky, 1984; Lindström *et al*., 2001; Ham *et al.,* 2006; Skelhorn & Rowe; 2006). However, the preliminary examinations showed that *N. senegalensis* isn't able to learn to avoid *O. fasciatus* after a bad experience. In fact, for the blind *Nephila,* there are no colourful bugs. However, it might be possible that several

successful alerting signals stimulate the various sense of the spider and thus reflect the chemical defence of the prey which in turn causes the learning behavior. The results of an experiment by Rowe & Guilford (1996) illustrated that avoidance of an offered coloured prey only showed up if an odour is also present.

Chemically defended insects in some cases release a disgusting odour when attacked, e.g. ladybirds are not only aposematic rather they contain a bitter tasting toxin and signal this by secreting a fluid that smells strongly of pyrazines (Moore *et al*., 1990). Indeed, pyrazines seems to be a common alerting signal in insect defence which functions for animals that rely on the olfactory sense. In order to test the influence of the spiders behaviour through possible occurring pyrazines or similar defensive odours in *O. fasciatus* reared on *A. incarnate*, further predation assays have to be performed in which two cardenolide rich *O. fasciatus* become offered *N. senegalensis* spiders successive. Performing this trial might explain the missing avoidance learning in the spiders so far.

The most essential feature of an alerting signal is that it has to be functional at a distance that ensures the survival of the prey (Moore *et al.,* 1990). Field observations with the cross orb-weaving spider, *Araneus diadematus,* demonstrated that toxic *Lygaeus equestris* have been rejected before contacting it (unpublished data). Besides *Nephila* also *Araneus* isn't able to visualize prey as being aposematic, however, it immediately removed the bug from the web by cutting the silk. This behavior could be the result of deterrent volatiles in combination with a learned avoidance which in turn of the spider provides an advantage through reduction of silk and energy. Referring to *Nephila* spiders detecting an alerting signal at an early stage could be a decisive advantage for both the spider which don't waste energy through performing test bites and for the bug that is protected before getting damage.

Apart from the function of several stimuli the memories of a bad experienced prey may only exist after the spiders were trained to associate through several repetitions. As it was shown in the memorability test by Ham *et al,* (2006) birds generally remembered to avid the prey with colour patterns they associate with unpalatabiliy, yet after a period of trays. To ensure the *Nephila* had the ability to decide through learning processes to avoid unpalatable Lygaeinae, several repetitions offering unpalatable bugs must be carried out in a separate trial.

Further, it is a widespread phenomenon that nearly all Heteroptera possess scant glands whose secretions primarily function in defence against predators (Aldrich, 1988). *O. fasciatus* also possess these metathoracis scant glands (MTGs), yet in a reduced form (Schaefer, 1972; Scudder & Duffery, 1972). The function of chemical compounds in MTGs however, showed no influence in deterrence of the spider *N. senegalensis* because cardenolide lacking bugs have been eaten immediately after contact.

Taken together our results demonstrate that *O. fasciatus* benefit from sequestration of cardenolides from the host plant *Asclepias incarnata* and obtain an effective defence against the invertebrate predator *N. senegalensis*. But there was no effect of aposematic color signal that implies avoidance learning and memorize retention. Several signals become directed against different groups of predators and divers hunting strategies. Whereas vertebrate predators such as birds were thought to be almost exclusively hunters by visual cues, some predators mainly identify potential prey primarily by means of olfactory and gustatory signals (Hostettler & Nentwig, 2006; Thomas *et al.,* 2008). It is necessary to perform additional assays in order to investigate whether besides an offensive hunting strategy avoidance learning is a natural instinct in *N. senegalensis.*

General Discussion and Outlook

Herbivorous insects have to cope with a stunning array of toxic secondary plant compounds which become ingested during the feeding process. In order to reduce or to overcome the potential damage, adapted herbivores have evolved complementary adaptations as a response to the chemical selection pressures. While some herbivores have developed strategies to avoid dangerous plant chemicals, or physiological and biochemical resistance mechanisms to overcome their toxic effect (Scudder & Meredith, 1982; Després *et al*., 2007; Capinera, 2008; Petschenka *et al*., 2013), sequestering specialists, i.e. those that selectively take up and accumulate plant chemicals, use these defensive substances for their own benefit against predators or pathogens (Nishida, 2002; Hartmann, 2004; Després *et al.,* 2007; Opitz & Müller, 2009). In order to be effective, the defence compounds must be able to interfere with molecular targets of cells, tissues or organs (Wink, 2009). Focusing on cardiac glycosides (CGs), these bitter tasting and emesis provoking plant compounds cause toxic effects in vertebrates and insects as neurotoxins (Malcolm, 1991; Wink, 2009).

Host plant use in the Lygaeinae

Some of the larger species of the hemipteran subfamily Lygaeinae are called “milkweed bugs” which reflects their worldwide common association with plants of the milkweed family Apocynaceae which is known to comprise many plant genera containing CGs (Scudder & Duffey, 1972). Approximately 80 % of the Lygaeinae use an apocynaceous plant as primary host. Whereas only a few species of herbivores utilize plants from more than three plant families, the majority is specialized to feed on few closely related plant species belonging to a single plant family or species similar in one or more properties such as biochemistry (Schoonhoven *et al*., 2005).

Given that Lygaeinae are the most species rich lineage within the Lygaeidae (sensu Henry, 1997) with more than 500 species compared to the sister groups Orsilinae (250 species) and Ischnorhynchinae (75 species), the adaptations to dietary CGs may represent a key innovation of this group. In general, it is assumed that species that are specialized in their food utilization are often more diverse than taxa including more generalist feeders because the rate of evolution is thought to be higher among specialists (Whitlock, 96).

Some Lygaeinae bugs however, occur on plants of other CG containing taxa including in families Ranunculaceae (Junior & Wichtl 1980; Winkler & Wichtl, 1986; Deckert, 2007), Asparagaceae (Vivas, 2012; Knittel *et al.,* 2013) or they are associated with Plantaginaceae (Péricart, 1998; Wachmann *et al*., 2007, Petschenka pers. obs.). The current

use of plants from non-apocynaceaous families most likely represents host shifts which were facilitated by the preadaptation of lygaeines to CGs. Remarkable however, species like *Tropidothorax leucopterus*, *Melanocoryphus albomaculatus*, *Spilostethus saxatilis, Lygaeus simulans* and *Horvathiolus superbus,* which in addition to the plant species mentioned above, are also closely associated with *Vincetoxicum hirundinaria* (Wachmann *et al.,* 2007) an apocynaceous host devoid of CGs (Dobler *et al.,* 1998). The former species in particular cannot survive without *V. hirundinaria* also known as Swallow-wort because they depend on it for growth and development. It is of interest to find out why, despite elaborated adaptations to GC in the Lygaeinae, particularly in *T. leucopterus* which possess a well-developed defence system, so many species of the subfamily use *V. hirudinaria* as host. Members of the genus *Vincetoxicum* contain the haemolytic glycoside vincetoxin and are highly poisonous and emetic to humans and mammals (DiTommaso *et al*., 2004). A mixture of steroid glycosides and phenanthroindolizidine alkaloids are characteristic constituents of 70 genera of Apocynaceae, including the species *Vincetoxicum hirudinaria* (Staerk *et al.,* 2000, 2005; Muola *et al.,* 2010). Further, the bitter tasting vincetoxin could be found next to CGs in apocynaceous species including *Asclepias syriaca* and *A. curassavica*. Wink (2009) classified the toxic effect of *Vincetoxicum* ingredients as strongly cytotoxic and neurotoxic. Through physiological studies testing the effect of dissolved compounds of *Vincetoxicum officinale* seeds on cardiac activity of frog hearts, Franzen (1929) could demonstrate that the substances have the same influence on heart beat and rythm as compounds of *Digitalis*. However, such biochemical studies focusing specifically on the mode of action of isolated compounds of *Vincetoxicum* seeds have not been repeated. It is tempting to speculate, that the structural properties of the vincetoxin molecule or related glycosides in the seeds are similar to CGs in their influence on the function of the Na/K-ATPase. Nevertheless, it could be shown that *T. leucopterus, L. equestris, S. saxatilis* species reared on *V. hirudinaria* are not worse protected than bugs raised on a CG rich diet (Sillen-Tullberg, 1982, 1985; Sillen-Tullberg *et al.,* 2000; Svadova *et al.,* 2010). It seems likely that due to the occurrence of similar chemistry e.g. vincetoxin in the former host plant species the switch to CG lacking Apocynaceae has been facilitated. Further, the presence of bitterness in the defensive secretions in species feeding on *V. hirudinaria* might act as an alarm signal in predators which is sufficient to get the same protection as the use of a CG diet. Nevertheless, the reason for the switch to *Vincetoxicum* is still open to speculation. In the case of *L. equestris* the second preferred host plant species is *Adonis vernalis,* a CG containing Ranunculaceae with a restricted distribution range, whereas *V. hirundinaria* is distributed over wide areas of continental Eurasia. The availability of a potential host plant may also explain current host use and distribution for the European *Arocatus longiceps* which probably changed host plant priority to a host plant (*Platanus*) that became widely distributed in Europe since the 17th

century, starting from an original occurrence in southeastern Europe. *A. longiceps* is common just as its host plant and originated from eastern Mediterranean regions, but has spread rapidly westwards (Barndt, 1995; Wachmann *et al.,* 2007).

Sequestration of – and Insensitivity against toxic cardenolides

Aposematically colored bugs of the Lygaeinae are not only adapted to feed on previously mentioned host plants further they are characterized to store cardenolides a class within the CGs for defensive purpose (Scudder & Duffey, 1972). It has to be mentioned that indeed cardenolides could be detected in many of these bugs but sequestration per se and adaptations against intoxication have only been found in a few species up to now (Dobler *et al*., 2012; Zhen *et al*., 20012). Therefore in a first approach (Chapter 1) I compared the uptake of two purified cardenolides, which represent a polarity range of these toxic compounds present in the natural died of the insects. The data obtained showed that cardenolide sequestration is restricted to the Lygaeinae and does not occur in outgroup species, however, two species of Lygaeinae also proved to be unable to sequester cardenolides. The absence of sequestration in the European *Arocatus* species *A. longiceps* and *A. melanocephalus* correlates with the life-history of these two species which are living and feeding on plants not known to produce cardenolides. Feeding on non-cardenolide containing hosts is most likely not the ancestral state in *Arocatus* as representatives of the genus in southern regions are well known to use CG plants as hosts or were even shown to possess sequestered cardenolides (von Euw *et al.*, 1971).

The presence of cardenolides in body parts of sequestering species necessitates mechanisms to prevent intoxication. Coadapted species need to be insensitive because the toxins should be in an active form to realize their impact on the next trophic level (Després *et al.*, 2007). As highly specifically acting substances, cardenolides binds to the Na/K-ATPase directly inhibiting its action. One possible mechanism consists in a modification of the target site in the Na/K-ATPase that has a strongly lowered binding affinity towards cardenolides. This phenomenon called target site insensitivity was observed in the monarch butterfly *Danaus plexippus* (Vaughan & Jungreis, 1977; Dobler *et al.,* 2012; Zhen *et al.,* 2012), in the grasshopper *Poekilocerus bufonius* (Al-Robai *et al.,* 1990), in *Chrysochus* leaf beetles (Labeyrie & Dobler, 2004) and in the milkweed bug *O. fasciatus* (Moore & Scudder, 1986; Dobler *et al.,* 2012). How sequestering Lygaeinae avoid cardenolide intoxication is demonstrated by our data (Chapter 1) on Na/K-ATPase activity under ouabain stress which indicate that target site insensitivity towards cardenolides is a common feature in the hemipteran subfamily Lygaeinae.

Even species, who do not normally encounter dietary cardenolides and do not store the toxins, still possess a modified insensitive Na/K-ATPase. These results lead us to conclude that target site insensitivity and sequestration of cardenolides are basal and plesiomorphic characters of the Lygaeinae which may stem from an originally host plant use.

Our *in vitro* investigations of the Na/K-ATPase of nervous tissues indicate that all Lygaeinae tested showed a relative uniform pattern of strong insensitivity towards oubain, i.e. a much higher ouabain-insensitive Na/K-ATPase (IC_{80} = $2.46*10^{-4}$M) than the outgroup species *Kleidoceris resedae* (IC_{80} = $2.06*10^{-7}$M) and a slightly higher insensitivity than the monarch butterfly (IC_{80} = $2.32*10^{-5}$M) (Petschenka *et al*., 2013). The preliminary molecular investigations of the Na/K-ATPase of several lygaeinae demonstrated that an amino acid substitution of asparagine for histidine at position 122 (N122H) is present in the first extracellular loop of the Na/K-ATPase of all species and is at least partly responsible for insensitivity in the *in vitro* enzyme assays (Dobler *et al.*, 2012; Dalla *et al.,* 2013). We have evidence that the possession of further amino acid substitutions at positions which are known to be involved in ouabain binding of Na/K-ATPase in the Lygaeinae lead to a further increased insensitivity. As previous studies could show up to nine different substitutions may be responsible for the lower cardenolide binding characteristics in CG adapted insects (Dobler *et al.*, 2012; Zhen *et al.,* 2012; Dalla *et al.,* 2013). The combined substitution of Q111T-N122H in *O. fasciatus* and Q111V-N122H in *D. plexippus* are known to reduce the inhibition by ouabain even more strongly compared to the wild-type occurring in *K. resedae*. Both combinations do not differ significantly from each other, yet in combination with a substitution of F786N and T797A in *O. fasciatus* may cause an even more strongly increased insensitivity. Enzyme assays performed with the single mutation T797A discovered in *L. kalmii,* a further Lygaeinae, showed a 250-fold increased resistance (Dalla *et al*., 2013). Therefore, it is likely that due to combinations of at least four potentially important substitutions at different positions the strongly increased resistance in the Lygaeinae can be explained.

Metabolic alteration of cardenolides

It is interesting to note that in general in *O. fasciatus* a high concentration of polar and an absence of apolar cardenolides was found (Duffey & Scudder, 1974; Yoder *et al.,* 1976; Duffey *et al.,* 1978; Moore & Scudder, 1985). Similar results could be obtained in *Syntomeida epilais*, *Empyreuma pugione*, and *D. plexippus* (Black, 1976; Seiber *et al*., 1980; Martin *et al*., 1992). The very polar compounds detected represent products arising from metabolic alteration in the species. Although apolar cardenolides may more emetic (Blum, 1981), Malcolm (1991) presumed that more polar cardenolides exhibit a lower mobility in the

haemocoel whereby they can better controlled and further they are easier to store. It appears plausible to prevent selfintoxication by minimizing the mobility and penetration of diffusible apolar cardenolides within the body tissues.

The study represented in Chapter 2 was undertaken in order to clarify certain aspects of the metabolic alteration of cardenolides in the Lygaeinae and to take conclusions about the mechanism by which these species are able to handle these toxic compounds. Metabolism of ouabain was not determined in any examined Lygaeinae. Digoxin however, was converted first to a metabolite (fraction I) more polar than ouabain but afterwards changed to a poorly more apolar cardenolide (fraction II) prod to two enzyme catalyzed reactions required for digoxin metabolism. According to our findings the storage of originally polar cardenolides (ouabain) should be less costly than storage of apolar cardenolides (digoxin) which necessitate an enzymatic conversion. The data presented here suggest the occurrence of at least one, most likely two enzymatically catalyzed reactions (Fig.1) forming a metabolic pathway to change apolar in rather polar cardenolides.

With regard to the toxic effects of cardenolides, it is important to differentiate between cardenolide sequestering species and those who are not. In non-sequestering *A. longiceps* and *A. melanocephalus* I could detect a highly polar metabolite (fraction I) in low amounts which disappeared rapidly may by non-selective excretion via the Malpighian tubules (Fig.1). In addition, excretion by the Malpighian tubules can be expected to reduce haemolymph levels of cardenolides as described in *Zonocerus variegatus* (Rafaeli-Bernstein & Mordue, 1978), *Drosophila melanogaster* (Torrie *et al.,* 2004), expected in *Manduca sexta* (Vaughan & Jungreis, 1977) and in *O. fasciatus* (Meredith *et al.,* 1984). The widespread occurrence of this feature suggests that insect Malpighian tubules generally are able to excrete cardenolides. Sequestering species in the Lygaeinae however, seem to possess a second enzyme which causes a amination reaction, expected at C-20 of the metabolite (fraction I) followed by a hydogenation. The metabolite of the postulated amination process (fraction II) is characterized by a lower polarity and could finally be detect in the defensive secretions of Lygaeinae bugs.

These results lead us to conclude that European *Arocatus* species not only lost the ability for sequestration as shown in Chapter 1, but also the ability to transform cardenolides into a storable form. It seems plausible to reduce a costly adaptation, such as an energy dependent metabolism which might be necessary to sequester cardenolides, if there are no cardenolides in the species diet anymore. Unexpected however, seems the maintaining of the fist metabolism in this species, because the transformation process is expected to be costly, too. These results suggest that involved enzymes are not specific for biotransformation of CGs, but rather they are engaged in diverse biochemical functions.

Generally, in species who are not adapted to use toxic compounds, it should be favorable to deactivate the toxins and to excrete them. Detoxification may be aided by specialized enzymes e.g. by polysubstrate monooxigenases (cytochrom P450 monooxigenase) that are rapidly induced by the presence of toxins. In general metabolic alteration of allelochemicals is a common mechanism to avoid intoxication (Després *et al*., 2007; Opitz & Müller, 2009). Cytochrom P450 enzymes transform toxins by oxidation into more polar compounds that are excreted or further metabolized (Feyereisen, 2005).

These enzymes may be widely distributed in the tissues because polar metabolites could be formed in Lygaeinae after ingestion as well as injection of apolar compounds. P450 gene expression, which have been found in insects' guts and fat bodies (rich sources of metabolism of substrates (Estela & Soulages, 2010)), may indicate that such an enzyme could also be involved in metabolic alteration of cardenolides in Lygaeinae (Feyereisen, 1999). Nevertheless, in agreement with the results reported by Scudder & Meredith (1982) metabolites could not be formed by isolated haemolymph, fat body or defence fluid so far.

With respect to the metabolic processes in the Lygaeinae we assume that alteration of apolar cardenolides as observed in *A. longiceps* and *A. melanocephalus* originated as a detoxification mechanism whereas further transformation as observed in *O. fasciatus, Horvathiolus superbus, Lygaeus equestris, L. simulans, L. kalmii, Spilostethus pandurus* and *Cosmopleurus fulvipes* represents an adaptation to accumulation and storage.

Uptake, transfer, and concentration of cardenolides

The phenomenon of cardenolide sequestration, per se, involves the uptake, transfer to, and concentration of cardenolides in the storage regions. Physiological studies on permeability of insect guts suggest that polar cardenolides such as ouabain require an energy dependent transport and presumably intestinal carriers in the gut epithelium which allow polar cardenolides to enter the haemolymph. Conversely, apolar cardenolides can be expected to cross the gut passively due to their physiological properties (Wright, 1960). Up to now the most comprehensive knowledge of sequestration processes in the Lygaeinae has been obtained for *O. fasciatus*. Several studies comparing the CG profiles of the large milkweed bug and its host plant species indicated that cardenolides of a wide polarity range were taken up through the gut (Yoder *et al.,* 1976; Duffey *et al.,* 1978; Scudder & Meredith, 1982b) whereas Scudder *et al.* (1986) mentioned that individual cardenolides are selectively sequestered and stored. The phenomenon of selective sequestration could also be observed in *D. plexippus* (Brower *et al.*, 1982), *Aphis nerii* (Rothschild *et al.,* 1970), *P. bufonius* (Von Euw *et al.,* 1967; Nahrstedt, 1982) and the Lygaeinae *Caenocoris nerii* and *Spilostethus pandurus* (von Euw *et al*., 1971).

In *O. fasciatus* it was found (Fig.1) that the uptake from the gut into the haemolymphe of apolar cardenolides occurred far more rapidly (77% in 30 min.) than the uptake of polar ones (3% in 30 min.) (Yoder *et al*., 1976; Scudder & Meredith, 1982b). This result, exactly as expected demonstrate that digoxin seem to cross the gut passively whereas, the uptake of rather polar ouabain into the body cavity is only possible via a carrier mechanisms. Nevertheless, the results by Yoder *et al*., 1976 and Scudder & Meredith, 1982b let us assume that gut uptake is unspecific and allows a broad spectrum of cardenolides to enter the haemolymph.

Further, the results presented here agree with those of Duffey *et al.* (1978) in so far as the uptake of polar CGs from the haemolymph into the storage space (50-70% in 72h) is faster than the transfer of metabolized apolar cardenolides (1-3% in 72h). My results clarify that a selective process seems to underlie the passage into the storage compartment. It might be expected that a transformation mechanism as shown for digoxin in Chapter 2 which is costly offers an advantage for storing apolar cardenolides. However, our data suggest that a selective carrier, a cardenolide carrier with substrate specificity might be present which preferred the transport of original polar CGs (e.g. ouabain) into the storage compartment.

This glycoside storage compartment is a specialized layer of the epidermis where sequestered cardenolides get enriched (Chapter 3). This special structure underlying the storage compartment of sequestered compounds, include vacuoles suggested to store cardenolides (Scuddee & Meredith, 1982a).

The study by Strauss *et al.,* (2013) demonstrates that a selective transfer takes place to regulate the uptake of plant compounds from the haemolymph into the secretory cells which in turn contain vacuoles transporting compounds to the storage reservoir in Chrysomelina larvae. With respect to the occurrence of a similar structure, it might be possible that as described in Strauss *et al.,* (2013) an ABC transporter in the Lygaeinae transfers cardenolides into intracellular vesicles which are thereon delivered in the glycoside storage compartment via exocytosis. Such a mechanism favors the re-fill of CGs after release of stored fluid without the need to destroy or excrete epithel cells which entails costs for regeneration of the cells. Further, the polarity dependent sequestration rates could be explained by a selective import into the epithelium of the Lygaeinae as well as described in Strauss *et al.,* (2013). In such case a lowered binding affinity of metabolites to carrier would be favor the passage of unchanged compounds which appears more plausible as described in Scudder *et al.,* (1986) that the uptake of CGs into the epithelial cells of the integument will be achieved by passive diffusion.

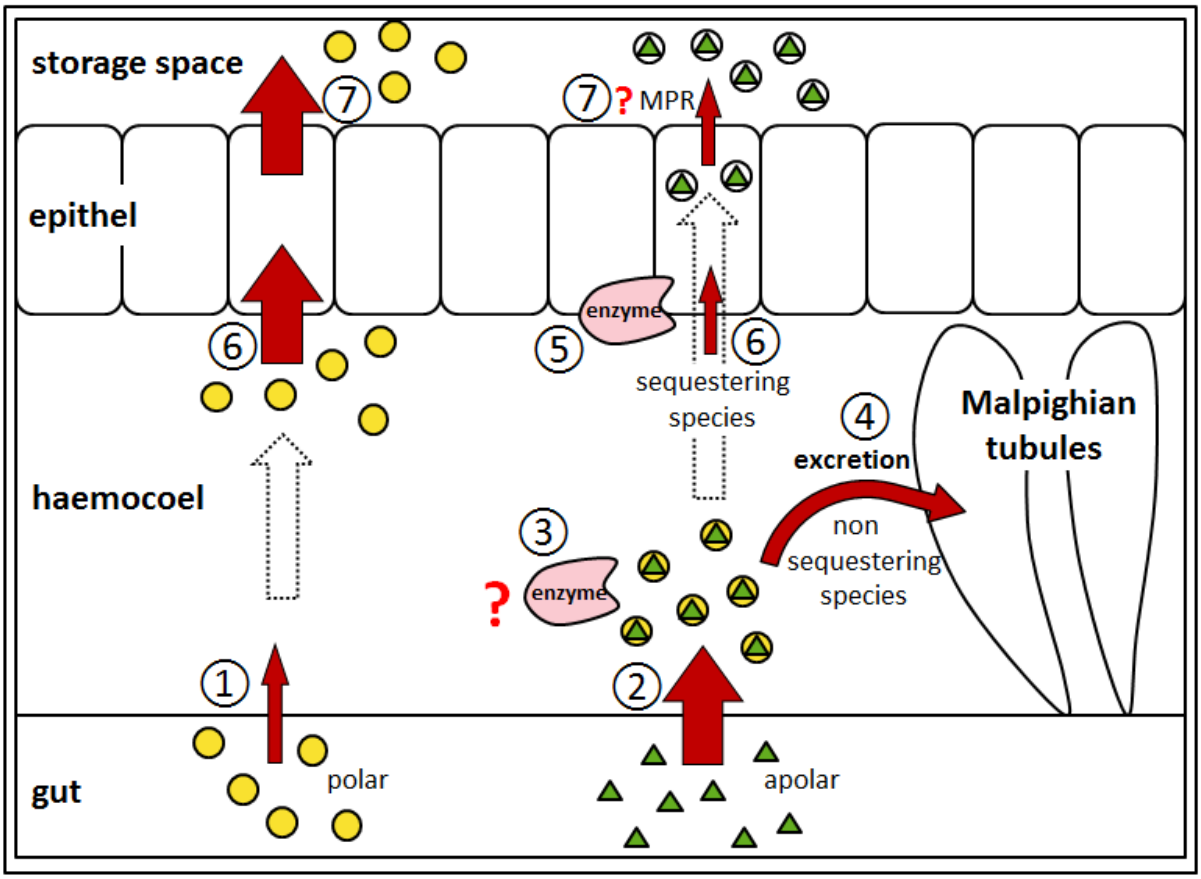

Fig.1 Schematic representation of the postulated sequestration process in the Lygaeinae of uptake, transfer and concentration of cardenolides into the storage space. (1) Ingested polar cardenolides can not cross the gut by simple diffusion but require an energy dependent transport (3% CGs in 30 min;) whereas apolar CGs may passively cross the gut membrane (77% CGs in 30 min) (2). In the haemocoel apolar cardenolides are enzymatically transformed into a very polar metabolite (3). Whereas non-sequestering species of the Lygaeinae excrete metabolized apolar cardenolides either by active or passive transport via the Malpighian tubules (4) in sequestering species a second, less polar metabolite is enzymatically formed out of the first polar product (5). Up to now it is unclear whether this reaction takes place in the haemocoel or only later in an epidermal cell. (6) The transport of glycosides from haemocoel to epithel relies on an unknown selective, possibly gradient-driven transporter which may be independent of polarity range. (7) In epithelial cells cardenolides are accumulated in intracellular vesicles maybe via a MRP transporter (Strauss *et al*., 2013).

Glycoside storage compartments for accumulation of cardenolides

The accumulation of cardenolides in the Lygaeinae takes place in a modified integument originally called dorolateral space (Scudder & Meredith, 1982a) which has been titled due to new findings in Chapter 3 as glycoside storage compartment (GSC). As a fluid filled storage compartment between epithelial cell layer and cuticle the GSC represents a unique mechanism developed for storage and use of CGs. Through a detailed description of the compartment and an extensive comparative approach we have been able to uncover the morphological features in the Lygaeinae developed as adaptation to CGs. The data obtained showed that the presence of a glycoside compartment in general represents an apomorphic feature of the subfamily Lygaeinae.

Besides the Lygaeinae, many sequestering species store plant toxins in defined compartments which include the cuticle, specialized tissues or glands where their potential toxic effects are confined and accordingly concentrations in the haemolymphe can be kept low (Blum *et al.,* 1990; Karban & Agrawal, 2002; Opitz & Müller, 2009). However, in some species the haemolymph contains remarkable amounts of defensive substances which are used in protection as well. As defensive action, at least five insect orders including mainly coleoptera perform reflex bleeding where insects present haemolymph, through integumental rupture (Wallace & Blum, 1971; Blum & Sannasi, 1974). Additional representatives are known from Plecoptera, Homoptera (Cercopidae), Lepidoptera and Hymenoptera (Symphyta) (Peck, 2000; Boevé, & Schaffner, 2003; Capinera, 2008). It is remarkable that plant derived toxins are often deposited in the integument or cuticle. Toxins can be stored in scales of the wings in Lepidoptera e.g. in Danainae (*D. plexippus*), Ctenuchidae (*Syntomeida epilais*) (Roeske *et al.,* 1975; Nickisch-Rosenegk *et al.,* 1990) or in specialized exocrine glands that are situated in thorax and abdomen of chrysomelid adults and larvae (Pasteels *et al.,* 1983, 89; Strauss *et al.,* 2013). The exosceleton provides an ideal site for storage of toxic compounds because it represents the barrier that a predator first is faced with.

Further, I could show that the reduction of cuticle thickness and the origin of a fluid releasing system are features of a stepwise evolution of which the presence of a mechanical toxin releasing system in *O. fasciatus* and *Tropidothorax leucopterus* represents the most developed mechanism. In some cases it is the mechanical action of the predator itself which causes the release of the defence substances. Through mechanical pressure on certain points of the surfaces of the adult bugs, single discrete droplets of distasteful fluid are secreted. Our data suggest however, that not all Lygaeinae possess the ability to emit droplets of cardenolide-rich secretion upon disturbance. In a previous study Scudder & Duffey (1972) described the occurrence of abdominal glycoside compartments in 57 Lygaeinae and justified a dichotomy due to the presence or absence of predefined releasing sites. However, they neglected to examine further features relevant to assign Lygaeinae with respect to how the insects use the chemicals. The present investigations pointed out how these species can be divided into three categories: (1) Species which are incapable to emit cardenolide-rich fluid at any point of body surface, (2) species whose ability is limited to release droplets only in thoracic regions and (3) those that possess thoracic and abdominal releasing sites. Species of the former category are well protected by confronting the attacking predator with a solution of cardenolides of many single released droplets whereas species without fluid exit points have reduced chances to survive an encounter of a predator since distastefulness first get noticed when the bug was crushed. In this case conspecifics of previously attacked prey are protected if a second prey will be released unharmed due to avoidance learning.

Cardenolides for defensive purpose

The process of learning and associating unpalatability with a certain kind of prey becomes more effective if it is equipped with a bright coloration. Frequently, chemical defences co-occur with conspicuous warning signals in insects. Most species of the Lygaeinae are colored bright red, orange and yellow in combination with a black pattern, with the exception of a few species from ground living genera (Slater & O'Donnell, 1995). The benefit of being conspicuously colored is that naive predators learn to avoid aposematic prey more quickly then cryptic prey (Gittleman & Harvey, 1980; Gambarele-Stille & Tullberg, 1999; Speed, 2000; Ripii *et al.,* 2001; Ham *et al.,* 2006; Exnarova *et al.,* 2006, 2008). Predators however, whose visual sense plays a very minor role can not differentiate between defended and non-defended prey on the basis of aposematic color patterns. Therefore, it is advantageous that chemical defenses are also frequently associated with specific odours or sounds which reveal their unpalatability or toxicity and encourage predators to learn to avoid distasteful prey. Predation assays performed with the not visually oriented golden orb-web spider *Nephila senegalensis* (Chapter 4) showed primarily that sequestered cardenolides from the host plant species *Asclepias incarnata* in *O. fasciatus* cause rejection by the spider. Furthermore, the results demonstrate that cardenolide sequestration of toxic host plant species is advantageous and ensures the survival of the attacked individual, yet experiments did not reveal any signs of avoidance learning so far. Moore *et al.,* (1990) assumed that CGs cannot serve as pre-sampling signals but rather act through their bitter taste as immediately post-sampling repellents. Thus in order to present existing chemical defence some aposematic insects release warning toxic fluid in combination with a strong odour (e.g. *Coccinella, Danaus, Zygaena*) (Rothschild *et al.,* 1984; Moore *et al.,* 1990; Marples *et al.,* 1996; Harborne, 2001). It is necessary in that case to test for the influence of a possible warning odour in defended bugs that stimulates the learn process in *Nephila* spiders.

The statement by Jeffry R. Aldrich that: "Milkweed bugs (Lygaeinae) are the butterflies of the bug world – black on red associated with poison may deter predators, but it attracts scientists", reflects only in part the actually importance of the bugs in studying plant-insect-interactions. Indeed, the large milkweed bug *O. fasciatus* became widely used in morphological, biochemical, physiological as well as molecular investigations. In general, recent work in insect-host plant interactions has mostly concentrated on single species relationships. With this thesis I have tried to do first steps towards a comparative approach to analyze the evolution of adaptations to CG in the subfamily Lygaeinae. Taken together, we showed that several Lygaeinae as mechanisms to prevent intoxication possess a

modification of the target site of Na/K-ATPase that has a strongly lowered binding affinity towards cardenolides. Besides these developed skilles, I could demonstrate that species of different genera are able to store two different polar ingested cardenolides. Given the uniform distribution of this trait all over our molecular phylogeny, it is likely that sequestration of cardenolides is a basal feature of the Lygaeinae. As a first step to explain the transformation mechanism in the Lygaeinae in detail, I cleared up that two separate reactions are involved in the metabolic pathway to change apolar in rather polar cardenolides. Through morphological investigations of thoracic compartments in several Lygaeinae and by mapping the observed morphological features on a recent phylogeny of the Lygaeinae I reported that the adaptation for storage and release of cardenolides evolved in a stepwise manner. Further, by performing predation assays with the golden orb-web spider *Nephila senegalensis* we showed primarily that sequestered cardenolides from the host plant species in *O. fasciatus* cause rejection by the spider. Finay, I could demonstrate a uniform pattern in terms of the resistance traits which might be due to a shared original host plant use. The occurrence of CG associations including cardenolide resistant Na/K-ATPases and cardenolide sequestration as probably basal features of the Lygaeinae as well as an frequent usage of worldwide distributed Apocynaceae suggest a very old association between these two taxa and adaptations may have evolved as basal features to cardenolides of Apocynaceae. Apart from few species which left the former host plants, to our knowledge all examined and aposematically colored species of Lygeinae use apocynaceous plants at least as one host and are able to sequester defensive compounds for their own benefits.

Nevertheless, it is necessary to elucidate the metabolic alteration of apolar cardenolides in detail which in turn, hopefully will provide information about the reaction sites and the involved metabolic enzymes. Moreover, none of the speculated selective or non-selective transporter utilized for cardenolide uptake or accumulation in the Lygaeinae have yet been characterized or localized. Furthermore, it remains unclear how many amino acid substitutions at which positions in the gene are responsible for the increased insensitivity in the whole subfamily. And finally it seems important to include the few non aposematic members of the subfamily in the analyses in order to clarify and to complete the story about the evolution of adaptations to cardiac glycosides in the hemipteran subfamily Lygaeinae.

Zusammenfassung

Verschiedenste phytophage Insekten, zu denen unteranderem auch einige Heteropteren zählen, haben den Umgang mit einer gewaltigen Menge sekundärer Pflanzenstoffe, die sich in ihren Wirtspflanzen finden, zu meistern. Da Insekten jedoch sehr vielseitige Innovatoren darstellen, sind sie in der Lage die verschiedensten pflanzlichen Abwehrstrategien durch Anpassungen zu überwinden.

Eine Stoffklasse, innerhalb dieser zum Teil hochtoxischen Substanzen, sind die Cardenolide, welche zu der Gruppe der Herzglykoside gezählt werden. Diese Herzgifte kommen in verschiedensten Pflanzenfamilien vor und wirken als spezifische Inhibitoren des tierischen Enzyms Na/K-ATPase, welches in eine Vielzahl physiologischer Funktionen involviert ist. Demzufolge müssen Arten, die auf cardenolidhaltigen Pflanzen fressen und diese sequestrieren, hochspezialisierte Anpassungen an diese Toxine aufweisen. Eines der bekanntesten Beispiele in dieser Richtung ist die große Milchkrautwanze *Oncopeltus fasciatus* (Heteroptera, Lygaeidae, Lygaeinae), die sich auf Samen verschiedenster *Asclepias* Arten spezialisiert hat. Die zu den Hundsgiftgewächsen (Apocynaceae) gehörige Pflanzengattung ist dafür bekannt hohe Mengen Cardenolide zu enthalten. Um sich vor der Vergiftung zu schützen besitzt *O. fasciatus* daher eine veränderte Na/K-ATPase, welche durch einen Aminosäure Austausch eine reduzierte Sensitivität gegenüber Herzglykosiden aufweist (target site insensitivity). Neben dieser sich entwickelten Eigenschafft gehört *O. fasciatus* zu den sequestrierenden Spezialisten, die die in der Lage sind selektiv Pflanzentoxine aufzunehmen, zu speichern um sie höchstwahrscheinlich zur eigenen Verteidigung zu verwenden. Nahezu alle Lygaeinae scheinen ebenfalls eine enge Bindung an Pflanzen der Familie Apocynaceae aufzuweisen. Diese Eigenschaft in Kombination mit der Warntracht, die bei den Lygaeinae zu finden ist, scheint möglicherweise mit der Nutzung der Cardenolide als Verteidigungssubstanzen in Verbindung zu stehen.

In einer vergleichenden Studie präsentieren wir die Anpassungsmechanismen, die sich bei den Lygaeinae entwickelt haben um eine Schädigung durch Cardenolide zu vermeiden. Wir versuchten herauszufinden, ob target site insensitivity der Na/K-ATPase und die Sequestration von Cardenoliden ein basales Merkmal der Hemipteren Unterfamilie sind. Doch um dies zu bewerkstelligen, wurde vorerst ein molekularer Stammbaum der Lygaeinae (20 Arten der Lygaeinae und 4 Außengruppen Taxa) konstruiert. Anschließend sequenzierten wir die Na/K-ATPase Gene von insgesamt 13 Arten (inklusive Außengruppe) und prüften die Aminosäuresequenz auf Substitutionen. Mit Hilfe eines physiologischen Assays wurde dann direkt die Cardenolid abhängige Sensitivität der Na/K-ATPase ermittelt. Es wurden 7 Arten der Lygaeinae und eine Außengruppenart (*Kleidocerys resedae*) auf ihre

Sensitivität gegenüber des Herzglykosides Ouabain getestet. Diese Studie zeigte, dass die Na/K-ATPase aller getesteten Arten mit Ausnahme der Außengruppe, hoch insensitiv gegenüber Ouabain ist. Die molekularen Untersuchungen der Na/K-ATPase zeigten dass alle getesteten Lygaeinae den gleichen Aminosäure Austausch an Position 122 aufweisen (Histidin gegen Asparagin), welcher für die Bindung der Herzglykosiden eine wichtige Rolle spielt. Anhand dieser Ergebnisse können wir vermuten, dass target site insensitivity ein einheitliches Merkmal der Lygaeinae zu sein scheint und vermutlich an der Basis derer entstanden ist.

Zusätzlich war es von großem Interesse herauszufinden, ob eine Aufnahme und Einlagerung in mögliche Körpersegmente ebenfalls ein einheitliches Merkmal darstellt. Für diesen Versuch haben wir die Aufnahme zweier unterschiedlich polarer, radioaktiv markierter Crdenolide (Ouabain und Digoxin) verglichen. Sieben der neun getesteten Individuen zeigten eine Fähigkeit zur Sequestration. Nur zwei europäisch beheimatete Arten aus der Gattung *Arocatus,* deren Wirtspflanzen keine Cardenolide enthalten, waren nicht in der Lage Cardenolide zu speichern. Betrachtet man jedoch das Einheitliche Vorhandensein dieses Merkmales über den Stammbaum hinweg, so zeigt sich das Sequestration ebenfalls ein grundlegendes Merkmal der Lygaeinae zu sein scheint.

Frühere vergleichende Studien haben gezeigt, dass *O.fasciatus* nicht exakt die Cardenolide sequestriert wie sie in seiner Wirtspflanze vorhanden sind. Es zeigte sich ehr eine Vorliebe für die Sequestration polarer Herzglykoside, die wie sich später herausstellte, das Ergebnis einer stofflichen Veränderung darstellen. In dieser Doktorarbeit verglichen wir verschiedene Arten der Unterfamilie an Hand ihrer Fähigkeit mit zwei unterschiedlich polaren Cardenoliden umzugehen. Wir verwendeten Ouabain und Digoxin, zwei Substanzen, die nicht natürlich in ihren Wirtspflanzen auftauchen, jedoch eine große polare Breite repräsentieren. Weiterhin analysierten wir das Spektrum an Metaboliten bei *O. fasciatus* über eine Zeitspanne von 170 Stunden nach der Injektion von Digoxin. Wir fanden heraus, dass dieses Cardenolid in der Wanze in mindestens zwei Metabolite transformiert wird, während Ouabain unverändert im Tier nachzuweisen ist. Mit Hilfe dieser Studie gelang es zu zeigen, dass die Veränderung von unpolarem Digoxin ein gnerelles Phänomen der Unterfamilie ist, welches möglicherweise als Anpassung an den Umgang und die Speicherung von Cardenoliden aus den Wirtspflanzen entstanden ist. Mit Hilfe der MALDI-TOF-MS Analyse Methode versuchten wir erste Schritte in der Aufklärung der Metaboliten, vorkommend in *O. fasciatus*, zu unternehmen.

O. fasciatus ist bekannt dafür enorme Mengen Cardenolide in einem speziellen Integument, welches als Dorsolateral-Raum bezeichnet wird, zu speichern. Als eine weitere morphologische Anpassung an den Nutzen von Herzglykosiden, finden sich Öffnungsstellen in der Cutikula von Thorax und Abdomen. Diese können durch ausgeübten Druck auf die

Körperoberfläche Herzglykosid-haltige Flüssigkeitstropfen abgeben, die dann als Verteidigung gegen Fraußfeinde eingesetzt werden. Die vorliegende Arbeit befasste sich mit der Untersuchung des thorakalen Speicherraumes bei den Lygaeinae. Im Detail untersuchten wir den Thorax von *O. fasciatus* und stellten anschließend vergleichende Studien bezüglich des Integuments weiterer Lygaeinae an. Für diese Untersuchungen nutzten wir zwei vergleichende Techniken: Histologische Serienschnitte und Mikro Computer Tomographie (µ- CT). Die gefundenen morphologischen Eigenschafften wurden an Hand des Stammbaums miteinander verglichen und es gelang uns zu zeigen, dass es sich bei der Entstehung des Speicherraums und der Öffnungsstellen um eine schrittweise Evolution innerhalb der Lygaeinae handelt.

Eine hohe Cardenolid Konzentration im Körper geht häufig einher mit einer aposematischen Färbung des Sequestrierers. Diese Warntracht stellt für Prädatoren ein deutliches Signal dar, welches dabei Hilft toxische von schmackhafter Beute zu unterscheiden. Nymphen und Adulti der Milchkrautwanze sind darauf spezialisiert vorwiegend auf *Asclepias*, einer Cardenolid-reichen Pflanze, zu fressen. In einer letzten Studie testeten wir die Wirkung von sequestrierten Cardenoliden auf die Radnetzspinne *Nephila senegalensis*. Der Prädatoren-Versuch zeigte sehr eindeutig, dass Wanzen, die auf Asclepias gefressen haben signifikant weniger getötet bzw. gefressen wurden als die Wanzen die auf Cardenolid-freiem Futter aufgezogen wurden. Diese Studie zeigt eindeutig den Vorteil sequestrierender Arten.

Summary

Phytophagous insects, including some heteroptera, have to cope with a stunning array of toxic secondary plant compounds encountered in their natural host plants. Insects, however, are the most versatile evolutionary innovators, as they are able to disarm multiple chemical plant defences through evolution of complementary adaptations.

One such powerful class of chemicals are the cardenolides (cardiac glycosides; CGs), distributed in several plant families. As high specifically acting substances, CGs bind to the animal enzyme Na/K-ATPase, directly inhibiting its action. This ion-pump is involved in a variety of physiological functions whereby species that feed on cardenolide-containing plants have to be highly specialized. Most renown in this context is the large milkweed bug *Oncopeltus fasciatus* (Heteroptera, Lygaeidae, Lygaeinae) being specialized on seeds of *Asclepias* species (Apocynaceae) which contain high amounts of toxic cardenolides. It possesses an altered form of the Na/K-ATPase which induces a reduced sensitivity by an amino acid exchange (target site insensitivity). Besides these developed skills to avoid dangerous plant chemicals, *O. fasciatus* belongs to sequestering specialists that selectively take up and accumulate plant chemicals, frequently for their own benefit against predation. Nearly all members of the subfamily Lygaeinae are also associated with apocynaceous plants, and hence the warning coloration occurring in this taxon also appears to be correlated with the use of cardenolides for defensive purposes.

In a comparative approach we were interested in identifying the adaptation which are evolved in order to reduce or to overcome the extensive damage of toxic cardenolides. In an initial investigation we tried to find out if target site insensitivity of Na/K-ATPase and cardenolide sequestration as strategy to use defensive compounds, are basal features of the hemipteran group. But to do so, we first constructed a molecular phylogeny of 20 Lygaeinae and 4 outgroups which enabled us to test the assumption mentioned previously. Afterwards we sequenced Na/K-ATPase genes of 13 species (including outgroup species) and analyzed amino acid sequences for substitutions. In addition, by using a physiological assay which allowed a direct measurement of Na/K-ATPase cardenolide sensitivity, seven Lygaeinae and one outgroup species (*Kleidocerys resedae*) were tested on their sensitivity towards ouabain. Molecular investigations of the Na/K-ATPase of several Lygaeinae demonstrated an amino acid substitution at position 122 (N122H) in the first extracellular loop of the Na/K-ATPase, a position which is critical for cardenolide binding.

Furthermore, this study revealed that Lygaeinae avoid cardenolide intoxication by the possession of a relative insensitive Na/K-ATPase caused by amino-acid substitutions. We could show that target-site-insensitivity is a common feature within the hemipteran subfamily

Lygaeinae. It is of interest to determine if Lygaeinae are able to selectively take up and accumulate plant chemicals in body cavities. Therefore, we compared the uptake of two purified radioactively labeled glycosides, ouabain and digoxin, by using feeding assays. Seven out of nine species of the Lygaeinae tested are obviously able to store both ingested cardenolides. Only European *Arocatus* species, whose lifestyle isn't oriented to dietary cardenolides, do not store the toxins. Given the uniform distribution of this trait all over our molecular phylogeny, we assume that sequestration of cardenolides is a basal feature of the Lygaeinae.

In general, in *O. fasciatus* there was found to be a high concentration of polar- and an absence of apolar cardenolides. The very polar compounds detected represent products arising from metabolic alteration in the species. We performed a comparative study in which 10 different Lygaeinae were tested for metabolic alteration of [3H]-labeled cardenolides (ouabain and digoxin) which represent a relative wide polarity range. Furthermore, we analyzed the alteration mechanism of digoxin in *O. fasciatus* in detail. Over a period of 170 hours after injection of digoxin, we found that the toxin was converted into individual fractions depending on time, whereas ouabain appeared to be unaltered. Here we report that metabolic alteration of apolar digoxin is a general phenomenon in the hemipteran subfamily which might be evolved as an adaptation to handling and accumulation cardenolides derived from host plant species. In addition, through use of MALDI-TOF-MS analysis we took the first steps in analyzing the structure of the second metabolite recovered in *O. fasciatus*.

O. fasciatus is examined to store large amounts of sequestered cardenolides in an unusual modified integument currently known as dorsolateral space. As additional morphological adaptation, special weak areas of the cuticle exist on the thorax and abdomen which rupture under pressure to release the cardenolide-rich droplets as a feeding deterrent towards predators. The present study reexamines the thoracic storage compartment of the large milkweed bug in detail and presents a comparative analysis of the integument as storage space in several species of the Lygaeinae. Therefore we used two complementary techniques: the histological section series and micro-computed tomography (µ-CT). By mapping the observed morphological features onto the recent phylogeny of the subfamily we here report that the adaptation for storage and release of plant compounds evolved in a stepwise manner.

The cardenolide concentrations in Lygaeinae bugs are frequently associated with their defensive or aposematic coloration, which is an essential warning signal for predators to distinguish between toxic and tasty prey. Larvae and adults of *O. fasciatus* are specialized to feed almost exclusively on milkweed species of the genus *Asclepias*, which contain high concentrations of toxic cardenolides. In this study we tested whether *O. fasciatus* gained improved protection by feeding on the toxic host plant vs. a nontoxic alternative. Therefore

we used naïve orb-weaving *Nephila senegaensis* (Araneae, Nephilidae) as predators. The predation trials suggest that the cardenolide containing bugs were killed significantly less than the bugs reared on a cardenolide free diet, which clarifies the effectiveness of the defence due to sequestered CGs. Furthermore, we investigated whether the spiders learn avoidance of unpalatable prey by signals conveyed non-visually.

Literature

AGRAWAL, A. A., PETSCHENKA, G., BINGHAM, R. A., WEBER, M. G., RASMANN, S. (2012). Toxic cardenolides: chemical ecology and coevolution of specializedplant-herbivore interactions. *Tansley Review*. 194, 28-45

ALDRICH, J. R. (1988). Chemical Ecology of the Heteroptera. *Ann. Rev. Entomol*.. 33, 211-238

AL-ROBAI, A. A., KHOJA, S. M., AL-FIFI, Z. I. (1990). Properties of ouabain-resistant Na+/K+-Transporting ATPase from the excretory system of *Poekilocerus bufonius*. *Insect Biochem*. 20(7), 701-707

AUKEMA, B., BOS, F., HERMES, D., ZEINSTRA, P. (2005). Nieuwe en interessante Nederlandse wantsen II, met een geactualiseerde naamlijst (Hemiptera: Heteroptera). *Nederlandse Faunistische Mededelingen.* 23, 37–76

AWMACK., C. S., LEATHER, S. R. (2002). Host plant quality and fecundity in herbivorous insects. *Annual Revies*. 47, 817-844

BARNDT, D. (1995). Bemerkungenzum Vorkommen von Arocatus-Arten und *Salda littoralis* (LINNAEUS, 1758) (Heteroptera: Lygaeidae et Saldidae) in Brandenburg und Berlin. *Märkische Ent. Nachr*. 10(2). 187-194

BERENBAUM, M. R., MILICZKY, E. (1984). Mantids and milkweed bugs: Efficacy of aposematic coloration against invertebrate predators. *Amrican Midland Naturalist*. 111(1), 62-68

BLACK, D. W. (1976). Studies on cardiac glycoside storage in moths. Ph.D. thesis, University of Miami

BLUM, M. S., SANNASI, A. (1974). Reflex bleeding in the lampyrid *Photinus pyralis*: Defensive function. *Journal of Insect Physiology*. 20(3), 451-460

BLUM, M. S. (1981). Chemical defenses of arthropods. Academic Press, London, UK

BOEVÉ, J. L., SCHAFFNER, U. (2003). Why does the larval integument of some sawfly species disrupt so easily? The harmful hemolymph hypothesis. *Oecologia*. 134, 104-111

BOUCIAS, G. G., PENDLAND, J. C. (1998). Principles of insect pathology. Kluwer Academic Publishers, Boston, USA 7pp

BOWERS, M. D. (1993). Aposematic caterpillars: Lifestyles of the unpalatable and warningly colored. In: N. Stampand T. Casey (eds.) Caterpillars: Ecological and Evolutionary Constraints on Foraging. *Chapman and Hall*. pp. 331-371

BROWER, L. P., VAN BROWER, J., CORVINO, J. M. (1967). Plant poisons in a terrestrial food chain. Proceedings of the National Academy of Sciences of the United Stated of America. 57(4), 893-898

BROWER, L. P. (1969). Ecological chemistry. *Sci. Amer*. 220(2), 22-29

BROWER, L. P., SEIBER, J. N., NELSON, C. J., LYNCH, S. P., TUSKES, P. M. (1982). Plant-determined variation in the cardenolide content, thin-layer chromatography profiles, emetic potenc of monarch butterflies, *Danaus plexippus* reared on the milkweed, *Asclepias eriocerpa* in california. *Journal of Chemical Ecology*. 8(3), 578-633

BURROWS, G. E., TYRL, R. J. (2013). Toxic plants of North America. Wiley-Blackwell, 2nd ed.

CAPINERA, J. L. (2008). Encyclopedia of Entomolofy. Springer Science + Business Media B.V. USA. 2nd edition

CASSIS, G., GROSS. G. F. (2002). Zoological Catalogue of Australia. Heteroptera. Pentatomomorpha. Volume 27.3B. CSIRO, Canberra 732 pp

CROYLE, M. L., WOO, AL., LINGREL, J. B. (1997). Extensive random mutagenesis analysis of the Na+/K+-ATPase alpha subunit identifies known and previously unidentified amino acid residues that alter ouabain sensitivity—implications for ouabain binding. *Eur J Biochem.* 248, 488–495.

DALLA, S., SWARTS, H. G. P., KOENDERINK, J. B., DOBLER. S. (2013). Amino acid substitutions of Na,K-ATPase conferring decreased sensitivity to cardenolides in insects compared to mammals. *Insect Biochemistry and Molecular Biology*. 43, 1109-1115

DECKERT, J. (2007). Die Ritterwanze Lygaeus equestris LINNAEUS, 1758-das Insekt des Jahres 2007 in Deutschland und Österreich (Heteroptera, Lygaeidae). *Entomologische Nachrichten und Berichte*. 51(1), 1-5

DESPRÉS, L., DAVID, J-P., GALLET, C. (2007). The evolutionary ecology of insect resistance to plant chemicals. *TRENDS in Ecology and Evolution.* 22(6), 298-307

DETZEL, A., WINK, M. (1995). Evidence for a Carrier in *Oncopeltus fasciatus* (Dassas) (Insecta: Hemiptera). *Zeitschrift für Naturforschung*. C50 (1-2), 127-134.

DITOMMASO, A., LAWLOR, F. M., DARBYSHIRE, S. J. (2004). The Biology of Invasive Alien Plants in Canada. 2. *Cynanchum rossicum* (Kleopow) Borhidi [= *Vincetoxicum rossicum* (Kleopow) Barbar.] and *Cynanchum louiseae* (L.) Kartesz & Gandhi [= *Vincetoxicum nigrum* (L.) Moench]. *Canadian Journal of Plant Science*, 243-263

DOBLER, S., DALOZE, D., PASTEELS, J. M. (1998). Sequestration of plant compounds in a leaf beetle`s defensive secretion: cardenolides in Chrysochus. *Chemoecology.* 8, 111-118

DOBLER, S., PETSCHENKA, G., PANKOKE, H. (2011). Coping with toxic plant compounds – The insect's perspective on iridoid glycosides and cardenolides. *Phytochemistry*, 72(13), 1593-1604

DOBLER, S., DALLA, S., WAGSCHAL, V., AGRAWAL, A. A. (2012). Community-wide convergent evolution in insect adaptation to toxic cardenolides by substitutions in the Na,K-ATPase. *PNAS*. 109(32), 13040-13045

DUSSOURD, D. E., EISNER, T. (1987). Vein-cutting behavior: insect counterploy to the latex defense of plants. *Science*. 237, 898-901

DUFFEY, S. S., SCUDDER, G. G. E. (1972). Cardiac glycosides in north American Asclepiadaceae, a basis for unpalatability in brightly coloured Hemiptera and Coleoptera. *J. Insect Physiol*., 18, 63-78

DUFFEY, S. S. & SCUDDER, G. G. E. (1974). Cardiac glycosides in *Oncopeltus fasciatus* (Dallas) (Hemiptera: Lygaeidae). I. The uptake and distribution of natural cardenolides in the body. *Canadian Journal of Zoology,* 52(2), 283-290

DUFFEY, S. S, BLUM, M. S., ISMAN, M. B., SCUDDER, G. G. E. (1978). Cardiac glycosides: a physiological system for their sequestration by the milkweed bug. *J. Insect Physiol*. 24, 639-645b

EBERHARD, W. G. (1982). Bihavioural characters for the higher classification of orb-weaving spiders. *Evolution*. 36, 1067-1095

EHRLICH, P. R., RAVEN, P. H. (1964). Butterflies and plants: A study in coevolution. *Evolution.* 18(4), 586–608.

ENDLER, J. A., MAPPES, J. (2004). Predator mixes and the conspicuousness of aposematic signals. *The American Naturalist*. 163(4), 531-547

ESTELA, L. A., SOULAGES, J. L. (2010). Insect fat body: energy, metabolism, and regulation. *Annu Rev Entomol*. 55, 207-225

EVANS, A. L., CASTORIADES, N., BADRUDDINE, H. (1986). Cardenolides in the defense of *Caenocoris nerii* (Hemiptera). *OIKOS,* 46, 325-329

EXNEROVA, A., STYS, P., FUCICOVA, E., VESELA, S., SVADOVA, K., PROKOPOVA, M., JAROSIK, A., FUCHS, R., LANDOVA, E. (2006). Avoidance of aposematic prey in European tits (Paridae): learned or innate?. *Behavioral Ecology*, 88(1),148-156

EXNEROVA, A., SVADOVA, K., FOUSOVA, P., FUCIKOVA, E., JEZOVA, D., NIEDERLOVA, A., KOPECKOVA, M., STYS, P. (2008). European birds and aposematic Heteroptera: review of comparative experiments. *Bulletin of Insectology,* 61 (1), 163-165

FEIR, D., SUEN, J. S. (1971). Cardenolides in milkweed plant and feeding by milkweed bug Hemiptera Lygaeidae. *Ann. Entomol. Soc*. 64, 1173-1174

FEYEREISEN, R. (1999). Insect P450 enzymes. *Annual Review of Entomology*. 44. 507-533

FRANZEN, G. (1929). Pharmakologische Untersuchungen über Semen Vincetoxici. *Archiv f. experiment. Path. U. Pharmakol*. 148, 211-217

FRICK, C., WINK, M. (1995). Uptake and sequestration of ouabain and other cardiac glycosides in *Danaus plexippus* (Lepidoptera: Danaidae): evidence for a carrier-mediated process. *Journal of Chemical Ecology.* 21(5), 557-575

FUTUYAMA, D. J., AGRAWAL, A. A. (2009). Macroevolution and the biological diversity of plants and herbivores. *PNAS*. 106(43), 18054-18061

GAMBARELE-STILLE, G., TULLBERG, B. S. (1999). Experienced chicks show biased avoidance of stronger signals: an experiment with natural colour variation in live aposematic prey. *Evolutionary Ecology*. 13, 579-589

GILBERT, M. T. P., MOORE, W., MELCHIOR, L., WOROBEY, M. (2007). DNA Extraction from dry museum beetles without conferring external morphological damage. PLOS ONE. 2(3). e272

GITTLEMAN, J. L., HARVEY, P. H. (1980). Why aren`t distasteful prey cryptic?. *Nature*, 286, 149-150

GLENDINNING, J. I. (1990). Responses of the three mouse species to deterrent chemicals in the monarch butterfly. II. Taste tests using intact monarchs*. Chemoecology.* 1, 124-130

GOVID, C. K., DANDY, J. W. T. (1970). The thoracic mechanism of the milkweed bug, *Oncopeltus fasciatus* (Heteroptera: Lygaeidae). *The Canadian Entomologist*. 102(9), 1057-1074

GUILFORD, T. (1990). The evolution of aposematism. Pages 23-62 in D. L. Evans and J. O. Schmidt, eds. Insect defenses: adaptive mechanisms and strategies of prey and predators. State University of New York Press, Albany

HAM, A. D., IHALAINEN, E., LINDSTRÖM, L., MAPPES, J. (2006). Does colour matter? The importance of colour in avoidance learning, memorability and generalization. *Behav Ecol Sociobiol.* 60, 482-491

HANSEN, O. (1984). Interaction of Cardiac Glycosides with (Na+ + K+)- Activated ATPase. A Biochemical Link to Digitalis- Induced Inotropy. *Pharmacological Reviews*, 36(3), 143-163

HÄNSEL, R., STICHER, O. (2007). Pharmakognosie-Phytopharmazie. Springer Medizin Verlag, 8. Auflage, München

HARBORNE, J. B. (2001). Twenty-five years of chemical ecology. *Nat. Prod. Rep*. 18, 361-379

HARTMANN, T. (2004). Plant-derived secondary metabolites as defensive chemicals in herbivorous insects: a case study in chemical ecology. *Planta*, 219, 1-4

HENRY, T. (1997). Phylogenetic Analysis of family groups within the infraorder Pentatomomorpha (Hemiptera: Heteroptera), with emphasis on the Lygaeidae. *Ann. Entomol. Soc. Am*. 90(3), 275-301

HIGGINS, L. (2008). Short Communications, Juvenile *Nephila* (Araneae, Nephilidae) use various attack strategies for novel prey. *The Journal of Arachnology.* 35, 530-534

HOLZIGER, F., FRICK, C., WINK, M. (1992). Molecular basis for the insensitivity of the Monarch (*Danaus plexippus*) to cardiac glycosides. *FEBS*, 314(3), 477-480

HOLZINGER, F., WINK, M. (1996). Mediation of cardiac glycoside insensitivity in the monarch butterfly (*Danaus plexippus*): Role of an amino acid substitution in the ouabain binding site of Na +,K +-ATPase. *Journal of Chemical Ecology*. 22(10), 1921-1937

HOSTETTLER, S., NENTWIG, W. (2006). Olfactory information saves venom during prey capture of the hunting spider *Cupiennius salei* (Araneae: Ctenidae). *Functional ecology.* 20(2), 369-375

HORISBERGER, J. D., KHAROUBI-HESSE, S. (2002) Functional differences between α subunit isoforms of the rat Na,K-ATPase expressed in *Xenopus* oocytes. *The Journal of Physiology. 539, 669-680*

HRISTOV, N., CONNER, W. E. (2005). Effectiveness of tiger moth (Lepidoptera, Arctiidae) chemical defenses against an insectivorous bat (Eotesucus focus). *Chemoecology.* 15, 105-113

HUELSENBECK, J. P., RONQUIST, F. (2001). MRBAYES: Bayesian inference of phylogeny. *Bioinformatics* 17:754-755.

ISMAN, M. B., DUFFEY, S. S., SCUDDER, G. G. E. (1977). Variation in cardenolide content of the lygaeid bugs *Oncopeltus fasciatus* and *Lygaeus kalmii kalmii* and of their milkweed hosts (*Asclepias spp*.) in central California. *J. Chem. Ecol.* 3(6), 613-624

JOBB, G. (2008). Treefinder version of April 2008. Available at http://www.treefinder.de/. Accessed 31 June 2011

JONES, F. M. (1934). Further experiments on coloration and relative acceptability of insects to birds. *Trans. R. Entomol. Soc. London*. 82, 443-453

JORGENSEN, P. L., HÅKANSSON, K. O., KARLISH, S. J. D. (2003). Structure and mechanism of Na,K- ATPase: functional sites and their interactions. *Annual Review of Physiology* 65(1), 817-849.

JUNIOR, P., WICHTL, M. (1980). *Phytochemistry*, 19, 2193

KARBAN, R., AGRAWAL, A. A. (2002). Herbivore offense. *Annual Review of Ecology and Systematics*, 33, 641-664

KNITTEL, D. N., KAMMERER, D. R., STINTZING, F. C. (2013). Characterization of phenolic compounds and cardiac glycosides from red sea squill (*Urginea maritima* (L.) Baker) by RP-HPLC-DAD-MSn. *Planta Med*. 79(21),

KRENN, L., KOPP, B. (1998). Bufadienolides from animal and plant sources. *Phytochemistry*, 48(1), 1-29

LABEYRIE, E., DOBLER, S. (2004). Molecular Adaptation of *Chrysochus* Leaf Beetles to Toxic Compounds in Their Food Plants*. Mol. Biol. Evol*. 21(2), 218–221

LANGFORD, D. S., BOOR, P. J. (1996). Oleander toxicity: an examination of human and animal toxic exposures. *Toxicology*. 109(1), 1-13

LAURSEN, M., YATIME, L., NISSEN, P., NATALYA U., FEDOSOVA, N. U. (2013).Crystal structure of the high-affinity Na+,K+-ATPase–ouabain complex with Mg2+ bound in the cation binding site*. PNAS.* 110(27), 10958–10963.

LARSEN, O. (1945). Bisher unbeachete wichtige Züge im Bau des Metathorax bei den Heteropteren. *K. fysiog. Sällsk. Lund. Förb.* 12(13), 1-15

LEVEY, B. (1983). Plant allelochemicals and the evolution of host plant relationships in the genus *Spilostethus*. London

LI, W, PETERSEN, R. A., SCHULER, M. A., BERENBAUM, M. R. (2002).CYP6B cytochrome P450 monooxygenases from *Papilio Canadensis* and *Papilio glaucus*; potential contributions of sequence diver-gence to host plant associations. *Insect Mol Biol*. 11, 543–551

LI, X., SCHULER, M. A., BERENBAUM, M. R. (2007). Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. *Annu Rev Entomol* 52, 231-253.

LINDSTEDT, C., EAGER, H., IHALAINEN, E., KAHILAINEN, A., STEVENS, M., MAPPES, J. (2011). Direction and strength of selection by predators for the color of the aposematic wood tiger moth. *Behavioral Ecology* 22:580–587.

LINDSTRÖM, L., ALATALO, R. V., LYYTINEN, A., MAPPES, J. (2001). Predator experience on cryptic prey affects the survival of conspicuous aposematic prey. *Proc. R. Soc. Lond. B.* 22. 268(1465), 357-361

LINGREL, J. B., ORLOWSKI, J., SHULL, M., PRICE, E. M. (1990). Molecular Genetics of Na,K-ATPase. *Prog Nucleic Acid Res Mol Biol.* 38, 37-89

LUCKNER, M., WICHTL, M. (2000). Digitalis. Wissenschaftliche Verlagsgesellschaft mbH Stuttgard

MALCOLM, S. B. (1989). Disruption of web structure and predatory behavior of a spider by plant-derived chemical defenses od an aposematic aphid. *Journal of Chemical Ecology.* 15(6), 1699-1716

MALCOLM, S. B. (1991). Cardenolide-mediated interactions between plants and herbivores In: Herbivores: their interactions with secondary metabolites (eds. Rosenthal A andBerenbaum MR). Academic Press, San Diego, California, USA.

MARPLES, N. M., VAN VEELEN, W., BRAKEFIELD, P. M. (1994). The relative importance of colour, taste and smell in the protection of an aposematic insect *Coccinella septempunctata*. *Anim. Behav*. 48, 967-974

MARPLES, N. M., ROPER, T. J. (1996). Effects of novel colour an smell on the response of naïve chicks towards food and water*. Anim. Behav*. 51, 1417-1424

MARTIN, R. A., LYNCH, S. P., BROWER, L. P., MALCOLM, S. B., VAN HOOK, T. (1992). Cardenolide content, emetic potency, and thin-layer chromatography profiles of monarch butterflies, *Danaus plexippus*, and their larval host-plant milkweed, *Asclepias humistrata*, in Florida. *Chemoecology* 3(1), 1-13

MAUS, C., PESCHKE, K., DOBLER, S. (2001). Pgylogeny of the genus *Aleochara* Inferred from mitochondrial cytochrome oxidase sequences (Coleoptera: Staphylinidae). *Molecular Phylogenetica and Evolution.* 18(2), 202-216

MEREDITH, J., MOORE, L., SCUDDER, G. G. E. (1984). Excretion of ouabain by Malpighian tubules of Oncopeltus fasciatus. *Am J Physiol Regulatory Integrative Comp Physiol*. 246, 705-715

MOORE, L. V., SCUDDER, G. G. E. (1985). Selective sequestration of Milkweed (*Asclepias sp.*) Cardenolides in *Oncopeltus fasciatus* (DALLAS) (Hemiptera: Lygaeidae). *Journal of Chemical Ecology*, 11(5)

MOORE, L. V., SCUDDER, G. G. E. (1986). Ouabain-resistant Na, K-ATPases and cardenolide tolerance in the large milkweed bug, *Oncopeltus fasciatus*. *J. Insect Physiol.* 32(1), 27-33

Moore, B. P., Brown, W. V., Rothschild, M. (1990). Methylalkylpyrazines in aposematic insects, their hostplants and mimics. *Chemoecology.* 1, 43-51

MUOLA, A., MUTIKAINEN, P., LILLEY, M., LAUKKANEN, L., SALMINEN, J.-P., LEIMU, R. (2010). Associations of plant fitness, leaf chemistry, and damage suggest selectionmosaic in plant-herbivoreinteractions. *Ecology.* 91(9), 2650-2659

MURAJI, M., TACHIKAWA, S. (2000). Phylogenetic analysis of water striders (Hemiptera: Gerroidea) based on partial sequences of mitochondrial and nuclear ribosomol RNA genes. Entomolo- gical. *Science*, 3, 615–626.

NAHRSTEDT, A. (1982). Strukturelle Beziehungen zwischen pflanzlichen uns tierischen Sekundärstoffen. *Journal of Medical Plant Research*. 44, 2-14

NATION, J. L. (2001). Insect physiology and biochemistry. CRC Press, Boca Raton, Florida, USA

NEVILLE, A. C. (1975). Biology of the arthropod cuticle. Springer, Berlin, Heidelberg, New York

NICKISCH-ROSENEGK, E., DETZEL, A., WINK, M., SCHNEIDER, D. (1990). Carrier-mediated uptake of digoxin by larvae of the cardenolide sequestering moth, *Syntomeida epilais* . *Naturwissenschaften.* 77(7), 336-338

NISHIDA, R. (2002). Sequestration of defensive substances from plants by Lepidoptera. *Annu. Rev. Entomol.* 47, 57-92

OLIVE, C. W. (1980). Foraging specialization in orb-weaving spiders. *Ecology*. 61(5), 1133-1144

OPITZ, S. E. W., MÜLLER, C. (2009). Plant chemistry and insect sequestration. *Chemoecology.* 19, 117-154

PASTEELS, J. M., ROWELL-RAHIER, M., BRAEKMAN, J. C., DUPONT, A. (1983). Salicin from host plant as precursor of salicylal- dehyde in defensive secretion of Chrysomeline larvae. *Physiological Entomol* 8, 307–314

PASTEELS, J. M., ROWELL-RAHIER, M., BRAEKMAN, J. C., DALOZE, D., DUFFEY, S. (1989). Evolution of exocrine chemical defense in leaf beetles (Coleoptera: Chrysomelidae). *Experientia* 45, 295-300

PARSONS, J. A.(1965). A digitalis-like toxin in the monarch butterfly, *Danaus plexippus* L. *J. Physiol.* 178, 290-303

PECK, D. C. (2000). Reflex Bleeding in Froghoppers (Homoptera: Cercopidae): Variation in Behavior and Taxonomic Distribution. *Annals of the Entomological Society of America.* 93(5). 1186-1194

PÉRICART, J. (1998). Hémiptéres Lygaeidae Euro-Méditerranéens. Volume 1. GénéralitésnSystématique: Premiére Partie. *Faune de Frnce*. 84, 1-468, 4 pls

PETSCHENKA, G., BRAMER, C., PANKOKE, H., DOBLER, S. (2011). Evience for a deterrent effect of vcardenolides on *Nephila* spiders. *Basic and Applied Ecology*, 12, 260-267

PETSCHENKA, G., OFFE, J., DOBLER, S. (2012). Physiological screening for target site insensitivity and localization of Na^+/K^+-ATPase in cardenolide-adapted Lepidoptera. *Journal of Insect Physiology.* 58(5), 607-612

PETSCHENKA, G., PICK, C., WAGSCHAL, V., DOBLER, S. (2013). Functional evidence for physiological mechanisms to cicumvent neurotoxicity of cardenolides in an adapted and a non-adapted hawk-moth species. *Proceedings of The Royal Society B*. 280, 20123089

POSADA, D., CRANDALL, K. A. (1998). MODELTEST: testing the model of DNA substitution. *Bioinformatics.* 14(9), 817-818

QIU, L. Y., KRIEGER, E., SCHAFTENAAR, G., SWARTS, H. G. P., WILLEMS, P. H. G. M., DE PONT, J. J. H. H. M., KOENDERINK, J. B. (2005) Reconstruction of the complete ouabain-binding pocket of Na,K- ATPase in gastric H,K-ATPase by substitution of only seven amino acids. *J Biol Chem* 280, 32349–32355.

RAFAELI-BERNSTEIN, A., MORDUE, W. (1978). The transport of the cardiac glycoside ouabain by the Malpighian tubules of *Zonocerus variegatus*. *Physiological Entomology*. 3, 59-63

REMOLD, H. (1962). Über die biologische Bedeutung der Duftdrüsen bei den Landwanzen (Geocorisae). *Z. Vergl. Physiol.*, 45, 636-694

REMOLD, H. (1963). Scent-glands of land-bugs, their physiology and biological function. *Nature*, 198, 764-768

RIIPI, M., ALATALO, R. V., LINDSTRÖM, L., MAPPES, J. (2001). Multiple benefits of grgariousness cover detectability costs in aposematic aggregations. *Nature*. 413. 512-514

ROBINSON, M. H., MYRIK, H. (1971). The predatory behaviour of the golden-web spider *Nephila clavipes* (Aranneae: Aranerdae). *Psyche.* 78, 123-139

ROBINSON, M. H., ROBINSON, B. (1973). Ecology and behaviour of the giant wood spider *Nephila maculata* (Fabricius) in New Guinea. *Smiths. Contr. Zool*. 149, 1-76

ROESKE, C. N., SEIBER, J. N., BROWER, L. P., MOFfiTT, C. M. (1975). Milkweed cardeno- lides and their comparative processing by monarch butterflies (*Danaus plexippus*). *Recent Adv. Phytochem*. 10, 93– 167

ROTHSCHILD, M., VON EUW, J., REICHSTEIN, T. (1970). Cardiac glycosides in the oleander aphid *Aphis nerii*. *J. Insect Physiol*. 16, 1141-1145

ROTHSCHILD, M., MOORE, B. P., BROWN. W. V. (1984). Pyrazines as warning odour components in the Monarch butterfly, *Danaus plexippus*, and in moths of the genera *Zygaena* and *Amata* (Lepidoptera). 23(4), 375-380

ROWE, C., GUILFORD, T. (1996). Hidden colour aversions in domestic chicks triggered by pyrazine odours of insect warning displays. *Nature.* 383, 520-522

ROWELL-RAHIER, M., PASTEELS, J. M. (1992). Third trophic level influences of plant allelochemicals in Herbivores: Their interactions with secondary plant metabolites, Volume 2: Evolutionary and Ecological Processeess. Academic Press, California, USA

SCHAEFER, C. W. (1972). Degree of metathoracic scent-gland development in the trichophorous Heteroptera (Hemiptera). *Ann. Entomol. Soc. Am*. 65, 810-r821

SCHOLZ, H., SCHMITZ, W. (1984). Positive inotropic effects of digitoxin- and digoxin-glucuronide in human isolated ventricular heart muscle preparations. *Cardiac Glycoside Receptors and Positive Inotropy*. 134-139

SCHOONHOVEN, L. M., VAN LOON, J. J. A., DICKE, M. (2005). Insect-plant biology. 2nd edn. University Press, Cambridge.

SCOTT, J. G., WEN, Z. (2001). Cytochromes P450 of insects: the tip of the iceberg. *Pest Management science*. 57(10). 958-967

SCOTT, J. G.(2008). 8 Insect cytochrome P450s: Thinking beyond detoxification. *Research Signpost.* 37, 117-124

SCUDDER, G. G. E., DUFFEY, S. S. (1972). Cardiag glycosides in the Lygaeinae (Hemiptera: Lygaeidae). *Can. J. Zool*. 50, 35-42

SCUDDER, G. G. E., MEREDITH, J. (1982a). Morphological basis of cardiac glycoside sequestration by *Oncopeltus fasciatus* (Dallas) (Hemiptera: Lygaeidae). *Zoomorphology.* 99, 87-101

SCUDDER, G. G. E., MEREDITH, J. (1982b). Permeability of the midgut of three insects to cardiac glycosides. *J. Insect Physiol*. 28(8), 689-694

SCUDDER, G. G. E., MOORE, L.V., ISMAN, M.B. (1986). Sequestration of cardenolides in *Oncopeltus fasciatus*: Morphological and physiological adaptations. *Journal of Chemical Ecology*, 12(5)

SEIBER, J. N., TUSKES, P. M., BROWER, L. P., NELSON, C. J. (1980). Pharmacodynamics of some individual milkweed cardenolides fed to larvae of the monarch butterfly (*Danaus plexippus* L.). *Journal of Chemical Ecology*. 6(2), 321-339

SHIMODAIRA, H., HASEGAWA, M. (1999). Multiple comparisons of log-likelihoods with applications to phylogenetic inference. *Mol. Biol. Evol.* 16, 1114-6.

SHIMODAIRA, K. (2002). An approximately unibased test pf phylogenetic tree selection. *Systematic Biology*. 51, 492-508

SILLEN-TULLBERG, B., WIKLUND, C., JÄRVI, T. (1982). Aposematic coloration in adults and larvae of *Lygaeus equestris* and its bearing on müllerian mimicry: an experimental study on predation on living bugs by the great tit Parus major. *OIKOS,* 39, 131-136

SILLEN-TULLBERG, B. (1985). Higher survival of an aposematic than of a cryptic form of a distasteful bug. *Oecologia,* 67, 411-415

SILLEN-TULLBERG, B., GAMBERALE-STILLE, G., SOLBRECK, C. (2000). Effects of food plant and group size on predator defence: differences between two co-occuring aposematic Lygaeinae bugs. *Ecological Entomology*. 25, 220-225

SKELHORN, J., ROWE, C. (2006). Predator avoidance learning of prey with secreted or stored defences and the evolution of insect defences. *Animal Behaviour.* 72, 827-834

SLATER, J. A., O'DONNELL, J. E. (1995). A Cataloque of the Lygaeinae of the World. New York: *New York Entomological Society*, 410 pp

SOLBRECK , C., KUGELBERG, O. (1972). Field Observations on the Seasonal Occurrence of Lygaeus eguestris (L.) (Het., Lygaeidae) with Special Reference to Food Plant Phenology. *End. Scand*. 3, 189-210

SPEED, M. P. (1993). Müllerian mimicry and the psychology of predation. *Animal Behaviour*. 45, 571-580

SPEED, M. P. (2000). Warning signals, receiver psychology and predator memory. *Animal Behaviour.* 60, 269-278

STADDON, B. W. (1979). The scent glands of Heteroptera. *Adv. Insect Physiol*., 14, 351-418

STADDON, B. W., DAROOGHEH, H. (1981). Allometry of C6 and C8 Alk-2-enals and Alka-2,4-dienals in the metathoracic scent gland of *Oncopeltus fasciatus. Comp. Biochem. Physiol.,* 68B, 593-598

STAERK, D., CHRISTENSEN, J.,LEMMICH, E., DUUS, J. O., OLSEN, C. E., JAROSZEWSKI, J. W. (2000). Cytotoyic activity of some phenanthroindolizidine N-oxide alkaloids from *Cynanchum vincetoxicum*. *J Nat Prod*. 63, 1584-1586

STAERK, D., NEZHAD, K. B., ASILI, J., EMAMI, S. A., AHI, A., SAIRAFIANPOUR, M., JAROSZEWSKI, J. W. (2005). Phenanthroindolizidine alkaloids from *Vincetoxicum pumilum. Biochemical Systematics and Ecology*. 33, 957-960

STEYN, P. S., VAN HEERDEN, F. R. (1998). Bufadienolides of plant and animal origin. *Natural Product Reports.*15(4), 397-413

STRAUSS, A. S., PETERS, S., BOLAND, W., BURSE, A. (2013). ABC transporter functions as a pacemaker for sequestration of plant glucosides in leaf beetles*. eLIFE*.01096

SVÁDOVA, K. H., EXNEROVÁ, A., KOPEÝKOVÝ, M., STYS, P. (2010). Predator dependent mimetic complexes: Do passerine birds avoid Central European red-and-black Heteroptera?. *Eur. J. Entomol*. 107, 349-355

TAYLOR, L. H. (1918). The Thoraic sclerites of Hemiptera and Heteroptera. *The Entomological Society of America.* 11(3), 225-254

THOMAS, R. S., GLEN, D. M., SYMONDSON, W. O. C. (2008). Prey detection through olfaction by the soil-dwelling larvae of the carabid predator *Pterostichus melanarius. Soil Biology and Biochemistry*. 40(1), 207-216

TORRIE, L. S., RADFORD, J. C., SOUTHALL, T. D., KEAN, L., DINSMORE, A. J., DAVIES, S. A., DOW, J. A. T. (2004). Resolution of the insect ouabain paradox. *Proceedings of the National Academy of Sciences of the United States of America*. 101, 13689-13693

VAUGHAN, G. L., JUNGREIS, A. M. (1977). Insensitivity of lepidopteran tissues to ouabain: physiological mechanisms for protection from cardiac glycosides, *J. Insect Physiol.* 23, 585-589

VIVAS, L. (2012). Algunos datos sobre ditribucion y biologia de Spilostethus furcula (Herrich-Schaeffer, 1850) (Hemiptera: Heteroptera: Lygaeidae) y clave para los ligeinos ibericos*. Fotografia Biodiversida*. 11, 59-74

VON EUW, J., FISHELSON, L., PARSONS, J. A., REICHSTEIN, T., ROTSCHILD, M. (1967). Cardenolides in a Grasshopper feeding on milkweeds. *Nature.* 214, 35-39

Von Euw, J., Reichstein, T., Rotschild, M. (1971). Heart Poisons (Cardiac Glycosides) in the Lygaeid bugs *Caenocoris nerii* and *Spilostethus pandurus. Insect Biochem.,* 373-384

WACHMANN, E., MELBER, A., DECKERT, J. (2007).Wanzen Bd. 3 Pentatomorpha 1. Pp. 272, Verl. Geock & Evers,Keltern

WALLACE, J. B., BLUM, M. S. (1971). Reflex Bleeding: A highly refined defensive mechanism in *Diabrotica* Larvae (Coleoptera: Chrysomelidae). *Annales of the Entomological Society of America*, 64(5), 1021-1024

WELLER, S. J., SIMMONS, R. B., CARLSON, A. L. (2004). Empyreuma species and species limits: Evidence from morphology and molecules (Arctiidae: Arctiinae, ctenuchini). *Journal of the Lepidopterists` Society*. 58(1), 21-32

WHITLOCK, M. C. (1996). The red queen beats the jack- of-all-trades: the limitations on the evolution of phenotypic plasticity and niche breadth. *Am. Nat*. 148, 65-77

WICHTL, M., JUNIOR, P. (1977). Strophanthidindigitalosidu nd Strophanthidingulomethylosid (Desglucocheiro toxin), zwei neue Cardenolidglykoside aus *Adonis vernalis* L.. *Arch. Pharm.* 310, 905-910

WINK, M., SCHNEIDER, D. (1990). Fate of plant-derived secondary metabolites in three moth species (Synthomis mogadorensis, *Synthomeida epilais* and *Creatonotus transiensis*). *J. Comp. Physiol.* B 160, 389-400

WINK, M. (2009). Mode of action and toxycology of plant toxins and poisonous plants. *Mitt. Julius Kühn-Instit.* 421, 93-112

WINKLER, C., WICHTL, M. (1985). Neue Cardenolide aus *Adonis vernalis*. *Planta Medica*. 68-70

WRIGHT, S. E. (1960). The metabolism of cardiac glycosides. In: American Lectures in Biochemistry and Biophysics (edited by Bladergroen W). Charles Thomas Publisher, Springfield, Illinois, USA

Yatime, L., Andersen, G. R. (2013). Structural insights into the oligomerization mode of the human receptor for advanced glycation end-products. *FEBS J.* 280(24), 6556-6568

YODER, C. A., LEONARD, D. E., LERNER, J. (1976). Intestinal uptake of ouabain and digitoxin in the milkweed bug, *Oncopeltus fasciatus*. *Experientia,* 32, 1549-1550

ZHEN, Y., AARDEMA, M. L., MEDINA, E. M., SCHUMER, M., ANDOLFATTO, P. (2012). Parallel molecular evolution in an herbivore community. *Science.* 337(6102), 1634-1637

Danksagung

In erster Linie danke ich meiner Betreuerin, Prof. Dr. Susanne Dobler für die Aufnahme in Ihr Labor vor 8 Jahren und für die großartige Gelegenheit meine Dr. Arbeit bei Ihr zu machen. Sie war in der ganze Zeit eine nette Wegbegleiterin voller Begeisterung für meine Arbeit. Insbesondere Danke ich Ihr aber für die großartige Hilfsbereitschaft und sehr gute Betreuung.

Weiterhin Danke ich meinem ehemaligen Arbeitskollegen und gutem Freund Georg Petschenka für die Themenfindung und meiner dadurch entstandene Begeisterung für die Wanzen. Aber vor allem für die Unterstützung und Motivation während der gesamten Zeit in der Arbeitsgruppe Dobler.

Großer Dank gilt insbesondere Dr. Jürgen Deckert, der durch die Beschaffung zahlreicher Wanzenarten viele Versuche erst möglich gemacht hat. Ich danke Ihm aber auch für die netten Inspirationen während der Kaffeerunden im Berliner Naturkundemuseum.

Ganz lieber Dank gilt auch Vera Wagschal für jedweilige Hilfe im Labor, für Ratschläge im Erstellen von Grafiken aber auch für die netten Pläuschen zwischendurch.

Herzlicher Dank, vor allem für die Hilfsbereitschaft, gilt der gesamten Arbeitsgruppe „Dobler“ mit der ich viel Spaß im Labor aber auch auf diversen Tagungen hatte.

Ich danke meiner liebsten Freundin Steffi für wöchentliche Motivationsschübe und inspirierende fachliche Austausche aber insbesondere dafür, dass sie immer für mich da ist.

Ich danke meinem guten Freund Martin für allerlei fachliche Unterstützung und eine lustige Zeit außerhalb der Uni.

Ich danke meinem Freund Robert für sein Verständnis für meine Arbeit und der wenigen Zeit die ich vor allem während des Schreibens hatte.

Aber vor allem Danke ich meinen Eltern für das Verständnis meiner Berufswahl, den jährlichen Insekten-Sammelausflügen und für jegliche Unterstützung während der gesamten Studienzeit.

DANKE!