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Sequestration of Plant Defense Compounds by Insects: From Mechanisms to Insect–Plant Coevolution

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Abstract

Plant defense compounds play a key role in the evolution of insect–plant associations by selecting for behavioral, morphological, and physiological insect adaptations. Sequestration, the ability of herbivorous insects to accumulate plant defense compounds to gain a fitness advantage, represents a complex syndrome of adaptations that has evolved in all major lineages of herbivorous insects and involves various classes of plant defense compounds. In this article, we review progress in understanding how insects selectively accumulate plant defense metabolites and how the evolution of specific resistance mechanisms to these defense compounds enables sequestration. These mechanistic considerations are further integrated into the concept of insect–plant coevolution. Comparative genome and transcriptome analyses, combined with approaches based on analytical chemistry that are centered in phylogenetic frameworks, will help to reveal adaptations underlying the sequestration syndrome, which is essential to understanding the influence of sequestration on insect–plant coevolution.

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INTRODUCTION

The ecology and evolution of herbivorous insects are strongly influenced by plant secondary metabolites that mediate both antagonistic and mutualistic interactions of plants with their environment (7, 29, 30). Many plant secondary metabolites are considered to be defense compounds because they deter herbivory, reduce food digestibility, or directly interact with molecular targets in non-adapted insects (33, 62). In response, insects have developed sophisticated morphological, behavioral, and physiological adaptations that enable them to exploit plants as a resource. The most complex syndrome of insect adaptations to plant toxins is sequestration, i.e., the ability to selectively accumulate plant defense compounds in the insect body for defense against natural enemies (26, 39, 66, 76). The hypothesis that insects, specifically aposematic butterflies, obtain defense compounds from larval food plants dates back to 1893 (37) and was finally shown in a series of studies with monarch butterflies, *Danaus plexippus*; their apocynaceous host plants; and bird predators in the 1960s and 1970s (15a, 57). To date, milkweed butterflies and the cardenolides that they sequester are an important research paradigm, but extensive research over the course of the past 50 years has revealed that sequestration is actually a common phenomenon among specialist herbivores, which are associated with specific host plant species containing various types of defense compounds (66, 70). In 2009, a comprehensive review summarized studies on insect sequestration, showing that “more than 250 insect species have been shown to sequester plant metabolites from at least 40 different families” (70, p. 117). Since then, sequestration of plant defense compounds has been demonstrated in more than 25 additional insect species (summarized in **Supplemental Table 1**). Because it is a complex biological phenomenon that involves multiple trophic levels (76, 78, 90, 112), sequestration has been investigated from very different perspectives and on different scales. In this article, we review our understanding of how plant defense compounds are acquired by insects, which resistance mechanisms enable insects to store these compounds in their bodies, and how sequestration has influenced insect–plant coevolution.

Supplemental Material >

ACQUISITION OF PLANT DEFENSE COMPOUNDS BY INSECTS

Sequestering insects have developed mechanisms that enable them to accumulate ingested plant defense compounds in various tissues, for example, in the hemolymph, in specialized defense glands, or in the integument. This accumulation is usually selective, which results from one or a combination of different mechanisms: (a) selective absorption across the gut, (b) selective transport within the body, (c) selective endogenous metabolism, and (d) selective excretion via the Malpighian tubules. Moreover, insects appear to regulate the levels and composition of defense compounds sequestered depending on the chemical profile of the food plant, e.g., glucosinolates and iridoid glycosides (5, 8, 108). However, we are just beginning to understand the importance of individual factors because sequestration mechanisms have been systematically studied in only a few insect species.

The first step in sequestration is the absorption of plant defense compounds from the gut lumen across the peritrophic matrix and gut epithelium into the hemocoel by passive diffusion or carrier-mediated transport, depending on the physicochemical properties of the compound. Absorption additionally depends on epithelial permeability, which is also mediated by the presence of efflux transporters that can prevent the absorption of compounds (19, 24, 92). For example, the cardiac glycoside digitoxin was proposed to passively diffuse across the midgut of the sequestering milkweed bug, *Oncopeltus fasciatus*, whereas the midguts of the desert locust, *Schistocerca gregaria*, and the American cockroach, *Periplaneta americana*, are impermeable to this compound (87). Thus, mechanisms that facilitate or prevent the absorption of plant defense compounds across the gut play a central role in the evolution of sequestration.

The absorption of plant defense compounds from the gut lumen presumably proceeds in a manner similar to that of nutrients, through transport across the midgut epithelium. Several insect gut transporters have been characterized (42), but transporters responsible for the uptake of polar plant defense compounds are still unknown. In addition, only a few studies have investigated the degree of uptake selectivity and determined precisely where defense compounds are absorbed. To address these questions, transport studies have been performed with synthetic thioglucosides, which resemble plant O-glucosides but resist hydrolysis by β -glucosidases, and dissected gut tissue of two closely related leaf beetle species, the sequestering poplar leaf beetle, *Chrysomela populi*, and the nonsequestering mustard leaf beetle, *Phaedon cochleariae* (22). All tested thioglucosides were absorbed into the gut tissues of both species, but the relative composition of compounds differed, suggesting that either import or export specificities vary between *C. populi* and *P. cochleariae*. In larvae of the six-spot burnet moth, *Zygaena filipendulae*, a stereoselective uptake of cyanogenic glycosides was demonstrated by feeding the larvae a mixture of synthetic α - and β -glucosides. Burnet moth larvae sequestered the β -glucosides and excreted the corresponding α -glucosides, indicating that transporters specific for β -glucosides enable the absorption of cyanogenic glycosides across the larval gut (111). Physiological studies with phloridzin, an inhibitor of sugar transport, also suggested a role for sugar transporters in cardenolide absorption by the midgut of *O. fasciatus* (21).

Another family of membrane proteins, the cytochrome P450 monooxygenases, were shown to be involved in nicotine transport from the gut into the hemolymph of the tobacco hornworm, *Manduca sexta* (49). Silencing of MsCYP6B46 reduced nicotine levels in the hemolymph compared to control larvae and increased susceptibility to predation by nicotine-sensitive wolf spiders. However, the mechanism by which MsCYP6B46 mediates nicotine absorption is not known.

After initial absorption, defense compounds are either stored in the hemolymph or deposited in specific tissues. Insects require mechanisms that enable transient and long-term storage, particularly of polar metabolites in the hemolymph, because these may be readily excreted by the Malpighian tubules (55, 56, 61). Indeed, it has become clear that the reabsorption of defense compounds in the Malpighian tubules represents an important mechanism that enables storage of defense metabolites in the hemolymph (61, 109). This mechanism was first described in the 1980s in the large milkweed bug. Passively secreted ouabain is reabsorbed from the Malpighian tubule lumen against the concentration gradient, preventing the loss of ouabain by excretion (61). Further support for an important role of the Malpighian tubules in sequestration was recently obtained in a study with the horseradish flea beetle, *Phyllotreta armoraciae*, which accumulates high concentrations of glucosinolates in the hemolymph (108, 109). Injection of a mixture of glucosinolates and nonhost glucosides into the beetle hemolymph revealed that only glucosinolates were retained in the body, whereas nonhost glucosides were excreted. Multiple glucosinolate-specific transporters belonging to the major facilitator superfamily (MFS) were responsible for glucosinolate reabsorption in the Malpighian tubules (109). These transporters, which represent the first specific transporters for plant defense compounds discovered in any sequestering insect, were identified by a comparative phylogenetic analysis of coleopteran MFS transporters, which revealed species-specific expansions of these transporters. Most glucosinolate-specific transporters were exclusively expressed in the Malpighian tubules, which indicates that *P. armoraciae* controls the glucosinolate levels and composition in the body by reabsorption. Findings in another sequestration model system, the sawfly genus *Athalia*, point in a similar direction. Iridoid glucoside-sequestering *Athalia* species accumulate much higher glucoside levels in the hemolymph as compared to glucosinolate-sequestering species (68), which instead rapidly metabolize and excrete sequestered glucosinolates (64, 69). One possible explanation is that iridoid glucoside-sequestering *Athalia* spp. have developed a reabsorption mechanism that enables them to accumulate high levels of

iridoid glucosides in the hemolymph, whereas glucosinolate-sequestering *Athalia* spp. cannot prevent glucosinolate excretion from the hemolymph. However, from an ecological perspective, it is not surprising that *Athalia* spp. do not store glucosinolates for a longer time. In contrast to other glucosinolate-sequestering specialists [flea beetles (8, 93), aphids (44, 99), and possibly bugs (2)], insect myrosinase activity has not been detected in *Athalia rosae* (64). Because glucosinolates themselves are not toxic, there is no obvious benefit to storing them in the body (68, 102). Instead, glucosinolate sequestration has been proposed to function as a detoxification mechanism in *Athalia* spp. (1, 102), which may represent a key step toward a sequestration syndrome.

Many insects store sequestered compounds in the integument, e.g., in cuticular cavities (34, 72), in subcuticular compartments (14, 86), or in exocrine defense glands, spatially separating bioactive metabolites from sensitive targets in the insect. In addition, defense compounds can be released without or with only minimal tissue damage. The presence of specific storage compartments facilitated the elucidation of transport processes involved in sequestration in the leaf beetle tribe Chrysomelina (22, 47, 48, 85, 97, 98). Chrysomelina larvae possess nine pairs of dorsally located exocrine defense glands, consisting of secretory cells connected to a chitin-coated reservoir by canal cells, from which they release deterrent secretions upon attack. Defense metabolites present in these secretions are either produced *de novo* or are derived from sequestered plant compounds. Independent of their origin, the precursors of defense metabolites are glucosides that are imported from the hemolymph into the gland reservoir, where they are activated by β -glucosidase and oxidase enzymes (80, 81).

Glucosides are accumulated with striking structural selectivity in the defense glands of sequestering (*C. populi* and *Phratora vitellinae*) and nonsequestering (*Hydrothassa marginella* and *Phratora laticollis*) Chrysomelina larvae (47). Molecular studies identified a transporter of the ATP-binding cassette (ABC) subfamily C that is specifically and highly expressed in defense glands and is required for salicin sequestration (97). Subcellular localization studies showed that this transporter is present in the membrane of storage vesicles in the secretory cells that release salicin into the gland reservoir by exocytosis (97). Because this ABC transporter has a broad substrate specificity, the selective transport step in this system must be the import of salicin from the hemolymph into the secretory cell. Proteomic profiling of membrane proteins in the defense glands identified a total of 27 membrane proteins, including 14 primary active transporters and 7 secondary active transporters. The best candidates for salicin import found with this approach are members of the sugar porter family (Transporter Classification Database 2.A.1.1), and indeed, silencing the expression of one of these sugar porters by RNA interference abolished the ability to produce defensive secretions (85).

Together, the physiological and molecular studies of glucoside transport in *C. populi* reveal one possibility for how insects can selectively sequester ingested plant defense compounds. The initial glucoside uptake across the gut is less selective (22), which enables a range of potential glucoside substrates to enter the hemolymph. Compounds are then either transported into defense glands or secreted into the Malpighian tubules to be metabolized and/or excreted. With one selective transport step (in *C. populi*, glucoside import into the secretory cell), selection acts on the substrate specificity of one or only a few transporters when insects are adapting to a new host plant with structurally similar defense compounds. Indeed, a less selective glucoside uptake into defense glands has already been demonstrated in the leaf beetle *Chrysomela lapponica*, which uses a broader spectrum of host plants and belongs to a more derived clade of chrysomelinae (48).

Changes in the substrate specificity of glucoside transporters may have played a role in a proposed host shift in the sawfly genus *Athalia* from iridoid glycoside-containing plants in the order Lamiales to glucosinolate-containing Brassicaceae. Comparative feeding studies with host and nonhost glucosides demonstrated that Brassicaceae-feeding *Athalia* species can sequester both

iridoid glycosides and glucosinolates, whereas Lamiales feeders only sequester iridoid glycosides (67). The ability of ancestral *Athalia* species to sequester iridoid glycosides has been hypothesized to represent a physiological preadaptation that facilitated the host shift to Brassicaceae.

Recently, an ABC transporter in the subfamily B has been implicated in the accumulation of cardenolides in defense glands localized in the pronotum and elytra of adult dogbane beetles (*Chrysobothris auratus*) (45). Of three ABCB transporters that were identified in the chrysomelid transcriptome, only ABCB2 was expressed in the elytra and stimulated by cymarin, a cardenolide present in the beetle's host plant *Apocynum cannabinum*. Moreover, phylogenetic analyses provided evidence that ABCB2 results from a recent gene duplication (45).

Despite their importance for understanding the evolution of sequestration mechanisms, the identification of only a few transporters of plant defense metabolites in insects remains a bottleneck. Unsurprisingly, the two largest transporter families in insects (ABC and MFS) have been shown to be involved in the sequestration of different classes of plant defense compounds. The increasing availability of sequenced insect genomes and transcriptomes will greatly facilitate the identification of additional candidates through comparative phylogenomic analyses, combined with tissue-specific expression analyses and reverse genetic approaches to investigate the role of candidate transporters *in vivo*.

INSECT RESISTANCE TO SEQUESTERED PLANT DEFENSE COMPOUNDS

Sequestration of plant compounds likely evolves under different selection pressures than those mediating the ingestion of plant toxins in the diet because many plant toxins are typically not absorbed into the body cavity but remain in the gut lumen (24), where they are degraded and excreted by defecation. Sequestered compounds, in contrast, are incorporated into the body tissues and will exert physiological effects on internal targets.

Insect adaptations to frequently sequestered plant cardenolides have been intensively studied during the past decade, in large part due to the integration of approaches from molecular biology and sequencing technologies (23, 43, 100, 113). Cardenolides, also known as cardiac glycosides, are specific inhibitors of the cation carrier Na^+/K^+ -ATPase, which is expressed in almost all animal cells and is involved in a great variety of essential physiological functions. Insects from at least seven orders (Heteroptera, Sternorrhyncha, Caelifera, Hymenoptera, Coleoptera, Diptera, Lepidoptera) have evolved resistant Na^+/K^+ -ATPases with key amino acid substitutions that decrease binding of cardiac glycosides to the enzyme (i.e., target site insensitivity) (23, 25, 43, 113). Remarkably, these substitutions often involve the same amino acids at homologous positions of the protein, providing a striking example of molecular convergence. Specifically, the replacement of asparagine with histidine at position 122 of the enzyme evolved independently at least seven times (25, 43). Moreover, the observed amino acid substitutions are frequently accompanied by gene duplications of the *Atp α* gene, which encodes the alpha subunit of Na^+/K^+ -ATPase that comprises the binding site for cardiac glycosides, suggesting that these substitutions have pleiotropic effects on vital functions of the protein (43, 79, 100, 113).

Interestingly, at least six insect lineages that evolved sequestration of cardenolides independently (i.e., all lineages known to sequester) also possess amino acid substitutions at positions relevant for cardenolide binding (43). Examples include the entire heteropteran Lygaeinae (approximately 600 described species), for which sequestration of cardiac glycosides and resistant Na^+/K^+ -ATPases most likely are ancestral traits (13); milkweed butterflies of the genus *Danaus* (77); pyrgomorphid grasshoppers (25); and chrysomelid leaf beetles (50). Although the extent to which individual amino acid substitutions (or combinations thereof) contribute to resistance in

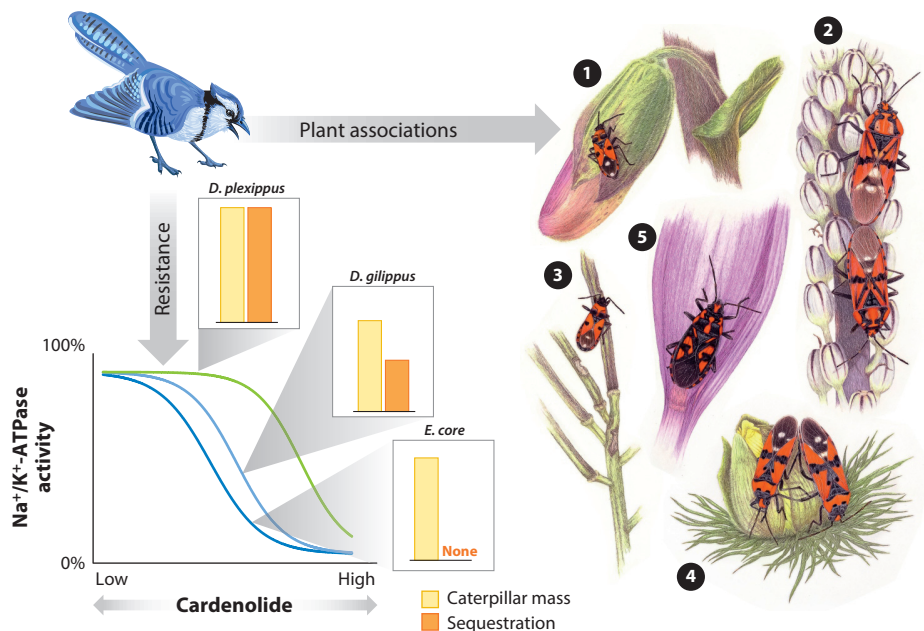


Figure 1

Sequestration as a vehicle of insect-plant coevolution. Sequestration of plant toxins in insects evolved as an adaptation for defense against predators (as indicated by the bird) and parasitoids (i.e., the third trophic level) (76, 96). Predators and parasitoids can spur insect-plant coevolution in at least two ways, either by selecting for resistance traits against specific classes of sequestered plant compounds (*left*) or by driving specialized interactions of insects with toxic plants (*right*). (*Left*) Cardiac glycoside (CG) resistance in the milkweed butterflies (Danaini) increased in a stepwise fashion, resulting in three forms of Na^+/K^+ -ATPase and exposing different resistance to the CG ouabain. Across eight species of milkweeds (*Asclepias* spp.), caterpillars of *Euploea core*, *Danaus gilippus*, and *Danaus plexippus* grew similarly on individual milkweed species and were not impaired by dietary CGs (75). The amount of sequestered CGs, in contrast, is positively correlated with the degree of CG resistance (75). (*Right*) Colonization of CG-producing plants by several species of milkweed bugs (Lygaeidae) was facilitated by preadaptation to CGs due to the ancestral association with CG-producing Apocynaceae and an existing sequestration syndrome. The polyphagous *Spilostethus saxatilis* evolved a specialized interaction with autumn crocus (*Colchicum autumnale*) and sequesters colchicine and related alkaloids (78). ① *Horvathiolus superbis* on *Digitalis purpurea* (Plantaginaceae). ② *Spilostethus pandurus* on *Urginea maritima* (Asparagaceae). ③ *Horvathiolus superbis* on *Erysimum crepidifolium* (Brassicaceae). ④ *Lygaeus equestris* on *Adonis vernalis* (Ranunculaceae). ⑤ *S. saxatilis* on *C. autumnale* (Colchicaceae). Drawings of milkweed bugs courtesy of Martina Zwanziger. Icon of a blue jay by Raven17 from iStock.

sequestering insects is not yet completely understood, some degree of target site insensitivity may be a prerequisite for sequestration.

During milkweed butterfly radiation, the resistance of the butterflies' Na^+/K^+ -ATPases increased in a stepwise fashion, resulting in three forms of Na^+/K^+ -ATPase with different levels of cardiac glycoside resistance (77) (Figure 1). In a comparison across three danaine species (*Euploea core*, *Danaus gilippus*, and *D. plexippus*), the extent of target site insensitivity (low, intermediate, or strong) had no effect on caterpillar growth upon dietary exposure to cardiac glycosides, but the amount of sequestered cardiac glycosides mirrored the level of resistance of the Na^+/K^+ -ATPase (75). Moreover, whereas *E. core* caterpillars are not harmed by orally administered cardiac glycosides, they are not able to cope with these compounds if they are injected into their hemocoel,

most likely due to a lack of a sufficiently resistant Na^+/K^+ -ATPase. These findings suggest that a resistant Na^+/K^+ -ATPase in milkweed butterflies is not required for tolerating cardiac glycosides in the diet, but rather represents an adaptation that is associated with the ability to sequester these compounds as defenses.

The lack of resistant Na^+/K^+ -ATPases in nonsequestering insects that feed on cardiac glycoside-containing plants (e.g., the arctiine moth *Cynia oregonensis* and the milkweed tussock, *Euchaetes egle*) (43) and the frequent association of cardiac glycoside sequestration and resistant Na^+/K^+ -ATPases suggest that sequestration and target site insensitivity of Na^+/K^+ -ATPase interact. Most interestingly, imaginal *Drosophila melanogaster* whose Na^+/K^+ -ATPase was genetically modified by CRISPR–Cas9 editing of the native *Atpa* gene to resemble the monarch butterfly phenotype contained cardiac glycosides obtained during larval development (43). This suggests that the resistance trait and the ability to sequester are mechanistically linked and may have evolved in concert.

While target site insensitivity against insecticides is observed frequently, examples involving toxic plant compounds that occur in insect diets naturally are very scarce (20). Besides cardenolide-resistant Na^+/K^+ -ATPases, a reduced electrophysiological response of the desheathed central nervous system in *M. sexta* to nicotine (63) and discrimination of arginyl-tRNA synthetase against the nonproteinogenic amino acid L-canavanine in the bruchid beetle *Caryedes brasiliensis* (9, 84) are the only other examples reported to date. However, the mechanisms of insect resistance to host plant toxins have been studied in relatively few systems (39), so it is likely that other examples will be found.

Although the ability of target site insensitivity to mediate resistance against plant toxins is obviously limited to toxins having a specialized mode of action (i.e., acting on a specific receptor), many plant toxins still fall into this category, e.g., pyrethrins and alkaloids acting on sodium channels, neurotransmitter receptors, and other neuronal targets or microtubules (106). Thus, the frequency of the evolution of target site insensitivity in natural systems and its putative association with sequestration should be studied more broadly to better understand the evolutionary drivers.

Sequestering insects must preserve the pharmacological activity of the sequestered compounds, and target site insensitivity is a suitable way to achieve this, in contrast to metabolic resistance, which involves alteration and detoxification of plant toxins (9). We thus propose that target site insensitivity is an important evolutionary driver of sequestration (or vice versa) because it simultaneously mediates tolerance and preserves the toxicity of sequestered compounds.

The glucosinolates, iridoid glycosides, cyanogenic glycosides, benzoxazinoids, and salicinoids that are sequestered by many insects (70, 83) represent two-component defenses that occur in plants as glycosylated inactive storage forms and require activation by β -glucosidases coming from the plant, the insect, or both (for a review, see 74). It is assumed that phloem-feeding insects circumvent the disruption of cells and ingest intact glucosinolates that are subsequently sequestered. Examples include hemipteran insects such as the aphids *Brevicoryne brassicae* and *Lipaphis erysimi* and the stink bug *Murgantia histrionica* (3, 15, 44). Phloem-feeding by sequestering hemipterans may therefore represent a preadaptive mode of resistance. However, it was recently shown for the whitefly *Bemisia tabaci* that phloem-feeding can result in the activation of glucosinolate protoxins in the host plant and that additional detoxification mechanisms are employed (58, 59). This suggests that avoidance of activation is not the only mode of resistance in sequestering hemipterans.

It was furthermore hypothesized that sequestration as such represents a resistance mechanism, preventing two-component plant defenses from being activated by β -glucosidases in the insect gut (74). In fact, a rapid uptake of glucosinolates in the anterior gut region of the glucosinolate-sequestering sawfly *A. rosae* was shown using matrix-assisted laser desorption ionization–mass

spectrometry imaging (1). In addition, myrosinase (i.e., the enzymes activating glucosinolates) activities were low in the anterior larval gut but increased along the gut, supporting the hypothesis that rapid uptake of intact glucosinolates into the body cavity prevents activation by myrosinases further along the larval gut (1). A rapid absorption of glucosinolates was also recently demonstrated in adult horseradish flea beetles (*Phyllotreta armoraciae*) (94). Nevertheless, some glucosinolates are hydrolyzed by the plant myrosinase during feeding and digestion in *P. armoraciae*, but without an apparent negative effect on beetle performance (94). While it is compelling to classify sequestration as a resistance mechanism that could also be involved in insects sequestering other two-component plant defenses, this hypothesis seems unlikely to apply in general, especially in insects that sequester defenses that do not require enzymatic activation, such as cardiac glycosides.

Similar to the avoidance of tissue disruption by phloem-feeding hemipterans and a leaf-snipping feeding mode (as opposed to leaf crushing, which is found in other insects) that is common across Lepidoptera and may minimize the activation of two-component plant defenses (6, 73), the alkaline midgut, which is a common feature in Lepidoptera and other Mecoptera (17), may represent a preadaptation to cope with two-component plant defenses. In the six-spot burnet moth (*Z. filipendulae*), a high gut pH (10.6) was interpreted as an adaptation to avoid activation of cyanogenic glycosides by inhibiting β -glucosidases from its host plant *Lotus corniculatus* (73). β -Glucosidases certainly represent an important target of selection to mediate resistance in insects feeding on plants producing two-component plant defenses (74), and the extent to which β -glucosidases are involved in resistance in sequestering insects needs further investigation. Interestingly, gut β -glucosidases from *Z. filipendulae* were found not to hydrolyze the cyanogenic glucosides linamarin and lotaustralin, which are present in *L. corniculatus* and are subsequently sequestered (74).

Among insects, sequestration of cyanogenic glycosides has to date been found only in Lepidoptera: Zygaenidae and especially Heliconiinae (Nymphalidae) (70, 110). There are two major resistance mechanisms in insects to tolerate hydrocyanic acid (HCN) based on the enzymes rhodanese (formation of nontoxic thiocyanates by transfer of sulfur to CN^-) or β -cyano-L-alanine synthase (formation of β -cyano-L-alanine) (10, 41, 95, 103), but it remains to be determined whether they play a role in the cyanogenic glucoside sequestration syndrome. For *Z. filipendulae*, endogenous production of cyanogenic glucosides, and therefore also resistance, probably preceded sequestration (34) and could in itself represent a preadaptation for the evolution of sequestration.

Another important class of compounds sequestered by insects from several orders and from various plant sources is pyrrolizidine alkaloids (PAs) (70). In plants, PAs occur as nontoxic N-oxides that are reduced to their corresponding protoxic free bases in the insect gut. PA toxicity is subsequently mediated by metabolic activation via cytochrome P450 monooxygenases forming pyrrolic metabolites that are cytotoxic and tumorigenic (88, 107). Sequestration of PAs was studied in detail in the cinnabar moth (*Tyria jacobaeae*), which possesses a flavin-dependent monooxygenase (FMO; senecionine N-oxygenase) that reconverts sequestered PAs into their corresponding N-oxides in the hemolymph (65). Within Lepidoptera, FMOs form a gene family with three members (FMO1–FMO3). Interestingly, the FMO1 gene underwent a further duplication event early in the arctiine moth lineage, resulting in a cluster containing the PA-specific FMOs of *T. jacobaea* and *Grammia geneura*. This gene duplication was interpreted as a key innovation that allowed for the evolution of the various adaptations of arctiines to PA-containing plants (88). Because sequestration of PAs probably evolved early in the Arctiinae (formerly Arctiidae, now included in the Erebidae), it seems likely that duplication of FMO1 and the evolution of enzymatic PA specificity are linked to the evolution of sequestration in the arctiines. Remarkably, a convergent scenario involving gene duplication leading to a PA N-oxygenating FMO was found in the pyrgomorphid grasshopper *Zonocerus variegatus* (105). Nevertheless, there are additional

mechanisms in PA-sequestering leaf beetles (*Oreina cacaliae*) that can prevent the activation of alkaloid N-oxides in the gut and efficiently absorb them into the body (38). Moreover, ingested tertiary alkaloids are detoxified by O-glucosylation (38). The great variation of biochemical mechanisms involved in the PA-sequestration syndrome indicates high evolutionary plasticity in this system.

Overall, sequestration of compounds from different chemical classes selected for highly predictable adaptations on the one hand, but for a variety of resistance mechanisms on the other, even within the same class of sequestered compounds. A more detailed understanding of resistance mechanisms on a comparative phylogenetic scale will be needed to obtain a general framework of the evolution of sequestration.

SEQUESTRATION AND INSECT-PLANT COEVOLUTION

From the plants' perspective, sequestration of plant toxins by specialist herbivores represents a backfiring of plant defenses, potentially increasing the insects' fitness if the toxins are sequestered as antipredator defenses. Consequently, sequestering insects should exert selection on plants to reduce defenses available for sequestration, as predicted by the defense de-escalation hypothesis (54, 60). Evidence for this hypothesis was recently found in the dogbane family (Apocynaceae), whose species are frequently attacked by milkweed butterfly caterpillars and represent the ancestral milkweed butterfly host plant family (54). Although the use of sequestered PAs as defenses and the use of PA derivatives as courtship pheromones may be ubiquitous throughout the Danaidae sensu lato (milkweed butterflies and clearwing butterflies), few of their apocynaceous host plants actually produce PAs. Instead of larval transfer of sequestered PAs from apocynaceous hosts to the adult stage, which is observed in only a few species, most Danaidae obtain PAs via adult pharmacophagy from distantly related plant families (12). It has been proposed that strong selection by PA-philic Danaini led to the reduction of PA production in many Apocynaceae and that pharmacophagy in adult danaines is a coevolutionary response to this loss of PAs (28). A macroevolutionary study found evidence for multiple losses of PA production in the so-called APSA clade of Apocynaceae, whose members comprise 98% of all known danaine host plants (54). While homospermidine synthase (HSS; a key enzyme for PA biosynthesis) evolved at the base of this clade, PAs are reported in only 4 out of the 16 lineages. Consistent with these findings, there have been four independent losses of an important HSS amino acid motif and one example of a nonfunctional HSS pseudogene that acquired a frameshift mutation resulting in a premature stop codon in the second exon. Based on these analyses, it was concluded that losses of PA production in the APSA clade occurred independently at least five times, supporting the hypothesis of defense de-escalation driven by milkweed butterflies (54).

There is accumulating evidence that sequestration of plant toxins for defense is an important driver of insect-plant associations and that dietary specialization and sequestration are evolutionarily linked (76). Among 70 tropical caterpillar species, dietary specialists were better protected against ants compared to generalists, implicating predators as an important evolutionary force selecting for host plant specialization (27). In tests using caterpillar and plant extractions, caterpillar unpalatability to predators could be attributed to the chemical composition of caterpillars, but there was no correlation between the antipredatory effect of the caterpillars and their host plant chemistry, suggesting that the caterpillars' defenses were not exclusively explained by plant chemistry. Nevertheless, palatability of plant extracts predicted protection of caterpillars from predators, indicating a direct effect of plant chemistry on caterpillar palatability (27). In consequence, sequestration could be one potential mechanism for the observed antipredator protection mediated by plant chemistry, and better protection in specialists than in generalists could indicate a higher prevalence of sequestration among specialized species.

That specialists sequester more efficiently compared to generalists has been shown in several systems (51, 53, 114). Caterpillars of the specialist buckeye butterfly (*Junonia coenia*, Nymphalidae) sequestered much higher concentrations of iridoid glycosides compared to the generalist arctiines *Estigmene acrea* and *Spilosoma congrua* (51). Similarly, the more specialized sawfly *Athalia liberta* (Tenthredinidae) sequestered higher amounts and structurally more diverse glucosinolates compared to its congener *A. rosae*, although the pattern of concentrations in the brassicaceous host plants was inverse (68). Along a gradient of dietary specialization, sequestration of cardenolides among four aphid species feeding on common milkweed (*Asclepias syriaca*) was lowest in the generalist *Myzus persicae*, higher in the more specialized species *Aphis asclepiadis* and *Aphis nerii*, and highest in the monophagous *Myzocallis asclepiadis* (114). Caterpillars of the generalist nymphalid butterfly *Vanessa cardui*, however, sequestered higher amounts of iridoid glycosides compared to *Anartia jatrophae*, which has a more restricted dietary spectrum, indicating that there is not always a clear correlation between the degree of dietary specialization and the concentrations of sequestered compounds (53).

Nevertheless, the pattern generally holds in phylogenetically controlled comparisons. Specialized species of *Heliconius* butterflies sequestered sevenfold higher amounts of simple monoglycoside cyclopentenyl cyanogens compared to generalists within the same clade (32). Similar results were found across eight *Heliconius* species caught in the wild, i.e., cyanide-based defenses were positively correlated with host plant specialization (4). This study, however, did not differentiate between sequestration and endogenous synthesis of cyanide-based defenses, both of which occur in *Heliconius* species (32). The same trend was found across 19 heliconiine species involving *Passiflora* generalists and specialists (18).

Within heliconiine butterflies, there are three categories regarding the sources of defensive cyanogenic glycosides: (a) endogenous biosynthesis of linamarin and lotaustralin, (b) sequestration of cyclopentenyl cyanogenic glycosides, and (c) both (18, 32). Interestingly, the highest concentrations of cyanogenic glycosides were found in species that either biosynthesized or sequestered only (18). Moreover, in species that both sequester and biosynthesize, sequestering species tended to have lower amounts of the biosynthesized cyanogenic glycosides linamarin and lotaustralin (18), suggesting a potential trade-off between the two strategies. Remarkably, within the sara-sapho *Heliconius* clade comprising some of the species sequestering the highest amounts of cyanide-based defenses, the ability to synthesize cyanogenic glucosides has been lost in several species (18). Future studies should focus on additional phylogenetic comparisons involving different insect taxa and classes of sequestered compounds to test whether there is a general link between dietary specialization and the extent of sequestration.

Sequestration and specialization may be linked not only at the level of species, but also within the ontogeny of individual species. For example, during their development, the spotted lanternfly (*Lycorma delicatula*) first forages on a wide range of host plants but finally narrows its dietary spectrum to its primary host, the tree-of-heaven (*Ailanthus altissima*, Simaroubaceae), from which the lanternflies sequester quassinoids such as ailanthone (91). Remarkably, this narrowing in diet is synchronized with a shift from black with white spots to red aposematic coloration. However, this color change also occurs independently of the diet. Importantly, lanternflies become unpalatable to birds only after switching to the tree-of-heaven, indicating that they only obtain sequestered defenses from their primary host, i.e., after they change from a generalist to a specialist dietary strategy (91). A similar mechanism may be found in many milkweed bug species, whose larval development is restricted to selected plant species that provide toxins for sequestration while the resulting adults forage broadly across many plant species (46, 78). In conclusion, although there are sequestering generalists, it seems likely that there is an intrinsic trade-off between generalist feeding behavior and sequestration.

One hypothesis of how generalist insects might tolerate a great diversity of structurally unrelated plant toxins is based on promiscuous mechanisms that could be mediated by degrading enzymes or active efflux barriers, both of which have broad substrate specificities (19, 24). In contrast, it was found that the generalist noctuid *Trichoplusia ni* responds to different plant defenses in specific ways, indicating that the mechanisms used by generalist insects to cope with a wide variety of plant defenses may be multifaceted and complex (40).

Although sequestration may be frequently associated with dietary specialization, several mechanisms have been proposed for how specialized sequestering lineages maintain the potential to carry out host shifts, implying that specialization and sequestration are not evolutionary dead-ends. Chrysomelina leaf beetles that originally produced autogenous monoterpene alkaloids and then specialized to sequester salicin have escaped specialization to Salicaceae by adopting a mixed defense strategy. Specifically, this clade of beetles (the *interrupta* group) esterify de novo–synthesized butyric acid with plant-derived alcohols, allowing them to expand their host plant range (100a). A similar mechanism exists in tropical leaf beetles of the genus *Platyphora* (71), which produce pentacyclic triterpene saponins from ubiquitous amyryns sequestered from several host plant families. However, some species evolved dual chemical sequestration and additionally sequester lycopsamine-type PAs, allowing host shifts along phytochemical bridges (i.e., to distantly related plants producing PAs convergently). Importantly, sequestration of PAs has only one evolutionary origin, emphasizing the indispensability of adaptations specific to sequestered plant toxins (101).

Recent studies suggest that host plant use in herbivorous insects is more adequately explained by similar host plant chemistry (host defense chase) than by a coevolutionary arms race, as would be indicated by a correlation of herbivore and host plant phylogenies (31). Interestingly, this scenario of resource tracking most likely relates to the use of plants not only as a dietary resource but also (and in some cases even more so) for the acquisition of plant-derived defenses. Nymphalid species in the tribe Melitaeini use 16 host plant families, 12 of which produce iridoid glycosides. It was found that the occurrence of iridoid glycosides was a more conservative trait than host plant taxonomy in the evolutionary history of host plant use in this group of butterflies (104). Because several species of the Melitaeini are known to sequester iridoid glycosides for defense, sequestration of these compounds could be a synapomorphic trait of this clade. Congruent with this hypothesis, the ancestral host plant of the Melitaeini was probably an iridoid glycoside–containing Plantaginaceae (104).

Similarly, milkweed bugs (Heteroptera, Lygaeinae) are predominantly associated with Apocynaceae, and cardiac glycoside–resistant Na^+/K^+ -ATPases and the ability to sequester these toxins are traits that most likely evolved at the base of this group (13). It was shown recently that the three milkweed bug species *Horvathiolus superbus*, *Lygaeus equestris*, and *Spilostethus pandurus* colonized plants in four botanical families that are phylogenetically disparate from the Apocynaceae but produce cardiac glycosides convergently (78) (**Figure 1; Supplemental Table 1**). Furthermore, all three species sequestered cardiac glycosides from these evolutionarily novel sources. Remarkably, another species, *Spilostethus saxatilis*, sequesters high amounts of colchicine and related colchicum alkaloids from meadow saffron (*Colchicum autumnale*). Sequestration of either cardiac glycosides or colchicum alkaloids mediated protection against insectivorous birds in the three species tested and additionally protected two out of four tested species against lacewing larvae (78). Several milkweed bug species are dietary generalists, and growth and development of *H. superbus*, *L. equestris*, and *S. saxatilis* was not dependent on the availability of toxin-containing seeds. In fact, growth on only toxin-containing seeds was inferior to growth on seed mixtures or pure sunflower seeds in some cases. Therefore, it is likely that specialized plant associations in this group were evolutionarily driven by the acquisition of defenses, and not by the use of novel dietary resources. Shifts to these plants in this case were probably facilitated by a pre-existing sequestration syndrome,

Supplemental Material >

such as the ability to sequester and tolerate cardiac glycosides. Remarkably, *S. saxatilis* evolved a novel resistance trait against colchicine but still possesses resistance against cardiac glycosides. This demonstrates that even highly specialized sequestering insects may still be able to switch to novel defenses with a completely different mode of action, probably facilitated by pre-existing traits of the sequestration syndrome, such as transport mechanisms that work for both cardiac glycosides and colchicum alkaloids.

The polyphagous arctiine caterpillars *G. geneura* and *E. acraea* have been classified as specialized generalists because they use specific plants to gain protection against parasitoids and are physiologically specialized to specific classes of compounds (89). This concept is very much in line with Petschenka et al.'s (78) findings on milkweed bugs that use particular plants to sequester defenses during phases of their life cycle but will forage broadly at different times. Consequently, these species could be classified as temporal specialists similar to the spotted lanternfly (91). Testing of 30 museum specimens of *S. saxatilis* from 21 locations across Europe and North Africa revealed colchicum alkaloids in each individual, suggesting that *S. saxatilis* is obligately associated with *C. autumnale* (the only natural European source of colchicine), although it is known to feed on seeds of >40 species from >15 botanical families (78). As outlined above, even dietary generalists can evolve adaptations specific to certain plant compounds, emphasizing that sequestration requires resistance traits different than those involved in feeding and that predators and parasitoids (i.e., the third trophic level) influence the coevolutionary arms race between insects and plants by selecting for these traits. Notably, sequestration does not protect herbivorous insects equally well against all kinds of natural enemies, and many sequestering lepidopterans experience high rates of parasitization (36, 52, 82). It has thus been proposed that sequestration evolved in response to selection pressure by generalist predators, which have also been shown to adapt to sequestered plant defenses in their insect prey (16, 112). The effects of sequestered plant toxins on predators, parasitoids, protozoans, and microorganisms certainly represent an important area of research that should be studied more broadly to provide a deeper understanding of the evolutionary forces selecting for sequestration.

CONCLUSIONS AND PERSPECTIVES

Sequestration is a complex and widespread insect defense strategy that influences interactions across multiple trophic levels and has most likely played a key role in the evolution of many insect–plant associations. Sequestration of the same types of plant compounds has repeatedly evolved in distinct insect lineages, providing opportunities to decipher commonalities and lineage-specific differences among sequestration syndromes, for example, with regard to molecular transport, resistance mechanisms, and ecological functions. Phylogenetically controlled comparative studies have revealed multiple paths leading to sequestration in insects, including (a) shifting from de novo synthesis to sequestering distinct defense compounds available in the food plant (11, 47) and (b) de novo synthesis in combination with sequestration of the same or structurally similar defense compounds (18, 34, 35). It is assumed that the metabolic cost of sequestration is lower than that of de novo synthesis, which could be one explanation for the prevalence of sequestration in at least some insect groups, for which sequestration could possibly represent an evolutionary key innovation. However, to date, very few studies have addressed this point experimentally (110, 115), most likely because such experiments require a comprehensive knowledge of the phylogenetic background and the natural history of the study system, which is often lacking. Sequestration research can be further advanced by studies of the molecular and physiological mechanisms involved to trace the evolutionary origin(s) of sequestration in a comparative phylogenetic framework. Moreover, with gene editing technology now available for nonmodel organisms, it will be possible to

manipulate the ability of insects to accumulate defense compounds to assess the costs and benefits associated with this syndrome of adaptations.

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