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How herbivores coopt plant defenses: natural selection, specialization, and sequestration

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We review progress in understanding sequestration by herbivorous insects, the use of plant chemical defenses for their own defense. We incorporate sequestration into the framework of plant–insect coevolution by integrating three hierarchical issues: (1) the relationship between dietary specialization and sequestration of plant defenses, (2) the physiological mechanisms involved in sequestration, and (3) how sequestration evolves via interactions between trophic levels. Sequestration is often associated with specialization, but even specialized sequestration is not an evolutionary dead-end. Despite considerable progress in understanding physiological mechanisms, detailed knowledge of how plant toxins cross the insect gut epithelium is still largely lacking. Sequestration is likely a major vehicle for coevolutionary escalation in speciose plant–insect–predator interactions, suggesting that a strictly bitrophic view is untenable.

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Introduction

Sequestration is a common phenomenon among herbivorous insects [1] and is defined as 'the selective uptake, transport, modification, storage and deployment of plant secondary chemicals for the insect's own defence' [2•]. While not all of these criteria are necessary for sequestration, the uptake of toxins typically connects the first trophic level (plants) via the second trophic level (insect herbivores) to the third trophic level (predators and parasitoids) and as such is an important force shaping ecological networks and evolutionary trajectories. From the perspective of such food chain interactions, the processes by which any chemical compound is acquired and used by an organism are essential to understand.

All heterotrophic organisms are exposed to a wealth of chemical compounds in their diet and selective uptake and incorporation of these compounds is vital. For example, nutrients are critically needed, while uptake of detrimental compounds like toxins should be avoided. This selectivity has natural limitations, as uptake of nutrients involves physical and biological mechanisms which are not absolute in their specificity [3]. Amino acid transporters, for instance, may also transport toxic non-protein amino acids [4,5] and the permeability of the gut epithelium for essential lipophilic compounds will also allow for diffusion of toxic organic molecules (Figure 1). In the context of antagonistic insect–plant coevolution, limits on the specificity of uptake have likely been the target of natural selection, both from the plants' and insects' perspective.

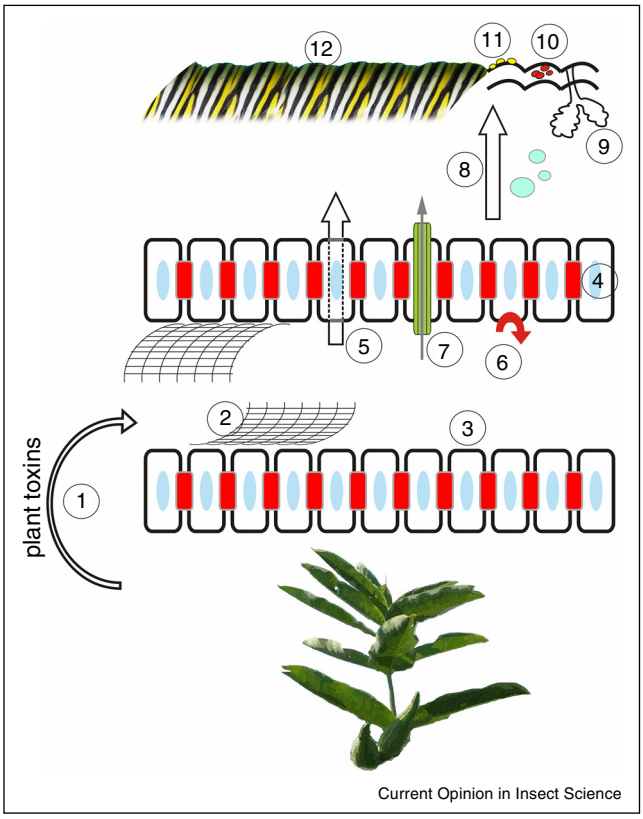
Selectivity of uptake can be realized by one or by a combination of many mechanisms. After ingestion, which itself can be selective based on gustatory discrimination, uptake of a compound across the gut epithelium can be prevented by either passive or active barrier mechanisms (e.g. diffusion barriers mediated by septate junctions or ATP-consuming efflux carriers like p-glycoproteins, see Figure 1) [6,7]. Moreover, a compound can be degraded in the midgut or in the body cavity and subsequently excreted via Malpighian tubules and defecation [2•]. Absorption of a compound into the haemocoel can be passive (e.g. by diffusion of lipophilic compounds) or could be mediated by active carriers. The interplay of these physical and biological parameters will determine how much of a compound will enter the body cavity and how much of it will be discarded. Accordingly, the extent of selectivity via various mechanisms provides the physiological template for sequestration.

Despite its base at the plant-herbivore interface, at its heart sequestration must be considered in a tri-trophic context, both in terms of ecological outcomes as well as evolutionary drivers of species interactions. Thus, in this paper we integrate a trophic perspective in the framework of sequestration and review recent progress in understanding (a) how sequestration relates to dietary specialization, (b) the physiological mechanisms of uptake and storage of plant toxins, and (c) how sequestration evolves in a tri-trophic context.

Sequestration and dietary specialization

Just like dietary specialization, sequestration also requires resistance traits specific to sequestered plant toxins, and

Figure 1



Physiological mechanisms involved in sequestration. Upon feeding plant toxins are ingested (1) and contact the peritrophic envelope (2). The peritrophic envelope may be involved in the process of sequestration as plant toxins could bind to the envelope and toxins could be retained in the gut. It thus will prevent some toxins from reaching the gut epithelium and prohibit sequestration. The gut epithelium (3) represents the next layer of selectivity and sequestration may be modulated by active and passive barrier functions as well as metabolism by degrading enzymes and selective uptake. Polar toxins cannot be sequestered passively as paracellular diffusion across the midgut epithelium is restricted by septate junctions (4). Non-polar (lipophilic) compounds can be sequestered passively as they can cross cell membranes (5). Proteins like efflux carriers (6) mediate an active barrier to prevent toxins from reaching the body cavity [6]. One possibility for how polar toxins can be sequestered is via specific carrier proteins (7) [16,21]. The involvement of carriers in sequestration of plant compounds from the gut lumen into the haemocoel has been suggested but has not been functionally demonstrated at the level of individual carriers. Within the haemolymph, sequestered plant metabolites can be metabolized [2]; potential binding to haemolymph proteins or sequestration into haemocytes has not been investigated to date. Some toxins are transported across the haemolymph (8) into glands (9) [20••], reservoirs [25] or into the integument [8•] where they are stored (10) or exposed to predators (e.g. in the form of droplets, (11) [26]). Protection mediated by sequestered plant toxins is often displayed by aposematic coloration (12). Although advertisement of protection/unpalatability can be signaled via other modalities, aposematic coloration points to the importance of visually oriented predators (e.g. vertebrates) as evolutionary drivers for sequestration.

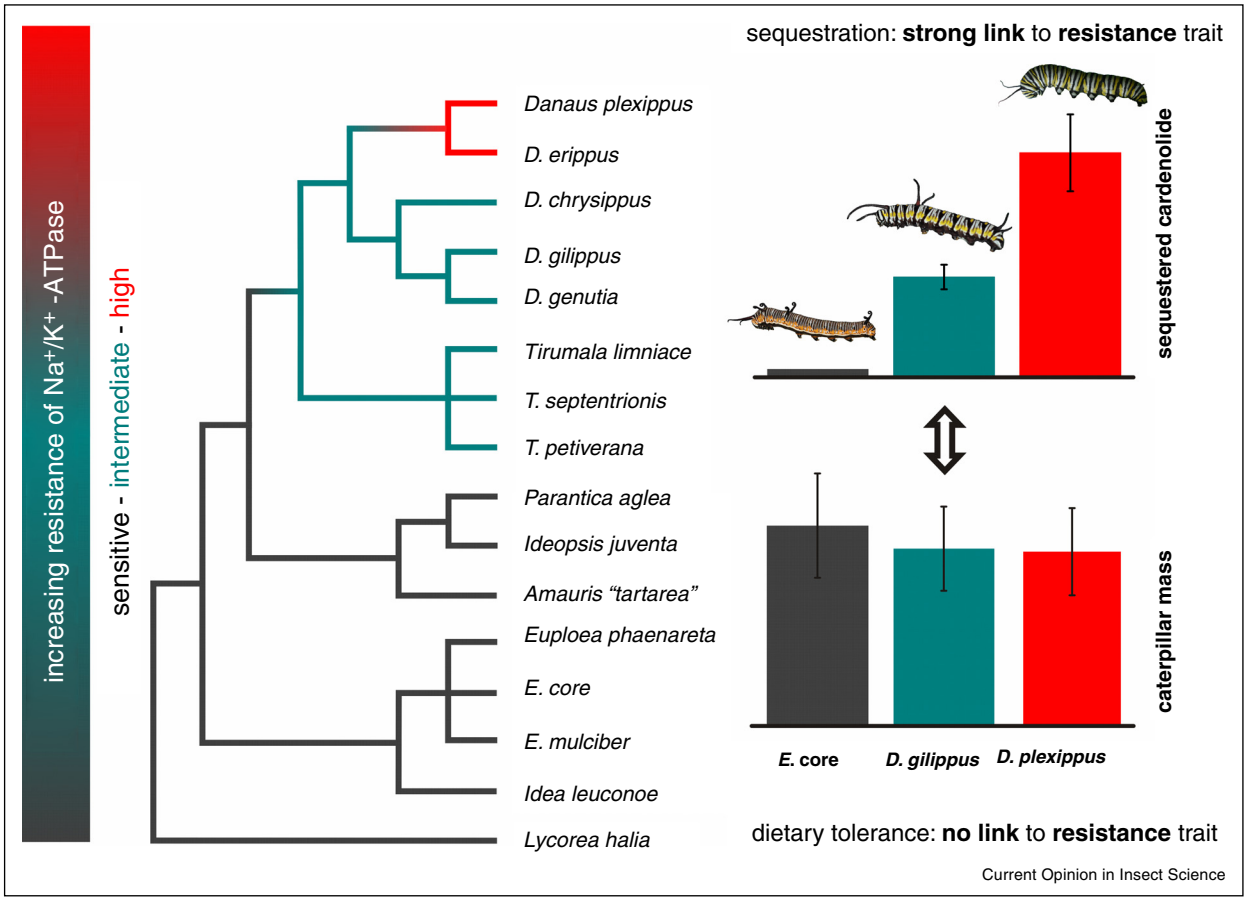
recent work has shown that these traits can differ from those primarily involved in eating toxic plants [8•]. Beyond simple consumption, sequestration requires adaptations for the transport, metabolism, and storage of the toxins, and often to advertise and deliver the same compounds (Figure 1). In contrast to overcoming a plant toxin simply to use a dietary resource, which could be driven by pairwise coevolution, sequestration is typically driven by predators and parasites (the third trophic level). Thus, sequestration provides a link for how higher trophic levels can engage in coevolutionary interactions (Figure 2, [8•,9]) and can influence host plant associations. As it is increasingly recognized that sequestration is a common phenomenon, it may well be a second vehicle of coevolutionary escalation between plants and insects causing resistance adaptations necessary to tolerate sequestered toxins.

A bitrophic coevolutionary view of species interactions suggests that specialization is a consequence of escalating evolutionary antagonism. Although it is unclear whether sequestration follows specialization or vice versa, the two traits are often coupled. In feeding trials involving a comparison of 70 tropical caterpillar species, Dyer [10] showed that specialist caterpillars are less palatable to ant predators compared to generalist caterpillars. On the basis of these results it was suggested that predation could be a substantial selective force for the evolution of specialized feeding behavior and sequestration. Indeed, ant choices were mediated by the chemical composition of caterpillars, which was clearly derived from host plant chemistry.

More focused experimental evidence on single classes of compounds also indicates a relationship between sequestration and the degree of dietary specialization. For example, Lampert and Bowers [11] and Lampert *et al.* [12] compared sequestration of iridoid glycosides between the specialist Buckeye caterpillar *Junonia coenia* and several more generalized feeders; the specialist sequestered dramatically more iridoid glycosides compared to the other species. The same pattern seems to hold for phloem sucking aphids. Züst and Agrawal [13•] recently demonstrated that among a gradient of dietary specialization comprising four aphid species on the milkweed host *Asclepias syriaca*, sequestration of cardenolides increased from the generalist *Myzus persicae* to the more specialized *Aphis asclepiadis* and *A. nerii*, and was highest in the monophagous *Myzocallis asclepiadis*.

The hypothesis that specialists sequester more efficiently than generalists also holds true in phylogenetically controlled comparisons. Engler-Chaouat and Gilbert [14] showed that several specialized species of *Heliconius* butterflies in the same clade sequester higher concentrations of simple monoglycoside cyclopentenyl cyanogens from their host plants (*Passiflora* spp.) as compared to *Passiflora* generalists fed the same plant species. These results suggest that herbivores with narrow diet spectra are more likely to sequester (or sequester more efficiently) as compared to those species which are less restricted in their host plant use.

Figure 2



Cardenolide resistance of Na^+/K^+-ATPase increased during the radiation of milkweed butterflies (three discrete levels, see [37], left panel). Sequestration of cardenolides in this clade is associated with increasing resistance of Na^+/K^+-ATPase (right panel, top graph). Caterpillars of *Euploea core* (gray bar), *Danaus gilippus* (blue bar), and *D. plexippus* (red bar) all have different Na^+/K^+-ATPases (*E. core* = sensitive, *D. gilippus* = intermediate resistance, *D. plexippus* = highly resistant). The level of sequestered cardenolides is mirrored by these discrete levels of resistance (*E. core* = none, *D. gilippus* = intermediate levels, *D. plexippus* = highest levels). In contrast, cardenolide resistance of Na^+/K^+-ATPase is not needed to tolerate cardenolides in the diet (right panel, bottom). Across eight species of milkweed comprising several species with high levels of cardenolides, caterpillar growth did not differ between caterpillar species and does not parallel the level of cardenolide resistance [8•]. Thus the third trophic level likely was the driver of the evolution of altered Na^+/K^+-ATPases in the milkweed butterflies.

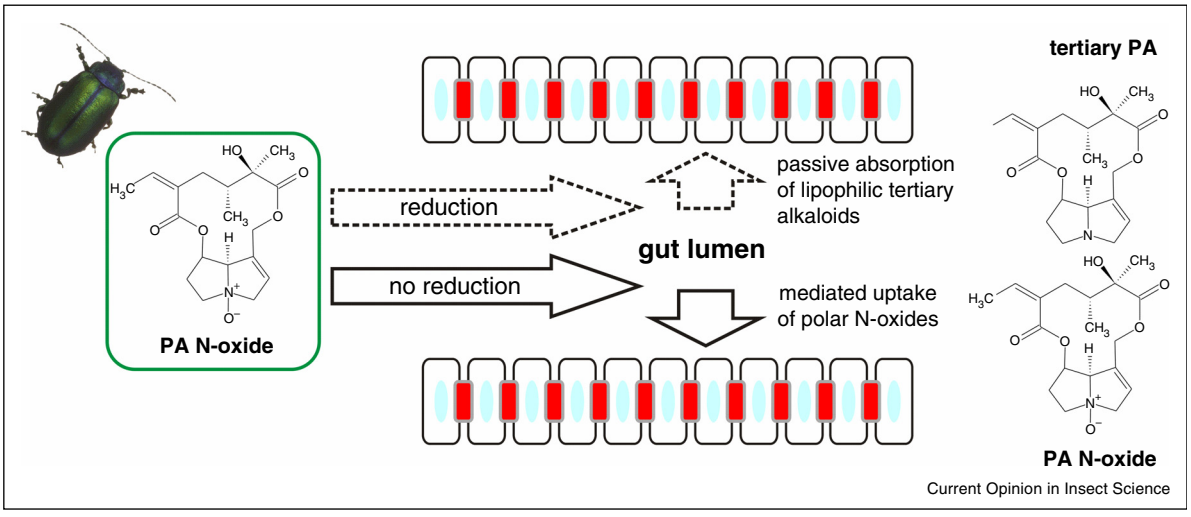
Importantly, this pattern could indicate that (1) sequestration is a selective force preventing herbivores from host shifts (as is the case for *Heliconius* [14]) or (2) specialized feeding behavior is the basis for the evolution of sequestration. Phylogenetic studies like the above mentioned are needed to distinguish these hypotheses. In another of the few such studies, sequestration appeared to be basal in the milkweed bugs (Heteroptera, Lygaeinae) [15] which then radiated on (or co-radiated with) plant species in the Apocynaceae sharing similar chemistry (and also on non-related host plants). A more detailed understanding of the physiological mechanisms involved in sequestration could explain why sequestration may lead to dietary specialization in some cases while it does not in others (e.g., passive mechanisms could be more promiscuous and carrier mechanisms more specialized).

Mechanisms of sequestration

Our knowledge of the physiological mechanisms underlying sequestration across the insect gut is currently lacking, and this critical knowledge will facilitate both our understanding of constraints on adaptation as well as the consistency of mechanisms involved in plant–insect coevolution. Remarkably, very few mechanisms of how plant toxins are transported from the gut into an insect's body have been definitively studied. Relatively detailed knowledge to date comes from insects sequestering pyrrolizidine alkaloids (PAs, Figure 3), where it was shown that sequestration of this one class of plant toxins involves different mechanisms (passive absorption versus carrier mediated transport).

Carrier mediated uptake across the gut epithelium has been proposed in additional systems [16–19] but the

Figure 3



Proposed mode for sequestration of pyrrolizidine alkaloids in insects. In plants, pyrrolizidine alkaloids (PA) are mostly produced and stored as N-oxides (green box) which are non-toxic. In the digestive tract of herbivores, N-oxides are typically reduced to (pro-toxic) tertiary lipophilic alkaloids which are passively absorbed into the body cavity by diffusion. Toxicity for vertebrates and insects is based on bioactivation of these tertiary alkaloids by cytochrome P450 enzymes [56]. Several insects have been shown to possess Flavin-dependent monooxygenases in their haemolymph which convert pro-toxic tertiary PAs back into non-toxic N-oxides [57]. As tertiary alkaloids are lipophilic, sequestration (i.e. uptake) of these toxins in *Creatonotos transiens* (Lepidoptera, Arctiidae) and *Zonocerus variegatus* (Orthoptera, Pyrgomorphidae) was assumed to occur passively (dashed arrows), representing the simplest way to realize sequestration of a plant toxin [58]. Passive uptake of a toxin, however, could still represent an adaptation if mechanisms which typically prevent uptake (e.g. active barrier mechanism) need to be down-regulated (see [6]). In the leaf beetle species *Longitarsus jacobeae*, *Oreina cacaliae* (top left), and *O. speciosissima* it has been conclusively shown that sequestered PA-N-oxides are absorbed intact (solid arrows) without being reduced to lipophilic tertiary alkaloids. As N-oxides are polar, it is likely that sequestration is carrier-mediated and cannot occur via simple diffusion across lipophilic cell membranes [59], which probably applies to other polar toxins as well. This case study of pyrrolizidine alkaloids demonstrates that different physiological mechanisms can be involved in the sequestration of the same class of compounds.

nature of such carriers has not yet been determined. The first candidate carrier involved in the process of sequestration (here transport of toxins into a gland) was recently elucidated by Strauss *et al.* [20••]. It was shown that in the leaf beetle *Chrysomela populi*, an ABC (ATP-binding cassette)-transporter mediates transport of the sequestered phenolglucoside salicin to the reservoir of a defensive gland where salicin is subsequently enzymatically converted into salicylaldehyde. ABC transporters have also been suggested to be involved in the transport of plant metabolites from the gut lumen into the haemocoel [21]. A member of the cytochrome P450s which is expressed in the midgut of *Manduca sexta* was suggested to be part of a multicomponent pump transporting nicotine into the larval haemocoel. It was hypothesized that MsCYP6B46 converts nicotine into a short-lived metabolite which in turn is shuttled into the haemolymph by a carrier mechanism and reconverted to nicotine [22••].

Using matrix-assisted laser desorption/ionization (MALDI) mass spectrometry imaging, uptake of glucosinolates in the midgut of the sawfly *Athalia rosae* has been shown to be a very rapid process which is likely an adaptation to prevent hydrolysis of glucosinolates in the midgut [23•] a mechanism which has also been proposed for iridoid glycosides [24]. In contrast, milkweed butterfly species of the genus *Danaus* apparently accumulate cardenolides in the midgut lumen while the non-sequestering species *E. core* may degrade the dietary toxins. Different ecological strategies (i.e. sequestering versus non-sequestering) and selectivity thus may start in the lumen of the midgut [8•] and might be mediated by physical mechanisms. To unravel the transport mechanisms underlying sequestration it will be necessary to integrate detailed insect physiology, including cell and tissue-based approaches, and molecular biology with variations in insect diet.

Upon reaching the haemocoel (see above), mechanisms are needed to transport sequestered toxins to their final destinations. Ways of advertisement can be different, as toxins can, for example, be released from glands (e.g. leaf beetles, [20••]), cuticular cavities (e.g. burnet moths [25]), or from special body spaces via segmental orifices (e.g. Lygaeinae [26]). Ejecting sequestered toxins upon predator threat could protect the individual insect from being eaten. In contrast, mere incorporation of toxins in the body (i.e., how monarch caterpillars sequester cardenolides) may not save the individual from being killed. This distinction probably has evolutionary implications for mimicry, individual selection, and kin selection. Besides

the advantage of direct display of toxicity to predators, toxin-ejecting structures also allow for compartmentalization which could be an important mode of resistance to reduce internal exposure to toxins [26,27]. In addition to spatial containment, the principal of chemical compartmentalization is also realized in the form of activated defenses, for example the glucosinolate–myrosinase system in aphids [28,29] and leaf beetles [30]. Here nontoxic glucosinolates are separated from myrosinases which together cause the release of toxic isothiocyanates. In addition to the more common ways of presenting sequestered toxins mentioned above, it has been shown recently that nicotine sequestered by caterpillars of *Manduca sexta* is 'exhaled' through larval spiracles to deter predacious wolf spiders [22••].

Although it seems clear that even for highly specialized herbivores there are likely some active (energy-consuming) and some passive (occurring by diffusion) mechanisms of sequestration, we currently lack a framework for predicting when such mechanisms are employed. Moreover, currently underexplored mechanisms (e.g. transcytosis, [31]) or physiological compartments (e.g. the peritrophic envelope, see [32]) may be involved in sequestration. For generalists, passive sequestration may be common, but we currently do not know whether such low level sequestration is effective against predators or if other agents of natural selection are acting on the interaction. Knowing the order of when specialization evolved and the mechanisms of sequestration will help resolve the factors that facilitated the evolution of specialization and its macroevolutionary consequences.

Evolution of sequestration

Theory has long predicted that specialized species will have few opportunities for speciation, in part due to narrow diet breadth. Although this idea, which dates back to the late 1800s proposed by Edward Drinker Cope, is intuitive, recent work has suggested that this may not be a general pattern [33]. Given the coupling of specialization and sequestration, it is reasonable to ask if sequestration is an evolutionary dead end, both in terms of opportunities for host shifts and for the uptake and storage of diverse plant toxins.

In the leaf beetle subtribe Chrysomelina, sequestration of plant compounds (salicin for the production of defensive salicylaldehyde) evolved two times independently from an ancestral self-producer [34]. Nonetheless, later diverging species evolved mixed defense strategies and host shifts. Also in the Chrysomelina, it was recently shown that β-glucosidases used to activate sequestered pretoxins show broad substrate selectivity, suggesting compatibility with different plant toxins and thus the physiological capability for host shifts [35]. In the milkweed bugs (Lygaeinae) sequestration of cardenolides is often associated with using cardenolide-producing host plants in the family Apocynaceae, but some species shifted to cardenolide-producing plants in unrelated families (e.g. *Lygaeus equestris* on the Ranunculaceae and *Horvathiolus superbus* on the Plantaginaceae) indicating that sequestration ability, or the traits associated with it, can drive host shifts [15]. In the same group, some species additionally switched to cardenolide-free hosts. Thus, in general, there are at least three reasons that specialization and sequestration are not an evolutionary dead end: sequestration can be lost, host shifts can be made on to distantly related plants with the same plant chemistry, or sequestration physiology may be promiscuous enough to coopt alternative defenses on unrelated plants.

Termonia *et al.* [36] suggested that switching from one group of host plants providing sequestered phytotoxins to new host plants producing different toxins for defense may be facilitated by a 'dual-defence system'. Tropical leaf beetles in the genus *Platyphora* for example are all defended by saponins which they produce from sequestered plant precursors (amyrins). One clade within *Platyphora* then evolved sequestration of lycopsamine-type pyrrolizidine alkaloids, while sequestration of saponins was retained in many species. We have recently shown that sequestration of cardenolides across the milkweed butterflies (Danaini) probably evolved in a stepwise-fashion [8•] and is associated with the three discrete levels of cardenolide resistance of Na^+K^+-ATPase which increased during the radiation of milkweed butterflies [37] (Figure 2).

While the physiological costs associated with sequestration were assumed to be lower than the production costs of autogenous defensive compounds [34], the picture emerging from the literature is equivocal. In *Heliconius*, sequestration of simple monoglycoside cyclopentenyl cyanogens is negatively correlated to the autogenous production of aliphatic cyanogens [14]. In milkweed bugs (Lygaeinae), the metathoracic gland is reduced and it has been suggested that sequestration superseded the defensive role of this organ. In addition, reduced selection pressure on methathoracic gland secretions here allowed this gland to secondarily evolve a sexual function [38]. Thus both traits seem to trade off, suggesting general costs with lower costs of sequestration. Nevertheless, sequestration does seem to produce some physiological burden on its own, probably due to autotoxicity of sequestered compounds [39–41]. In the lygaeine species *Arocatus longiceps* and *A. melanocephalus*, the ability to sequester was lost after host shifts to plants devoid of cardenolides, again suggesting a cost of sequestration [15]. On the level of the expression of enzymes involved in processing sequestered plant compounds, cost may [42] or may not be present [43].

Sequestration of plant toxins was shown to have an adverse effect on predators for the first time by Brower

et al. [44], who fed cardenolide sequestering monarch butterflies to blue jays. Since then the ecological benefit of sequestration for protection from predators and parasitoids and even self-medication by sequestered plant compounds was tested in multiple systems [45–48]. Clearly, predators, parasitoids or other antagonists like bacteria, fungi, and viruses must represent the selective forces for maintaining and increasing sequestration. Interestingly, several studies show that sequestered toxins which are known to deter predators are inefficient against parasitoids. Smilanich *et al.* [47] have demonstrated that increasing concentrations of sequestered iridoid glycosides reduced melanin encapsulation (a mechanism to fight parasitoids) of implanted silica beads in caterpillars of *Junonia coenia* (Lepidoptera, Nymphalidae) (see also [49]). On the basis of these findings it was suggested that sequestration trades off with immunity against parasitoids, a novel hypothesis for tradeoffs among resistance to different types of enemies.

In a broad comparison, Gentry and Dyer [50] suggested that caterpillars which are well defended against predators are used heavily by parasitoids as enemy-free space. Besides other defensive traits, sequestered caterpillar chemistry was associated with high rates of parasitation, suggesting that well defended hosts are advantageous for a parasite as they are less likely to be eaten together with the host by a predator. Thus, vertebrate predators may be the more important selective force for the evolution of sequestration compared to parasitoids (see also [51–53]). However, there is also contrary evidence demonstrating that sequestration protects insects from parasitoids (e.g. [48]), indicating that sequestration is not only driven by predators. Additionally, defensive chemicals increased the life span of monarch butterflies infected by the sporozoan parasite *Ophryopcystis elektroscirrha* [54] and sequestration of chlorogenic acid in *Manduca sexta* has also been shown to help against bacterial infections [55]. Feeding on a diet supplemented with chlorogenic acid increased the number of circulating haemocytes independent of infection with bacteria. Thus, the beneficial effects of sequestration observed might not be based on direct toxicity of the compound to bacteria, but rather mediated indirectly by stimulating the caterpillars' immune system. This latter notion points to the fact that interactions between plant toxins, insect herbivores, and the third trophic level are complex, and truly encompass community ecology.

Conclusion

Sequestration in herbivorous insects is an important mediator between the first and the third trophic level. This process of taking up toxins seems to be associated with dietary specialization and thus shapes plant–insect-associations. It furthermore requires novel resistance adaptations to tolerate sequestered toxins and therefore represents a second dimension of coevolutionary escalation. To understand sequestration and its evolution, we require both a physiological perspective of herbivores, but also an understanding of species interactions at three trophic levels.

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