Ecology and Evolution of Secondary Compound Detoxification Systems in Caterpillars

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Monarch caterpillar (*Danaus plexippus*) on showy milkweed (*Asclepias speciosa*) in Oakland, CA. Photo by Noah K. Whiteman.

Introduction

Ecological specialization generates and maintains biological diversity through evolutionary divergence between populations and subsequent coexistence between species (Allio et al. 2021; Braby and Trueman 2006; Gloss et al. 2016; Wiens et al. 2015). Dietary specialization typifies the life histories of most Lepidoptera (Forister et al. 2015), nearly all species of which are herbivorous (Wagner and Hoyt, Chapter "On Being a Caterpillar: Structure, Function, Ecology, and Behavior"). This form of ecological specialization is driven by both bottom-up (host plant quality and

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defenses) and top-down (enemies) selective forces (Lawton and McNeill 1979; Bernays and Graham 1988). In either case, specialization revolves around so-called plant secondary compounds – those chemicals not typically required for primary plant growth, maintenance, and reproduction – although some clearly are used by plants as signaling molecules within defense pathways (Clay et al. 2009). Plants produce an enormous diversity of secondary chemicals, and the *raison d'être* of many of these is that they function as toxic anti-feedants (Fraenkel 1959). A paradox is that these same toxins can become co-opted by specialized arthropods, including Lepidoptera, as host-finding cues, feeding/oviposition stimulants (or anti-stimulants, in the case of compounds to which the insect is not adapted), and defensive mechanisms for the arthropods themselves. The biology of lepidopteran larvae (caterpillars) has played a central role in the development of the field of coevolution. Foundational papers on the topic, including ones by Ehrlich and Raven (1964) and Berenbaum (1983), focus on patterns of host use in caterpillars as they relate to secondary chemistry.

Dietary specialization in Lepidoptera requires the ability to mitigate the toxic effects of these secondary compounds, which we broadly define as detoxification. In this chapter, we focus on detoxification strategies deployed by specialized caterpillars for exemplar toxins at two ends of the mode of action spectrum: cardiac glycosides (CGs) and glucosinolates (GSLs). Studies of these two classes of toxins have been foundational for our understanding of plant-caterpillar interactions (Fig. 1).

One mode-of-action strategy for plant toxins is to target highly conserved essential proteins or even specific amino acid residues found in animals but not in plants. The targeting of proteins used in nervous and circulatory systems is particularly widespread. Among such toxins, the best studied are the CGs, which bind to the first extracellular loop of the sodium/potassium ATPase (Na+/K+-ATPase; Fig. 1b). CGs contain three structures: a steroid core, a 5-(cardenolides) or 6-(bufadienolides) membered lactone ring, and sugar residue(s). These toxins evolved in ca. 60 genera from 12 plant families as well as in toads (Bufonidae) and fireflies (Lampyridae; Agrawal et al. 2012). Because plant genomes do not encode a copy of the Na+/K+-ATPase, they do not suffer from its toxic effects.

The process of detoxification in all animals, not just insects, can be divided into three phases of xenobiotic metabolism: phase I is the functionalization step of detoxification characterized by oxidation, hydrolysis, and reduction reactions; phase II is the conjugation step in which lipophilic compounds are converted into more hydrophilic ones to facilitate excretion or sequestration; and in phase III excretion takes place (Amezian et al. 2021; Nakata et al. 2006). As we will discuss later, strategies to detoxify CGs that involve proteins active in these phases have evolved in several insects. However, an important alternative strategy in Danainae butterflies and other herbivores specialized on CG-producing plants involves target site insensitivity (TSI). TSI describes a biophysical phenomenon in which the toxic ligand fails to bind (or binds poorly) to the target site owing to "alteration in structure or accessibility" (Berenbaum 1986 citing Brooks 1976). Several insects have evolved to sequester CGs from their host plants in response to pressure from the

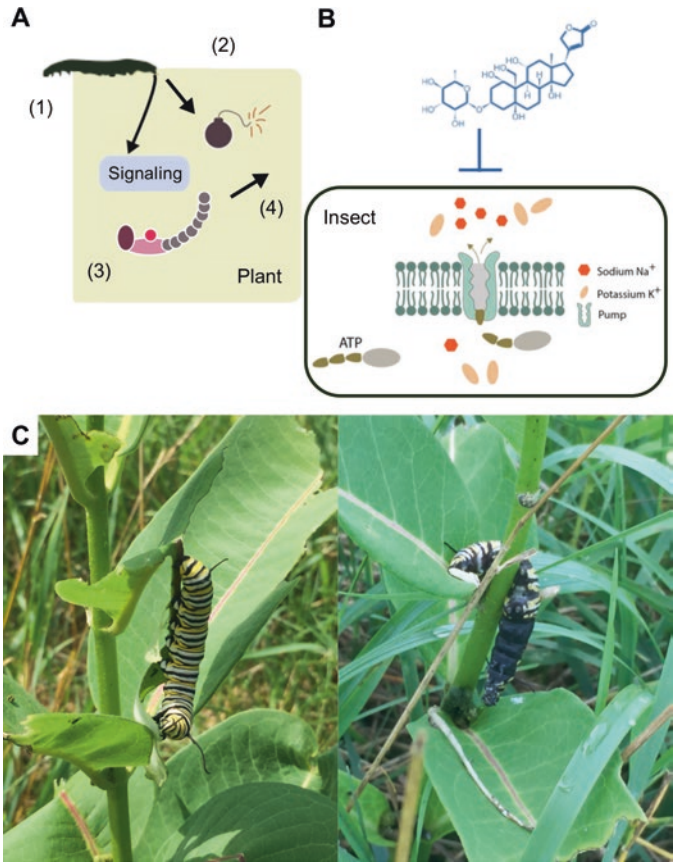

Fig. 1 (**a**) Upon attack by caterpillars (1), plants activate defense responses. In the case of Brassicaceae species, a reservoir of aliphatic glucosinolates (GSLs) is turned into toxic isothiocyanates (ITCs), activating the "mustard oil bomb" (2). In plants from all families, an intricate signaling network regulates production of heightened levels of defensive chemicals on top of a pre-made reservoir of stored chemicals. Such plant immune responses are activated after plants recognize the onset of attack through cell-surface and intracellular receptors (3). Brassicaceae species in the genus *Erysimum* produce toxic cardiac glycosides (CGs) in addition to producing GSLs (Züst et al. 2020). CGs are further produced by milkweeds and other Apocynaceae, plus species in 11 other plant families (Agrawal et al. 2012; 4). (**b**) CGs derive their toxicity from blocking activity of the caterpillars' sodium/potassium ATPases (Na+/K+-ATPases). (**c**) Caterpillars of the monarch butterfly engage in leaf vein-cutting or laticifer clipping behavior. On the left, a caterpillar cut the main mid-vein of a milkweed leaf and can now feed on a leaf with impaired defensive capabilities. On the right, a caterpillar died from exposure to CG-rich latex before it could disable this highly effective defensive barrier. (Cartoons by Simon C. Groen and Sophie Zaaijer, photos by Simon C. Groen)

third trophic level, in some cases by co-opting through gene duplication phase III drug transporters that originally evolved to remove CGs, which we will elaborate upon below.

At the other end of the spectrum, many plants produce non-toxic precursor glucoside molecules that are hydrolyzed, upon tissue damage, to toxic antiherbivore compounds by one or more β-glucosidases stored elsewhere (Fig. 1a). However, this reaction can yield toxins that are also auto-toxic to plants (Morant et al. 2008). Cyanogenic glycosides and their evolutionary derivatives, the GSLs, are well-studied examples of precursors relevant to caterpillars, as are iridoid and benzoxazinoid glucosides. GSLs are found only in plant species of the Brassicales and in the distantly related tropical tree genera *Drypetes* and *Putranjiva* (Malpighiales: Putranjivaceae; Rodman et al. 1998). As such, GSLs are used as host-finding/oviposition cues and feeding stimulants for many specialized insects. Interactions between GSLs and *Pieris* spp. gave rise to the field of chemical ecology, owing to Verschaffelt's 1910 study in which GSLs painted on non-host leaves stimulated feeding by *Pieris* spp. caterpillars, the first *bona fide* experiment showing that plant secondary compounds could be co-opted in this way. Some of the more toxic hydrolysis products of aliphatic GSLs, derived primarily from the amino acid methionine, are the isothiocyanates (ITCs), which give wasabi and other mustards their peppery and pungent taste. ITCs are general toxins that widely target nucleophilic residues such as exposed cysteine and lysine residues in proteins as well as DNA. In this case, full TSI could not evolve because the toxin is so promiscuous. Instead, a common strategy in Brassicaceae-feeding insects is to "disarm" the mustard oil bomb by preventing the formation of the ITCs through desulfation of the GSLs (e.g., in *Plutella* spp.) or diversion of hydrolysis products to nitriles (e.g., in *Pieris* spp.); this has occurred in both cases through a process of gene duplication and neofunctionalization (see references below). In generalists, or more recently derived specialists, the main route of GSL detoxification is a metabolically expensive strategy: the use of phase II detoxification enzymes (specifically glutathione *S*-transferases). Bacterial symbionts are able to hydrolyze ITCs, potentially facilitating colonization of GSL-bearing plants. Indolic GSLs, derived from the amino acid tryptophan, do not form stable ITCs, but rather are hydrolyzed into compounds that are oxidized by phase I enzymes. Thus, four of the principle means of detoxification (TSI, modification via phase I, conjugation via phase II, and excretion via phase III enzymes) can be subsumed by CGs and GSLs and will now be the subject of more detail.

We will use these two toxin classes to illustrate the different mechanisms by which caterpillars interact with toxins in general but will extend our discussion to other life stages, toxins, and plant-insect interactions to indicate potentially general mechanisms or to supplement known gaps in knowledge of how caterpillars interact with GSLs and CGs. We will start by providing an overview of functionally described proximate mechanisms of detoxification in Lepidoptera and then use this as a platform for diving into what is known about ultimate evolutionary patterns of Lepidoptera in response to their plant hosts.

Proximate Mechanisms of Detoxification

Resistance to host plant toxins evolves through different behavioral, physical, and physiological mechanisms including avoidance of toxin ingestion, reduced penetration through surface membranes such as the cuticle and gut lining, TSI, and active detoxification through metabolic enzymes (Li et al. 2007). These mechanisms often can be found in combination, providing a multi-tiered protection against toxins (Beran et al. 2018).

Behavioral

Studies across Lepidoptera and beyond have established functional roles for members of at least five chemoreceptor gene families in mediating behavioral avoidance of, or attraction to, plant odors and tastants that act as chemical signals. Chemical sensing starts through binding of an external ligand (e.g., a plant volatile) to receptor proteins that are located in the dendritic membrane of chemosensory neurons, such as those found in antennae (peripheral events). This interaction is then translated into an electrical cue to the central nervous system. Most of the chemoreceptors expressed in insect sensory organs are members of three main families, the gustatory, ionotropic, and odorant receptors (GRs, IRs, and ORs, respectively; Depetris-Chauvin et al. 2015). Added to these are receptors from the transient receptor potential (Trp) and degenerin/epithelial sodium channel (DEG/ENaC) or pickpocket (ppk) families, as well as the insect orphan G-protein-coupled DmXR protein (Depetris-Chauvin et al. 2015). Although members of these latter families are tightly involved in chemoreception in the main genetic model insect, the "fruit" fly *Drosophila melanogaster* (Benton et al. 2006, 2009; Matsuura et al. 2009; Mitri et al. 2009; Scott et al. 2001; Zelle et al. 2013), DEG/ENaC and DmXR orthologs have not yet been functionally described in Lepidoptera. Because of this, we will not discuss these further.

Olfactory Receptors

Insects detect a wide set of plant volatiles through expressing ORs in olfactory sensory neurons. OR function relies on an obligate partner, Orco, which is an OR itself (Benton et al. 2006). Indeed, knocking out Orco with CRISPR gene editing leads to largely disrupted foraging and oviposition behaviors of juvenile and adult moths toward host plants, as was observed for the silkmoth *Bombyx mori* (Bombycidae), the tobacco hawkmoth *Manduca sexta* (Sphingidae), and the Egyptian cotton leafworm *Spodoptera littoralis* (Noctuidae; Fandino et al. 2019; Koutroumpa et al. 2016; Liu et al. 2017). In one moth species, the importance of ORs in host plant detection was narrowed down to the level of an individual OR: CRISPR knockout

individuals for Or42 in the cotton bollworm *Helicoverpa armigera* (Noctuidae) were impaired for host detection because they could not sense phenylacetaldehyde (Guo et al. 2021). ORs also form one of the mechanisms through which at least adult insects may perceive ITCs. In the diamondback moth *Plutella xylostella* (Plutellidae), ITCs stimulate oviposition by gravid females, and this response relies on the combined activity of Or35 and Or49 (Liu et al. 2020).

Ionotropic Receptors

A second class of receptors involved in sensing a wide set of plant volatiles is that of the IRs, which do not depend on Orco function (Benton et al. 2009). There is currently no evidence for a role of IRs in mediating caterpillar responses to ITCs or other volatile chemicals emitted from plants. However, a functional genetic study in *M. sexta* observed that adult females are deterred from ovipositing on two host plants, *Nicotiana attenuata* and *Datura wrightii*, when plants are already occupied by a feeding caterpillar from the same species or another such as *S. littoralis* (Zhang et al. 2019a). This avoidance behavior is displayed upon detection of the caterpillar frass-emitted carboxylic acids 3-methylpentanoic acid and hexanoic acid and mediated through Ir8a, which was verified through abolishing Ir8a function using CRISPR (Zhang et al. 2019a).

Gustatory Receptors

With one recent exception involving *Pieris rapae* and GSLs (Yang et al. 2021a, b), the GRs that insect taste sensilla express have only been functionally described in Lepidoptera when sensing chemicals not considered defensive chemicals. In *Plutella xylostella*, which specializes on GSL-containing plants, caterpillars made foraging decisions partially based on sensing the canonical plant hormones brassinolide and 24-epibrassinolide via Gr34 (Yang et al. 2020). This was functionally verified through RNA interference/RNA silencing (RNAi) of *Gr34* expression (Yang et al. 2020). That GRs can have dramatic effects on plant acceptance or rejection by caterpillars was demonstrated for larvae of the mulberry (*Morus alba*) specialist *B. mori*, where knocking out *Gr66* with CRISPR led to the acceptance of a wide variety of plant species unrelated to mulberry when foraging. This stood in stark contrast to foraging patterns of wild-type *B. mori* caterpillars, which retained a strong feeding preference for mulberry (Zhang et al. 2019c).

Transient Receptor Potential Channels

One of the main mechanisms by which insects and other animals may sense ITCs and other, often bitter, electrophilic plant compounds with deterrent effects is through transient receptor potential (Trp) channels (Kang et al. 2010). Functional

genetic studies in *D. melanogaster* revealed the Trp channels TrpA1 and Painless to be involved in sensing ITCs, as knockout mutant flies showed a reduction in aversive responses to ITCs (Al-Anzi et al. 2006; Kang et al. 2010). Although more studies are needed in Lepidoptera, for now at least, we know that TrpA1 and Painless are expressed in sensory organs of the Brassicaceae specialist *P. rapae* (Mao et al. 2020) and that one of the "model" ITCs, allyl ITC (AITC), activates the TrpA1 channel in the generalist *Helicoverpa armigera* (Wei et al. 2015). Furthermore, there is functional evidence that TrpA1 is involved in tasting bitter compounds in caterpillars of *Manduca sexta* (Afroz et al. 2013).

Non-receptor Chemosensory Gene Families

Before reaching a herbivore's chemoreceptor, plant compounds travel through the lymph that fills the sensilla housing the dendrites of chemosensory neurons. This sensillar lymph contains a variety of water-soluble proteins, including members of two closely related families, the odorant-binding proteins (OBPs) and chemosensory proteins (CSPs; Vieira and Rozas 2011). Although these proteins are highly abundant, much about them is still unknown. Most likely, OBPs and CSPs mediate the solubilization and transport of generally hydrophobic odorants through the sensillar lymph and thereby regulate the sensitivity of the olfactory system (Leal, 2013; Vieira and Rozas 2011).

OBPs and CSPs typically contain six and four positionally conserved cysteine residues, respectively, which could have particular ecological relevance in Brassicaceae specialists such as *Plutella xylostella*. The exposed cysteines could make OBPs vulnerable to attack by reactive electrophiles such as the ITCs that mustard plants produce. A study of another Brassicaceae specialist, the fly *Scaptomyza flava* (Drosophilidae), observed a striking loss of OBPs (Gloss et al. 2019b). Losses were particularly apparent within the Plus-C OBP subfamily whose member genes encode six additional cysteine residues compared to other OBPs (Zhou et al. 2004), which might render them even more vulnerable to ITCs. Loss of OBPs may in this scenario contribute to a lower sensitivity of Brassicaceae specialists to the deterrent effects of ITCs.

On the other hand, OBPs and CSPs may have a detoxification function in the strict sense if they can remove harmful ligands such as ITCs from the peripheral nervous system. Moreover, expression of OBP and CSPs is not restricted to the olfactory tissues; they may also participate in detoxification of plant defensive chemicals in other tissues such as the gut (Bautista et al. 2015), although this still awaits experimental support (Pelosi et al. 2018). Such potential multiple functions in xenobiotic responses make it difficult to formulate predictions for how OBPs may evolve in response to the presence of host plant-derived ITCs. When characterizing the genomes of Lepidoptera that are Brassicaceae specialists, such as *Plutella xylostella*, and those of Lepidoptera that are not, such as the monarch butterfly (*Danaus plexippus*), there is no obvious difference in the number of OBPs in their genomes: 38 and 32, respectively (Cai et al. 2020; You et al. 2013; Zhan et al. 2011).

A similar pattern was visible for the CSP gene family, with 31 CSPs for *P. xylostella* and 34 CSPs for the monarch (You et al. 2013; Zhan et al. 2011).

While there is at least some mechanistic knowledge of how caterpillars sense potential host plants from the Brassicaceae that give rise to ITCs, virtually nothing is known about how herbivores sense plants that store less reactive toxins such as CGs (Agrawal et al. 2021). It will be fascinating to find out more about the molecular mechanisms that give rise to complex adaptive behaviors such as the leaf vein-cutting behavior displayed by larvae of the monarch and several other herbivores of milkweeds, including the milkweed tussock moth *Euchaetes egle* (Arctiidae) (Dussourd and Eisner 1987). Via a process of elimination, a series of experiments suggested that polar (water-soluble) CGs or non-CG chemicals might stimulate this behavior in monarch caterpillars (Helmus and Dussourd 2005). Deactivating the latex-containing canals in veins of milkweed leaves (which contain concentrated CGs) reduces exposure to toxic CGs, making this a life or death matter (Fig. 1c).

Prevention of Defense Response Induction

While behaviors such as selection of host plants and tissues as well as laticifer clipping are effective ways to avoid or, in the case of certain specialist herbivores, perhaps seek exposure to toxic plant defensive chemicals, there are further mechanisms that have evolved to prevent activation of plant defenses upon engagement of lepidopterans with host plants. Through expressing enzymes with immuno-suppressive effects on the host plant, caterpillars could actively stop plants from inducing toxin production upon feeding. One widespread mechanism is for caterpillars to produce glucose oxidase in their saliva (Eichenseer et al. 2010). Glucose oxidase is the most highly abundant salivary enzyme in *H. zea* and other caterpillars, converting D-glucose and molecular oxygen to D-gluconic acid and hydrogen peroxide (Musser et al. 2002). The hydrogen peroxide in turn elicits a burst of salicylic acid (SA) production by the host plant, which suppresses the synthesis of higher levels of defensive chemicals through interference with plant defensive signaling by jasmonic acid (JA) and ethylene (Fig. 1a; Diezel et al. 2009). JA/SA antagonism and its modulation by ethylene likely evolved in the last common ancestor of angiosperms (Groen and Whiteman 2014; Thaler et al. 2012a, b). The conserved nature of JA/SA antagonism may partially explain the pattern that caterpillars of highly polyphagous species were more likely to possess relatively high levels of glucose oxidase activity than caterpillars from more specialized species (Eichenseer et al. 2010).

Another mechanism of preventing plant production of defensive chemicals is to evade molecular detection of attack by plant receptor proteins that survey plant cells (Fig. 1a; Ngou et al. 2021; Yuan et al. 2021a, b). A particularly well-studied example can be found in the interaction between cowpea (*Vigna unguiculata*) and caterpillars. This plant activates production of defensive chemicals upon recognition of so-called inceptin-related peptides, present in caterpillar oral secretions, which are

peptides derived from chloroplastic ATP synthase γ-subunit proteins (Schmelz et al. 2012; Steinbrenner et al. 2020). While these active inceptins are generated when caterpillars of generalist herbivores such as the fall armyworm *Spodoptera frugiperda* (Noctuidae) are attacking cowpea, they are not generated when larvae of the legume-specializing velvet bean caterpillar (*Anticarsia gemmatalis*) feed on the plant. A functional screen of inceptin amino acid building blocks identified that unlike the main inceptin found in all other Lepidoptera examined (Vu-In; +ICDINGVCVDA−), the oral secretions of *A. gemmatalis* caterpillars predominantly contained an inactive, C-terminal truncated peptide (Vu-In−A; +ICDINGVCVD−), which also functioned as an effective antagonist of Vu-In-induced responses (Schmelz et al. 2012).

Diversion Strategies for Precursor Toxins

If defensive chemicals are already stored constitutively, as is the case for the mustard oil bomb and other toxins that are released upon β-glucosidase-mediated hydrolysis of stored precursor glucoside molecules, an alternative strategy to prevent toxin formation is to modify the precursors or divert the hydrolytic process. Prevention of ITC formation could have strong effects on caterpillar survival, growth, and development time, as was shown definitively for the small cabbage white *Pieris rapae* in feeding experiments with microencapsulated formulations of allyl ITC, its precursor allyl GSL, and myrosinase (Agrawal and Kurashige 2003).

One effective way through which several specialists on Brassicaceae disarm the mustard oil bomb and prevent ITC formation is to remove the sulfate group in GSLs using sulfatase enzymes (GSSs; Ratzka et al. 2002). This removal renders myrosinases ineffective, as they cannot use desulfo-GSLs as substrates and are competitively inhibited by sulfate (Ratzka et al. 2002). This mechanism has evolved in *Plutella xylostella*, whose genome encodes three GSSs with distinct expression patterns and substrate specificity patterns in response to dietary GSLs (Heidel-Fischer et al. 2019). Two of these gene copies evolved under positive selection while acquiring their new GSL desulfation capabilities (Heidel-Fischer et al. 2019). As a further testament to the importance of GSSs for *P. xylostella* fitness when feeding on GSL-containing host plants, larvae experienced reduced survival and slower development when GSSs were knocked out using CRISPR (Chen et al. 2020).

A second diversion mechanism of the mustard oil bomb has evolved in the pierid butterflies. Upon caterpillar feeding and concomitant GSL degradation, nitrile-specifier proteins (NSPs) in the gut redirect the GSL hydrolysis reaction away from formation of ITCs and toward formation of nitriles, which are subsequently excreted with the feces (Wittstock et al. 2004, Wheat et al. 2007). The genes involved in GSL and ITC production in Brassicales plants and members of the NSP gene family in pierids show evidence of evolving in an escalating evolutionary arms race pattern (Berenbaum and Feeny 1981). Key innovations are linked to gene and genome

duplications and shifts in diversification rates, followed by gradual changes in trait complexity that appear to have been facilitated by allelic turnover (Edger et al. 2015).

Physical Barriers (Peritrophic Membrane)

The peritrophic membrane or matrix (PM) (see Wagner and Hoyt, Chapter "On Being a Caterpillar: Structure, Function, Ecology, and Behavior") is a semi-permeable chitinous matrix that lines the midgut of caterpillars and most other insects. The PM not only serves to protect the midgut epithelium from microorganisms and mechanical damage, but also from large plant defensive chemicals such as CGs, including the highly polar CG digitoxin (Barbehenn 1999, 2001). A study in *Helicoverpa zea* observed that the PM reduced hydrogen peroxide in the midgut, acting as a physical antioxidant (Summers and Felton 1996).

The PM in insects is formed through binding between chitin fibrils and PM proteins with multiple chitin binding domains (CBDs). Multi-CBD chitin binding proteins form the two major types of structural proteins in the PM alongside the insect intestinal mucin proteins. While CRISPR knockout mutants for mucin proteins in the cabbage looper *Trichoplusia ni* did not perform worse when fed a diet of GSL-containing cabbage leaves than wild-type caterpillars (Wang and Wang 2020), mucin proteins are involved in protecting caterpillars of *Plutella xylostella* against the harmful effects of terpenoids such as (3E)-4,8-dimethyl-1,3,7-nonatriene (DMNT; Chen et al. 2021). DMNT repressed expression of *PxMucin* in the larval midgut, and knock-down of this gene led to PM rupture and caterpillar death. These harmful effects of DMNT were both direct and indirect, since DMNT-induced damage to the PM led to further costly imbalances in the midgut microbiome of caterpillars (Chen et al. 2021).

Another constituent protein of the PM is the chitin-binding protein Peritrophin A. Insect herbivores show enhanced expression of this gene when jasmonic acid-mediated defensive signaling and production of reactive oxygen species are active (Groen et al. 2016; Mittapalli et al. 2007; Whiteman et al. 2011). The chitin fibrils and glycoproteins present in the PM are further targeted by a group of carbohydrate-binding proteins known as lectins. Indeed, a study dissecting the PM from the *Spodoptera littoralis* midgut showed distinct abnormalities in the PM with disrupted microvilli structures owing to lectin binding (Vandenborre et al. 2011).

A second set of important physical barriers are transepithelial diffusion barriers such as septate junctions in the midgut and the hemolymph (or blood)-brain barrier (BBB), which is also known as the perineurium (Petschenka et al. 2013). Septate junctions limit solute passage through intercellular spaces in epithelia. One of the proteins that has been implicated in maintaining junctional activity is the Na+/K+-ATPase β subunit encoded by the gene *Nrv2* in *D. melanogaster* (Paul et al. 2003, 2007). This epithelial barrier function is independent of its role in Na+/K+-ATPase pump activity. The presence of the junctions, combined with a lack of active uptake mechanisms for hydrophilic substances, which cannot permeate lipid

bilayer membranes passively, can provide at least some protection against polar CGs such as ouabain (Dobler et al. 2015; Petschenka et al. 2013; Rubin et al. 1983). However, to prevent lipophilic defensive chemicals (e.g., the apolar CGs digoxin and digitoxin) from penetrating the midgut and the BBB, active detoxification mechanisms that counteract passive diffusion of the compounds through the lipid bilayers are necessary, which we will discuss below.

Target Site Insensitivity

Physiological investigations of the monarch butterfly provided early evidence of the existence of a Na+/K+-ATPase (the target of CGs) with dramatically lowered sensitivity (increased resistance) to CGs (Holzinger et al. 1992; Holzinger and Wink 1996). Molecular investigations demonstrated that this insensitivity may be explained in the monarch butterfly, at least in part, by an amino acid substitution of asparagine for histidine at position 122 (N122H) of the Na+/K+-ATPase's alpha subunit (Holzinger et al. 1992; Holzinger and Wink 1996). This form of molecular substitution that alters the toxin's binding potential to the enzyme is called "target site insensitivity" (TSI). By screening all Na+/K+-ATPase transmembrane domains involved in CG binding, a pair of studies detected the presence of the same substitution in five distantly related insect species representing a total of at least four independent origins across a phylogenetic distance of 300 million years (Dobler et al. 2012; Zhen et al. 2012). Remarkably, these screens also identified other amino acid substitutions associated with TSI of the Na+/K+-ATPase to CGs.

However, it was unknown if these substitutions could be sufficient for conferring resistance at the whole-organism level in a way that is beneficial, i.e., adaptive, for the animal. A follow-up study embarked on reconstructing possible mutational paths linked to CG insensitivity by comparing protein sequences of the CG binding site between the monarch butterfly and other animals with CG-rich diets to those of animals not regularly encountering dietary CGs (Weinreich et al. 2006, Karageorgi et al. 2019). Many evolutionary paths involved mutations in binding site residues 111, 119, and 122 (Karageorgi et al. 2019). A subset of these paths, including the monarch's, were then introduced into the genome of *D. melanogaster* through single-base edits using CRISPR (Gratz et al. 2014; Lin et al. 2014; Port et al. 2014; Groen and Whiteman 2016; Karageorgi et al. 2019). Since *D. melanogaster* is not specialized on a CG-rich diet, Karageorgi and co-workers (2019) tested whether the mutations conferred CG insensitivity at the neurophysiological and whole-organism levels.

A series of fly lines was engineered that represents steps in the evolution of CG insensitivity as observed in the lineages of the monarch butterfly and other CG-resistant species (Karageorgi et al. 2019). Mutating residues Q111 and N122 caused nervous system dysfunction, and co-introduction of A119S limited these deleterious side effects (Karageorgi et al. 2019). At the neurophysiological and whole-organism levels, flies with insensitivity mutations at sites 111 and 122 were

highly resistant to CGs, just as the monarch is. Again, co-introducing A119S was important by enhancing the resistance-conferring effects of these insensitivity mutations (Karageorgi et al. 2019). Overall, residue S119 unlocked adaptive paths to resistance through interactive effects (epistasis) with sites 111 and 122 (Weinreich et al. 2006, Karageorgi et al. 2019), a result confirmed independently (Taverner et al. 2019).

TSI is a particularly effective strategy in response to toxins with narrow target ranges such as CGs, where a single or few TSI mutations have the potential for producing large fitness consequences. However, toxins such as ITCs, other reactive electrophiles, and reactive oxygen species have a wide target range, and it is unknown if insensitivity of at least some of the target sites has the potential to evolve in response to such toxins.

We explored whether this could be the case by taking a comparative genomics approach for a Brassicaceae-specialized herbivorous fly, *Scaptomyza flava*. For such a comparative analysis, we could not work with lepidopteran herbivores because herbivory evolved too long ago and the availability of genomic data is still relatively limited (Groen and Whiteman 2016). In the analysis we used data from *D. melanogaster* and further leveraged available protein biochemistry data from human biomedical science studies where interactions between GSL breakdown products and target proteins were studied functionally. We find that *S. flava* orthologs of genes that encode proteins targeted by GSL breakdown products in humans evolve faster than orthologs of human genes that do not encode such proteins (Fig. 2). It will be interesting to see if similar polygenic patterns of presumptive TSI have evolved in lepidopteran specialists on Brassicaceae such as *Pieris* spp. and *Plutella* spp.

Detoxification

Alongside the behavioral changes, structural barriers, immunosuppressive mechanisms, and TSI to prevent or negate the toxic effects of plant defensive chemicals, caterpillars may actively detoxify and metabolize these compounds through a conserved set of enzyme families. These enzymes are active not only at the interface of plant cells and caterpillar mouthparts as part of the insect's saliva (Rivera-Vega et al. 2017a,b) but also in tissues such as the gut, the BBB, and the Malpighian tubules (Li et al. 2007).

The three phases of detoxification in animals, as defined earlier, are each characterized by the activity of certain ubiquitous enzyme families, and we will review these below. Caterpillars of different species harbor distinct subsets of these enzyme families, and in most cases specific plant defensive chemicals can only be metabolized by a small number of detoxification enzymes (Heidel-Fischer and Vogel 2015).

Over the last 10–20 years, genomics and transcriptomics studies have provided evermore comprehensive insights into xenobiotic metabolism of caterpillars. One comparative genomics study found that among lepidopteran species feeding on

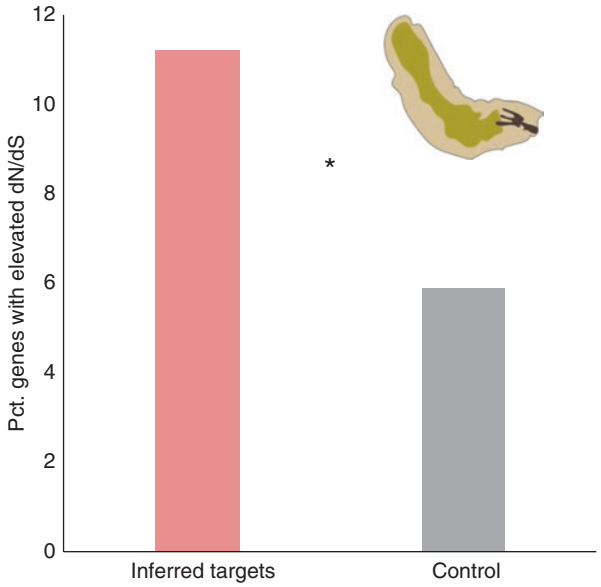

Fig. 2 Genes encoding proteins putatively targeted by GSL breakdown products display accelerated evolution in the Brassicaceae-specialized herbivorous fly *Scaptomyza flava*. Inferred putative targets of GSL breakdown products in *S. flava* and its one-to-one orthologs in *D. melanogaster* relatives were determined via orthology with human proteins that have functionally verified interactions with these products using the PantherDB database (Mi et al. 2013). Then, for each set of single-copy orthologous *Scaptomyza* and *Drosophila* genes, amino acid sequences from five species were aligned in MUSCLE: *S. flava*, *D. grimshawi*, *D. mojavensis*, *D. virilis*, and *D. melanogaster* (Gloss et al. 2019b). Using PAML v4.5's codeml module (Yang 2007), branch site tests for accelerated ratios of the number of non-synonymous substitutions per non-synonymous site (dN) to the number of synonymous substitutions per synonymous site (dS), dN/dS, were run for all terminal branches (Yang 1998), which has been described in more detail previously (Gloss et al. 2014). We define "accelerated" as being part of the top 5% tail of dN/dS values. Asterisk indicates a significant difference ($P < 0.05$) in the number of putative targets of GSL breakdown products with accelerated ratios of dN/dS (inferred targets) versus the number of putative non-targets with accelerated ratios of dN/dS (control) using a chi-square test. (Cartoon of *S. flava* larva by Sophie Zaaijer)

photosynthesizing plant tissue, highly polyphagous species had higher numbers of genes encoding cytochrome P450 monooxygenase (CYP450; phase I), carboxyl/choline esterase (CCE; phase I), and glutathione *S*-transferase (GST; phase II) genes (Gloss et al. 2019a; Rane et al. 2019). These genes are collectively among the most important in detoxification sensu stricto because they transform toxins into less toxic molecules.

Comparative gene expression studies in which transcriptomes have been sequenced in caterpillars reared on genetically manipulated crucifer plants, such as the model plant *Arabidopsis thaliana*, have shown how generalists and specialists appear to use different strategies to try to cope with the mustard oil bomb. In the tobacco budworm *Heliothis virescens*, a generalist, 3,747 transcripts were

differentially expressed when feeding on plants with intact GSL production compared to engineered plants with disrupted production, whereas only 254 transcripts were differentially regulated in a specialist, the large cabbage white *Pieris brassicae* (Schweizer et al. 2017). Moreover, twice as many transcripts were upregulated rather than downregulated in *H. virescens*, while these proportions were similar (i.e., 50:50) in *P. brassicae*. Several canonical detoxification genes were strongly induced in *H. virescens* by the presence of GSLs in host plants (up to 30-fold), including 17 CYP450s and 9 CCEs (phase I), as well as 7 ABC transporters (phase III; Schweizer et al. 2017). In *P. brassicae*, on the other hand, a member of the NSP gene family, known to divert GSL breakdown toward less toxic nitriles (see above), was regulated by GSLs, plus a homologue of *GSTD1* (Schweizer et al. 2017), which efficiently catalyzes the conjugation of reduced glutathione (GSH) with ITCs in the dipteran herbivore *Scaptomyza nigrita* (see below; Gloss et al. 2014).

Although similar experiments with genetically engineered host plants are not yet possible for milkweed herbivores, transcriptomes of monarch caterpillars reared on *Asclepias curassavica* and *A. incarnata*, two species that differ substantially in CG concentrations, have been measured. Monarch larvae differentially expressed several hundred genes when feeding on these different hosts, including numerous phase I, II, and III detoxification genes, suggesting that these genes play a role in monarch toxin resistance and sequestration (Tan et al. 2019a, b).

Transcription of xenobiotic metabolism genes is regulated by a signaling network with at least five different pathways through it, each initiated by different classes of receptors: (1) the membrane-localized G protein-coupled receptors; (2) cyclic adenosine 3′,5′-monophosphate (cAMP)-response element binding protein (CREB), which is a bZIP family transcription factor and requires phosphorylation by environment-responsive mitogen-activated protein kinase (MAPK) cascades to initiate signaling; (3) Cap'n'collar isoform C/Kelch-like ECH associated protein 1 (CncC/Keap1), which is another bZIP family transcription factor and ortholog of Nuclear factor erythroid-derived 2-related factor 2 (Nrf2) found in mammals; (4) the basic helix-loop-helix (bHLH)-Per-ARNT-Sim (PAS) domain-class transcription factor aryl hydrocarbon receptor (AhR), which heterodimerizes with the AhR nuclear translocator (ARNT) before binding to xenobiotic response elements (XRE) in target gene promoters to activate their transcription; and 5) the nuclear receptor (NR) superfamily transcription factor Hormone receptor-like in 96 (HR96), which is related to genes encoding the Steroid and Xenobiotic Receptor (SXR) and Constitutive Androstane Receptor (CAR) in vertebrates (Amezian et al. 2021; Li et al. 2021a, b). Both CAR and SXR may translocate to the nucleus upon activation and subsequently dimerize with Retinoid-X-Receptor (RXR) to enhance target gene transcription (Amezian et al. 2021).

Which receptors initiate signaling depends partly on the solubility of the plant defensive chemicals the insect encounters. ITCs and another GSL breakdown product, indol-3-carbinol (I3C), are relatively lipophilic, and after passing through the cell membrane, they can elicit a burst of reactive oxygen species (ROS) directly or indirectly. This in turn can activate transcription through CncC/Keap1 (Nrf2) interaction with the antioxidant response element (ARE) in promoters of downstream

detoxification genes such as CYP450s and GSTs (Chen et al. 2018; Giraudo et al. 2015; Li et al. 2021a, b).

CGs, on the other hand, occur in a range of polarities and, therefore, solubilities. In addition to being perceived through their inhibition of the Na+/K+-ATPase, there are hints they could be perceived by intracellular receptors, which may depend on the solubility of individual CGs. Although polar, water-soluble plant defensive compounds, including several alkaloids such as nicotine, cannot passively diffuse through membranes and may thus be perceived by membrane-localized receptors such as GPCR (Amezian et al. 2021; Li et al. 2021a, b; Yang et al. 2020), polar CGs have not been connected with this mechanism. Certain polar compounds, including the CG ouabain, can be actively transported into cells via transmembrane transporters such as organic anion transporter peptides (Groen et al. 2017; Wink 2018). Polar CGs, along with lipophilic membrane-permeable CGs such as digitoxin, might then be perceived by intracellular receptors. However, while in mammals the relatively polar CG digoxin interacted with the nuclear receptor RORγT, this was not the case for its distant ortholog in insects, the steroid-sensing receptor DH3/Hr3 (Ahmed et al. 2020; Huh et al. 2011). Genetic screening in the model insect *D. melanogaster* may be the most efficient way forward for identifying if there is an intracellular receptor for CGs in insects in addition to the Na+/K+-ATPase at the cell membrane (Groen and Whiteman 2016).

We will now go into more depth regarding the multiple families of canonical insect xenobiotic metabolism genes.

Phase I: Oxidation, Hydrolysis, Reduction

Here, the goal is to provide an overview of the role that phase I enzymes, principally CYP450s, play in mediating detoxification of plant secondary compounds encountered by lepidopteran larvae. We then narrow our discussion to focus on their role in CG and GSL detoxification.

CYP450s are membrane-localized enzymes with important roles in metabolizing a variety of chemicals, ranging from steroid hormones to fatty acids to vitamins. The monooxygenases achieve this by adding oxygen atoms to target chemicals, using heme as a co-factor. A critical part of the heme group is an iron atom, which is activated by a conserved cysteine residue (Feyereisen 2012). The oxygenated substrates typically become more water-soluble and more amenable to being targeted by enzymes in subsequent phases of the detoxification process (which is why they are called phase I).

CYP450s are critical for successful detoxification of a range of plant defensive chemicals, and particularly well-studied members of the CYP450 family in this regard are those of the CYP6 clade. Members of the CYP6AE clade show a bloom (expansion in gene number through duplications) in Lepidoptera (Dermauw et al. 2020). Silencing or knocking out CYP6AE genes in the cotton bollworm *H. armigera* impairs caterpillar tolerance toward the cotton toxin gossypol (Mao et al. 2007) and the furanocoumarin xanthotoxin that is found in plants from the Rutaceae and

Apiaceae (Wang et al. 2018), respectively. In particular, CYP6AE19 was shown to metabolize xanthotoxin, but not as efficiently as the P450 CYP6B1 from the black swallowtail *Papilio polyxenes*, a specialist on furanocoumarin-containing plants (Wang et al. 2018). *P. polyxenes* caterpillars can tolerate dietary furanocoumarin concentrations of up to 1% using CYP6B1, its paralogue CYP6B3, and other CYP6Bs as detoxifying enzymes (Berenbaum and Zangerl 1993; Cohen et al. 1992; Hung et al. 1995; Wen et al. 2003). CYP6B1 and -3 probably evolved toward subfunctionalization under independent purifying selection after the duplication event that gave rise to both, and now display different efficiencies with which they metabolize different types of furanocoumarin (Wen et al. 2006). A similar pattern of subfunctionalization under selection apparently occurred in the parsnip webworm *Depressaria radiella* (formerly *D. pastinacella)*, which has an even narrower host range (restricted to Apiaceae) than *P. polyxenes*, with at least two CYP450s (CYP6AE89 and CYP6AB3) efficiently metabolizing a variety of different furanocoumarins (Calla et al. 2020; Li et al. 2004a, b; Mao et al. 2006, 2007a, 2008). Going in the other direction, away from specialization and toward more generalized host plant ranges, substrate specificities of CYP450s in *Papilio* spp. were broader in the oligophagous species *P. multicaudatus* than in the specialist *P. polyxenes* and broader still in the polyphagous species *P. glaucus* and *P. canadensis*; this was linked to the relative abundance of furanocoumarin-producing plants in the diet (Li et al. 2003; Mao et al. 2007b).

In the context of handling toxic GSL breakdown products, it appears that CYP6B enzymes can process I3C as a substrate, which is one of the major derivatives of indole GSLs. Caterpillars of the generalist moth *H. virescens* showed enhanced transcription of CYP6B8 and several other CYP6AE and CYP6AB genes after encountering GSLs, including I3C (Schweizer et al. 2017). Comparison of the homolog of CYP6B8 in another generalist, *H. zea* (which has a wide host range and occasionally encounters GSLs), with CYP6B1 from the Rutaceae and Apiaceae specialist *P. polyxenes* (which practically never encounters GSL-producing plants), showed that while CYP6B1 did not metabolize the indole GSL breakdown product I3C, CYP6B8 did (Li et al. 2004a, b). CYP6B8 further metabolized a number of other chemically diverse plant defensive compounds including quercetin, flavone, chlorogenic acid, rutin, and xanthotoxin (Li et al. 2004a, b). The latter compound is one of the defensive chemicals abundant in hosts of *P. polyxenes*, and indeed, CYP6B1 of the specialist had a 30-fold higher metabolic clearance rate toward xanthotoxin than CYP6B8 (Li et al. 2004a, b), pointing to a trade-off between breadth and efficiency in terms of substrate handling for these CYP450s.

There is some evidence that CGs may also be substrates for CYP450s (Marty and Krieger 1984). However, the identity of individual CYP450s that may metabolize CGs in caterpillars from the monarch and other milkweed herbivores is currently unknown. Two studies that compared transcriptomes of monarch caterpillars reared on host plants with different CG profiles revealed suites of CYP450s that were differentially expressed (Gonzalez-De-la-Rosa et al. 2020; Tan et al. 2019a), potentially narrowing down the set of candidate CYP450s that may be involved in processing CGs. It has recently been established that an enzymatic reduction step is

critical for detoxification of the toxic CG voruscharin, produced by one of the monarch's main host plants *Asclepias curassavica* (Agrawal et al. 2021). After a first non-enzymatic step in which voruscharin is converted to uscharidin, a step facilitated by the alkaline pH of the gut milieu (Berenbaum 1980), this compound is then enzymatically reduced to the more polar and less toxic CGs calactin and calotropin (Agrawal et al. 2021; Marty and Krieger 1984; Seiber et al. 1980). Oxidoreductases such as CYP450s are candidates for carrying out this step, as indeed, CYP450s such as the Halloween genes have well-studied roles in facilitating molecular alterations of plant-derived steroids that are chemically related to CGs to synthesize molting hormones (Gilbert 2004; Seiber et al. 1980).

The carboxyl/cholinesterases (CCEs) form another functionally diverse superfamily of enzymes. These hydrolyze carboxylic esters to their component alcohols and acids. Although CCEs have been studied less intensively than P450s, evidence has been found for a role of CCEs in targeting host plant defensive chemicals. In caterpillars of *Depressaria radiella*, CCEs are involved in processing plant-derived aliphatic esters in the midgut (Zangerl et al. 2012). Furthermore, in adults of the generalist moth *Spodoptera littoralis*, two CCE genes, *SlCXE7* and *SlCXE10*, were found to degrade the plant volatile (Z)-3-hexenyl acetate in the antennae, but it is unclear which of these genes could have a role in processing volatile cues in the larval stage as well (Durand et al. 2010, 2011). It is further unknown if CCEs could be involved in processing GSLs, GSL breakdown products, or CGs. However, transcriptomic studies have identified a number of CCEs that are responsive to the presence of dietary GSLs in *Heliothis virescens* (Schweizer et al. 2017) and to host plants with different CG contents in the monarch (Gonzalez-De-la-Rosa et al. 2020; Tan et al. 2019a).

Phase II: Conjugation

In the second phase, the products of the first phase or, often, the toxins themselves are conjugated to other molecules. The enzymes that catalyze these reactions are various transferases such as GSTs, many of which are regulated by the Keap1-Nrf2-ARE signaling pathway. Perhaps their best-studied detoxification mechanism is the conjugation reaction with GSH. Conjugation neutralizes reactive nucleophile sites of plant defensive chemicals. It can further increase their solubility in water, thereby facilitating their excretion from cells in phase III.

GST-mediated detoxification can happen through the metabolism of secondary products generated from other detoxification enzymes (phase II). It can also occur directly during phase I as an alternative to P450- or CCE-mediated detoxification. Despite their central role in processing a range of plant defensive chemicals, GSTs appear not to have undergone a gene family-wide expansion in the Lepidoptera (You et al. 2015).

GSTs play an important role in the detoxification of ITCs in caterpillars of generalist species that have not evolved specialized mechanisms to prevent ITC formation, such as GSL desulfation through GSSs in *Plutella* spp. and diversion of GSL

breakdown toward nitriles under the influence of NSPs in *Pieris* spp. Although mechanistic evidence is still being gathered, it appears that GST-mediated ITC detoxification occurs via a series of enzymatic steps known from mammalian studies as the mercapturic acid pathway (Traka and Mithen 2009). This pathway starts with activity of GSTs, generating ITC conjugates with GSH, cysteinylglycine (CysGly), and Cys, which end up as conjugates with an N-acetylcysteine group through the action of N-acetyltransferases (Traka and Mithen 2009). The last step deserves particular attention. While ITCs leave the mammalian body in urine and bile as N-acetylcysteine conjugates, such conjugates have not been observed in caterpillar frass, despite detection of all conjugates from intermediate steps in the pathway (Jeschke et al. 2016, 2017, 2021; Schramm et al. 2012). It is currently unclear if lepidopteran genomes do not encode the required enzymes, whether such enzymes are perhaps not expressed at the caterpillar stage, or if the enzymatic reaction may be impeded by the relatively high pH of the caterpillar midgut milieu (Berenbaum 1980; Schramm et al. 2012).

Thus far, ITC detoxification via GSTs and the mercapturic acid pathway has been studied in a variety of generalists (e.g., *Helicoverpa armigera*, *Mamestra brassicae*, *Spodoptera* spp., *Trichoplusia ni*) and Brassicaceae specialists, but also in a specialist on legumes: *Anticarsia gemmatalis*. A comparative study of GST activity in response to the presence of dietary ITCs showed that in the highly polyphagous species *Spodoptera frugiperda*, GSTs metabolize a wide range of ITCs (Wadleigh and Yu 1988). This range becomes progressively narrower in GSTs of *T. ni*, which is less polyphagous and metabolizes only allyl and benzyl ITC, and *A. gemmatalis*, which does not typically encounter ITCs and metabolizes only benzyl ITC. These comparisons suggest that GST substrate specificity may evolve according to the proportion of GSL-containing plant material in the diet (Wadleigh and Yu 1988).

This study and subsequent studies further identified that GST levels are induced, not only when ITCs are present in the diet, but also when indole GSL-derived I3C and indole-3-acetonitrile are present in the diet (Li et al. 2007; Wadleigh and Yu 1988). In the generalist *Spodoptera litura*, expression of the epsilon-class GST (*Slgste1*) in the midgut was responsive to the formation of ROS induced by I3C (Chen et al. 2018). Induction of expression was regulated by binding of SlNrf2 to an antioxidant response *cis*-regulatory element in the *Slgste1* promoter. This was functionally verified through RNAi on *SlNrf2*: caterpillars with silenced *SlNrf2* showed reduced expression of *Slgste1*, lower levels of peroxidase reactions by GSTs, and reduced cell viability in response to treatment with I3C (Chen et al. 2018).

Although a specialist such as *Pieris rapae* does not rely mainly on GST- and GSH-dependent detoxification to handle dietary GSLs, it may have additional adaptations to prevent oxidative damage that could still be induced by non-ITC breakdown products of GSLs. *P. rapae* individuals show genetic variation in/near *Glyoxalase 1* (*Glo1*), encoding a lactoyl-GSH lyase that is linked to caterpillar performance on *Arabidopsis thaliana* plants (Nallu et al. 2018). As part of the glyoxalase pathway, Glo1 neutralizes toxic by-products of metabolism, using GSH in the process.

In addition to clade-specific defensive chemicals such GSLs, GSTs have also been found to provide protection against more widely occurring toxins. The compound 12-oxophytodienoic acid (12-OPDA), which is part of the jasmonate family and also acts as a signaling molecule (Groen et al. 2013), has a reactive α,β-unsaturated carbonyl structure. It easily adds cellular nucleophiles, making OPDA potentially toxic for herbivores. The glutathione *S*-transferase GST16 inactivates 12-OPDA in the insect gut by isomerization to inactive *iso*-OPDA in *Helicoverpa armigera* (Shabab et al. 2014), and GST family members perform the same function in a suite of other generalist moth larvae (Dabrowska et al. 2009).

A more recently identified family of genes acting in phase II detoxification is that of the UDP-glycosyltransferases (UGTs; Ahn et al. 2012). UGTs may catalyze conjugation of sugars with lipophilic plant defensive chemicals, which increases water solubility of the toxins and makes it easier for them to be processed further in subsequent phases of detoxification. UGTs show lineage-specific expansions within the Lepidoptera and appear to play an important role in the xenobiotic response (Ahn et al. 2012).

While not yet studied in the context of GSLs and ITCs, a role for UGTs has been identified for caterpillar detoxification of three other classes of toxins that share certain properties with ITCs. The first class is represented by capsaicin from peppers (*Capsicum* spp.), which in mammals and *D. melanogaster* is perceived by Trp receptors as are ITCs (Li et al. 2020a, b). Although it is unknown if Trp receptors are involved in capsaicin perception in Lepidoptera as well, capsaicin does have a deterrent effect on feeding and oviposition in *Helicoverpa* spp. moths (Ahn et al. 2011a). Interestingly, these species all appear to employ UGT-mediated glucosylation as a means of capsaicin detoxification, including not only the generalists *H. armigera* and *H. zea* but also the specialist *H. assulta*, despite the latter showing higher capsaicin tolerance levels (Ahn et al. 2011a,b).

The second class is exemplified by the sesquiterpene dimer gossypol from cotton, which, not unlike ITCs, is able to cross membranes passively as an apolar chemical, deriving its toxicity from damaging amino acids in proteins. Gossypol toxicity occurs through interaction between its highly reactive aldehyde groups and amino acids, while six phenolic hydroxyl groups lend it additional toxicity. Enzymatic essays with insect cells expressing UGT41B3 and UGT40D1 from the generalist *Helicoverpa armigera* showed that these UGTs can glycosylate gossypol to diglycosylated gossypol isomers, a process which may be involved in detoxification in vivo (Krempl et al. 2016).

The third class is formed by benzoxazinoid glycosides, which are produced by a subset of monocots, including maize. Benzoxazinoids are indole-derived defensive chemicals whose aglucone breakdown products delay caterpillar growth and survival. *Spodoptera frugiperda* detoxifies these aglucones through UGT-mediated reglucosylation. In the process, the chemical is inverted compared to its original benzoxazinoid glycoside state as found in the host plant. This inverted glucosylation ensures that the benzoxazinoids cannot be turned into the toxic aglucone form by either plant or insect ß-glucosidases again, making the detoxification strategy effective for enhancing caterpillar fitness (Maag et al. 2014; Wouters et al. 2014).

In all of these examples, more work will be necessary to narrow down the mechanistic involvement of UGTs to the levels of individual genes and the enzymes for which they code. Lastly, UGTs are enriched in the transcriptome of monarch caterpillars compared to the transcriptomes of the pupal and adult life stages (Ranz et al. 2020). Although it was speculated that these UGTs may have a role in the detoxification of milkweed host toxins such as CGs, this has not yet been studied functionally (Ranz et al. 2020).

Phase III: Excretion

Enzymatic reactions in phases I and II make plant defensive chemicals available for the last phase of the detoxification process, if they were not already available as water-soluble compounds. In this last phase, phase III, the compounds become substrates of several diverse sets of transporters from multiple gene families and subfamilies. Activity of these transporters is particularly important in three tissue types where they shunt away plant defensive chemicals and/or their processed derivatives: the gut, the BBB, and the Malpighian tubules. We will now focus on two classes of transporters that are expressed in all three of these tissues.

The first class is formed by the multidrug transporters (Mdrs), which are also known as P-glycoproteins and B-type ABC transporters (Dermauw and Van Leeuwen 2014). Tissue-specific gene expression measurements and staining with Mdr-specific antibodies detected the presence of Mdrs in the midguts of generalist herbivores as well as CG-adapted insects (Dobler et al. 2015; Petschenka et al. 2013). Mdr expression is further enriched in the Malpighian tubules (Chahine and O'Donnell 2009; Dow and Davies 2006), where efflux capacity increases dramatically upon toxin exposure (Chahine and O'Donnell 2009). The regulation of Mdr expression appears to be coordinated with that of genes involved in earlier phases of xenobiotic detoxification (Chahine and O'Donnell 2011). Lastly, Mdrs are expressed in the BBB across all of the animal kingdom (Hindle and Bainton 2014). Physiological assays, complemented with reverse genetic studies, have established that Mdrs act as active diffusion barriers to apolar CGs such as digoxin in Lepidoptera, other insects, and vertebrates (Gozalpour et al. 2013; Petschenka et al. 2013; Groen et al. 2017).

Interestingly, knockout mutants of *Mdr50* in *D. melanogaster* are compromised in their digoxin resistance (Groen et al. 2017). The putative monarch orthologs show interesting properties: (1) the monarch orthologs appear to have undergone a bloom compared to orthologs in caterpillars that do not regularly encounter dietary CGs (Fig. 3); and (2) expression of these genes is upregulated on a diet containing CG-rich milkweeds (Gonzalez-de-la-Rosa et al. 2020). If the role of Mdr50 is conserved in the monarch butterfly, this might provide a mechanism for the monarch to minimize exposure to apolar CGs by reducing their entry from the midgut to the hemolymph. Excluding apolar CGs such as the thiazolidine ring-containing voruscharin from the hemolymph could have important fitness consequences. This CG is the most abundant CG in one of the monarch's main milkweed hosts, *Asclepias*

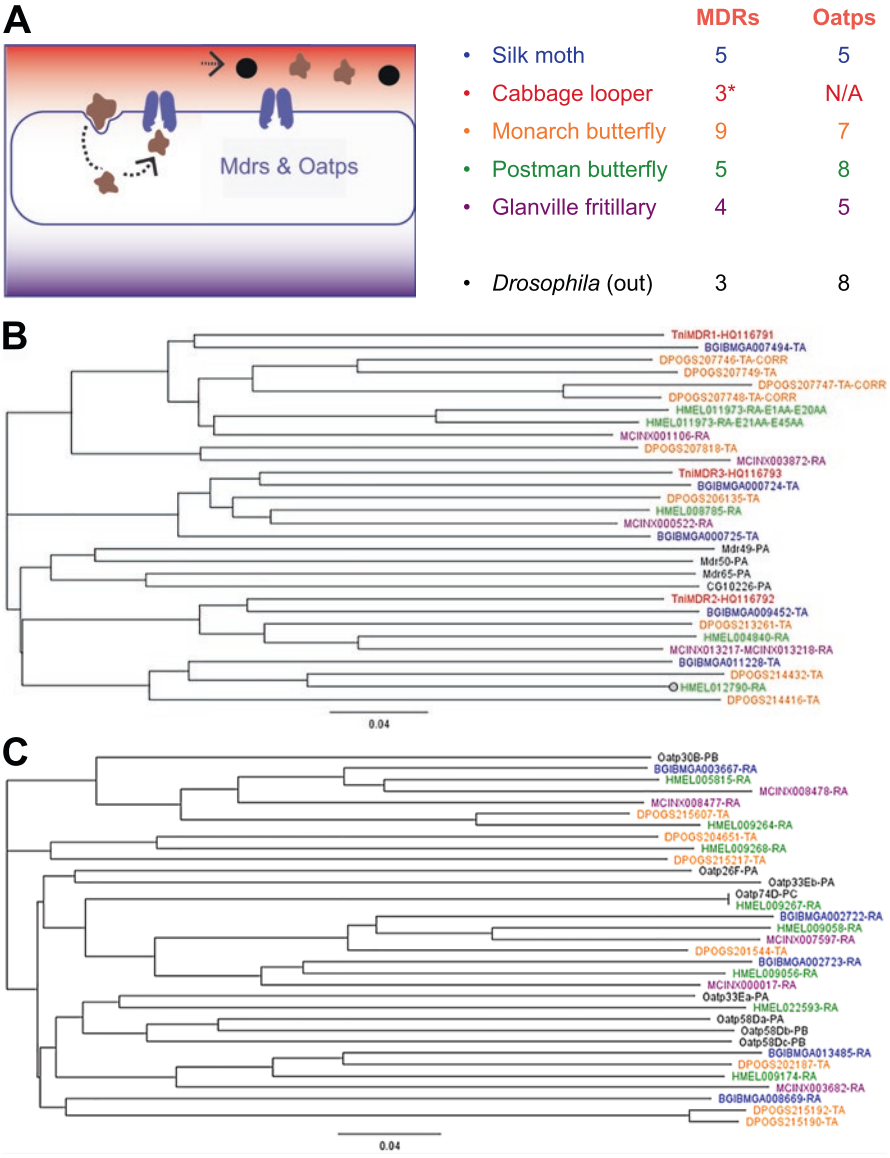

Fig. 3 (**a**) In addition to TSI-conferring substitutions in the Na+/K+-ATPase, monarch caterpillars may resist CG toxicity by excluding CGs (black and brown compounds) from the sensitive nervous tissue by ABC transporters and organic anion transporting polypeptides (purple transmembrane proteins) that are mainly active in the midgut, blood-brain barrier (depicted), and Malpighian tubules. This mechanism is particularly important for protecting the nervous tissue (purple area) against apolar CGs, which can cross membranes passively, by transporting these back into the hemolymph (red area), whereas polar CGs (black) can be kept out to some extent through tight junctions between cells. (**b**) Orthologs of *D. melanogaster* Mdr50 (a B-type ABC transporter) may have experienced a gene bloom in the monarch butterfly (*Danaus plexippus*) relative to the silk moth (*Bombyx mori*), the cabbage looper *Trichoplusia ni*, the postman butterfly (*Heliconius melpomene*), and the Glanville fritillary (*Melitaea cinxia*). The asterisk at the cabbage looper indicates that its genome may encode more than three Mdrs. (**c**) A duplication was also detected for the monarch ortholog of Oatp33Eb (see text for details). (Cartoon by Sophie Zaaijer)

curassavica, accounting for 40% of leaf CGs, and its abundance was negatively correlated with caterpillar growth (Agrawal et al. 2021). It will also be interesting to study Mdrs more closely in caterpillars of species such as *Empyreuma pugione* and *Daphnis nerii*. These species specialize on CG-bearing plants, but do not have known TSI substitutions in their Na+/K+-ATPases. Indeed, in vitro analyses of enzyme activity in the presence of increasing CG concentrations indicate that their Na+/K+-ATPases are highly sensitive to CGs (Petschenka and Dobler 2009; Petschenka et al. 2012, 2013; Petschenka and Agrawal 2015). This sensitivity suggests they may have evolved alternative mechanisms of handling dietary CGs, which may include efflux through Mdrs (Petschenka et al. 2013).

A second class of transporters is formed by the organic anion transporting polypeptides (Oatps). Many of these transporters show strong expression in the BBB and midgut (Hagenbuch and Stieger 2013; Hindle and Bainton 2014), while some are highly expressed in the Malpighian tubules (Torrie et al. 2004). Like Mdrs, the expression of Oatps is coordinated with that of other enzymes involved in xenobiotic detoxification. Besides their role in this process, Oatps are also involved in the metabolism and efflux of endogenous solutes (Dow and Davies 2006). In vitro and in vivo reverse genetic screens on *D. melanogaster* established that a subset of Oatps prevent polar CGs such as ouabain from interfering with Na+/K+-ATPase function (Groen et al. 2017; Torrie et al. 2004). The Oatps provide a baseline level of protection against CGs in insects not specializing on CG-containing diets. These transporters may have provided a substrate for natural selection to work upon in insects that transitioned to feeding on CG-producing host plants (Groen et al. 2017).

Although the Oatp family and the superfamily of solute carrier transporters they belong to, the SLC22 organic cation/anion/zwitterion transporters, underwent an expansion in the Lepidoptera (Denecke et al. 2020), the absolute number of Oatps does not appear to have changed in the monarch (Fig. 3). However, there has been a duplication of the monarch ortholog of *D. melanogaster* Oatp33Eb, and a fly knockout mutant of Oatp33Eb (an Oatp that is typically expressed in the gut system) showed the lowest lethal dose of ouabain of several Oatp mutants compared to wild-type flies (Groen et al. 2017). It will be interesting to find out if these monarch Oatps are indeed involved in dealing with dietary CGs.

Which transporters allow herbivores on Brassicaceae to expel ITCs and other GSL breakdown products has not been determined. However, evidence from biomedical studies suggests that instead of B-type ABC transporters (P-glycoproteins or Mdrs), it is likely the C- and G-type ABC transporters that may be important. Like B-type transporters, the C-type transporters are full ABC transporters with at least 12 transmembrane domains and a nucleotide-binding domain that has ATPase activity (Dermauw and Van Leeuwen 2014). In human cells, Multidrug resistance protein1 (MRP1 or ABCC1) mediates efflux of AITC, BITC, PEITC, and sulforaphane as conjugates with GSH and cysteinylglycine (Callaway et al. 2004; Hu and Morris 2004; Zhang and Callaway 2002), whereas its subfamily relative MRP2 (ABCC2) transports the GSH-conjugated form of PEITC (Ji and Morris 2005a).

Unlike B- and C-type transporters, Breast cancer resistance protein (BCRP or ABCG2) is a half transporter, and besides the nucleotide binding domain with

ATPase activity, it contains only six transmembrane domains. BCRP transports the unchanged form of PEITC, without conjugation to molecules such as GSH (Ji and Morris 2005b). Future functional studies may find out if B-, C-, and/or G-type transporters may be involved in GSL detoxification in caterpillars as well.

Microbial Interactions

With important caveats (e.g., that many caterpillar individuals may lack a resident gut microbiome), microbes associated with caterpillars and their immediate host plants may have important modulating effects on the different mechanisms caterpillars use for dealing with plant defensive chemicals.

Chewing herbivores could benefit from microbes through at least two mechanisms. One is through the sometimes immunosuppressive effects of microbes on the host plant when deposited via oral secretions (regurgitant derived from the foregut) or the saliva (Grant 2006). Experiments with the Colorado potato beetle (*Leptinotarsa decemlineata*) demonstrated that larvae benefitted from the suppressive effects of oral secretions containing *Pseudomonas* and *Enterobacter* spp. bacteria on antiherbivore defenses in one of the host plants, tomato (*Solanum lycopersicum*; Chung et al. 2013). Immunosuppression by bacteria in oral secretions has more recently also been found to occur for *Spodoptera frugiperda* caterpillars, particularly when the herbivores deposited *Pantoea* spp. bacteria on tomato host plants (Acevedo et al. 2017). It is not yet known if bacteria in caterpillar saliva, as opposed to regurgitant oral secretions (Grant 2006), could also influence the outcome of plant-herbivore interactions. However, it is interesting that salivary glands of *Trichoplusia ni* are enriched for a distinct bacterial flora compared to other organs that open directly into the digestive system, including the mandibular glands, the Malpighian tubules, and the midgut itself, and that *Pseudomonas* bacteria were one of the enriched genera (Lawrence et al. 2020).

A second mechanism of microbial effects on caterpillar fitness, and one that has been studied somewhat more extensively, is through modification of plant defensive chemicals by enzymes derived from microbes (Mason et al. 2019a). At an extreme, entire microbes become internalized in herbivore cells in an endosymbiotic relationship. More commonly, however, single microbial genes end up in the herbivore genome through horizontal gene transfer (Hansen and Moran 2014). In this scenario, a microbe-herbivore association becomes fixed and microbe-produced detoxification enzymes are now indirectly derived from microbes (Mason et al. 2019a). This has happened relatively frequently in clades of herbivores such as piercing/sucking insects and chelicerates (Hansen and Moran 2014; Wybouw et al. 2018; Greenhalgh et al. 2020). In the whitefly *Bemisia tabaci*, the herbivore genome even had a host plant-derived phenolic glucoside malonyltransferase gene incorporated that allows detoxification of phenolic glycosides (Xia et al. 2021). Genomic analysis of three lepidopteran herbivores (*Bombyx mori*, *Heliconius melpomene*, and *Danaus plexippus*) revealed that horizontal transfer events had occurred ca. 12 times

per species and that at least some of the genes with putative origins from bacteria or fungi were transferred prior to the formation of many herbivore species (Sun et al. 2013). Several of the genes encode enzymes that are potentially involved in metabolizing amino acids, starch, and sugar, and some might be involved in detoxification of host plant defensive chemicals (Li et al. 2011). In one well-studied example, all lepidopteran genomes examined contain orthologs of bacterial β-cyanoalanine synthase/cysteine synthase (*CAS*/*CYS*) genes, which is probably the result of an ancient horizontal gene transfer event from methylobacteria in the ancestor of all Lepidoptera (Wybouw et al. 2014, 2016). Caterpillars of a variety of species show inducible CAS activity upon encountering plant-produced cyanide in their diet. Functional studies in the Brassicaceae specialist *Pieris rapae* showed that CAS enzymes convert this toxic defensive chemical via a cross-reaction with cysteine into the less toxic products β-cyanoalanine and hydrogen sulfide (Witthohn and Naumann 1987; Meyers and Ahmad 1991; Stauber et al. 2012; Van Ohlen et al. 2016).

Yet, many of the relevant associations between microbes and caterpillars fall toward the more plastic/labile end of the spectrum (Mason et al. 2019a). Unlike herbivores with piercing/sucking mouthparts (Hansen and Moran 2014), caterpillars appear to lack a resident gut microbiome (Hammer et al. 2017). They probably derive a large proportion of their gut microbiome from their diet (Hammer et al. 2017) and may even obtain much of it from the soil (Hannula et al. 2019). In addition to this lack of specificity in caterpillar gut microbiomes, there also remains much to be discovered about whether and how caterpillars may receive benefits from microbes in dealing with host plant defenses (Hammer and Bowers 2015). Observations on fitness outcomes of interactions between caterpillars and internal, non-disease causing microbes show a continuum from positive, to neutral, to negative. Caterpillars of *Anticarsia gemmatalis* showed better survivorship and growth when their gut microbiome was left intact (Visôtto et al. 2009), while suppressing gut bacteria had no detectable effect on fitness in *Manduca sexta* (Hammer et al. 2017). A negative effect of gut microbes was observed in *Spodoptera frugiperda* caterpillars feeding on maize plants. When a defensive protease (Mir1-CP) produced by maize damaged the peritrophic matrix, gut bacteria from the genera *Enterobacter*, *Enterococcus*, and *Klebsiella* then penetrated this protective barrier, invaded the hemocoel, and exacerbated the negative fitness consequences of the maize protease on the caterpillars (Mason et al. 2019b). It will be fascinating to see if such interactive effects of plant defenses and microbial infections occur more generally.

Several studies have assessed mechanisms of how gut microbes may affect detoxification of ITCs and GCs. Although more work on caterpillars is needed, experiments across various species of chewing insects (and humans) have identified bacteria that metabolize these defensive chemicals. Among the gut microbiota of the cabbage stem flea beetle *Psylliodes chrysocephala*, the bacterial genera *Pantoea*, *Pseudomonas*, and *Acinetobacter* were associated with degradation of ITCs (Shukla and Beran 2020). However, only *Pantoea* spp. had measurable effects on ITC detoxification in follow-up experiments (Shukla and Beran 2020), despite the fact that strains of *Pseudomonas* bacteria produce enzymes that detoxify ITCs (Fan et al.

2011) and can suppress plant defenses locally and systemically (Groen et al. 2013, 2016). Separate studies on the human gut microbiome identified that the bacterium *Eggerthella lenta* carries a "CG reductase" operon that metabolizes CGs (Koppel et al. 2018). Taken together, these studies show that gut microbes have the potential to play a role in ITC and CG detoxification, but much more research will be needed to determine if the microbiome may perform similar functions in the guts of caterpillars that feed on toxic host plants.

Ultimate Causes of the Evolution and Maintenance of Detoxification Mechanisms

A salient discussion of the genomic and phenotypic targets of selection associated with how herbivorous insects interact with plant defensive chemicals requires identification of the agents of selection. Selection on insect herbivores is applied by both bottom-up agents (e.g., the host plants that are fed on) and top-down agents (e.g., predators and parasites; Price et al. 1980). Comparison between different species of herbivores and between herbivores and their non-herbivorous relatives can reveal genotypic and phenotypic signatures of selective pressure by each of these agents.

Bottom-Up Agents of Selection

Host plant species are typically polymorphic for the production of defensive chemicals, and the same is true for many counter-adaptations in insects. Such coinciding patterns of trait distributions are hypothesized to be the consequence of coevolutionary dynamics (Flor 1956; Ehrlich and Raven 1964; Karasov et al. 2014; Stahl et al. 1999).

These dynamics can be subdivided into distinct classes according to several criteria, a main one being if dynamics show directionality or whether instead they are fluctuating (Hall et al. 2020; Woolhouse et al. 2002). When directionality is present, the dynamics often resemble "arms races," which may, for example, result in escalation of plant defensive chemical production over generations and counter-adaptations by herbivores (Ehrlich and Raven 1964; Dawkins and Krebs 1979; Kareiva 1999; Van Valen 1973). As part of arms race dynamics, successive selective sweeps are likely to occur, purging alleles that are non-adaptive in the participating species. However, depending on fitness costs associated with evolving traits and the genetic architecture of these traits, polymorphisms can be maintained over short to longer periods of time. When polymorphisms are stably maintained, the dynamics appear as "trench warfare" (Stahl et al. 1999). On the other hand, costs may also drive selection and evolutionary dynamics to fluctuate, favoring different traits or trait values during different episodes of selection (Hall et al. 2020). This could result in

fluctuations in the frequencies of alleles involved in regulating the traits (Speed et al. 2015).

The presence and nature of fitness costs associated with traits under selection can thus play an important role in determining which type of coevolutionary dynamics populations of herbivores and their host plants will follow over time. On the plant side, the production of toxins can be constrained by several different types of costs: 1) opportunity costs may arise if toxin production in early life stages diminishes subsequent plant growth vigor and competitive ability (Coley et al. 1985; Züst et al. 2011); 2) metabolic costs are incurred when toxins are produced (Bekaert et al. 2012; Gershenzon 1994); 3) allocation costs may cause growth and/or reproduction to be reduced when limited resources are spent on toxin production (Simms 1992); 4) toxin production can carry genetic costs depending on the presence and level of genetic correlation with other traits, for example, via genetically hardwired signaling networks (Groen et al. 2020; Züst and Agrawal 2017); and 5) production of toxins effective against one herbivore genotype may have negative fitness consequences on interactions with other genotypes or other species and thus carry ecological costs. For example, producing toxins effective against a generalist herbivore may harm mutualistic interactions with pollinators or increase plant susceptibility to specialist herbivores (Strauss et al. 1999). Although fitness costs have been notoriously difficult to measure (Bergelson and Purrington 1996; Koricheva 2002), it appears that at least in some environmental contexts, GSL and CG production may incur costs to plants (Stowe and Marquis 2011; Züst et al. 2015).

On the herbivore side, the types of costs associated with detoxification can be divided into similar classes. While in plants costs and benefits of toxin production will be influenced by the probability of encountering certain herbivores, costs and benefits of detoxification in herbivores will be influenced by the chance that dietary toxins will be encountered (Després et al. 2007). Perhaps they have not received as much attention from scientists in terms of theoretical framework development and experimental work as the costs on the plant side (Després et al. 2007; Karban and Agrawal 2002).

Behavioral avoidance of toxin ingestion by searching for hosts or tissues with lower toxin levels comes with opportunity costs in the form of spending time searching or actively manipulating the host plant to subvert activation of defenses. These costs will increase as well-defended plants increase in population frequency (Després et al. 2007; Karban and Agrawal 2002). Another set of costs that increase as hosts produce more toxins are the metabolic and allocation costs as herbivores spend energy on detoxification (Després et al. 2007). Costs of handling plant toxins have thus far been established for several toxin-herbivore combinations in the Lepidoptera, including GSLs in *Pieris rapae* and *Helicoverpa armigera* (Agrawal and Kurashige 2003; Wang et al. 2021; Jeschke et al. 2021), nicotine in *Spodoptera eridania* (Cresswell et al. 1992), furanocoumarins in *Depressaria pastinacella* (Berenbaum and Zangerl 1994), and CGs in the monarch (Seiber et al. 1980; Zalucki et al. 2001; Agrawal 2005; Rasmann et al. 2009; Tao et al. 2016; Agrawal et al. 2021).

As a general pattern, herbivores combine several of the mechanisms described in the previous section to deal with plant defensive chemicals: e.g., behavioral

avoidance of toxin ingestion is regularly associated with enzymatic detoxification. The monarch combines laticifer clipping behavior with enzymatic detoxification of and TSI to CGs (Agrawal et al. 2021; Dussourd and Eisner 1987; Marty and Krieger 1984; Seiber et al. 1980), while a generalist herbivore on Brassicaceae such as *Helicoverpa armigera* combines searching for low-level GSL areas of leaves with GSL detoxification via the mercapturic acid pathway (Jeschke et al. 2021; Shroff et al. 2008). However, it is unknown if such trait co-occurrences arise from environment-imposed, phenotypic, or genetic constraints (Després et al. 2007). Theoretical modeling has shown that such combined strategies may confer fitness advantages when traits are associated with ever-rising costs and the probability of ingesting certain toxins is low (Vacher et al. 2005). Genetic costs may be particularly pronounced when TSI-conferring mutations evolve, especially when the target proteins of toxins are active in the nervous system. TSI-conferring mutations can incur costs when they lower the efficiency of a protein in the herbivore (Després et al. 2007). We have observed this in experiments with *D. melanogaster*, when substitutions conferring TSI of the Na+/K+-ATPase to CGs that have evolved in the monarch and other specialists on milkweeds were introduced in flies (Karageorgi et al. 2019; Taverner et al. 2019). While the substitutions heightened insect resistance to CGs, they also appear to have caused pleiotropic nervous system defects. These potential defects were ameliorated through epistasis when accompanied by a facilitating or compensatory substitution near the TSI-conferring substitutions in the first extracellular loop of the Na+/K+-ATPase (Karageorgi et al. 2019; Taverner et al. 2019). Contrary to other toxin resistance traits, the costs of TSI are fixed, i.e., they do not change with the probability of dietary toxin ingestion (Després et al. 2007). However, these costs can be modulated through epistatic interactions with genetic variation elsewhere in the herbivore genome and by environmental fluctuations.

A second general pattern is that costs and benefits of toxin resistance traits in herbivores can be phenotypically plastic. Generalists, and to a lesser extent specialists, are presented with highly variable levels and diverse combinations of toxins both across and within host plant species (Després et al. 2007). The within-species variability is partially under genetic control by the plant and partially by factors such as the plant's phenological stage and fluctuations in biotic and abiotic factors it encounters. To the extent that this is controlled by genetics (Fig. 1a), such variability may be an evolved plant strategy that follows the moving target theory or, perhaps more likely, the optimal defense theory, since it is thought to increase costs for the herbivore to acclimate its gut milieu and other traits as cocktails of dietary toxins change in composition, causing the herbivore population to always be chasing moving fitness optima (Wetzel and Thaler 2016; Li et al. 2020a, b). A study with artificial diets with variable levels of the furanocoumarin xanthotoxin presented to caterpillars of the generalist *Trichoplusia ni* provides support for this notion that toxin level variability suppresses herbivore performance (Pearse et al. 2018).

In response to variable toxin levels in host plants, generalists have evolved toxin-induced avoidance behaviors and both constitutive and induced production of detoxification enzymes (Després et al. 2007). TSI, on the other hand, is typically

restricted to specialist herbivores that use it alongside more generalized toxin resistance mechanisms. The use of more than one resistance mechanism may confer robustness to the efforts of specialists to deal with host plant toxins. This strategy might also prevent specialization from becoming an evolutionary "dead end" if host plant populations dwindle, in which case shifts to novel host plants might be necessary (Termonia et al. 2001).

Examples from specialists on CG-producing plants illustrate how herbivore adaptations to the presence of certain toxins in their host plants may facilitate shifts to other plant species producing those toxins. Our reconstructions of host plant usage of herbivorous insects revealed that in three independent instances among the Coleoptera, Diptera, and Lepidoptera, close relatives of specialists on CG-producing plants in the Apocynaceae were feeding on *Solanum* spp. (Solanaceae) plants (Fig. 4; Begon 1975; Brown 1987; Schoville et al. 2018). Intriguingly, the species feeding on *Solanum* spp. hosts all possess one or more substitutions in the Na+/K+-ATPase that confer TSI to CGs (Karageorgi et al. 2019). While only a subset of Solanaceae plant species are known to produce CGs like the Apocynaceae, Solanaceae produce saponins such as glycoalkaloids and steroidal glycosides (Pomilio et al. 2008), and there is some evidence that these may inhibit Na+/

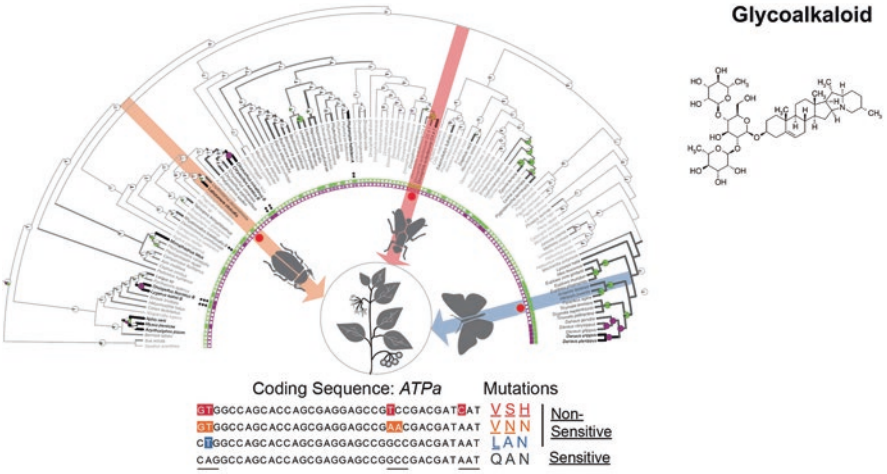

Fig. 4 Mutations in three codons of the Na+/K+ATPase alpha subunit gene *ATPa* (highlighted in the sequences above the sequence of *D. melanogaster* as a reference species without target site insensitivity mutations in the bottom) have evolved at least three times (red dots) in insects from different orders that feed on plant species of the nightshade family (Solanaceae) (center). These insects are weakly or completely non-sensitive to the steroidal toxins that the plants produce. The known species are the nymphalid butterfly *Mechanitis polymnia* (blue, with mutations causing codon changes to amino acids L, A, and N at positions 111, 119, and 122 of the Na+/K+ATPase alpha subunit, respectively, appearing as if the mutations were introduced into the *D. melanogaster* sequence), the "fruit" fly *D. subobscura* (red, with mutations causing codon changes to amino acids V, S, and H), and the Colorado potato beetle *Leptinotarsa decemlineata* (orange, with mutations causing codon changes to amino acids V, N, and N). (Cartoon by Sophie Zaaijer)

K+-ATPase as well (Blankemeyer et al. 1995). This sets up a potential mechanism of cross-resistance that could facilitate host switches between Solanaceae and CG-producing Apocynaceae plants, which may be facilitated further by the activity of conserved, generalized toxin resistance mechanisms such as the expression of multidrug transporters in the midgut and BBB of all of these species (Fig. 3; Dobler et al. 2015; Groen et al. 2017). Indeed, the milkweed butterfly clade (Danainae) is the sister group of Ithomiinae, which are specialists on the Solanaceae. The latter clade includes *Mechanitis polymnia*, which has a somewhat CG-insensitive Na+/K+-ATPase (Petschenka et al. 2013; Karageorgi et al. 2019). It is likely that host switching between Solanaceae and Apocynaceae has occurred (Brown 1987).

Although fluctuating dynamics and "trench warfare" dynamics are yet to be studied in the context of plant-herbivore interactions (Gloss et al. 2013), dynamics that resemble arms races have been examined in several plant-lepidopteran herbivore study systems. Among them are the well-studied interactions between Brassicaceae plants and their herbivore communities, which include *Pieris* spp. (Edger et al. 2015; Griese et al. 2021); between milkweeds and their herbivore communities, including monarch and queen butterflies (Agrawal and Fishbein 2008; Agrawal et al. 2021); and between wild parsnip and the herbivores *Depressaria pastinacella* and *Papilio polyxenes* (Berenbaum and Feeny 1981; Berenbaum and Zangerl 1998).

Potential mechanisms for how arms race dynamics may lead to co-diversification between herbivore and host plant species have been studied in most detail for the pierid butterflies and their Brassicales host plants (Edger et al. 2015). Here we review the herbivore side of these interactions. *Pieris* spp. contain the NSPs, which are part of the NSP-like gene family that also includes the NSP paralog, the major allergen (MA) protein. These proteins are unique to pierids and are related to the single domain major allergen (SDMA) proteins, which are generally expressed in the gut systems of caterpillars (Fischer et al. 2008). Like NSPs, MAs can also disarm the mustard oil bomb (Edger et al. 2015). Based on experimental work and comparative analyses, it appears that the *Pieris* spp. maintain a breadth of potential host plant species while specializing on a smaller subset of hosts through gene duplications and subsequent sub- or neo-functionalization of NSPs and MAs. While NSPs show more stable expression, they have experienced positive selection related to specialization on different host plants with unique GSL profiles (Heidel-Fischer et al. 2010; Okamura et al. 2019a,b). MAs showed GSL-inducible expression but were more evolutionarily stable and are perhaps involved in detoxification of those GSLs that are produced more commonly among the host plants of the Pieridae (Okamura et al. 2019a,b). Like the NSPs, the horizontally transferred CAS/CYS enzymes also underwent further duplication in *Pieris* spp. and other species feeding on cyanogenic plants compared to lepidopteran species not feeding on such plants (Li et al. 2021a, b). This may have further facilitated the ability of *Pieris* spp. to handle the formation of equimolar levels of cyanide upon the breakdown of GSLs to nitriles (Steiner et al. 2018). In particular, the number of BSAS genes encoding the CAS/CYS enzymes showed a stepwise increase as species specialized onto Brassicaceae host plants with BSAS2, which shows high affinity for cyanide, generally present in all Lepidoptera; while BSAS3 is restricted to the Pieridae, and

BSAS1 is restricted even further to the Pierinae (Herfurth et al. 2017). The CAS/CYS enzymes may be complemented in their role of cyanide detoxification by two rhodaneses, which may add robustness to the detoxification process. The rhodanese-encoding genes, *TST1* and *TST2*, differ in their expression, subcellular localization, and kinetic properties and are the result of a rhodanese family expansion in the Pieridae (Herfurth et al. 2017; Steiner et al. 2018).

However, such arms race dynamics between Brassicales specialists and their host plants are not a given: *Plutella xylostella*'s genome encodes three GSSs that stem from duplications of insect arylsulfatases (Heidel-Fischer et al. 2019). Each GSS has distinct expression patterns in response to dietary GSLs and mediates desulfation of different types of GSLs with varying efficiency. Rather than showing signatures of arms race coevolution early after duplication from an arylsulfatase gene and evolving in a stepwise manner, copies of GSS genes neofunctionalized in parallel under positive selection caused by the herbivore's host shift to GSL-producing plants while gaining their novel detoxification functions (Heidel-Fischer et al. 2019).

Interestingly, aside from *D. radiella*, all Lepidoptera in these examples are multivoltine (Hazel 1977; Berenbaum and Zangerl 1991; Brower 1998; Fei et al. 2014; Agrawal 2017; Moranz and Rahman et al. 2019). The herbivores thus have the potential to evolve faster than their host plants, which have no more than one generation per year. This discrepancy sets up an apparent paradox: how are host plants able to prevent losing out in these arms races? A first potential reason might be that defense or, alternatively, loss of susceptibility is relatively more straightforward for the host plant than using a plant as a host is for the herbivore (Thompson 1986). For example, the most abundant sterol in herbivorous insects is cholesterol, but insects rely on plant-produced sterols to synthesize it. Changes in sterol profiles may not have apparent fitness consequences in host plants (Corbin et al. 2001) but could provide effective loss of susceptibility to herbivores, with relative cholesterol levels and larval survival deteriorating the most in a host plant specialist (Jing et al. 2012, 2013). A second potential reason is that escalation of arms races comes with the production of novel defenses by host plants, and being able to combine defensive traits may give plants an evolutionary advantage (Gilman et al. 2012; Speed et al. 2015). A third potential reason is that coevolution can be diffuse. For example, because of its migratory lifestyle, the monarch butterfly encounters multiple species of milkweed hosts. This may pose a limitation to the monarch for evolving more efficient mechanisms of handling the CGs and other toxins produced by any one milkweed species (Agrawal et al. 2021). A fourth potential reason is that herbivores are attacked by natural enemies in the form of pathogens, parasites, parasitoids, and predators, and top-down control by these organisms may dampen the negative effects that herbivore populations may have on host plant populations. It is possible that natural selection becomes less efficient if effective population sizes are reduced. Finally, interactions between the first and third trophic levels lead to trade-offs that prevent herbivores from adapting strictly to plant defenses. We will now take a more detailed look at the effects of these top-down agents of selection on herbivore-plant interactions.

Top-Down Agents of Selection

Organisms that are natural enemies of caterpillars and other lepidopteran life stages not only form independent agents of selection by consuming their prey partially or wholely (Bernays 1997) but also influence caterpillar fitness in conjunction with bottom-up, host plant-derived agents of selection (Bernays and Graham 1988; Lill et al. 2002; Thaler et al. 2012a, b; Kaplan et al. 2014; Singer et al. 2014). For these effects to occur, caterpillars do not need to experience attack directly; even the perceived threat of attack may cause caterpillars, including *Pieris rapae* and the monarch, to become less efficient at dealing with plant defensive chemicals (Lund et al. 2020; Lee et al. 2021). In addition, plant toxin level variability may not only affect herbivore performance from the bottom-up but may influence top-down selection as well. *Trichoplusia ni* caterpillars ingesting higher dietary levels of the furanocoumarin xanthotoxin were attacked at lower rates by the parasitoid wasp *Copidosoma floridanum* (Paul et al. 2020). Interactive top-down and bottom-up effects can even be modulated further by viruses, microbes, and parasites of the natural enemies, showing the ecological complexities (Harvey et al. 2003; Tan et al. 2018).

Among Brassicales specialist herbivores, the effects of plant-produced GSLs and their breakdown products on multi-trophic interactions appear to be species dependent. For example, the performance of an endoparasitoid *Diadegma semiclausum* was negatively correlated with GSL concentrations, as the wasp developed better when caterpillars of its host *Plutella xylostella* were actively detoxifying GSLs via desulfation (Sun et al. 2020). In contrast, the performance of the endoparasitoid *Hyposoter ebeninus* was positively correlated with higher GSL concentrations of the Brassicaceae plants that their hosts, caterpillars of *Pieris rapae* and *Spodoptera exigua*, were feeding on (Kos et al. 2012). The authors speculated that this may have been caused by negative effects of plant GSLs on caterpillar immunity against the parasitoid (see also Smilanich and Muchoney, Chapter "Host Plant Effects on the Caterpillar Immune Response").

Interactive effects between host plant defensive chemicals and the insect immune system were also invoked to explain population-specific patterns of selection on immunity genes in the monarch butterfly (Tan et al. 2019a, b). While the North American population of monarchs predominantly uses the common milkweed *Asclepias syriaca* as larval host plant, caterpillars of monarch populations outside North America typically feed on other milkweed species, including *A. curassavica*. This species and other alternative milkweed hosts outside North America contain higher CG concentrations. Such elevated CG levels are known to affect the success rate of infection by the protozoan parasite *Ophryocystis elektroscirrha* (Sternberg et al. 2012; Gowler et al. 2015; Tao et al. 2016) and may also influence performance of other pathogens, predators, and parasites of the monarch (Brower et al. 1967, 1968). The use of dietary CGs in defense against attack could in principle lead to relaxation of selection on the monarch's immune system genes, especially when their expression is accompanied by costs (de Roode et al. 2013; Gerardo et al. 2010; Parker et al. 2011).

One mechanism by which the monarch and many other specialist herbivores on a variety of host plants minimize fitness losses from attack by natural enemies is through sequestration of plant defensive chemicals (see Bowers, Chapter "Sequestered Caterpillar Chemical Defenses: From "Disgusting Morsels" to Model Systems"). However, sequestration of these chemicals comes with a set of challenges. For chewing herbivores such as caterpillars, this is particularly true in the case of sequestering plant-produced, non-toxic precursor glucoside molecules such as GSLs that are hydrolyzed by plant-derived β-glucosidases upon herbivore feeding. Herbivores would need to leave GSLs intact if they are to evolve GSL storage and the ability to set up their own mustard oil bomb. Perhaps not surprisingly, the first well-studied instance of GSL sequestration was for an aphid species that specializes on Brassicaceae, *Brevicoryne brassicae* (Kazana et al. 2007). As a piercing-sucking herbivore, it can leave at least the aliphatic GSL intact, allowing it to store GSLs in its body. It further produces its own myrosinase enzyme in separate compartments, which is brought in contact with the GSLs upon wounding, thereby effectively setting itself up as a "booby trap" to predators and parasites. However, chewing herbivores, including caterpillars, may not have easy access to this option, given the amount of tissue disruption they bring about. Sequestration of intact and/or modified GSL by chewing herbivores has thus far only been reported outside Lepidoptera: in larvae of the sawfly *Athalia rosae* (Hymenoptera; Müller et al. 2001) and in the flea beetle *Phyllotreta armoraciae* (Coleoptera; Sporer et al. 2021). In the sawfly, GSL breakdown in the gut appears to be prevented by rapid GSL uptake across the epithelium, which may be facilitated by low activity of plant myrosinases in the anterior gut (Abdalsamee et al. 2014). The flea beetle appears to employ similar mechanisms and may have an additional mechanism to reduce activity of plant myrosinases in the gut to trace levels (Sporer et al. 2021). Intriguingly, *P. armoraciae* can supercharge GSL sequestration via 13 putative sugar porters in the major facilitator superfamily (MFS) that import GSLs (Yang et al. 2021a, b). These proteins, dubbed glucosinolate-specific transporters (GTRs), show expression predominantly in the Malpighian tubules, and silencing them via RNAi showed that GTR activity in the tubules enabled the beetles to sequester high GSL levels in their hemolymph (Yang et al. 2021a, b). Characterization of sugar transporters has started in the moths *Bombyx mori* and *Helicoverpa armigera* (Govindaraj et al. 2016; Yuan et al. 2021a, b), and it will be interesting to see their characterization in Brassicaceae-specializing lepidopterans such as *Pieris* spp. and *Plutella* spp. It could also be fruitful to study ABC transporters in caterpillars of Brassicaceae specialists since at least one of these broad-spectrum transporters, the C-type ABC transporter MRP, has already been shown to mediate toxin sequestration in another beetle, *Chrysomela populi* (Strauss et al. 2013).

While Brassicales specialists such as *Pieris brassicae* and *P. rapae* do not appear to sequester intact GSLs (Müller et al. 2003), *P. brassicae* caterpillars do show attack-induced production of an intensely green regurgitant (that likely contains high levels of nitriles), which has been shown to act as a deterrent to *Myrmica rubra* ants. These observations further suggest that nitriles may have a defensive role for *P. brassicae* and could come with adaptive benefits (Müller et al. 2003). Sequestration

of nitriles might even bring more benefits to herbivores in some interactions with natural enemies than the ability to release ITCs. When GSL desulfation in *Plutella xylostella* was disrupted by silencing its *GSS* genes via RNAi, the caterpillars systemically accumulated ITCs (Sun et al. 2019). Not only did the ITCs impair caterpillar development, but the larvae were still efficiently captured and eaten by the lacewing *Chrysoperla carnea*, a predator able to degrade ingested ITCs via the mercapturic acid pathway (Sun et al. 2019).

Specialists on CG-producing plants may have easier paths to evolve sequestration since these dietary toxins come to the herbivores in stable form. A series of different studies over the last 50 years using a variety of approaches have elucidated an important part of the genetic, molecular, and physiological mechanisms underlying CG sequestration in the monarch. Several studies with monarch butterflies reared on milkweeds (including *Asclepias curassavica* and *A. fruticosa* as host plants) demonstrated that the monarch may selectively avoid sequestration of more toxic apolar CGs such as voruscharin, a compound to which its Na+/K+-ATPase is sensitive, despite the monarch's TSI mutations. The monarch preferentially sequesters the less toxic polar CGs calotropin and calactin, compounds to which the TSI mutations provide >50-fold relative increase in resistance (Reichstein et al. 1968; Roeske et al. 1976; Seiber et al. 1980, 1983; Cheung et al. 1988; Groeneveld et al. 1990; Malcolm 1990; Nelson 1993; Malcolm 1995; Petschenka et al. 2018; Jones et al. 2019; Agrawal et al. 2021). The monarch achieves this biased sequestration in part through converting voruscharin into calotropin and calactin via non-enzymatic and enzymatic steps (Agrawal et al. 2021; Marty and Krieger 1984; Seiber et al. 1980) and through transporting CGs via as-of-yet unknown carriers (Frick and Wink 1995). New experimental work should identify these CG carriers in the monarch; past studies have identified a set of candidate carriers. Kowalski and coworkers recently identified that the B-type ABC transporters ABCB1-3 may allow the dogbane beetle *Chrysochus auratus*, a specialist on the CG-producing plant *Apocynum cannabinum*, to sequester calotropin and other CGs (Kowalski et al. 2020). Interestingly, the most efficient transporters of calotropin were ABCB2 and -3, which are most closely related to *D. melanogaster* Mdr50 (Groen et al. 2017). It is precisely in orthologs of Mdr50 that we observed a gene bloom in the monarch genome (Fig. 3). From data produced by several population genetic/genomic studies, it can be observed that the monarch population does not seem to show genetic variation for the TSI mutations (Aardema et al. 2012; Zhan et al. 2014, Pierce et al. 2016), but does show genetic variation for sequestration (Freedman et al. 2020). It will be interesting to see if this genetic variation may be found in and around genes that code for CG detoxification enzymes, CG carriers, and/or other proteins that may be involved in sequestration.

Evolution of the substitutions in the monarch's Na+/K+-ATPase that confer TSI to many, but not all, CGs appears to have followed arms race dynamics (Aardema et al. 2012; Petschenka et al. 2013; Petschenka and Agrawal 2015; Pierce et al. 2016). The latest escalation (at least as far as major effect substitutions in the first extracellular loop are concerned) was the addition of substitution N122H. This step was most likely linked to CG sequestration, rather than merely

coping with the toxins as part of the diet (Petschenka and Agrawal 2015). N122H was not necessary for protecting caterpillars against CG toxicity when toxins were ingested with the diet, but the substitution allowed tolerance of CGs when hemolymph with sequestered CGs from the monarch was injected into the body cavity (Petschenka and Agrawal 2015). Intriguingly, not all CG-sequestering lepidopteran species have evolved accompanying TSI substitutions. For example, larvae of several species of arctiid moths sequester CGs, but their Na+/K+-ATPases do not harbor TSI substitutions (Petschenka et al. 2012; Petschenka and Agrawal 2015). This suggests that costs of N122H and other TSI substitutions may be high and would need to be offset by compensatory mechanisms and/or ecological benefits. Our own and our collaborators' work with *D. melanogaster* has shown that the monarch's TSI substitutions indeed come with substantial costs in the form of imbalances in nervous system functioning (Karageorgi et al. 2019). Exactly how the monarch nullifies these deleterious side effects is unknown, but one mechanism is the evolution of a facilitating substitution in the form of A119S that offsets the negative pleiotropic consequences of N122H (Karageorgi et al. 2019). For sequestration to evolve, other (potential) costs need to be overcome. Agrawal and colleagues recently measured significant CG sequestration costs for monarch caterpillars that were evident in reduced growth rates (Agrawal et al. 2021). Slower growth may have been caused by the burden of energetic costs that selective detoxification and transport mechanisms may incur (Després et al. 2007). Ultimately, the sum total of all costs needs to be lower than the ecological benefits of sequestration in the form of lower predation rates, which will depend on local environmental constraints (Després et al. 2007). Reduced predation due to sequestration is certainly possible for the monarch in at least some locations and conditions as several studies with natural enemies have shown (Brower et al. 1967, 1968), and this fits within a more general pattern that toxin-sequestering specialists are measurably better defended against predation than generalists (Zvereva and Kozlov 2016).

A meta-analysis of 159 publications on the costs and benefits of toxin accumulation in herbivores further revealed that chemical defenses were generally beneficial when herbivores are threatened by generalist predators, but not when threatened by specialist predators or generalist and specialist parasitoids (Zvereva and Kozlov 2016) (see also Singer et al., Chapter "Predators and Caterpillar Diet Breadth: Appraising the Enemy-Free Space Hypothesis"). Furthermore, chemical defenses were more effective against vertebrate predators, particularly birds, compared to invertebrate predators (Zvereva and Kozlov 2016). Studies with different types of natural enemies of the monarch show patterns that are broadly consistent with this (Brower et al. 1967, 1968, 1985; Fink and Brower 1981; Fink et al. 1983; Brower and Calvert 1985; Brower 1988; Glendinning et al. 1988; Glendinning and Brower 1990; Glendinning 1992; Koch et al. 2003; Rafter et al. 2013; Hermann et al. 2019; Stenoien et al. 2019).

One important mechanism through which sequestering species may enhance the benefits of sequestration is evolving aposematism (see also Bowers, Chapter "Sequestered Caterpillar Chemical Defenses: From "Disgusting Morsels" to Model

Systems"). The monarch and other sequestering specialist herbivores have evolved warning coloration as a corollary to their accumulation of protective toxins that serves to advertise the herbivores' toxicity and can prevent attacks from happening, especially when vertebrate predators are a threat (Zvereva and Kozlov 2016). A first population genomic study has identified part of the genetic basis of the monarch's orange-and-black warning coloration (Zhan et al. 2014). Future studies may more fully characterize the genetic architecture of the monarch's CG detoxification- and sequestration-related traits and determine the extent of genetic correlation with its aposematic colorations. In this way, the evolution of the monarch's mechanisms to deal with bottom-up and top-down selection pressures can be understood more completely.

The herbivores on which we have focused, the Brassicaceae specialist pierid butterflies and the milkweed butterflies, and their mechanisms of handling host plant-produced toxins are fitting illustrations of broader patterns concerning the role of defensive chemical detoxification and sequestration for caterpillars to navigate interactions with selective agents at lower and higher trophic levels. A meta-analysis of 112 studies found that effect sizes of top-down selection pressures were generally larger than those of bottom-up selection pressures (Vidal and Murphy 2018). However, for specialist chewing herbivores, this pattern was turned upside down, which suggests that mechanisms such as the sequestration of host plant defensive chemicals in defense against natural enemies could have alleviated top-down selection pressures. An illustration of this pattern was found in a study of the insect community around *Brassica nigra* and *B. oleracea* plants: in this community, where specialist herbivores were more abundant than generalists, bottom-up selection had a larger influence on herbivore abundance than top-down selection (Kos et al. 2011).

Finally, it is interesting to contemplate the role that climate change may play in influencing the ecology and evolution of detoxification phenotypes *sensu lato*. For example, experimental increases in temperature raised cardenolide levels in foliage of *A. curassavica*, a species now widespread in the southern USA, that may be causing a reduction in the proportion of migrating monarchs (Faldyn et al. 2018). There is some concern that, owing to the fitness reduction monarchs experienced when feeding on plants grown in experimentally warmed conditions, these butterflies could become caught in an ecological trap. Adult female monarchs in the southern USA prefer to oviposit on *A. curassavica*, and as the climate warms, so too should cardenolide levels rise in these plants. Although higher cardenolide levels tend to enhance protection from natural enemies, there are also costs to sequestration, and overall, this could reduce average fitness of monarchs in these populations. Unconsidered by Faldyn et al. (2018) is the potential role for an evolutionary response in such scenarios. Adaptation in the populations of monarchs facing higher cardenolide concentrations owing to warming conditions could produce any variety of adaptations, including reduced preference for *A. curassavica*, mitigation of the higher cardenolide levels physiologically, and/or increased resistance or tolerance of cardenolides that are particularly toxic. On the other hand, higher temperatures directly reduce fitness as well (York and Oberhauser 2002). This one example

highlights the difficulty in predicting the impacts of climate change at the plant-insect nexus. More research in this area is certainly needed, especially in the area of adaptation per se.

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