Tansley review

Toxic cardenolides: chemical ecology and coevolution of specialized plant–herbivore interactions

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Contents

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Summary

Cardenolides are remarkable steroidal toxins that have become model systems, critical in the development of theories for chemical ecology and coevolution. Because cardenolides inhibit the ubiquitous and essential animal enzyme Na^+/K^+-ATPase, most insects that feed on cardenolide-containing plants are highly specialized. With a huge diversity of chemical forms, these secondary metabolites are sporadically distributed across 12 botanical families, but dominate the Apocynaceae where they are found in > 30 genera. Studies over the past decade have demonstrated patterns in the distribution of cardenolides among plant organs, including all tissue types, and across broad geographic gradients within and across species. Cardenolide production has a genetic basis and is subject to natural selection by herbivores. In addition, there is strong evidence for phenotypic plasticity, with the biotic and abiotic environment predictably impacting cardenolide production. Mounting evidence indicates a high degree of specificity in herbivore-induced cardenolides in *Asclepias*. While herbivores of cardenolide-containing plants often sequester the toxins, are aposematic, and possess several physiological adaptations (including target site insensitivity), there is strong evidence that these specialists are nonetheless negatively impacted by cardenolides. While reviewing both the mechanisms and evolutionary ecology of cardenolide-mediated interactions, we advance novel hypotheses and suggest directions for future work.

I. Historic background and introduction

Plant produced cardenolides are a group of remarkable chemical compounds (Fig. 1, Box 1), at once responsible for the poisoning of livestock and the treatment of countless people with congestive heart failure. These same compounds have been applied to poison arrows in human warfare and mediate antagonistic ecological interactions between plants and herbivores. Cardenolides are a group of diverse steroids derived from triterpenoids, almost certainly evolved as a defense, primarily in plants (but also in animals). In turn, specialist insects are known to sequester cardenolides from plants (history reviewed by Malcolm, 1995). Because cardenolides have a very general and potent mode of action against animals, and their likely role in the narrowing of the diet breadth of hundreds of species of specialist insect herbivores, they have been a model in chemical ecology research for nearly 50 yr. In particular, there is no known or even hypothesized 'primary' function of cardenolides in terms of plant resource acquisition and allocation, and thus they make an excellent model of 'secondary' compounds to study.

Here we will primarily focus on cardenolide ecology and their effects in the new world genus *Asclepias* (Apocynaceae), as much of the chemical ecology research to date has concentrated on this group. Carl Linnaeus named this plant genus of some 130 milkweed species after the Greek God of healing *Asklēpiós*, son of Apollo, because of its long use as a medicinal group of plants. The historical development of *Asclepias* spp. and monarch butterflies (*Danaus plexippus*) as a textbook case of the intersection of biology and chemistry began with a call by E. B. Poulton to explain the unpalatability of monarchs in 1914 (elaborated by Malcolm, 1995). Since then, tremendous progress has been made in identifying cardenolides in plants and butterflies during the 1960s (Brower *et al.*, 1967; Reichstein *et al.*, 1968), discovering the diversity of compounds in insects and plants in the 1970s (Brower *et al.*, 1972; Duffey & Scudder, 1972; Roeske *et al.*, 1976), and detailed analyses of their allocation in plants and insects in the 1980s (Nelson *et al.*, 1981; Brower *et al.*, 1984; Malcolm *et al.*, 1989). Finally, the 1990s saw a major breakthrough with the discovery of a molecular mechanism by which monarchs have reduced sensitivity to cardenolides (Holzinger *et al.*, 1992; Holzinger & Wink, 1996). Cardenolides continue to be widely studied in several laboratories across the globe, with major thrusts toward the understanding of plant defense and multitrophic interactions, applications in agriculture and medicine, and mechanisms of binding to the sodium–potassium pump.

II. Diversity of cardenolide forms

Cardenolides are a chemical class within the cardiac glycosides, a group comprising two main classes of compounds which differ in the structure of their aglycone (Box 1, Fig. 1). Cardenolides have a five-membered lactone group in the β position at C17, whereas the other group, the bufadienolides, first discovered as skin poisons in toads, have a six-membered lactone ring at this position (Fig. 1). Plants can produce both cardenolides and bufadienolides, as do various species of animals such as toads (Bufonidae) and insects (Lampyridae and Chrysomelidae) (Van Oycke *et al.*, 1987; Krenn & Kopp, 1998; Luckner & Wichtl, 2000). The minimum structure of cardenolides that is necessary

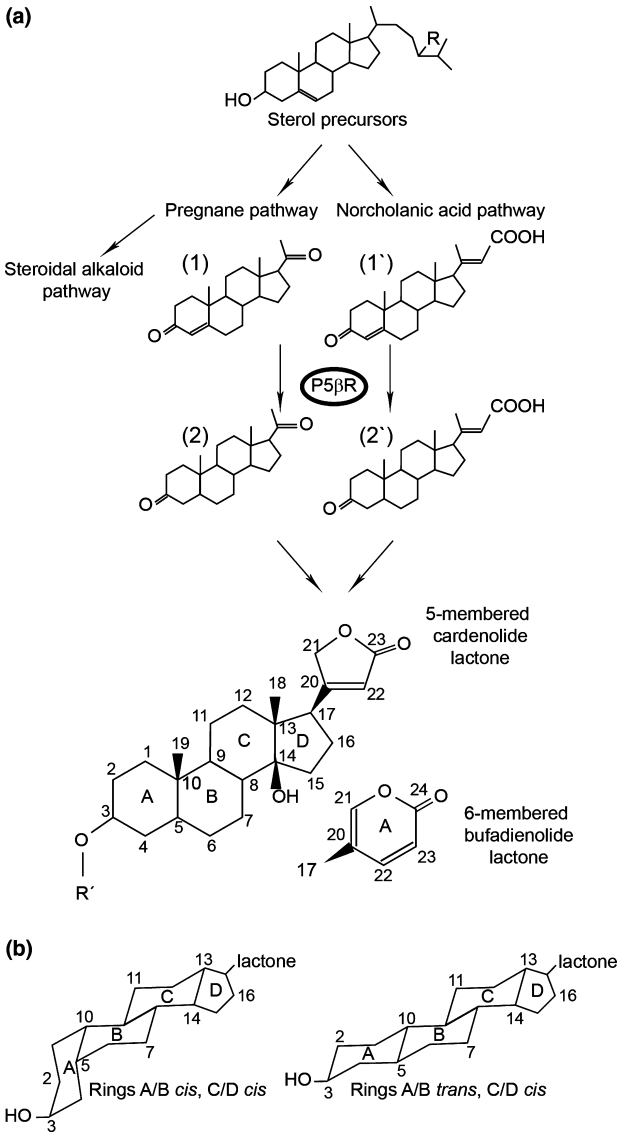

Fig. 1 Schematic of cardenolide production from phytosterols and steroidal structures (based on Malcolm, 1991; Bauer *et al.*, 2010). (a) Two putative pathways are shown leading to the production of cardenolides. Pregnane glycosides and steroidal alkaloids are additional endpoints which are also likely to be defensive. Circled is the enzyme progesterone 5b-reductase (P5bR), a key enzyme involved in the stereo specific reduction of the $\Delta^{4,5}$-double bond of a steroid precursor to produce 5b-Cardenolides (Bauer *et al.*, 2010). At the bottom, the skeleton structure of a cardenolide is shown, composed of the core steroid (four fused rings), the lactone group, and a glycoside group typically attached at position 3 (R′). Bufadienolides have a six-membered lactone group at position 17 instead. R′ represents one or more sugars in glycosides or H in genins. (b) *cis* (e.g. of strophanthidin- and digitoxigenin-based cardenolides) and *trans* (e.g. of uzarigenin-based cardenolides) configurations between A and B rings. The *cis* configuration is shown as bent and *trans*-configuration as flat. 1 = progesterone, 2 = 5b-pregnane-3,20-dione, 1′ = 23-nor-4,20(22)E-choladienic acid-3-one, 2′ = 23-nor-5b -chol-20(22)*E*-enic acid-3-one.

Box 1 Definitions of chemical terms relevant to cardenolides.

Cardenolide:	a 23-carbon structure with three components: a steroid backbone structure of four fused C rings, a five-membered lactone group (a butenolide) at C-17, and a carbohydrate or sugar moiety attached to C-3 of the first carbon ring (Fig. 1).
Bufadienolide:	compounds that differ from cardenolides only in that they are characterized by a six-membered lactone ring at C-17. Both types of compounds are produced in plants and animals.
Genin/ Aglycone:	the backbone steroid and lactone group of a cardiac glycoside; variation in this backbone is typically associated with altered number or positions of OH groups (CH3-groups, epoxides and others). In the strict sense, genins are not cardiac glycosides because the latter require one or more sugar moieties.
Cardiac glycoside:	the parent group of compounds, including both cardenolides and bufadienolides, typically containing structural groups derived from sugars.
Pregnane:	steroids with four fused rings and carbons present at positions 1–21 and potential precursors to cardenolides. Pregnane glycosides occur alongside cardenolides in many Apocynaceae.
Steroidal alkaloid:	alkaloids are typically alkaline compounds containing a nitrogen atom as part of the carbon ring. Steroidal alkaloids found in the Apocynaceae typically contain the backbone steroid structure of four fused C rings in addition to carbon rings containing the nitrogen; however, unlike most alkaloids, the nitrogen is not obtained from amino acids, and thus are sometimes called pseudoalkaloids.

for receptor recognition (and therefore toxicity to animals) is the steroid nucleus 5β,14β-androstane-3β14-diol (Repke, 1985). Lactone rings and sugar moieties (the glycoside side chains) are dispensable structures for biological activity (Glynn, 1957; Repke, 1985), but are nonetheless predicted to play a primary role in the interaction with Na^+/K^+-ATPase (Paula *et al.*, 2005).

Cardenolides from the former family Asclepiadaceae, now subsumed in the Apocynaceae, possess features that distinguish them from others in the Apocynaceae and *Digitalis* spp. (Plantaginaceae). Typically among *Asclepias* there is a *trans* configuration of rings A and B in the steroidal skeleton (whereas there is a *cis* configuration in other cardenolides) and a cyclic bridge to a single sugar moiety (Fig. 1; Malcolm, 1991). Chromatographic analysis of leaf and root extracts of > 50 *Asclepias* showed a high diversity of separation patterns of individual cardenolides along a polarity gradient (Fig. 2). Interestingly, across the genus, it appears that plants favor the production of either relatively more apolar or more polar compounds, but avoid having cardenolides with midpolarities (Fig. 2). Additionally, species having higher concentrations of total cardenolides tend to have fewer polar compounds on average, thus having a smaller number of polar cardenolides overall (Rasmann & Agrawal, 2011a). In evolutionary terms, we have hypothesized that more toxic species not only have high concentrations of cardenolides, but also more apolar forms, which are more easily absorbed in the insect hemolymph (Rasmann & Agrawal, 2011a) (Table 1).

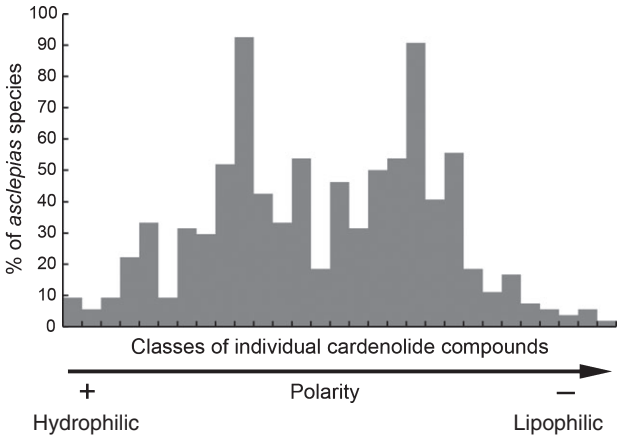

Fig. 2 Frequency distribution of cardenolides collected from 52 *Asclepias* and 2 *Gomphocarpus* species. Shown is the percent of plant species producing each cardenolide, with identifications binned by polarity. Polarity was estimated by high performance liquid chromatography (HPLC) analyses as the retention time before elution from the column with a gradient of increasingly nonpolar solvent. Although originally 82 individual cardenolide peaks were identified, to reduce errors in judgment when differentiating individual peaks, here, each distribution class is the sum of three consecutive peaks (29 total categories). The key result is the typical bimodal distribution of cardenolides in *Asclepias* species. Data from Rasmann & Agrawal (2011a).

III. Biosynthesis

The C23 steroids containing a butenolide ring at C-17 (5β-cardenolides) are derived from mevalonic acid via phytosterol and pregnane intermediates (Fig. 1). The reaction converting sterols into 5β-cardenolides such as digitoxigenin was summarized recently by Bauer *et al.* (2010). Pregnenolone is thought to be formed by a mitochondrial cytochrome P450-dependent side chain cleaving enzyme (P450scc, CYP11A in animals) (Lindemann & Luckner, 1997), although no evidence of such a P450 has been found in plants so far (Ohnishi *et al.*, 2009). Subsequently, pregnenolone has to be modified via several steps and condensed with a C2 unit to yield the 5β-cardenolide genin (digitoxigenin, Fig. 1). The most common sequence of the individual biosynthetic steps leading to 5β-cardenolides is not yet clear and more than one pathway may be operative (Maier *et al.*, 1986; Kreis *et al.*, 1998). Indeed, it was suggested that a metabolic 'grid' rather than a pathway should be used to imagine cardenolide biosynthesis (Kreis & Müller-Uri, 2010). As mentioned earlier, although mostly recognized as typical plant compounds, cardiac glycosides may also be produced via the cholesterol pathway in animal tissues. Functioning as mammalian hormones (Schoner, 2002), they are often structurally identical to those found in plants, demonstrating high amounts of convergent evolution.

In *Digitalis*, cardenolides can be interconverted between the primary (with glucose at the end of the sugar chain, the form principally stored in the cell vacuoles) and the secondary type (without glucose) (Wojciechowski, 2003). Secondary cardenolides are glucosylated by cytoplasmatic glucosyltransferases and actively transported into the vacuole by a primary glycoside-translocase (Luckner & Wichtl, 2000). The glucose attached

Table 1 Plant and environmental factors that impact the toxicity of cardenolides (after Rasmann & Agrawal, 2011a)

Factors influencing cardenolide toxicity	Resulting effect	Evidence
Concentration in leaves	Dose-dependent effects, even on specialist insects. Adaptation to cardenolides (target site 'insensitivity') typically only reduces the binding affinity of the toxin to the target	Zalucki *et al.* (2001); Agrawal (2004); Rasmann *et al.* (2009b); Green *et al.* (2011)
Concentration in latex	Rapidly mobilized high dose delivered to the point of attack.	Agrawal & Konno (2009)
Inducibility	Dose-dependent effects, with initial feeding by the herbivore resulting in greater concentrations of the toxic compounds (with specific toxic compounds being selectively induced)	Rasmann *et al.* (2009b); Bingham & Agrawal (2010)
Diversity of compounds	Chemical mixtures that are difficult to deal with; also a diversity of cardenolide structures may impact the active uptake in sequestering species	Detzel & Wink (1995)
Polarity	Less polar cardenolides are more able to cross membranes	Malcolm (1991); Rasmann *et al.* (2009b)
Structure of aglycone/genin	Different substitution patterns result in different inhibition of the Na^+/K^+-ATPase	Hoch (1961); G. Petschenka & S. Dobler, (unpublished)
Stucture of glycoside	Sugars attached to the aglycone/genin (Fig. 1, Box 1) may enhance inhibition of the Na^+/K^+-ATPase	Hoch (1961); G. Petschenka & S. Dobler, (unpublished)
Nutritional environment	When cardenolides are coupled with low nutritional quality, herbivores may experience greater toxicity per unit growth	No tests for cardenolides
Temperature	Binding of cardenolides to Na^+/K^+-ATPase is temperature-dependent	No tests for cardenolides
Formulation	Physical environment modulates absorption	No tests for cardenolides

Note that insect adaptation and processing of cardenolides are not indicated here, but are outlined in the text and Fig. 7.

could enhance the polarity of the cardenolide and therefore prevent its passive efflux out of the vacuole. In *Digitalis*, two enzymes have been shown to control this glucosidation, being principally responsible for the transport of cardenolides across cell membranes (Kreis & May, 1990). The conversion of primary to secondary cardenolides can thus explain their presence in various tissues of the plants such as the latex of all milkweeds (Agrawal *et al.*, 2008) and the nectar of some milkweeds (J. S. Manson *et al.*, unpublished).

IV. Cardenolide variation among plant parts

Although best studied in leaves, cardenolides can occur in all plant tissues. Work on foxglove indicates that cardenolides are stored in cell vacuoles (Löffelhardt *et al.*, 1979; Kreis *et al.*, 1990; Kreis & May, 1990), but this has not been confirmed in Apocynaceae. Comparative studies across different plant parts show tissue specificity in cardenolide expression in Zingiberaceae (Afolabi *et al.*, 2011), Apocynaceae (e.g. Isman *et al.*, 1977a; Malcolm, 1991; Abe & Yamauchi, 1994; Rasmann & Agrawal, 2011a), and Liliaceae (Schrutka-Rechtenstamm *et al.*, 1985). Indeed, there is tremendous variation in cardenolide concentrations of even closely allied plant parts. For example, among stem parts (epidermis, cortex, vasculature, and pith), cardenolide concentrations vary over fivefold in *A. syriaca* (Fordyce & Malcolm, 2000). Within seeds of *A. syriaca*, cardenolides are most concentrated in the embryos, the parts on which Lygaeid seed predators feed (Vaughan, 1979). Nonetheless, latex appears to be where plants concentrate cardenolides to the highest degrees. Zalucki *et al.* (2001) found up to 90 times higher concentrations of cardenolides in latex than in leaves of *A. humistrata*. In *A. eriocarpa*, relative concentrations of four cardenolides increase from roots to leaves to stems to latex (Nelson *et al.*, 1981).

Despite strong variation among plant parts, there are some conserved patterns for concentrations of cardenolides in different plant tissues. Seiber *et al.* (1982) found a positive correlation between concentrations of cardenolides in leaves and latex across

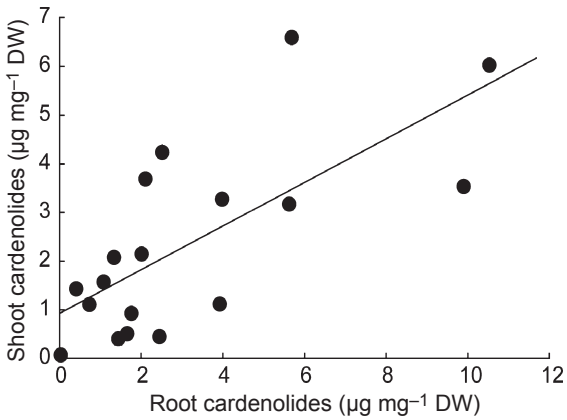

Fig. 3 Relationship between root and shoot cardenolides. Shown are the total concentrations of cardenolides in roots and shoots across 18 *Asclepias* species. Data from Rasmann & Agrawal (2011a). The line shows a strong correlation in cardenolide production across species (n = 18, r = 0.701, P = 0.001).

seven *Asclepias* species. Similarly, leaf and nectar cardenolides were positively correlated across 12 species of *Asclepias* (J. S. Manson *et al.*, unpublished). And finally, Rasmann & Agrawal (2011a) found a positive relationship between leaf and root cardenolides in 18 species of *Asclepias* (Fig. 3). Nonetheless, across the 18 species, we found that only 5% of the specific cardenolide peaks were shared between the above- and below-ground tissues, suggesting highly independent regulation among plant parts. Nelson *et al.* (1981) also found differences in the polarity and structure of individual compounds in roots, leaves, and stems of *A. eriocarpa*. Whether this is due to different selection pressures by herbivores is an open question. Although it is highly probable that plants can differentially regulate cardenolides to plant parts, there may be physiological and developmental constraints as well. For example, latex, which typically contains highly concentrated cardenolides, does not contribute to the cardenolides of roots or nectar in *Asclepias*.

V. Phylogenetic distribution of cardenolides

The > 500 characterized cardiac glycosides (cardenolides and bufadienolides) are endogenously produced in *c.* 60 genera and 12 families of the angiosperms (Singh & Rastogi, 1970; Kreis & Müller-Uri, 2010). A survey of the literature suggests that cardiac glycosides have a higher prevalence in phylogenetically younger angiosperm orders, including the rosids (Crossosomatales, Myrtales, Celastrales, Malpighiales, Fabales, Rosales, Brassicales, Malvales) and the asterids (Gentianales, Lamiales, Solanales, Asterales). About half of the genera reported so far belong to the Gentianales, including the cardenolide-rich Apocynaceae. Nonetheless, cardiac glycosides have been reported from monocots (Poales, Asparagales, Liliales) as well as from basal eudicots (Ranunculales), suggesting some disjunction in the phylogenetic distribution (summarized from Krenn & Kopp, 1998; Luckner & Wichtl, 2000). Unfortunately, little is known about the distribution of species with cardenolides within phylogenetic groups. For example, among the legumes (Fabaceae), reviews have frequently only cited two genera as containing cardenolides (usually in the seeds) (Malcolm, 1991; Wink *et al.*, 2009). However, a recent literature search indicates that at least 12 genera contain cardenolides, and these span nearly all legume tribes (A. A. Agrawal, unpublished).

Given their wide distribution across the angiosperms, cardenolides are a striking example of convergent evolution. Bauer *et al.* (2010) attempted to infer phylogenetic conservatism and homology of cardenolide biosynthesis in the angiosperms using enzymatic analyses. Early steps in the biosynthesis of 5β-cardenolides from sterols include the production of progesterone, which is mediated by the enzyme progesterone 5β-reductase (P5βR) (Gartner *et al.*, 1990; Gartner & Seitz, 1993) (Fig. 1). When comparing plant species belonging to five different angiosperm orders (Brassicales, Gentianales, Lamiales, Malvales and Solanales), 5β-reductase (P5βR) orthologs were present in both cardenolide-free and cardenolide-producing species, demonstrating that the enzyme is conserved and widespread. This pattern suggests that cardenolide biosynthesis could represent an ancient pathway, with multiple evolutionary losses and reversals. Additionally, substrate conversion analyses revealed low specificity in the production of the progesterone precursor (Bauer *et al.*, 2010). These findings highlight a possibly dormant pathway in noncardenolide-producing plants, potentially explaining their sometimes idiosyncratic distribution (i.e. in *Erysimum* (Brassicaceae), Rodman *et al.*, 1982; Hugentobler & Renwick, 1995).

Our recent phylogenetic analyses of cardenolides have yielded insights into previously untested hypotheses about the evolution of plant defense (Rasmann & Agrawal, 2009). For example, we have found evolutionary trends (declines) in the production of cardenolides as *Asclepias* diversified (Agrawal & Fishbein, 2008; Agrawal *et al.*, 2009a). These declines were associated with adaptive radiation, as species-rich lineages of *Asclepias* underwent a proportionately greater reduction in cardenolides (quantitatively and qualitatively) relative to species-poor lineages. The rate of trait change was most rapid early in the diversification of the genus (Agrawal *et al.*, 2009a), and we found evidence of macroevolutionary coordination in allocation to different biochemical pathways (Agrawal *et al.*, 2009b). This last point was exemplified by positive correlated evolution between cardenolides and flavonoids, which are potentially linked via the acetate-malonate pathway (Groeneveld *et al.*, 1990b).

1. Distribution of cardenolides and alkaloids in the Apocynaceae

The Apocynaceae has classically been considered a toxic plant family. The family is distributed worldwide and contains over 400 genera. Given that cardenolides are best known and most widely distributed in the Apocynaceae, we took advantage of the well-developed phylogenetic and chemical information to assess the phylogenetic distribution of cardenolides in the family. Because alkaloids represent another well-known and potent group of secondary plant compounds in the Apocynaceae, we reconstructed evolutionary patterns of both alkaloids and cardenolides (Fig. 4). In particular, most of the alkaloids in the Apocynaceae have a steroidal backbone, although other forms, including indole, phenanthroindolizidine, pregnane, pyridine, pyrrolizidine, and vinca type alkaloids, can be found.

We constructed a 'mega-tree' (Webb & Donoghue, 2005) of the Apocynaceae using a set of published molecular phylogenies for different sections of the family and mapped on the distribution of cardenolides and alkaloids (Fig. 4). Our analysis revealed a broad taxonomic distribution of both alkaloids and cardenolides in the Apocynaceae. In particular, 75% of the surveyed genera contained alkaloids while 27% contained cardenolides. In the majority of genera, cardenolides occurred in genera that also had alkaloids, with only five genera having cardenolides alone. The phylogenetic distribution of toxins (reconstructed using maximum parsimony methods) suggests that both cardenolides and alkaloids originated several times independently in Apocynaceae (Fig. 4). While alkaloids appear also to have been lost several times, cardenolides showed a maximum of two losses, assuming all species in the genus that have the toxins are monophyletic.

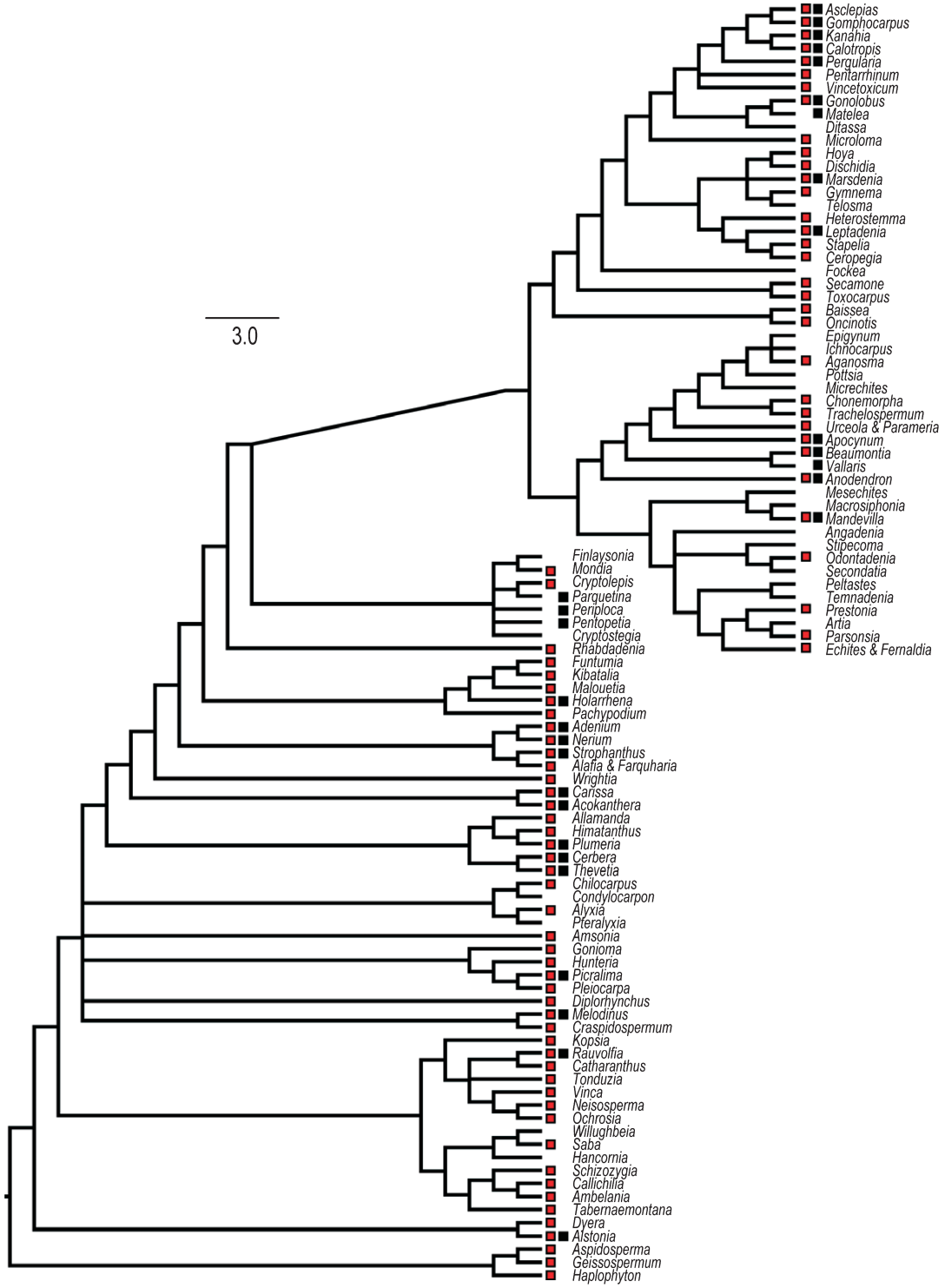

Fig. 4 A mega-tree (Webb & Donoghue, 2005) cladogram of the Apocynaceae with the presence of cardenolides (black squares) and alkaloids (red squares) displayed next to genera for which they have been reported in the literature. The mega-tree is simply a compilation of published phylogenies, stitched together to provide a broad representation of relationships across the family. Here, the molecular phylogeny of Livshultz *et al.* (2007) was used for the backbone, and resolution and new taxa information were added from four independent phylogenetic studies (Ionta & Judd, 2007; Rapini *et al.*, 2007; Simoes *et al.*, 2007; Livshultz, 2010). The long offset branch near the top of the phylogeny is simply for compacting the figure. The original mega-tree contained 170 genera (*c.* 40% of the family), and was subsequently pruned to include only genera with sufficient information on the presence or absence of cardenolides and alkaloids (113 genera). The Apocynaceae contains a diversity of different alkaloid types (not distinguished in the figure). In order to determine which genera to include, we conducted bibliographic searches (using Google Scholar and Web of Science) with the following keywords singly paired with the genus name: cardenolide, cardiac, alkaloid, pregnane, and terpene. We excluded genera for which there was no bibliographic information; genera were labeled as 'absences' when cardenolides and alkaloids were searched for but not found, or when published information about phytochemistry was available but no information about alkaloids or cardenolides was presented. Our approach relies on a lack of bias in the literature for testing plant species for the presence of these phytochemicals.

VI. Geographic distribution of cardenolides

Cardenolides have been found in plants growing in a wide range of habitats, across six continents, and in tropical and temperate regions. However, few surveys have compared the prevalence of cardenolides across geographic regions. It has long been hypothesized that plants from lower latitudes consistently experience greater amounts of herbivory and therefore should evolve to invest in higher levels of defenses than related plants from higher latitudes. In the only phylogenetic test of this hypothesis to date, we studied 49 species of *Asclepias* for geographic clines in the production of cardenolides (Rasmann & Agrawal, 2011a). Indeed, we found that *Asclepias* species from tropical regions are better defended by cardenolides than more temperate species. In particular, the inducibility of cardenolides (i.e. the amount increased in response to insect herbivory) was significantly correlated with latitude, with higher inducibility evolving in the tropics. We also investigated other aspects of cardenolide chemistry and found that the production of cardenolides showed positive correlated evolution with cardenolide diversity; that greater cardenolide investment by a species was accompanied by decreased chemical polarity; and that instead of trading off, constitutive and induced cardenolides showed positive correlated evolution. Analyses of shoot cardenolides (and their induction by monarch butterfly caterpillars) were concordant with root cardenolides (and their induction by specialist *Tetraopes* beetles) for these patterns (Rasmann & Agrawal, 2011a). Thus, milkweed species from lower latitudes appear better defended with higher inducibility, greater diversity, and lower polarity of cardenolides. This pattern was not biased by evolutionary history and appears to have repeatedly evolved in *Asclepias*.

Within species, patterns of defense investment across geographical clines have also been suggested (Schemske *et al.*, 2009). In a survey of natural populations of *A. syriaca*, Malcolm (1995) found an east–west gradient in production of cardenolides, but this relationship was relatively weak. A reanalysis of these data, split by an east–west and north–south transect, showed some evidence for a cline of increasing cardenolides at higher latitudes (Hunter *et al.*, 1996), a pattern opposite to that predicted and found across *Asclepias* species. To address this same cline in plant cardenolide production, we established a field common garden experiment with collections from 22 populations of *A. syriaca* spanning over 10 degrees of latitude (from New Brunswick, Canada to North Carolina, USA, Woods *et al.*, 2012). Such a common garden analysis allows for the assessment of genetically based differences among populations. While populations significantly varied in cardenolide concentrations (ranging from 0.138 to 0.597 μg mg^{-1} dry mass in the first year of growth and 0.811 to 1.82 μg mg^{-1} dry mass in the second year of growth) and the specific peaks present (Table 2), we did not find strong evidence for a cline in cardenolide production. In the first year (2008) we reported a marginal population-level correlation of latitude with cardenolides ($n = 22$, $P < 0.1$, higher total cardenolide concentrations at higher latitudes), but we found no relationship in the second year. Nonetheless, we did find a cline in latex exudation (again, higher amounts at higher latitudes), and given that cardenolides are highly concentrated in latex (Seiber *et al.*, 1982), they may indirectly contribute to higher resistance at high latitudes. Together, these data suggest that defense within *A. syriaca* shows a gradient (higher defense at high latitudes) counter to that found across *Asclepias* species (higher defense at low latitudes).

VII. Ecological genetics of cardenolide production

Within a species, heritable variation in cardenolide production has been most extensively studied in the common milkweed *A. syriaca*. We have found cardenolides to be heritable when we measured the number of distinct cardenolides, concentrations of the three most commonly appearing cardenolides, and total concentrations across several experiments (Table 2). In addition, these results were robust to measures of leaves and roots, and tissues from seedlings and more mature plants (Table 2). Within-population genetically based variation usually spans two- to threefold differences in total cardenolides, and this has been replicated in other laboratories and *A. syriaca* collections from distinct geographic regions (e.g. Vannette & Hunter, 2011a). The three most consistently observed cardenolide peaks in *A. syriaca* (Table 2) appear to be coregulated. For example, when we reanalyzed the dataset from Bingham & Agrawal (2010), the three most abundant peaks were consistently positively phenotypically correlated ($n = 289$, $Ps < 0.001$, $r \approx 0.40$ for each of the three correlations, with no interactions with treatment – Expt 5 in Table 2). There is no evidence for tradeoffs in the production of different cardenolides. In genetic analyses (on 20 family means), only two peaks (5.2 and 18.4) were positively correlated, and only in herbivore-damage treatments ($n = 20$, $Ps < 0.02$, $r \approx 0.57$ in the monarch or *Euchaetes* treatments, but no other genetic correlations between the peaks were significant – Expt 5 in Table 2). Thus, the environment (i.e. herbivore damage), at least somewhat dictates cardenolide expression.

VIII. Environmental regulation of cardenolide production

There is a history of experiments and controversial theory associated with the impacts of resource availability on allocation to defense in plants (Koricheva, 2002). Although there are limited data, evidence from several different systems indicates that abiotic conditions do, indeed, impact the production of cardenolides. For example, in *Digitalis lanata*, exposure of plants to CO_2 enrichment (from 350 to 1000 ppm) enhanced foliar cardenolide concentration by 60–80%, while water stress (an 86% reduction in leaf water potential) resulted in a 20–30% decline in cardenolides (Stuhlfauth *et al.*, 1987). This effect was reversed for *A. syriaca*, with CO_2 enrichment (from 385 to 770 ppm) either reducing cardenolides (in two genotypes) or having no effect (in three genotypes) (Vannette & Hunter, 2011a). In a wild mustard (*Erysimum cheiranthoides*) which produces two cardenolides (erysimoside and erychroside), nitrogen fertilization resulted in reduced cardenolide concentrations (Hugentobler & Renwick, 1995). Finally, in experiments with *Asclepias curassavica*, there was some indication that N : P : K fertilization reduced cardenolide production while ozone fumigation enhanced cardenolides

Table 2 A summary of seven experiments designed to examine the genetic and environmental impacts on cardenolide production in common milkweed *Asclepias syriaca*

	N	Total # of peaks	Total cardenolides	5.2 min	13.7 min	18.4 min
(1) 22 populations from across eastern North America in a field common garden						
Population	130	*	*	*	*	*
Range		1–7	0.1–1.5	0–0.9	0–0.5	0–0.3
(2) Induction experiment with 10 genetic families in a growth chamber						
Genetic family	166	*	*	*	*	*
Induction treatment (three herbivores + control)		*	*	ns	*	*
Range		5–9	0.6–2.0	0–2.3	0–0.6	0–0.1
(3) Induction and light intensity experiment with 10 genetic families in the field						
Genetic family	153	*	*	ns	*	*
Induction treatment (monarch + control)		*	ns	ns	ns	ns
Shade treatment		*	ns	ns	*	ns
Induction by shade interaction		ns	ns	ns	ns	ns
Range		1–6	0–1.7	0–1.1	0–0.3	0–0.3
(4) Paired comparison of natural field plants in full sun or deep shade at 24 sites						
Shade environment	48	ns	*	*		ns
Site		ns	*	ns		*
Range		1–7	0.4–3.7	0.2–2.3	Absent	0–0.1
(5) Induction experiment with 20 genetic families in the field						
Genetic family	289	*	*	*	*	*
Induction treatment (two herbivores + control)		ns	*	*	*	*
Family × induction interaction		*	*	*	ns	*
Range		3–10	0.1–4.3	0–0.9	0–0.6	0–0.4
(6) Root induction experiment with six genetic families (three high and three low) in the field						
Genetic family	89	*	*	*		*
Induction treatment (*Tetraopes* + control)		ns	*	ns		ns
Family × induction interaction		*	ns	ns		†
Range		1–9	0.1–1.2	0–0.2		0–0.3
(7) Root induction experiment with 11 genetic families in a growth chamber						
Genetic family	93	*	*	*	*	*
Induction treatment (*Tetraopes* + control)		ns	ns	ns	ns	*
Family × induction interaction		†	*	ns	*	*
Range		3–11	0.2–2.1	0–0.1	0–0.2	0–0.3

Results shown are for the number of distinct cardenolides peaks, total cardenolides, and the three cardenolide peaks (identified by their respective retention time in a 35 min high-performance liquid chromatorgraphy run) that were in nearly all samples. The range of cardenolide values is given across all samples in $\mu g\ mg^{-1}$ dry mass. Across all experiments, the peak at 5.2 min was 33%, at 13.7 min was 6%, and at 18.4 min was 11%, of the total sample, respectively. *N* indicates sample size for each experiment. Chamber experiments 2 and 7 were conducted on seedlings (typically 5 wk of age); field experiments 1, 5, and 6 were on first-year plants grown from seed, typically 7–10 wk of age; field experiment 3 was conducted on second-year plants early in the growing season; and field experiment 4 was conducted on mature plants in natural populations. Induction typically increased the diversity and concentration of cardenolides, while shading typically decreased the diversity and concentration of cardenolides. Results are indicated as: *, $P < 0.05$; †, $P < 0.1$; ns, $P > 0.1$. Data from experiments 2–4 are unpublished studies from A. A. Agrawal's laboratory, while experiment 1 is from Woods *et al.* (2012), 5 is from Bingham & Agrawal (2010), and experiments 6 and 7 are from Rasmann *et al.* (2011).

(Bolsinger *et al.*, 1991; Couture *et al.*, 2010). Despite inconsistencies in the direction of effect, it is clear that environmental conditions do strongly impact cardenolide production.

To generally assess the impact of plant fertilization on foliar cardenolides, we conducted an experiment on 12 closely related *Asclepias* species (Fig. 5). N : P : K fertilization decreased cardenolide concentrations (on a dry mass basis) by 45%, but this effect was highly variable among species (species by fertilization interaction: $F_{11,141} = 2.286$, $P = 0.013$, Fig. 5). If cardenolide concentrations are calculated on a fresh mass basis, however, this interaction term is no longer significant ($P = 0.318$), and the overall impact of fertilization (decreasing cardenolide concentrations by 47%) is consistent across all species (suggesting complex interactions with water content among species) (A. A. Agrawal, unpublished). Reduced light environments (i.e. shade imposed by trees in the field) have the same impact as fertilization on cardenolides for *A. syriaca* (Table 2, A. A. Agrawal *et al.*, unpublished). Assessed on a fresh or dry mass basis, naturally or experimentally shaded plants had 21% lower cardenolides than plants in the full sun.

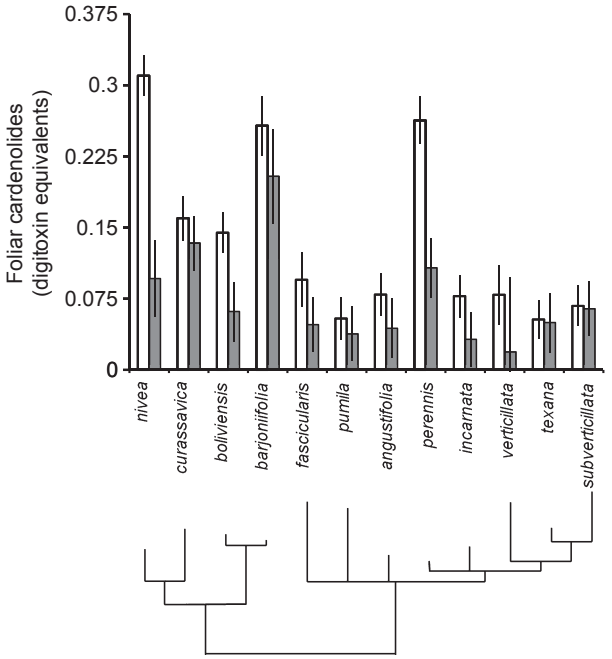

Fig. 5 Impact of fertilization on mean ± SE foliar cardenolide concentrations in 12 *Asclepias* species (Series Incarnatae, molecular phylogeny shown below species names) (A. A. Agrawal, unpublished). Plants were grown from seed in a common growth chamber for 1 month and fertilized plants were given a weekly application of N : P : K 21 : 5 : 20, 150 ppm N (fertilized, closed bars; control, open bars). In this analysis, the species × fertilization interaction was significant, concordant with a lack of a response in some species. Cardenolide analysis was conducted using a spectrophotometric analysis (Agrawal, 2005).

These results on the abiotic regulation of cardenolides raise two important and general issues for the study of plant defense against insects. First, environmental manipulations can have profound impacts on foliar traits, such as water content, which will have an impact on the concentration of toxins experienced by herbivores. Although secondary compounds like cardenolides are often reported on a dry mass basis for empirical convenience, it is unclear what the most relevant measure is in terms of plant allocation and the toxins experienced by herbivores. Indeed, this warrants further investigation. Secondly, manipulations such as fertilization can have a strong impact on plant nutritional quality. In our experiment (Fig. 5), as in others (Couture *et al.*, 2010) fertilization decreased the carbon : nitrogen ratio of leaves by over 50% (averaged across all species), mostly via increased N in fertilized plants. Thus, fertilized plants have leaves with twice the nutrients and half the toxins of unfertilized leaves, and hence a remarkably low toxin to nitrogen ratio, potentially providing a very high-quality resource for herbivores. Such impacts appear to have dramatic effects on multitrophic interactions (Couture *et al.*, 2010; Mooney *et al.*, 2010). Shaded leaves also show higher leaf nitrogen and lower cardenolides than leaves in full sun. Thus, experiments specifically manipulating the environment and measuring multiple plant traits and insect bioassays would be especially informative. In the context of adaptive defense, the regrowth ability of such plants may also be highly informative, as under some conditions plants may favor growth (or tolerance of herbivory) over resistance.

IX. Biotic induction of cardenolides

Most of the work on cardenolide induction has been on the common milkweed *A. syriaca*. Malcolm & Zalucki (1996) provided the first suggestion that herbivore damage may induce elevated concentrations of cardenolides, and in their initial study, mechanical damage (hole punches) was imposed on field plants of *A. syriaca*, and a time course of cardenolide concentrations was followed over 6 d and compared with the initial measure (at time zero). In the 24 h following damage, cardenolides increased to nearly threefold constitutive concentrations, but relaxed to control values 5 d later (Malcolm & Zalucki, 1996). However, this remarkably fast induction (evidenced within 10 min) was likely the effect of increased latex flow to the areas that were damaged, as cardenolide synthesis could not have occurred so quickly. In addition, work discussed later in this review shows that mechanical damage typically induces a more distinct (and much weaker) effect on cardenolides than real herbivore damage. Fordyce & Malcolm (2000) showed that *A. syriaca* naturally attacked by the stem weevil *Rhyssomatus lineaticollis* had lower, and more nonpolar, cardenolides compared with unattacked plants. Whether weevils induced this difference or it was related to their selection of host plants has not been resolved, but either cause would be quite interesting. These enticing suggestions of cardenolide induction, coupled with strong ecological impacts of initial herbivory on subsequent damage (i.e. induced resistance) and insect community structure on milkweeds (Van Zandt & Agrawal, 2004a,b; Agrawal, 2005), paved the way for more detailed studies of induced cardenolide responses.

Very small amounts of herbivory by monarch caterpillars (i.e. < 5% leaf tissue loss) consistently result in substantial increases in foliar cardenolides of *A. syriaca* (Table 2, Mooney *et al.*, 2008; Bingham & Agrawal, 2010; Vannette & Hunter, 2011a). Cardenolide induction is associated with an endogenous jasmonate burst (Agrawal, 2011) and can be triggered by exogenous jasmonate application (Mooney *et al.*, 2008; Rasmann *et al.*, 2009b). This induction is systemic, typically ranges from a 15 to a 30% increase, and has been observed as soon as 3 d following damage. It is unclear how long elevated concentrations of cardenolides last, although cardenolide induction has been observed as long as 10 d after the imposition of herbivory. Mechanical leaf damage does not induce changes in cardenolides, at least when measured several days after treatment (Mooney *et al.*, 2008).

Among leaf-chewing herbivores tested (two beetles and two lepidopterans), there was little specificity in cardenolide elicitation, but one herbivore, adult *T. tetraophthalmus*, failed to cause induction with leaf damage (Mooney *et al.*, 2008; Bingham & Agrawal, 2010). Nonetheless, *T. tetraophthalmus* larvae induced cardenolide production (alongside induced volatile organic compounds) in *A. syriaca* roots (Rasmann *et al.*, 2011), suggesting that different plant tissues may have divergent induction responses. There is also strong evidence that *A. syriaca* genotypes exhibit differential induction. Finally, induction responses can

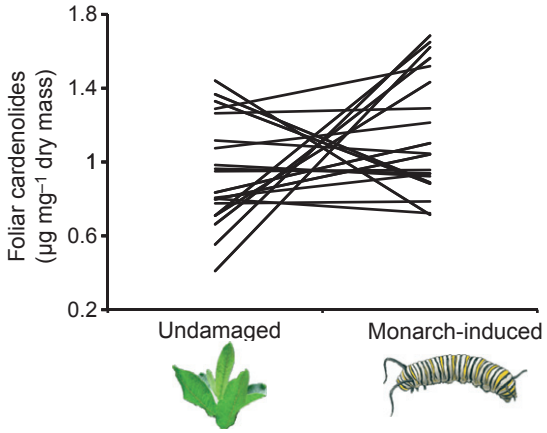

Fig. 6 A tradeoff among constitutive and induced cardenolides among 20 full-sibling families of *Asclepias syriaca* from a single population in Ithaca, NY (USA) (data from Bingham & Agrawal, 2010). Although there was a 25% overall increase in cardenolides in monarch-damaged plants, genetic families varied significantly in their induction. In particular, it appears that *A. syriaca* can produce a maximum concentration of cardenolides, and this is achieved through different genotypic strategies. The fact that the highest constitutive cardenolide genotypes decrease concentrations following damage is surprising, but has been consistently observed in our experiments (Bingham & Agrawal, 2010; Rasmann *et al.*, 2011; A. A. Agrawal, unpublished).

vary across closely related species, as monarch-induced cardenolides have now been studied in some 50 species of *Asclepias*, and induction is consistently higher in tropical milkweeds than in their close relatives from higher latitudes (Rasmann *et al.*, 2009a; Rasmann & Agrawal, 2011a).

Theories of plant defense predicted that constitutive and inducible resistance should negatively covary within and across species (Koricheva *et al.*, 2004). In both above- and below-ground tissues, we have repeatedly found a negative genetic correlation between constitutive and induced cardenolides of *A. syriaca* (Bingham & Agrawal, 2010; Rasmann *et al.*, 2011; and two unpublished studies from A. A. Agrawal's laboratory) and a positive association between constitutive cardenolides and induction across species (Rasmann *et al.*, 2009a; Rasmann & Agrawal, 2011a). It appears that *A. syriaca* genotypes produce a maximum concentration of cardenolides that is modulated by different genotypic strategies: increasing low concentrations following damage, maintaining intermediate concentrations following damage, or decreasing cardenolide concentrations after damage (Fig. 6). Among *Asclepias* species, species have evolved the ability to produce different amounts of cardenolides (Agrawal *et al.*, 2009a,b), and as total cardenolide production has changed, so too has inducibility in a proportional manner. This pattern suggests that species variation in production of cardenolides is greater than variation in allocation patterns to constitutive and induced resistance, leading to the hypothesis that tradeoffs between constitutive and inducible cardenolides may be present within each species (Agrawal *et al.*, 2010).

1. Induction caused by aphids

Aphids on milkweeds appear not to cause cardenolide induction in the same way that chewing herbivores do. There is no evidence for induction of cardenolides in *A. syriaca* by *Aphis nerii* (Zehnder & Hunter, 2007; Mooney *et al.*, 2008) or *A. asclepiadis* (A. A. Agrawal, unpublished, $F_{2,76} = 1.126$, $P = 0.330$). Martel & Malcolm (2004) studied induction by *A. nerii* on *A. curassavica* over 5 d of feeding. Compared with uninfested controls, plants with relatively few aphids (one to 20) showed a > 50% decline in cardenolide concentrations. Nonetheless, at higher aphid densities, cardenolides increased almost twofold compared with controls. Thus, *A. curassavica* appears to have a density-dependent reduction followed by induction of cardenolides in response to *A. nerii* feeding. This induction was consistent across five daily samples and was systemic throughout the plant, although cardenolides were, on average, 30% higher on infested leaves compared with paired uninfested leaves on the same plant. In a longer-term experiment with *A. nerii* on *A. viridis*, Zehnder & Hunter (2007) reported a 43% decline in cardenolides from low- and high-density treatments. Both of these studies also examined induction in *A. incarnata*, but found very low cardenolide concentrations, and little impact of aphid feeding (Martel & Malcolm, 2004; Zehnder & Hunter, 2007). Thus, aphids appear to have impacts on the cardenolides of milkweeds, but their effects are not straightforward. In addition to induction, aphids can cause dramatic impacts on plant water relations and amount of foliar carbohydrates, which could indirectly impact cardenolide concentrations.

2. Microbial induction of cardenolides

In both *Digitalis* and *Asclepias*, preliminary work indicates that root infection affects foliar cardenolides. For example, Manero *et al.* (2003) showed that infection of *D. lanata* roots by *Bacillus* spp. bacteria induced both growth and cardenolides in above-ground tissues. For *A. syriaca*, root infection by two mycorrhizal fungal species (*Scutellospora pellucida* and *Glomus etunicatum*) yielded divergent responses: the former enhanced plant growth and foliar cardenolides, while the latter had no effect (Vannette & Hunter, 2011b). Root infection with a mixture of four *Glomus* strains caused *A. cryptoceras* to significantly increase concentrations of total root cardenolides, although this effect was not found in 13 other *Asclepias* species (S. Rasmann & R. L. Vannette, unpublished). These results highlight two major research gaps. First, cardenolide induction should be examined within plant modules (above ground or below ground) to assess the potential for adaptive cardenolide-mediated responses to infection, especially in response to established mutualistic or pathogenic microbes. And secondly, to our knowledge, there are no studies of induced resistance to microbes in cardenolide-containing plants (and hence, no information on the independent and joint action of cardenolides with more commonly studied antimicrobial agents; across 14 species of *Asclepias*, Rasmann & Vannette also did not find a relationship between cardenolide concentration and average numbers of arbuscules in roots). However, given that cardenolides appear to have activity against bacteria, fungi, viruses and protozoa (Jacobsohn & Jacobsohn, 1985; Akhtar *et al.*, 1992; Lefevre *et al.*, 2010; Bertol

et al., 2011), further investigations of cardenolide-mediated interactions between plants and microbes is certainly warranted.

X. Mode of action and toxicity of cardenolides

1. Specificity of cardenolide action

Unlike many other toxic plant compounds, cardenolides act in a highly specific manner, as they are specific inhibitors of the ubiquitous animal enzyme Na^+/K^+-ATPase, a cation pump which actively transports Na^+ out of the cell and K^+ into it. The Na^+ and K^+ gradients established by Na^+/K^+-ATPase are important for essential physiological processes such as maintenance of membrane potentials and secondary active transport (Jorgensen *et al.*, 2003). The specific binding site for cardenolides is located on the α-subunit of the enzyme. Cardenolides bind from the extracellular side with the lactone ring deeply inserted into the transmembrane domain and with the sugar moiety facing the extracellular medium (Ogawa *et al.*, 2009; Yatime *et al.*, 2011). Binding of ouabain to Na^+/K^+-ATPase relies on hydrogen bonds between amino acids of the protein and hydroxyl groups of the cardenolide (De Pont *et al.*, 2009). However, van der Waals and electrostatic interactions are also likely to play a role in binding of cardenolides to Na^+/K^+-ATPase (Paula *et al.*, 2005).

2. Physicochemical factors influencing cardenolide toxicity

Physicochemical properties of the diverse cardenolide forms such as polarity impact direct gut uptake, distribution in the body, and excretion. Moreover, it is likely that the form in which the toxin is administered (i.e. in leaf pieces, latex, phloem sap, etc.) will also impact toxicity because intestinal absorption is biased by interactions between cardenolides and other molecules in the gut solution. For example, cardenolides which are entangled in pieces of chewed leaves must be extracted and therefore potentially leave the gut in feces without exerting a toxic effect. The matrix in which the toxin is delivered also affects toxicity: for example, a high oil content in the food may facilitate the uptake of lipophilic compounds (Duffey, 1980). Indeed, midgut absorption should also be influenced by the physical form in which a cardenolide molecule reaches the gut epithelium (e.g. solution, emulsion) (see Duffey, 1980 for further physicochemical factors).

The chemical environment is also likely to influence cardenolide toxicity. For example, it has been known for some time (e.g. Glynn, 1957) that the inhibitory effect of cardenolides is antagonized by K^+ ions. Because lepidopterans (and perhaps other phytophagous insects) often have very high K^+ concentrations in their hemolymph (Florkin & Jeuniaux, 1974), cardenolide toxicity on insects may be less acute than predicted by their chemical attributes alone (Vaughan & Jungreis, 1977).

3. Physiological factors influencing cardenolide toxicity

If absorption of cardenolides in insects is a carrier-mediated process, as has been suggested for sequestering species like *D. plexippus* and *Oncopeltus fasciatus* (Detzel & Wink, 1995; Frick & Wink, 1995), the uptake of a single cardenolide form into the body cavity is likely to be influenced by other cardenolide forms (e.g. by competition due to the affinity of a hypothetical carrier being higher to one cardenolide than to another cardenolide form, see Frick & Wink, 1995). As in vertebrates or nematodes (Broeks *et al.*, 1995; Yao & Chiou, 2006), the insect midgut probably contains efflux carriers like p-glycoprotein (Murray *et al.*, 1994; G. Petschenka & S. Dobler, unpublished; Lanning *et al.*, 1996). This carrier is known to transport cardenolides such as digoxin in mammals (Mayer *et al.*, 1996) and it is plausible that it forms an active efflux barrier in the insect midgut, which may prevent cardenolides from entering the body cavity (Fig. 7). Such transport may represent an additional cost of cardenolides for herbivores.

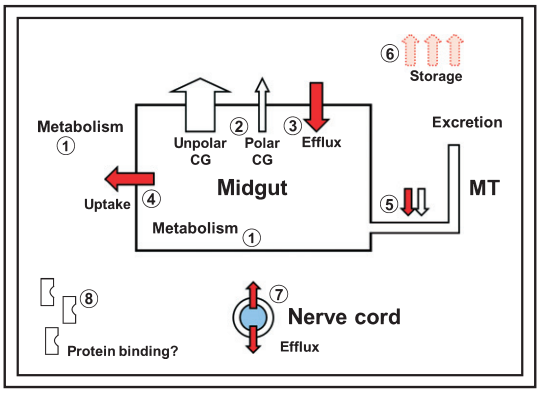

Fig. 7 The possible fate of cardenolides (CG) in insects. Shown are active processes (solid red arrows), potentially active processes (dotted red arrows), and passive processes (clear arrows) for cardenolide transport across membranes. The outer frame represents the insect integument. (1) Ingested cardenolides may be metabolized during their gut passage or within the body cavity (Seiber *et al.*, 1980; Abe *et al.*, 1996). (2) Nonpolar cardenolides can more easily (and passively) cross the gut membrane than polar forms. (3) Cardenolides which enter gut cells may be removed by efflux carriers like p-glycoprotein (PGP), whose occurrence in the lepidopteran midgut has been suggested (Murray, 1996; Lanning *et al.*, 1996). (4) Active carrier-mediated transport of cardenolides via the gut membrane in sequestering species like *Danaus plexippus*, *Syntomeida epilais* and *Oncopeltus fasciatus* (von Nickisch-Rosenegk *et al.*, 1990; Detzel & Wink, 1995; Frick & Wink, 1995). (5) Reduction or regulation of hemolymph cardenolides could be achieved by excretion via the Malpighian tubules (MT). Excretion of ouabain can occur either actively or passively (Rafaeli-Bernstein & Mordue, 1978; Meredith *et al.*, 1984; Torrie *et al.*, 2004). (6) Sequestration of cardenolides into the integument (*D. plexippus*, Brower & Glazier, 1975) or into the extradorsal space (lygaeid bugs, Scudder *et al.*, 1986) might reduce hemolymph concentrations. (7) Access of cardenolides to nerve cord Na^+/K^+-ATPase (light blue) may be prevented by the perineurium (G. Petschenka & S. Dobler, unpublished), which constitutes a diffusion barrier for polar cardenolides and which contains efflux carriers for extruding nonpolar cardenolides. (8) A further unconfirmed possibility is that stored hemolymph cardenolides bind to proteins, affecting their toxicity.

4. Molecular diversity of cardenolides and toxicity

Although it is known that structurally different cardenolides exert quantitatively different toxic effects (e.g. Rasmann *et al.*,

2009b), the precise structure–function relationships have not been well studied in insects (although they are known for vertebrates). For example, considering the steroid nucleus, the configuration of the A/B and C/D ring junctions (Fig. 1) is likely important for toxicity. Whereas the *cis* configuration at the C/D ring junction is mandatory, the *cis* configuration at both junctions renders cardenolides most toxic. Where cardenolides possess a *trans* configuration at the A/B junction (which are most widely distributed among *Asclepias* species) there is a predicted weaker toxicity (Hoch, 1961). In an ecological sense this might imply that *Asclepias* cardenolides are less toxic than cardenolides originating from other plant families. In addition, cardenolide (but not bufadienolide) monosides (i.e. having one sugar) are usually more active than their genins or biosides (i.e. having two sugars). Successive removal of terminal glucose from bi- or triosides can lead to a stepwise increase in toxicity (at least in mammals) (Hoch, 1961). Removal of sugar (β-glucose) is reported to occur in the arctiid *Syntomeida epilais* (Black, 1976) and the monarch butterfly (as well as glucoside formation, Seiber *et al.*, 1983) and might represent a detoxification mechanism.

XI. Direct and indirect effects of cardenolides on specialists and generalist insect herbivores

A given cardenolide form is predicted to exert differential toxicity depending on the way it is delivered. For example, the cardenolide ouabain is reported not to be absorbed in the midgut of locusts and cockroaches and therefore might not produce a toxic effect if administered orally. Nonetheless, body cavity injection of minute amounts (*c.* 1/150 of the amount introduced into the midgut for the locust and *c.* 1/1000 for the cockroach) can be lethal (Scudder & Meredith, 1982; Moore & Scudder, 1986). As discussed later, injections avoid gut processing and selective absorption (see also Table 1), and thus we do not consider studies that have measured cardenolide toxicity by injecting insects in this review. One of the relatively few reports of acute intoxication of herbivorous insects by orally delivered cardenolides is the study by Dussourd & Hoyle (2000), who demonstrated that *Trichoplusia ni* caterpillars suffered from ingestion of cardenolide solutions or latex of *A. curassavica*. Chronic toxicity upon oral exposure was also shown by Karowe & Golston (2006) and Rasmann *et al.* (2009b), when the cardenolide digitoxin was fed to caterpillars of *Lymantria dispar* (a generalist) or *D. plexippus* (a specialist), respectively. Individually isolated cardenolides from *Anodendron affine* (Apocynaceae) reduced growth of the silkworm, *Bombyx mori*, when fed in artificial diets (Fukuyama *et al.*, 1993). Cohen (1983) found no effect of the cardenolide digitoxin when painted on low cardenolide leaves and fed to fifth instar monarchs. Nonetheless, two nonadapted noctuids, the fall armyworm (*Spodoptera frugiperda*) and the velvetbean caterpillar (*Anticarsia gemmatalis*), were negatively impacted by digitoxin in growth and survival, respectively (Cohen, 1983).

Cardenolides have also been demonstrated to deter feeding, typically in nonadapted insects using laboratory choice tests. For example, cabbage loopers (*T. ni*) were deterred in feeding by two cardenolides (digitoxin and cymarin) and the larvae did not habituate to these compounds (Akhtar & Isman, 2003). Each of six cardenolides from *Gomphocarpus sinaicus* and *Pergularia tomentosa* deterred feeding by *Spodoptera littoralis* (Green *et al.*, 2011), while two cardenolide standards, digoxin and digitoxin, were not deterrent. *Pieris rapae* larvae, which specialize on Brassicaceae, were deterred by the cardenolides found in the treacle mustard, *Erysimum cheiranthoides* (Sachdev-Gupta *et al.*, 1993). For monarch butterfly caterpillars, choice tests revealed no deterrence by digitoxin and ouabain, but cymarin was somewhat deterrent (Vickerman & de Boer, 2002). Finally, some generalist predators of cardenolide-containing insects (including spiders and mice) are deterred by the compounds (Malcolm, 1989; Glendinning, 1992; Petschenka *et al.*, 2011). In summary, despite high degrees of variability, a wide range of animals show preingestive (gustatory) sensitivity to cardenolides.

Several studies have taken an indirect or correlative approach to examine the impacts of cardenolides on insects under relatively natural conditions. In two field observational studies of *D. plexippus* on *Asclepias humistrata*, caterpillar survival was negatively correlated with foliar cardenolide content (which varied nearly fourfold among individual plants) (Zalucki *et al.*, 1990; Zalucki & Brower, 1992). Additionally, in two separate experiments, differences in cardenolide content (between leaves of different ages or leaves that had severed or intact laticifers) were associated with differences in larval performance (Zalucki & Brower, 1992; Zalucki *et al.*, 2001). In particular, this latter result suggests that cardenolides were causally linked to insect performance. In a field common garden with 23 genotypes of *A. syriaca*, *D. plexippus* caterpillars grew more slowly on higher cardenolide genotypes than those on lower cardenolide genotypes; the amount of latex exudation and cardenolide concentration in leaves were not correlated in this study (Agrawal, 2005). Across genotypes of *A. syriaca* and 18 *Asclepias* spp., higher concentrations of cardenolides resulted in higher mortality for the specialist root herbivore, *T. tetraophthalmus* (Rasmann *et al.*, 2011; Rasmann & Agrawal, 2011b).

Effects of cardenolides on Hemiptera appear to be more variable. When *O. fasciatus* is reared on different seed collections of *A. syriaca* or on seeds of different *Asclepias* spp., its body cardenolide content proportionally reflects the seed cardenolide content; nonetheless, in neither case was there any correlation between cardenolide concentration and measures of insect performance (Isman, 1977; Vaughan, 1979). Using 18 species of *Asclepias*, and controlling for other factors such as leaf water content, nitrogen, and trichomes, Agrawal (2004) found that population growth rate of the aphid *A. nerii* (a specialist sequestering insect) was negatively correlated with cardenolides. Given the several other hemipteran species that specialize on *Asclepias* spp. with different life history strategies, much work could still be done to compare their induction, sequestration, and tolerance of cardenolides (Mooney *et al.*, 2008; Smith *et al.*, 2008).

XII. Cardenolides and insect oviposition

All the data on cardenolides and adult insect choices come from work on butterflies, primarily from work on *D. plexippus*. In

correlative analyses, Zalucki and colleagues conducted painstaking field observations of oviposition and compared the cardenolide content of leaves that received eggs with those that were assessed, but not chosen, for oviposition. For example, in field populations of *Gomphocarpus fruticosus*, Oyeyele & Zalucki (1990) reported that *D. plexippus* oviposited on plants that were, on average, 20% lower in foliar cardenolides than the overall population average; the bulk of the eggs (70%) were laid on low-cardenolide plants. This pattern mirrors data for *D. plexippus* on *A. humistrata* in three separate datasets, again with butterflies choosing relatively low-cardenolide plants (Cohen & Brower, 1982; Zalucki *et al.*, 1990; Zalucki & Brower, 1992). For *A. curassavica*, butterflies also preferred to lay on lower cardenolide individuals, and this relationship was linear (Zalucki *et al.*, 1990). Thus, although patterns of oviposition have often been interpreted as monarchs ovipositing on 'intermediate' cardenolide-containing plants, balancing the need for sequestration with toxicity of cardenolides (Malcolm, 1995), the general result from these analyses appears that *D. plexippus* prefers to oviposit on the low end (but not lowest) of cardenolide individuals.

It is still unclear, however, whether *D. plexippus* adults can directly perceive foliar cardenolides, and it is unknown if *Asclepias* leaves contain cardenolides on the surface.

XIII. Target site insensitivity

The term insensitivity is used here because of convention, but can only be interpreted in a relative sense, and it does not indicate complete insensitivity (Fig. 8). Target site insensitivity towards cardenolides was first described for the monarch butterfly (*D. plexippus*) by Vaughan & Jungreis (1977). Using *in vitro* assays, these authors found the Na^+/K^+-ATPase of *D. plexippus* to possess substantially reduced sensitivity towards the standard cardenolide ouabain compared with nonadapted lepidopteran species. Holzinger *et al.* (1992) reported the molecular basis for

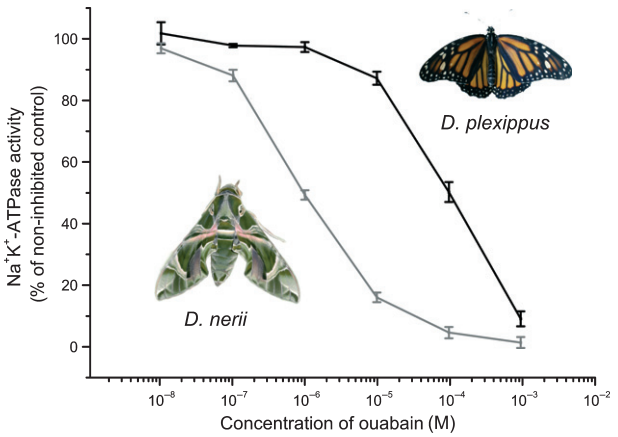

Fig. 8 Inhibition of lepidopteran Na^+/K^+-ATPase by the polar cardenolide ouabain. Shown is a comparison of a preparation from *Danaus plexippus* which demonstrates greater target site insensitivity than *Daphnis nerii*. Both insects are specialists on cardenolide-containing plants, although only the former sequesters cardenolides. Data for *D. nerii* (n = 4) are from (Petschenka & Dobler, 2009) while data for *D. plexippus* (n = 2) are from G. Petschenka & S. Dobler (unpublished).

the monarch's target site insensitivity to be the substitution of asparagine by histidine at position 122 of the Na^+/K^+-ATPase. Target site insensitivity to cardenolides may thus be demonstrated either in *in vitro* assays using Na^+/K^+-ATPase preparations (Vaughan & Jungreis, 1977; Moore & Scudder, 1986; Al-Robai, 1993; Fig. 8) or by molecular investigation of the enzyme's amino acid sequence (Holzinger & Wink, 1996; Labeyrie & Dobler, 2004). Reconstruction of the amino acid sequence allows one to check for substitutions known to confer resistance, as the Na^+/K^+-ATPase sequences are typically highly conserved, spanning most invertebrates and vertebrates. Substitutions at these positions potentially change the physicochemical properties of the binding site, thereby altering the sensitivity of the Na^+/K^+-ATPase to cardenolides (Croyle *et al.*, 1997).

Apart from the work on monarchs, target site insensitivity to cardenolides has been shown to occur in several additional insects from four different insect orders: *O. fasciatus* (Heteroptera: Lygaeidae, Moore & Scudder, 1986), *Poekilocerus bufonius* (Orthoptera: Pyrgomorphidae, Al-Robai, 1993), *Chrysochus auratus* and *Chrysochus cobaltinus* (Coleoptera: Chrysomelidae, Labeyrie & Dobler, 2004) and in an agromyzid fly *Liriomyza asclepiadis* (Diptera: Agromyzidae) mining in the leaves of *A. syriaca* (Dobler *et al.*, 2011). This widespread occurrence suggests that insensitivity has evolved independently several times. In addition, 11 species representing four insect orders, but all feeding on cardenolide-containing plants, possess a substitution at position 111 (S. Dobler *et al.*, unpublished), a position whose substitution is part of the molecular basis of the ouabain insensitivity of the rat Na^+/K^+-ATPase (Croyle *et al.*, 1997; De Pont *et al.*, 2009).

We emphasize that target site insensitivity is a relative phenomenon. At nearly millimolar cardenolide concentrations, which naturally occur in the hemolymph of monarch caterpillars (Nishio, 1980), even the 'insensitive' Na^+/K^+-ATPases of *D. plexippus*, *O. fasciatus* and *P. bufonius* showed significant inhibition (Vaughan & Jungreis, 1977; Moore & Scudder, 1986; Al-Robai, 1993; Fig. 8). And as discussed earlier, there is both direct and indirect evidence that specialized herbivores like *D. plexippus* are indeed negatively impacted by ingested cardenolides. Differential binding affinity and inhibitory potency between diverse cardenolides and Na^+/K^+-ATPase are based on molecular features of the cardenolide molecule (ligand) (Paula *et al.*, 2005) and on molecular properties of the Na^+/K^+-ATPase (receptor) (De Pont *et al.*, 2009). Additionally, several cardenolide-adapted and cardenolide-sequestering lepidopterans were found not to possess cardenolide-insensitive Na^+/K^+-ATPases, suggesting that target site insensitivity is not the only way to cope with dietary cardenolides (Petschenka, 2010; S. Dobler *et al.*, unpublished).

XIV. Alternative mechanisms of cardenolide resistance

It seems that even nonadapted insects possess mechanisms which often render them highly resistant to cardenolides. Caterpillars of *Manduca sexta*, which do not experience cardenolides in their

natural diet, can tolerate ouabain injections that bring their hemolymph to *c.* 10^{-3}M of this cardenolide (Vaughan & Jungreis, 1977). Additionally, Scudder & Meredith (1982) found the midgut of the generalist insects *Schistocerca gregaria* and *Periplaneta americana* to be impermeable to digitoxin. Since digitoxin is a nonpolar cardenolide, it was expected to cross epithelia by diffusion; but this result suggests the existence of a gut barrier to nonpolar cardenolides. There are several plausible mechanisms which might be involved in such a barrier (Fig. 7). First, Barbehenn (1999) showed that the peritrophic envelope can reduce absorption of digitoxin via the midgut. Digitoxin was reported to form micelles which could not pass the meshes of this protein cage. Secondly, in the malpighian tubules of *Drosophila melanogaster*, Na^+/K^+-ATPase is protected from ouabain by organic anion transporters (Torrie *et al.*, 2004). And finally, P-glycoprotein-like efflux carriers appear to occur in the insect midgut of many insects (Murray *et al.*, 1994; G. Petschenka & S. Dobler, unpublished; Lanning *et al.*, 1996). Since P-glycoprotein transports the cardenolide digoxin in the mammalian gut, it is possible that efflux carriers maintain a physiological barrier to the uptake of cardenolides in the insect midgut as well. Finally, our data support the idea that an active efflux mechanism for digoxin exists at the lepidopteran nerve cord and prevents the cardenolide from reaching the susceptible target site Na^+/K^+-ATPase (Petschenka, 2010).

To avoid polar cardenolides like ouabain (which cannot cross membranes by diffusion), herbivores may not require energy-dependent barrier mechanisms. Ouabain was shown not to enter the lepidopteran nerve cord where Na^+/K^+-ATPase is highly expressed (Rubin *et al.*, 1983; Petschenka, 2010). More generally, ouabain tends to have reduced (or no) impact on herbivores compared with digitoxin (Rasmann *et al.*, 2009b). For sequestering insects, it may well be that it is less costly to store relatively more polar cardenolides. This hypothesis is supported by the observation that cardenolides are converted into more polar forms during sequestration (Black, 1976; Seiber *et al.*, 1980; Brower *et al.*, 1984; Martin *et al.*, 1992) or that polar cardenolides are preferentially sequestered compared with nonpolar cardenolides (Frick & Wink, 1995). Finally, we also expect that metabolism of cardenolides may play a role in detoxification. Cardenolides are substrates to cytochrome P450 monooxygenases (Marty & Krieger, 1984) and glycosidases (Black, 1976), and modification of the cardenolide molecule which are relevant for specific binding to Na^+/K^+-ATPase could well lead to inactivity. Some evidence indicates that such metabolism of cardenolides occurs in lygaeid bugs (Scudder & Meredith, 1982; Scudder *et al.*, 1986).

XV. Cardenolide sequestration

Sequestration of dietary cardenolides is known from members of several insect orders (Fig. 9): Lepidoptera: Danaidae (Brower *et al.*, 1984), Arctiidae (Black, 1976; Nishio, 1980; Cohen & Brower, 1982); Coleoptera: Chrysomelidae (Dobler *et al.*, 1998; Isman *et al.*, 1977b), Cerambycidae (Duffey & Scudder, 1972; Nishio *et al.*, 1983); Homoptera: Aphididae (Rothschild *et al.*, 1970); Heteroptera: Lygaeidae (Duffey & Scudder, 1972; Duffey *et al.* 1978); Orthoptera: Pyrgomorphidae (von Euw *et al.*, 1967). Nonetheless, other insects on cardenolide-containing plants do not sequester (Fig. 9). For example, the oleander hawkmoth (*Daphnis nerii*) and the danaine butterfly *Euploea core* both feed on cardenolide-rich oleander, but are relatively poor sequesterers of cardenolides compared with others (Marsh *et al.*, 1977; Rothschild *et al.*, 1978; Abe *et al.*, 1996).

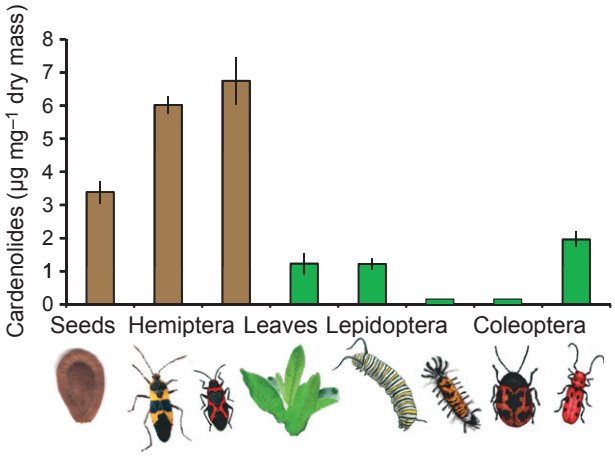

Fig. 9 Estimates of cardenolide sequestration in six milkweed herbivores (two from each of three orders of insects) collected or reared on *Asclepias syriaca* in Ithaca (NY, USA): *Oncopeltus fasciatus*, *Lygaeus kalmii*, *Danaus plexippus*, *Euchaetes egle*, *Labidomera clivicollis*, and *Tetraopes tetraophthalmus*. The Hemipterans are seed feeders while the rest are leaf chewers; *T. tetraophthalmus* larvae feed on roots, which have comparable cardenolide concentrations to leaves. Shown are the means and standard errors for five samples (each sample was 50–100 mg of tissue, typically comprising several animals). All insects were pupal or adult stages, were starved for 2 d, and were analyzed using high-performance liquid chromatography following Rasmann *et al.* (2009b). Images are not shown to scale.

In monarch butterflies, sequestered cardenolides are dependent on host plant characteristics and larval developmental stage, and the extent of sequestration dictates the extent of protection from bird predators (Brower *et al.*, 1972; Brower & Moffitt, 1974; Roeske *et al.*, 1976). Cardenolides in monarch wings are nearly twice as concentrated as those in the rest of the body, and are especially concentrated in the wing-scales (Nishio, 1980). Nonetheless, relatively little is known about cardenolide sequestration and arthropod predation. Although spiders are deterred by cardenolides (Petschenka *et al.*, 2011), and sequestered cardenolides can affect web-building and prey capture (Malcolm, 1989). *A. nerii* sequesters higher concentrations of cardenolides than its congener, *A. asclepiadis*, and this translates into a preference of predators for *A. asclepiadis* (Mooney *et al.*, 2008). Mantids experience some toxic effects of ingesting sequestered cardenolides, but nonetheless appear to regulate their intake to avoid chronic toxicity (Berenbaum & Miliczky, 1984; Paradise & Stamp, 1993). Our own observations of sequestering aphids *A. nerii* and butterflies *D. plexippus* in natural populations is that a diversity of invertebrate predators frequently prey upon these insects. Thus, although the reinforcing nature of aposematism and toxicity can clearly cause predators to learn to avoid cardenolide-containing insects, we argue that the actual ecological impact of sequestration is not well understood.

So far, the physiological basis of cardenolide sequestration is largely unknown. Scudder & Meredith (1982) postulated a physical (passive) system for cardenolide sequestration in *O. fasciatus*, which favors the nonpolar cardenolide digitoxin over the polar cardenolide ouabain. Nonetheless, for the high accumulation of cardenolides into the dorsolateral space of this species, the situation is reversed, and ouabain, as well as the polar metabolites of digitoxin, are preferred over digitoxin (Yoder *et al.*, 1976; Scudder *et al.*, 1986). Additionally, Detzel & Wink (1995) found the uptake of digoxin into midgut cells of *Oncopeltus* to be inhibited by metabolic poisons, again suggesting an active process. Uptake was decreased by the presence of the structurally related cardenolide convallatoxin, suggesting a selective (carrier-mediated) process. There is also evidence for an active and selective uptake in midgut cells of *D. plexippus* and the arctiid *S. epilais* (von Nickisch-Rosenegk *et al.*, 1990; Detzel & Wink, 1995). Despite this evidence, the nature of these putative carriers is completely unknown.

It is interesting that two cardenolide forms, calotropin and its configurational isomer calactin (Seiber *et al.*, 1980), are apparently favored among the plants' cardenolide spectrum with respect to sequestration or storage (or both) by *D. plexippus*. Both of these compounds are the dominant cardenolides stored by the monarch when the caterpillars feed on *A. curassavica* or *A. fruticosa* (Seiber *et al.*, 1983; Groeneveld *et al.*, 1990a). Again, calotropin and calactin are the only cardenolides present in the defensive spray of the pyrgomorphid grasshopper *P. bufonius* (von Euw *et al.*, 1967). This might mean that calotropin and calactin are at the same time favored by cardenolide carriers, and that they are products of insect metabolism, as demonstrated by Seiber *et al.* (1980). These compounds, which are some of the most effective cardenolides to induce animal emesis (Duffey, 1977), could in addition possess advantageous properties for storage (Seiber *et al.*, 1980), or avoiding of autointoxication.

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