Na\textsuperscript{+}/K\textsuperscript{+}-ATPase resistance and cardenolide sequestration: basal adaptations to host plant toxins in the milkweed bugs (Hemiptera: Lygaeidae: Lygaeinae)

Christiane Bramer\textsuperscript{1}, Susanne Dobler\textsuperscript{1}, Jürgen Deckert\textsuperscript{2}, Michael Stemmer\textsuperscript{4} and Georg Petschenka\textsuperscript{1,3}

\textsuperscript{1}Biozentrum Grindel, Martin-Luther-King-Platz 3, 20146 Hamburg, Germany
\textsuperscript{2}Museum für Naturkunde, Leibniz-Institut für Evolutions- und Biodiversitätsforschung, Invalidenstrasse 43, 10115 Berlin, Germany
\textsuperscript{3}Department of Ecology and Evolutionary Biology, Cornell University, Corson Hall, Ithaca, NY 14853, USA
\textsuperscript{4}Am Stux, 53572 Unkel, Germany

Despite sequestration of toxins being a common coevolutionary response to plant defence in phytophagous insects, the macroevolution of the traits involved is largely unaddressed. Using a phylogenetic approach comprising species from four continents, we analysed the ability to sequester toxic cardenolides in the hemipteran subfamily Lygaeinae, which is widely associated with cardenolide-producing Apocynaceae. In addition, we analysed cardenolide resistance of their Na\textsuperscript{+}/K\textsuperscript{+}-ATPases, the molecular target of cardenolides. Our data indicate that cardenolide sequestration and cardenolide-resistant Na\textsuperscript{+}/K\textsuperscript{+}-ATPase are basal adaptations in the Lygaeinae. In two species that shifted to non-apocynaceous hosts, the ability to sequester was secondarily reduced, yet Na\textsuperscript{+}/K\textsuperscript{+}-ATPase resistance was maintained. We suggest that both traits evolved together and represent major coevolutionary adaptations responsible for the evolutionary success of lygaeine bugs. Moreover, specialization on cardenolides was not an evolutionary dead end, but enabled this insect lineage to host shift to cardenolide-producing plants from distantly related families.

1. Introduction

Coevolution in plant–herbivore interactions involves the production of plant defences and insect counter strategies. From the insects’ perspective, diet specialization, resistance to plant toxins, sequestration of these toxins and aposematic coloration may often be coupled as an adaptive strategy [1,2]. Yet, quite remarkably, we have little understanding of the macroevolution of such strategies and how interacting traits facilitate tolerance and sequestration of host plant toxins. In particular, the genetic, physiological and ecological basis of host use in closely related species needs to be assessed in a phylogenetic context in order to understand the evolution of insect resistance traits, potential costs and patterns of host shifts.

Plants in the Apocynaceae produce toxic cardenolides (aka cardiac glycosides) [3], which are specific inhibitors of the ubiquitous animal enzyme Na\textsuperscript{+}/K\textsuperscript{+}-ATPase, a cation carrier essential for a variety of physiological functions [4,5]. Due to the ubiquity of their target, cardenolides are considered universal toxins potentially affecting any animal species. On a broad scale, several studies have shown that a community of distantly related insects evolved the same strategies to overcome two major defence traits of Apocynaceae, latex and cardenolides [6–8]. By contrast, within insect lineages, our knowledge on the evolution of these insect traits is very limited.

The milkweed bugs (Hemiptera: Lygaeidae; Lygaeinae, ca 550 species) are seed predators which are well known for their aposematic black and red...
oloration and seem to be commonly associated with apocy-
enadeous host plants on five continents [9–13]. The thick
walls, wide spacing and rapid seed dispersal of Asclepias
seed pods (and probably also fruits of other genera of Apo-
cynaceae like Gomphocarpus and Calotropis) may be
adaptations which have been selected for by seed predators
like milkweed bugs [14], supporting the ecological signifi-
cance of the interaction between lygaeine bugs and
Apocynaceae. The close relationship between these insects
and the Apocynaceae is furthermore supported by the
assumption that North American Asclepias and the associated
Lygaeus species share the same evolutionary origin as their
distribution matches [15].

The large milkweed bug (Oncopeltus fasciatus (Dallas, 1852))
and the small milkweed bug (Lygaeus kalmii Stål, 1874) are well
known to sequester cardenolides from Asclepias seeds [16]
which protect them against predator attacks [17]. Oncopeltus
fasciatus has been shown to possess a specialized, double
layered epidermis forming the so-called dorsolateral space
where cardenolides absorbed into the body are concentrated
and stored. Upon squeezing (e.g. caused by a predator
attack), special weak areas of the cuticle rupture and droplets
of cardenolide-rich fluid are released [18]. Besides O. fasciatus
and L. kalmii, several other Lygaeinae have also been shown
to sequester [19] or contain (as dried museum specimens) car-
denolides [9]. In addition, O. fasciatus was shown to possess a
Na+/K+-ATPase which is highly resistant to cardenolides
(target site insensitivity) [20]. Both, O. fasciatus and L. kalmii,
share a modified form of Na+/K+-ATPase carrying specific
amino acid substitutions which lead to reduced sensitivity
to cardenolides [7,8,21]. Both adaptations, cardenolide
sequestration and target site insensitivity may be involved in
interactions across three trophic levels, allowing for use of
Asclepias seeds as a food resource and facilitating storage of
these toxins in the body cavity as a defence against predators.
Accordingly, the milkweed bugs provide an ideal model to
study the macroevolution of these two traits involved in
adaptation and probably coevolution.

To trace the macroevolution of both traits, we construc-
ted a molecular phylogeny of 20 lygaeine species plus four
outgroups (two Lygaeidae, one Oxycarenidae and one Pyrhrho-
coridae). Using in vitro assays of hemipteran Na+/K+-ATPase,
we tested seven species of Lygaeinae for target site insensitiv-
ity. The ability to sequester cardenolides was assessed by
feeding assays with radioactively labelled cardenolides in
nine species. Based on these data, we reconstruct the origin
of cardenolide resistance and sequestration in the Lygaeinae
and investigate whether target site insensitivity and cardeno-
lide sequestration are maintained in species no longer
encountering the toxins in their host plants. By combining
our data, we conclude that the secondary use of unrelated
but cardenolide-containing host plants in other families, such
as Adonis vernalis (Ranunculaceae), Digitalis purpurea (Plantagi-
naceae) or Urginea maritima (Asparagaceae) can be explained
by pre-existing adaptations to cardenolides.

2. Material and methods

(a) Construction of molecular phylogeny

Our sampling included 20 species of Lygaeinae from four conti-
nents (electronic supplementary material, table S1) which based
on our taxonomic knowledge represent the phylogenetic
relationships within the subfamily. Species were selected so as
to represent well-established host associations and assumed tox-
onomic bread by one was limited by species availability. Our
sampling comprises species using Apocynaceae as hosts as well
as species which exclusively use host-plants from other families.
Some species were represented by more than one individual (indi-
cated by Roman numerals in figure 1). In addition, DNA was
sequenced from Klototroca reseda (Panzer, 1797) and Belolochilus
numenius (Say 1832), species belonging to closely related subfami-
lies in the Lygaeidae (Icarchirotiinae and Orsillinae), and
Pyrhrhocoris apterus (Linnaeus, 1758) and Oxyacanthus lavaterae
(Fabricius, 1787) (Pyrhrhocoridae and Oxyacanthidae) as representa-
tives of more distant relatives [22]. The target sequences were
1714 bp from the 3’ half of the mitochondrial cytochrome oxidase
subunit I and II genes (COI/II) including the tRNA leucine gene
(tRNAleu) between them and 507 bp of the large nuclear riboso-
mal subunit (28S rDNA). DNA was extracted from fresh
(preserved at −20°C), ethanol preserved (98%) or dried specimens
either by the DNeasy Tissue Kit (Qiagen) or a DNA extraction
system for dried museum material described by Gilbert et al.
[23]. DNA vouchers were deposited at the Zoological Research
Museum Alexander Koenig, Bonn, Germany.

The target sequences were amplified by standard polymerase
chain reaction protocols. To generate homologous sequences for
the 28S rDNA fragment, we used the primers described by
Muraji & Tachikawa [24]. 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. [25] and Weller et al. [26].

Complementary strands of single individuals were edited
and aligned using SEQUENCHER v. 4.6 (Gene Codes Corpora-
tion, Ann Arbor, MI). The final 2221 bp alignment consisted of
1714 bp of the mitochondrial COI/II and tRNAleu genes and
of 507 bp of the nuclear 28S gene obtained for 35 individuals.

Phylogenetic reconstructions were carried out using maximum-
likelihood (ML) and Bayesian inference. Prior to likelihood ana-
lyses, best-fit models of nucleotide substitution were selected
with likelihood ratio tests as implemented in MODELTEST v. 3.7
[27]. Models of sequence evolution and parameters were estimated
for each gene partition separately. ML analyses were performed
with TREEFINDER [28] using a GTR + G model of sequence evolution
for all partitions. Tree searches were started from five trees derived
by a random walk of 10 nearest neighbour interchange steps
around a centre tree (neighbour joining tree) generated by PAUP
v. 4.0b10 [29]. The robustness of the ML tree was evaluated by
bootstrap analyses with 1000 replicates using the same programme.

Bayesian analysis was conducted with MrBayes v. 3.0b4 [30],
fitting a GTR + I + G model to each of the four data positions.
Substitution parameters were estimated separately for each
gene partition. Two independent runs were carried out with
four parallel Markov chain Monte Carlo chains of 1 million
generations and trees sampled every 200 generations.

(b) Lygaeinae specimens for sequestration assays and

in vitro analysis of Na+/K+-ATPase

Adult specimens of Lygaeinae were obtained from the field or if
the number of specimens was not sufficient maintained and pro-
gated for a few generations in laboratory cultures. As in the
phylogenetic analysis, we used species of the families Pyrhrho-
coridae, Bertytidae and also Lygaeidae from a non-lygaeine
subfamily as outgroup comparisons. All outgroup species used
here and in the phylogeny are not reported to use Apocynaceae
as hosts.

For sequestration and Na+/K+-ATPase assays with Cosmo-
pleurus fuliceps (Dallas, 1852), Horvathiolus superbus (Pollich,
1781), Lygaeus equestris (Linnaeus, 1758), L. kalmii, Lygaeus simul-
lans Deckert, 1985, O. fasciatus and Sploidosthous panduris
Figure 1. Maximum-likelihood (ML) tree of the subfamily Lygaeinae based on the combined dataset of COI, COII, tRNALeu and 28S genes. Values above branches indicate ML bootstrap support values (1000 replicates) and Bayesian posterior probabilities (500,000 generations): only values more than 50% are shown. The black branches represent three lygaeid species from subfamilies other than the Lygaeinae (Ischnorrhynchinae, Orsillinae) and *P. apterus* (Pyrrhocoridae) as well as *O. lavaterae* (Oxycarenidae) which were used as outgroups. Capital letter A marks the evolutionary origin of target site insensitivity and cardenolide sequestration, capital letter B the loss of the ability to sequester cardenolides. Evidence for sequestration indicated by (S) was taken from the literature [9], experimental evidence for sequestration from this study is given by $S^+$, $S^-$ indicates lack of sequestration ability. (Online version in colour.)
ATPase does not reach 50% inhibition within the range of inhibitor concentrations used. Assays were performed as described in Petschenka et al. (1998). Cardenolide sensitivity of Na+/K+-ATPase was determined after 0, 5, 10, 15 and 20 min incubation (reactions stopped after 0, 5, 10, 15 and 20 min) using a Na+/K+-ATPase assay of seven lygaeine species tested (IC50 values are calculated based on the fitted curves as lygaeine Na+/K+-ATPase tested under all reaction conditions (A. l. C. fulvipes, M. rufescens and P. apterus) with radioactively labelled cardenolides. As plants typically produce several cardenolides with a wide polarity range, we used the polar [3H]-ouabain and the relatively non-polar cardenolide [3H]-digoxin (both Perkin Elmer LAS GmbH, Rodgau, Germany) to cover a part of this range. Both cardenolides probably do not occur naturally in the host plants of Lygaeinae but were used due to their commercial availability. Individuals were immobilized with a lasso made of dental floss (figure 2, inset) and their proboscis was manually introduced into a 2 μl droplet of 5% sucrose solution containing 5 μM [3H]-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 in individuals kept for only 72 h before analysis differences were resolved less clearly and data showed larger variation (see the electronic supplementary material, figure S1) which might be due to cardenolides still present in the gut. After 10 days, specimens 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 by vortex stirring. After centrifugation, an aliquot (200 μl) of the supernatant was added to 3 ml scintillation cocktail (Ultima Gold, Perkin Elmer) to quantify radioactivity (amount of [3H]-cardenolide absorbed into the body cavity) with a liquid scintillation counter (Wallac 1409). In addition, the residual radioactivity on the parafilm used as a feeding support and the radioactivity of the drinking solution (2 μl aliquots) were determined to calculate the amount taken up and the percentage of radioactivity actually stored by the hemipteran specimens. Ninety-five per cent confidence intervals of means were calculated in JMP® Pro v. 10.0.2 (SAS institute Inc.). All feeding experiments (each species was tested for ouabain and digoxin, separately) were repeated with three to 13 specimens (see figure 2 for sample sizes).

(c) In vitro assay of Na+/K+-ATPase
To test for the occurrence of target site insensitivity to cardenolides, we assayed Na+/K+-ATPase of seven lygaeine species and one lygaeid outgroup species in vitro. Na+/K+-ATPase assays were performed as described in Petschenka et al. (2001). Briefly, brains and thoracic ganglia of hemipteran specimens (killed and stored at −80°C) were dissected under deionized water, pooled (see table 1 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 200 μl water, vortex stirring and incubation for 10 min in a chilled ultrasonic bath. Undissolved residues were removed by centrifugation at 5000 g (3 min). Protein content of Na+/K+-ATPase preparations was determined using the method of Bradford (1976) and subsequently adjusted to provide a total amount of 6 μg protein per Na+/K+-ATPase reaction. Cardenolide sensitivity of Na+/K+-ATPase was determined by photometric quantification of inorganic phosphate released from ATP by Na+/K+-ATPase at different concentrations of ouabain over a period of 20 min at 37°C. To test for linearity, we measured a time course of Pi release over the period of incubation (reactions stopped after 0, 5, 10, 15 and 20 min) using a Na+/K+-ATPase preparation of O. fasciatus under all reaction conditions (10−4 to 10−8 M plus controls) and found that Pi release was always linear (see [31] and the electronic supplementary material, figure S2). Linearity under all conditions ensures that ouabain inhibition curves are not biased by nonlinear Pi release over time under different incubation regimes.

(d) Sequestration assay
To assess the ability to sequester cardenolides, we fed nine lygaeine species in six genera and three outgroup species (K. resedae, M. rufescens and P. apterus) with radioactively labelled cardenolides. As plants typically produce several cardenolides with a wide polarity range, we used the polar [3H]-ouabain and the relatively non-polar cardenolide [3H]-digoxin (both Perkin Elmer LAS GmbH, Rodgau, Germany) to cover a part of this range. Both cardenolides probably do not occur naturally in the host plants of Lygaeinae but were used due to their commercial availability. Individuals were immobilized with a lasso made of dental floss (figure 2, inset) and their proboscis was manually introduced into a 2 μl droplet of 5% sucrose solution containing 5 μM [3H]-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 in individuals kept for only 72 h before analysis differences were resolved less clearly and data showed larger variation (see the electronic supplementary material, figure S1) which might be due to cardenolides still present in the gut. After 10 days, specimens 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 by vortex stirring. After centrifugation, an aliquot (200 μl) of the supernatant was added to 3 ml scintillation cocktail (Ultima Gold, Perkin Elmer) to quantify radioactivity (amount of [3H]-cardenolide absorbed into the body cavity) with a liquid scintillation counter (Wallac 1409). In addition, the residual radioactivity on the parafilm used as a feeding support and the radioactivity of the drinking solution (2 μl aliquots) were determined to calculate the amount taken up and the percentage of radioactivity actually stored by the hemipteran specimens. Ninety-five per cent confidence intervals of means were calculated in JMP® Pro v. 10.0.2 (SAS institute Inc.). All feeding experiments (each species was tested for ouabain and digoxin, separately) were repeated with three to 13 specimens (see figure 2 for sample sizes).

3. Results
(a) Phylogeny of Lygaeinae
In the Bayesian and the ML analyses, the subfamily Lygaeinae was recovered as a monophyletic group. The ML tree
generated with a GTR + G model fitted to each gene partition (figure 1) and the tree derived from the Bayesian analysis supported without any conflicts the same topology. Of the 11 genera, all individuals of the same species from different populations clustered together and were supported by high bootstrap values. In all genera represented by more than one species, species clustered together. However, the genera Spilostethus and Lygaeus are paraphyletic with respect to other species. In Spilostethus, Haemobaphus concinnus (Dallas, 1852) is included while in Lygaeus, L. equestris and L. simulans are strongly supported as monophyletic group but the new world species L. kalmii branches off before the remaining Lygaeus and Spilostethus species and does not seem to belong to the same genus. 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 nertii (Germar, 1847) at the base of this clade. In the second major group, C. fulvipes appears with strong support as the most basal lineage and sister group to the remaining Lygaeinae. The relationship between the genera Horvathiolus, Melanocoryphus, Graptostethus, Tropidothorax and O. fasciatus, on the other hand, is less well supported. No previous phylogenetic analyses with a similar coverage of the Lygaeinae exist that could provide additional support for the resolution of this part of the tree [22,33].

**Figure 2.** 3H-cardenolide-sequestration in nine Lygaeinae and three outgroup species 10 days after oral administration. Bars indicate the proportion of stored cardenolides (digoxin, blue (dark), ouabain, green (light); means ± 95% CI, error bars projecting below zero were cut for clarity). The total amount ingested was set to 100%. The picture of a feeding L. kalmii illustrates the method used to force the hemipteran specimens to drink the test solutions. (Online version in colour.)

### (b) Ouabain resistance of Na\(^+\)/K\(^+\)-ATPase *in vitro*

Na\(^+\)/K\(^+\)-ATPases of all seven Lygaeinae tested here showed a highly similar characteristic of *in vitro* inhibition by ouabain (figure 3). Their Na\(^+\)/K\(^+\)-ATPase was highly resistant to cardenolides and nearly not affected over three orders of magnitude of ouabain concentration (10\(^{-8}\) to 10\(^{-5}\) M). At 10\(^{-3}\) M ouabain, where non-adapted Na\(^+\)/K\(^+\)-ATPases are typically completely inhibited [34], the enzyme preparation of all Lygaeinae tested still showed about 70% remaining activity. IC\(_{50}\) values for all species are presented in table 1. Lygaeine Na\(^+\)/K\(^+\)-ATPase is much more resistant to ouabain (cardenolides) than Na\(^+\)/K\(^+\)-ATPase of the monarch butterfly (figure 3). In comparison, the lygaeid (non-lygaeine) outgroup species used in this study, *K. resedae*, is highly
Lygaeinae correlated with the origin of target site insensitivity ((A) in figure 1) and a secondary loss of sequestration in *A. longiceps* and *A. melanocephalus* (B) in figure 1).

**Figure 3.** *In vitro* inhibition of Na\(^+/\)K\(^+-\)ATPase by ouabain. Each data point represents the mean of three biological replicates ± s.d. (a) Na\(^+/\)K\(^+-\)ATPase of seven species of Lygaeinae (top set of curves) versus Na\(^+/\)K\(^+-\)ATPase of the outgroup species *K. resedae* (dashed line). A.L., *A. longiceps*; S.p., *S. pandanus*; L.k., *L. kalmii*; O.f., *O. fasciatus*; L.e., *L. equestris*; T.l., *T. leucopterus*; H.s., *H. superbus*. (b) Comparison of the non-adapted Na\(^+/\)K\(^+-\)ATPase of *Drosophila melanogaster* (dashed line, G. Petschenka 2012, unpublished data) with the cardenolide-resistant Na\(^+/\)K\(^+-\)ATPase of *D. plexippus* (dotted line, data from [31]) and *O. fasciatus* (solid line).

**4. Discussion**

We reconstructed the macroevolution of two adaptations to host plant toxins, cardenolide resistance and sequestration. We found that both traits are probably basal features of the Lygaeinae. Our findings demonstrate that the coevolutionary response of herbivores to plant toxins and their use as an acquired defence involves a combination of traits not just a single adaptation. As several insect species with cardenolide-resistant Na\(^+/\)K\(^+-\)ATPase were also shown to sequester the toxins, the two traits might often be linked and are probably co-adaptive (e.g. *Chrysochus auratus*; *Crysomelidae*; *Poeckilocerus bufonius*; *Pyrgomorphidae*; *Danais plexippus*; *Nymphalidae*; [41–45]). However, the leaf beetle *Labidomera clivicollis* which feeds on *Asclepias* species without sequestering cardenolides but still has a Na\(^+/\)K\(^+-\)ATPase bearing resistance conferring substitutions [7] and cardenolide-sequestering arctiid moths (e.g. *Empyreuma pugione*) with sensitive Na\(^+/\)K\(^+-\)ATPases [34] indicate that both traits are not obligatorily linked.

We found that seven of the nine Lygaeinae tested here store the orally ingested cardenolides ouabain and digoxin in their body. This supports earlier findings of Scudder & Duffey [9] who detected cardenolides in dried museum specimens of more than 20 genera of Lygaeinae. The lack of sequestration in *A. longiceps* and *A. melanocephalus* found here is in line with the life history of these two species which live on plants not known to produce cardenolides (*Platanus*: Platanaceae, or *Ulmus*: Ulmaceae, respectively). Feeding on non-apocynaceous hosts is most likely a derived...
state in this genus, as *Arocatus* species in Australia are well known to use apocynaceous plants (*A. aenesceus*, *A. chiasmus*, *A. contintus*, *A. montanus* and *A. rusticus* feed on *Arumia*, *Asclepias*, *Gomphocarpus*, *Nerium* and *Parsonia* species, respectively [35]). As *A. longiceps* and *A. melanocephalus* are recovered as sister species in a monophyletic group with *A. aenesceus* and the cardenolide-sequestering *A. rusticus* [9] and this group is moreover in a sister group relationship with the cardenolide-sequestering *C. neri* [19], we assume that the ability to sequester cardenolides was lost in the branch leading to the two European *Arocatus* species. This finding may indicate that physiological adaptations which are necessary to sequester cardenolides might be costly and are reduced if not needed. Comparatively small amounts of ouabain (but only marginal amounts of digoxin) sequestered are reduced if not needed. Comparatively small amounts of ouabain observed here showed the same ouabain resistance as observed here (S. Dalla & S. Dobler 2014, unpublished results in an enzyme showing similar resistance to ouabain observed here resembles the one described by Moore & Scudder [20]. Remarkably, we have not found a stepwise pattern of evolution of *Na*+/K+/ATPase resistance as detected in milkweed butterflies [31] but rather strong resistance even in the species placed on the earliest branches in our phylogeny. Interestingly, cardenolide resistance of milkweed bug *Na*+/K+/ATPase is about 10-fold higher than cardenolide resistance of *D. plexippus* *Na*+/K+/ATPase (based on the IC₅₀ of ouabain). This remarkable difference could be an adaptation to seed feeding of milkweed bugs as *Asclepias* seeds in some species have been reported to have much higher cardenolide concentrations than leaves [55]. Moreover, the higher level of resistance might provide the physiological basis for the much higher concentrations of sequestered cardenolides in milkweed bugs as compared with monarch butterflies [3].

Previous analyses of *Na*+/K+/ATPase α gene sequences (the subunit of *Na*+/K+/ATPase where cardenolides bind to) of *L. kalmii* and *O. fasciatus* have shown that both species possess amino acid substitutions at positions known from structural analyses and mutagenesis of the mammalian gene to confer resistance to cardenolides [7,8]. Furthermore, transcriptome analyses of both species evidenced the presence of three copies of the relevant *Na*+/K+/ATPase α gene. Based on divergence estimates, it was assumed that many lygaeine species possess the same set of *Na*+/K+/ATPase copies [8]. This is in good agreement with the homogeneity of our *in vitro* ouabain inhibition assays in seven species. All of the three ATPase α gene copies share a replacement of the conserved asparagine by histidine at position 122 [8], a substitution which has been shown to significantly lower the sensitivity of the *Na*+/K+/ATPase to ouabain [7,21,56]. Our preliminary analyses of gene transcripts in the Lygaeinae investigated here (see the electronic supplementary material, table S1) corroborate the presence of this histidine residue in all species, however, only a combination of four substitutions, glutamine⁸⁸⁸threonine + asparagine¹⁰¹ histidine + phenylalanine⁴⁸⁶asparagine + threonine⁷⁸⁸alanine, as observed in the ATPase αC copy [8] results in an enzyme showing similar resistance to ouabain as observed here (S. Dalla & S. Dobler 2014, unpublished data). Which of the three *Na*+/K+/ATPase α copies is preponderantly expressed in the nervous tissue is still an open question, yet it is plausible that all species express the same set of genes with identical amino acid substitutions in their *Na*+/K+/ATPases. We can assume that the series of gene
duplications and amino acid substitutions must have arisen by stepwise evolution just as in milkweed butterflies, but only a broader sampling of other basal lygaeines and more closely related outgroups can potentially reveal traces of this process.

Our finding of insensitive Na⁺/K⁺-ATPase(s) expressed in the nervous system of the Lygaeinae suggests that Na⁺/K⁺-ATPase in the hemipteran nervous system is not protected by the perineurium as was suggested for cardenolide-sequestering Lepidoptera that nevertheless possess sensitive Na⁺/K⁺-ATPases [34,57]. As Na⁺/K⁺-ATPase is essential for the generation of neural action potentials and is heavily expressed in insect nervous tissues [5,34], selection pressure by cardenolide-containing host plants must probably result in either of these two protective mechanisms.

We have shown that two traits associated with the beneficial use of a host plant toxin most likely form a co-adaptive strategy. Our findings indicate that sequestration of toxins need to be accompanied by other adaptations to compensate for the cost which otherwise would arise when plant toxins are incorporated into an insect’s body. Given the evolutionary success of milkweed bugs, the ecological benefit achieved by sequestration not only seems to outweigh potential costs of these additional adaptations but also could, on the other hand, lead to a dual increase of fitness. For the milkweed bugs, it was suggested that sequestration of host plant toxins may have superseded the role of the metathoracic scent gland which is generally involved in defence in the Hemiptera. In this case, the insects not only only save the production of endogenous defensive compounds but the gland even secondarily adopted a sexual function [58]. Evolutionary trade-offs like this could well exist in other groups of Hemiptera and have also been shown to occur in other groups of herbivorous insects [1].

**Data accessibility.** Data from Na⁺/K⁺-ATPase and sequestration assays were deposited on Dryad (doi:10.5061/dryad.1707a). All sequences generated for this study have been deposited under GenBank accession no. LN623642–LN623676 and LN614530–LN614584.

**Acknowledgements.** We are greatly indebted to Prof. Anurag Agrawal (Cornell University, Ithaca, NY, USA), Dr David Britton (Australian Museum, Sydney, Australia), Michael Falkenberg and Dr Robert Trusch (Staatliches Museum für Naturkunde, Karlsruhe, Germany), Lara Flucht (University of Hamburg, Germany), Dr Stefan Küchler (University of Bayreuth, Germany), Klaus Liebenow (Brandenburg an der Havel, Germany), Kai Schütte (Zoologisches Museum, Hamburg, Germany) and Prof. Klaus Schönitz and Bärbel Stock-Dietl (Zooloogische Staatssammlung, München, Germany) for providing hemipteran specimens. We thank Vera Wagschal (University of Hamburg, Germany) for sequencing Na⁺/K⁺-ATPase genes and the Landesumweltamt Brandenburg (Potsdam, Germany) for issuing hemipteran permits. We furthermore thank Prof. Anurag Agrawal (Cornell University, Ithaca, NY, USA) for commenting on and improving this manuscript.

**Funding statement.** This work was supported by the German Research Foundation (DO 527/5-3 and PE 2059/1-1) and the Templeton Foundation.

**References**


6. Dussourd DE, Eisner T. 1987 Vein-cutting behavior: potential costs of these additional adaptations but also could, benefit achieved by sequestration not only seems to outweigh the evolutionary success of milkweed bugs, the ecological compensation for the cost which otherwise would arise when toxins need to be accompanied by other adaptations to compensate for the cost which otherwise would arise when plant toxins are incorporated into an insect’s body. Given the evolutionary success of milkweed bugs, the ecological benefit achieved by sequestration not only seems to outweigh potential costs of these additional adaptations but also could, on the other hand, lead to a dual increase of fitness. For the milkweed bugs, it was suggested that sequestration of host plant toxins may have superseded the role of the metathoracic scent gland which is generally involved in defence in the Hemiptera. In this case, the insects not only only save the production of endogenous defensive compounds but the gland even secondarily adopted a sexual function [58]. Evolutionary trade-offs like this could well exist in other groups of Hemiptera and have also been shown to occur in other groups of herbivorous insects [1].