

# Sequestration of Defenses against Predators Drives Specialized Host Plant Associations in Preadapted Milkweed Bugs (Heteroptera: Lygaeinae)

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**ABSTRACT:** Host plant specialization across herbivorous insects varies dramatically, but while the molecular mechanisms of host plant adaptations are increasingly known, we often lack a comprehensive understanding of the selective forces that favor specialization. The milkweed bugs (Heteroptera: Lygaeinae) are ancestrally associated with plants of the Apocynaceae from which they commonly sequester cardiac glycosides for defense, facilitated by resistant Na<sup>+</sup>/K<sup>+</sup>-ATPases and adaptations for transport, storage, and discharge of toxins. Here, we show that three Lygaeinae species independently colonized four novel nonapocynaceous hosts that convergently produce cardiac glycosides. A fourth species shifted to a new source of toxins by tolerating and sequestering alkaloids from meadow saffron (*Colchicum autumnale*, Colchicaceae). Across three milkweed bug species tested, feeding on seeds containing toxins did not improve growth or speed of development and even impaired growth and development in two species, but sequestration mediated protection of milkweed bugs against two natural predators: lacewing larvae and passerine birds. We conclude that physiological preadaptations and convergent phytochemistry facilitated novel specialized host associations. Since toxic seeds

did not improve growth but either impaired growth or, at most, had neutral effects, selection by predators on sequestration of defenses, rather than the exploitation of additional profitable dietary resources, can lead to obligatory specialized host associations in otherwise generalist insects.

**Keywords:** milkweed bugs, coevolution, sequestration, cardiac glycoside, specialization, antipredator defense.

## Introduction

Herbivorous insects show tremendous variation with regard to dietary specialization. While it is a long-standing assumption that phytochemicals may restrict and direct the evolution of host plant use (Ehrlich and Raven 1964), the explicit role of phytochemicals as drivers of host plant associations has been revealed in only a few systems (Denno et al. 1990; Becerra 1997; Berenbaum 2001; Termonia et al. 2001; Murphy and Feeny 2006). As generalist herbivores encounter many more plant secondary compounds in their diet than specialists, broader detoxification capabilities of generalists may trade off with host plant use efficiency, while specialists may optimize their consumption of a single resource (Dethier 1954; Ehrlich and Raven 1964; Scriber and Feeny 1979; Montandon et al. 1987; Singer 2008). Beyond optimized dietary tolerance, specialization also

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appears to be evolutionarily linked with the sequestration of plant toxins to provide a defense against the third trophic level (Dyer 1995; Engler-Chaouat and Gilbert 2007; Lampert and Bowers 2010; Züst and Agrawal 2015; Petschenka and Agrawal 2016). In fact, predators that drive the occupation of enemy-free spaces are typically hypothesized to select for increased specialization (Brower 1958; Bernays and Graham 1988; Singer 2008). However, while the close link between dietary tolerance and sequestration of specialist herbivores is widely recognized (Opitz and Müller 2009; Erb and Robert 2016; Petschenka and Agrawal 2016), the extent to which sequestration, rather than dietary tolerance, could drive the evolution of insect–host plant associations has rarely been addressed (Camara 1997; Termonia et al. 2001, 2002).

Even though dietary specialization and sequestration of plant toxins have been hypothesized to lead to an evolutionary dead end (Kelley and Farrell 1998; Termonia et al. 2001), there is evidence that ecological specialization does not necessarily prevent host use expansion (Termonia et al. 2001). In addition, recent research indicated that sequestration requires resistance traits different from those required to merely cope with dietary toxins (Petschenka and Agrawal 2015), suggesting that selection by predators or parasitoids (i.e., the third trophic level) opens an additional arena for coevolutionary escalation (Petschenka and Agrawal 2015, 2016). Consequently, a rigorous analysis of coevolution between plants and specialized insects requires the integration of adaptations underlying bitrophic interactions with adaptations driven by higher trophic levels (Petschenka and Agrawal 2015, 2016; Huber et al. 2016).

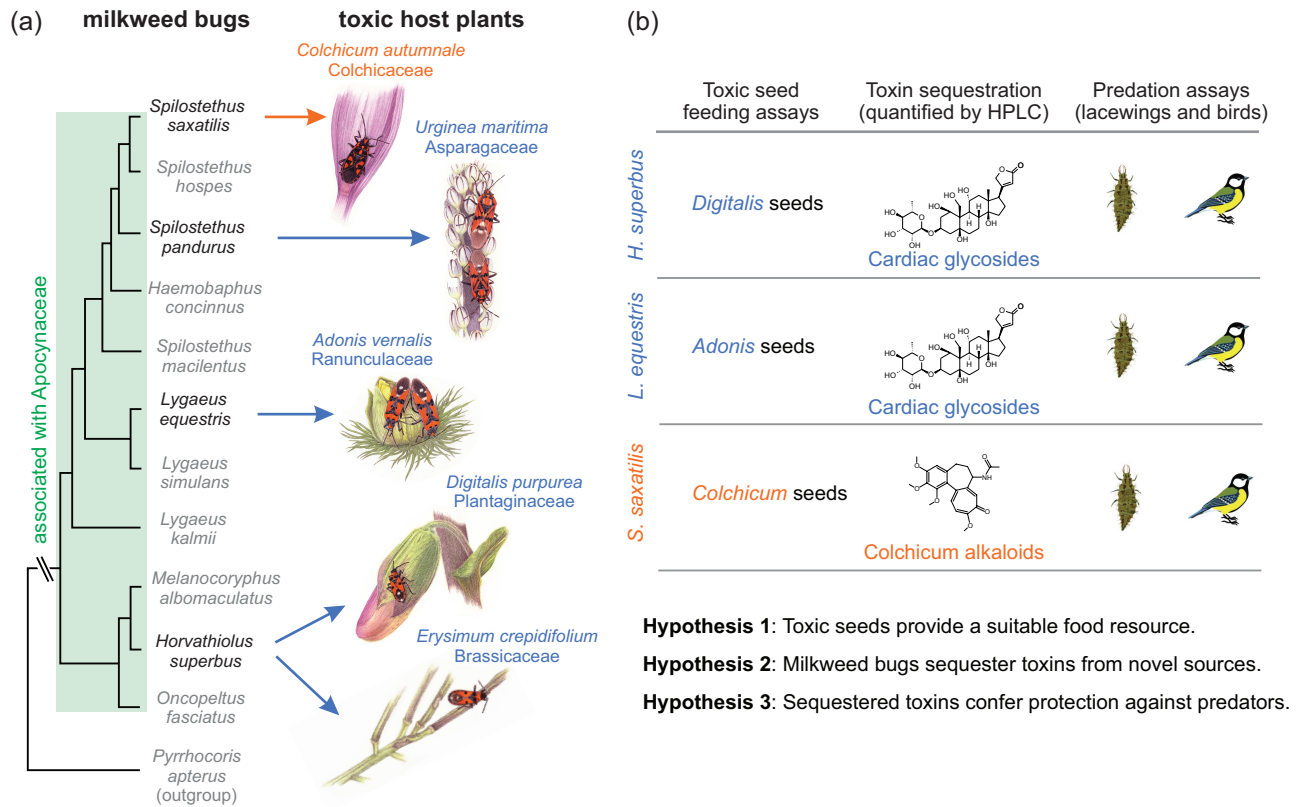
Here, we used milkweed bugs (Hemiptera: Heteroptera: Lygaeinae) as a model system to test hypotheses about the evolutionary drivers leading to specialized associations with particular plant species. The Lygaeinae comprise about 600 primarily seed-eating species (fig. S1; figs. S1–S11 are available online) that are well known for their predilection of plants in the Apocynaceae worldwide (Scudder and Duffey 1972; Slater and Sperry 1973; Slater 1985; Péricart 1998). Milkweed bugs typically exhibit a red and black aposematic coloration, and in addition to defensive scent glands typical for Heteroptera (Schuh and Slater 1995), several species have been shown to acquire defenses against predators from their host plants (Berenbaum and Miliczky 1984; McLain and Shure 1985; Evans et al. 1986; Tullberg et al. 2000). Upon attack, many milkweed bug species release sequestered toxins in a defensive secretion from a specialized storage compartment of the integument (the dorsolateral space; Scudder et al. 1986; Bramer et al. 2017). The large milkweed bug (*Oncopeltus fasciatus* (Dallas, 1852)) in particular has been studied in detail with regard to sequestration of cardiac glycosides, which it derives from the seeds of milkweeds in the Apocynaceae genus *Asclepias* (Duffey et al. 1978).

Cardiac glycosides are important defense metabolites of plants in the Apocynaceae and evolved convergently in at least 11 additional botanical families (Agrawal et al. 2012). Both compound subtypes, the cardenolides and the bufadienolides, are specific inhibitors of the ubiquitous animal enzyme  $\text{Na}^+/\text{K}^+$ -ATPase. Specialized insects from at least six taxonomic orders, including several milkweed bug species (Dobler et al. 2012, 2015; Zhen et al. 2012), tolerate cardenolides by expressing  $\text{Na}^+/\text{K}^+$ -ATPases with several amino acid substitutions that mediate a high degree of cardenolide resistance in vitro (target site insensitivity; Moore and Scudder 1986; Bramer et al. 2015). In addition to resistance, sequestration requires accumulation of toxins from the dietary resource, and milkweed bugs possess an as yet unidentified mechanism for the transport of toxins across the gut epithelium.

In summary, milkweed bugs possess a suite of traits related to sequestration and defense that includes aposematic coloration, resistant  $\text{Na}^+/\text{K}^+$ -ATPases, and mechanisms for accumulation, storage, and release of toxins. This suite of traits may also function as a physiological preadaptation facilitating the sequestration of novel toxin compounds. For example, the milkweed bug *Neacoryphus bicrucis* (Say, 1825) sequesters pyrrolizidine alkaloids (McLain and Shure 1985), a class of compounds unrelated to cardiac glycosides.

Sequestration of cardiac glycosides by milkweed bugs was described for several species feeding on Apocynaceae (Von Euw et al. 1971; Duffey and Scudder 1972), and it was suggested that sequestration of cardiac glycosides, target site insensitivity of  $\text{Na}^+/\text{K}^+$ -ATPase, and an association with apocynaceous plants are ancestral traits of the entire clade (Scudder and Duffey 1972; Bramer et al. 2015). Nevertheless, despite being specialized to cardiac glycosides, several milkweed bug species are dietary generalists and feed on seeds from a great variety of plant families. The Palearctic species *Lygaeus equestris* (Linnaeus, 1758), for example, was observed feeding on more than 60 plant species from roughly 20 families (Solbreck and Kugelberg 1972). Even though dietary breadth can vary among species and larval instars to some extent (Solbreck and Kugelberg 1972; Péricart 1998), this large number of potential host plants is in stark contrast to the narrow set of species that produce cardiac glycosides for sequestration. The extreme prevalence of cardiac glycoside sequestration in milkweed bugs thus suggests an important role of selective pressure exerted by higher trophic levels in shaping milkweed bug–host plant associations.

Remarkably, several Palearctic species of Lygaeinae are associated with cardiac glycoside-producing plants that are phylogenetically disparate from the Apocynaceae (fig. 1). *Horvathiolus superbus* (Pollich, 1779) seems to depend on *Digitalis purpurea* (Plantaginaceae) at least in parts of its distributional range (Ollivier-Schricke 1984; Péricart 1998). In



**Figure 1:** Associations of milkweed bugs and toxic plants used as a study system and major hypotheses tested. *a*, Clade of milkweed bugs comprising all species investigated in this study. Milkweed bugs are ancestrally associated with species of the plant family Apocynaceae (indicated by green box) from which they commonly sequester cardiac glycosides for defense against predators (Bramer et al. 2015). We studied three milkweed bug species that independently associated with phylogenetically disparate plant species producing cardiac glycosides convergently (indicated by blue arrows and botanical names) and a fourth milkweed bug species that specialized on *Colchicum autumnale* producing colchicum alkaloids (orange arrow and name). Phylogenetic relationships based on Bramer et al. (2015). *b*, Using three milkweed bug species as model systems, we tested three major hypotheses to unravel the evolutionary driver underlying the observed associations with toxic plants. Drawings of milkweed bugs on plants by Martina Zwanziger. Drawing of great tit from iStock.com/insima. HPLC = high-performance liquid chromatography.

addition, we found this species using the cardenolide-producing *Erysimum crepidifolium* (Brassicaceae) as a host plant in a *Digitalis*-free habitat. Early instars of *L. equestris* larvae in Sweden feed almost exclusively on the cardenolide-producing Ranunculaceae *Adonis vernalis* (Solbreck and Kugelberg 1972; Junior and Wichtl 1980), which is also an important host plant elsewhere (Péricart 1998). Finally, the generalist milkweed bug *Spilostethus pandurus* was recorded on the Asparagaceae *Urginea maritima* (Vivas 2012), which produces cardiac glycosides of the bufadienolide type (Steyn and van Heerden 1998).

Surprisingly, *Spilostethus saxatilis* (Scopoli, 1763), a closely related species that uses a great variety of host plants (Péricart 1998; Banar 2003; Vivas 2012), is not known to typically visit cardiac glycoside-producing plants. However, we and others (Péricart 1998; Hotová Svádová et al. 2013) have often observed this species on flowers and fruits of meadow saffron (*Colchicum autumnale*, Colchicaceae),

which is highly toxic owing to the production of colchicine and related alkaloids. Colchicum alkaloids inhibit polymerization of tubulin (Ravelli et al. 2004), thus showing a mode of action that is different from cardiac glycosides. Nonetheless, according to its evolutionary history, *S. saxatilis* may be preadapted by possessing some traits of the “sequestration suite,” including aposematic coloration, storage and release of toxins, and a putative mechanism for transport.

We used a suite of three insect-plant interactions (fig. 1) to test a set of hypotheses about the potential evolutionary drivers that selected for the observed host plant associations. Specifically, we tested (1) whether seeds from evolutionarily novel toxic host plants provide a valuable food resource, (2) whether milkweed bugs sequester cardiac glycosides or colchicum alkaloids from these evolutionarily novel sources, and (3) whether sequestered toxins confer protection against insect and avian predators. Furthermore, we tested for colchicine resistance in *S. saxatilis* and

assessed whether this species is obligatorily associated with *C. autumnale* across a wide part of its distributional range by screening museum specimens for the occurrence of colchicum alkaloids.

On the basis of our findings, we suggest that sequestration of plant toxins as a defense against predators mediates specialized associations of milkweed bugs with specific host plant species. The preadaptations for sequestration of cardiac glycosides and their convergent occurrence within distantly related plants most likely facilitated new host associations. Furthermore, a subset of these preadaptations may have facilitated the shift to new host plants with functionally distinct but highly potent toxins. We thus demonstrate that species that are dietary generalists under a bitrophic perspective may nonetheless be highly specialized on plants that provide defenses against the third trophic level.

## Material and Methods

### *Origin and Maintenance of Bugs Used for Experiments*

Eggs of milkweed bugs used in all of the experiments were from laboratory colonies maintained on toxin-free food for several generations, with the exception of *Spilostethus saxatilis*, which cannot be maintained successfully long-term and for which we used eggs obtained from adults in the field (Berghausen, Germany). Founding individuals for laboratory colonies of *Lygaeus equestris* were collected in Brandenburg, Germany; individuals of *Horvathiolus superbus* were collected in Eberbach, Germany; and individuals of *Spilostethus pandurus* were collected in Portugal. *Oncopeltus fasciatus* used as controls for predation assays and for the assessment of colchicine resistance were from a long-term laboratory colony at the University of Hamburg (collected in 2008 in Ithaca, NY, and mixed with individuals collected in 2014 in Urbana-Champaign, IL). Bugs were raised on husked sunflower seeds and supplied with water in Eppendorf tubes plugged with cotton wool, as well as pieces of cotton wool for oviposition. Colonies were maintained in environmental chambers at 28°C and 60% humidity at a 16L:8D cycle. *Pyrrhocoris apterus* (Linnaeus, 1758) used as a nonadapted out-group for injection assays were collected in the field (Giessen, Germany) and used directly or after 1 day of maintenance in the laboratory on linden seeds. Exact locations and details (if available) are given in the supplemental PDF, available online.

### *Seed Mixture Experiments to Assess Growth and Sequestration*

To assess whether inclusion of *Digitalis*, *Adonis*, or *Colchicum* seeds (hereafter, “toxic seeds”) in the dietary spectra affects growth and sequestration of toxins, we reared

larvae of *S. saxatilis*, *L. equestris*, and *H. superbus* under four dietary treatments. Starting with preweighed first-instar larvae, bugs were raised on pure sunflower seeds (positive control), a seed mixture comprising 11–15 natural host plant species to reflect the broad dietary spectra of *S. saxatilis* and *L. equestris* (table S1; tables S1–S3 are available online), the identical seed mixture supplemented with toxic seeds, or toxic seeds only. To establish species-specific seed mixtures, we selected natural host plant species according to literature data or our own field observations. Toxic seeds were selected on the basis of natural associations of the bug species with toxic plants in the field. Specifically, we used seeds of *Digitalis purpurea* for *H. superbus*, seeds of *Adonis vernalis* for *L. equestris*, and seeds of *Colchicum autumnale* for *S. saxatilis*.

Untreated, ripe seeds were either obtained commercially or collected in the field (*D. purpurea*; Eberbach 2016). Seed mixtures consisted of 11 host species from four botanical families for *S. saxatilis* and 15 host species from seven botanical families for *L. equestris*. Because of the lack of natural history data for *H. superbus*, we used the same natural seed mixture for *H. superbus* as for *S. saxatilis*. Within seed mixtures, we standardized proportions of individual plant species according to mass. Growth experiments were carried out in spatially randomized Petri dishes in a growth chamber (Binder KBWF 240, Tuttlingen, Germany) under the following conditions: 16L:8D photoperiod, 26°C (*S. saxatilis*) or 28°C (*L. equestris* and *H. superbus*), and 60% humidity over the course of 3 weeks. We lined Petri dishes (60 mm × 15 mm, with vents; Greiner Bio-One;  $n = 11$  for all species and diets) with filter paper and added three first-instar larvae to each dish. Petri dishes were supplied with a water source (see above) and 140.7 mg ( $\pm 0.72$  SE; *S. saxatilis*), 145.1 mg ( $\pm 1.03$  SE; *L. equestris*), or 140.1 mg ( $\pm 0.69$  SE; *H. superbus*) of seeds.

We recorded body mass weekly by anesthetizing all bugs in a Petri dish with CO<sub>2</sub> and weighing them jointly. In addition, we recorded survival of bugs weekly. After the experiment, we transferred one (for *S. saxatilis* and *L. equestris*) or three (for *H. superbus*) bugs from each Petri dish to fresh sunflower seeds for a period of 2 weeks to clean potentially remaining toxins from guts. Note that most bugs had not yet reached the adult stage at the time of transfer; thus, the time spent on sunflower seeds during the adult stage varied, and some bugs were still larvae at the time of analysis (see table S2 and the “Supplementary Results” section of the supplemental PDF). Finally, bugs were frozen at  $-80^{\circ}\text{C}$ , freeze-dried, weighed, and analyzed to quantify sequestered toxins via high-performance liquid chromatography (HPLC) as described below ( $n = 10$  or  $11$  per species and diet for both feeding and sequestration assays).

Growth of bugs on different seed mixtures, approximated as body mass (i.e., the total weight of all bugs

per Petri dish divided by the number of remaining individuals) after 3 weeks of feeding, was analyzed by ANCOVA using the standard least squares method in JMP (ver. 13; SAS Institute, Cary, NC). Body masses were  $\log_{10}$  transformed to achieve homogeneity of variances and normality of residuals. Dietary treatment was used as a main effect, and initial mass of the bugs was included as a covariate. Two individuals of *L. equestris* (*Adonis* treatment) and of *H. superbus* (*Digitalis* treatment and seed mixture plus *Digitalis* treatment) were statistical outliers, but their exclusion did not affect the direction or significance of effects. In addition to comparing final body mass after 3 weeks of feeding, we also modeled growth as a continuous process (see the supplemental PDF). We analyzed developmental time across treatments by comparing the number of days ( $\log_{10}$  transformed) after which at least one bug per Petri dish (mostly after being transferred to sunflower seeds) reached the adult stage using ANOVA in JMP ( $n = 11$  for *L. equestris* for all treatments;  $n = 9$  for *H. superbus* for the seed mixture without *Digitalis* seeds and  $n = 10$  for all other treatments). We omitted this analysis for *S. saxatilis*, since on the *Colchicum* diet, only one individual reached adulthood during the time of observation.

#### Sampling of Milkweed Bugs to Assess Sequestration under Field Conditions

To assess sequestration of toxins under natural conditions, adult milkweed bugs were collected in the field from habitats with natural stands of their toxic host plants (i.e., *C. autumnale* for *S. saxatilis*, *Urginea maritima* for *S. pandurus*, *A. vernalis* for *L. equestris*, *D. pupurea* and *Erysimum crepidifolium* for *H. superbus*; figs. 1, S1). Details on sampling and notes on natural history observations are given in the supplemental PDF. Fieldwork in protected areas was permitted by the responsible agencies (see the acknowledgments). After the bugs were brought to the lab, they were maintained on sunflower seeds and water provided in Eppendorf tubes plugged with cotton wool under ambient conditions to purge remaining toxins from their guts. After 14 days ( $\geq 12$  days for *S. pandurus*), bugs were frozen at  $-80^{\circ}\text{C}$  and freeze-dried for chemical analysis as described below.

#### Preparation of Insect Specimens for HPLC Analysis

We determined the concentration of plant toxins sequestered by individual milkweed bugs with HPLC with diode array detection (HPLC-DAD). We added 1 mL of methanol (HPLC grade) containing 0.01 mg of the internal standard digitoxin (Sigma-Aldrich, Taufkirchen, Germany) to freeze-dried specimens of *L. equestris* and *S. pandurus*. For *H. superbus*, digitoxin was replaced by oleandrin (PhytoLab, Vestenbergsgreuth, Germany) because of the natural occurrence of digi-

toxin in *Digitalis*. For specimens of *S. saxatilis* and *S. pandurus*, we used no internal standard and quantified colchicum alkaloids and bufadienolides with an external calibration curve (see below). After the addition of  $\sim 900$  mg of zirconia beads (Roth, Karlsruhe, Germany), specimens were homogenized in a FastPrep-24 homogenizer (MP Biomedicals, Eschwege, Germany) for two 45-s cycles at a speed of 6.5 m/s. After centrifugation at 16,100 g for 3 min, supernatants were transferred to fresh 2-mL plastic vials (Sarstedt, Nümbrecht, Germany). Extractions were repeated once more with pure methanol. Pooled supernatants were evaporated under nitrogen gas. Subsequently, we resuspended samples by adding 100  $\mu\text{L}$  of methanol (200  $\mu\text{L}$  for field-collected *S. pandurus* and *S. saxatilis*) and agitating in the FastPrep-24 homogenizer (45 s, 6.5 m/s) without beads to facilitate dissolution of dried residues. Finally, samples were centrifuged (16,100 g, 3 min) and filtered into HPLC vials using ROTILABO syringe filters (nylon, 0.45  $\mu\text{m}$ ; Roth, Karlsruhe, Germany). Eggs obtained from *S. saxatilis* ( $n = 5$ ; pools of 7, 18, 22, 4, and 7 eggs) females collected in Berghausen on May 5, 2016, and from field-collected *L. equestris* (Lebus,  $n = 3$ ; pools of 27, 28, and 63 eggs) were freeze-dried and extracted as described above with  $2 \times 500 \mu\text{L}$  or  $2 \times 1$  mL of methanol, respectively. Details on harvesting of hemolymph and defensive secretion (i.e., clear droplets released at the integument upon attack) of *S. saxatilis* as well as the preparation of dried museum specimens and plant seeds for chemical analysis are described in the supplemental PDF.

#### HPLC Analysis of Cardenolides, Bufadienolides, and Colchicum Alkaloids

We injected 15  $\mu\text{L}$  of extract into an Agilent 1100 series HPLC system, and compounds were separated on an EC 150/4.6 NUCLEODUR C18 gravity column (3  $\mu\text{m}$ , 150 mm  $\times$  4.6 mm; Macherey-Nagel, Düren, Germany). Cardenolides and bufadienolides were eluted at a constant flow of 0.7 mL/min at  $30^{\circ}\text{C}$  with an acetonitrile- $\text{H}_2\text{O}$  gradient as follows: 0–2 min at 16% acetonitrile, 25 min at 70% acetonitrile, 30 min at 95% acetonitrile, 35 min at 95% acetonitrile, 37 min at 16% acetonitrile, and reconditioning for 10 min at 16% acetonitrile. We recorded ultraviolet (UV) absorbance spectra from 200 to 400 nm with a diode array detector. Peaks with symmetrical absorption maxima between 216 and 222 nm were interpreted as cardenolides, integrated at 218 nm, and quantified according to the peak area of the known concentration of the internal standards digitoxin or oleandrin. Peaks with a symmetrical absorption maximum of 300 nm were interpreted as bufadienolides. For bufadienolide analysis, we used an external calibration curve based on proscillaridin A (PhytoLab, Vestenbergsgreuth, Germany).

Colchicum alkaloids were eluted at a constant flow of 0.7 mL/min at 30°C with an acetonitrile–0.25% phosphoric acid gradient as follows: 0–2 min at 10% acetonitrile, 10 min at 40% acetonitrile, 15 min at 80% acetonitrile, 16 min at 10% acetonitrile, and reconditioning for 5 min at 10% acetonitrile. UV absorbance spectra were recorded from 190 to 400 nm by a diode array detector. Peaks with absorption maxima at 245 and 350 nm resembling the absorption spectra of colchicine were recorded as colchicosides and quantified at 350 nm. Colchicine equivalents were calculated according to an external colchicine (Roth, Karlsruhe, Germany) calibration curve.

Analysis of chromatograms was carried out with the Agilent ChemStation software (ver. B.04.03). Details on the evaluation of individual data sets are described in the supplemental PDF. Individual compounds were identified by comparisons of UV spectra and retention time with commercial reference compounds and liquid chromatography–mass spectrometry (details are described in the supplemental PDF).

For analyzing sequestered toxins during the seed feeding assays, we used Welch's test (JMP) and the Games-Howell post hoc test (see <http://www.biostathandbook.com>) on  $\log_{10}$ -transformed data to evaluate differences across treatments, since data for *L. equestris* and *S. saxatilis* did not meet the assumption of equal variance. For this analysis, we excluded all data for treatments of *H. superbus* and *L. equestris* raised without toxic seeds, as these lacked sequestered cardenolides.

#### *Behavioral Assays with Lacewings*

We used larvae of the lacewing *Chrysoperla carnea* (Neuroptera: Chrysopidae) to test the effect of sequestered plant metabolites against an arthropod predator. Although information about natural predators of milkweed bugs is very limited, neuropteran larvae were reported as common predators of first- and second-instar larvae of *O. fasciatus* in the field (Sauer and Feir 1972). For these experiments, eggs of *L. equestris*, *H. superbus*, and *S. pandurus* were transferred to either sunflower seeds (nontoxic controls) or toxic seeds (*A. vernalis* for *L. equestris*, *D. purpurea* for *H. superbus*, and *U. maritima* for *S. pandurus*). Petri dishes were supplied with a water source as described above. After at least 3 days (*S. pandurus*) to 5 days (*H. superbus*) in a growth chamber (Binder KBWF 240) with a 16L:8D photoperiod at 28°C and 60% humidity, first- to third-instar larvae (mainly first and second instar) were presented individually to a lacewing larva (second or third instar). Larvae hatched from eggs of field-collected *S. saxatilis* were maintained on *C. autumnale* seeds for at least 4 days as described above. Because of the maternal transfer of colchicum alkaloids into the eggs, we used *O. fasciatus* larvae raised on sunflower seeds as a nontoxic negative control for these

assays. We saved at least five individuals of each species from both treatments (toxic seeds and nontoxic sunflower seeds) to assess the amount of toxins sequestered using HPLC-DAD as described above.

Lacewing larvae that had not ever consumed toxin-containing food were obtained commercially (Sautter and Stepper, Ammerbuch, Germany), maintained individually on *Sitotroga* (Lepidoptera: Gelechiidae) eggs (Katz Biotech AG, Baruth, Germany) for 3–5 days at room temperature, and starved for 2 days before the experiments. In a first set of experiments focusing on bug survival, one milkweed bug larva was exposed to one lacewing larva in a Petri dish (50 mm × 15 mm, with vents) and observed until the lacewing larvae attacked for the first time. If the first attack was not successful (i.e., if the lacewing larva released the milkweed bug larva after a short period of probing, 52 s on average, SD = 92;  $n = 24$  across all trials), lacewing larvae were removed, and milkweed bug larvae were provided with a sunflower seed and water and checked for survival the next day. We excluded trials in which lacewing larvae did not attack bug larvae over the time of observation. The proportion of surviving milkweed bug larvae after the first attack by a lacewing between treatments was compared using two-tailed Fisher's exact test in JMP.

In a second set of experiments, we tested whether sequestered toxins have a deterrent effect on lacewing feeding after successful attack (i.e., if the lacewing larvae initiated feeding on the milkweed bug larvae). For *L. equestris*, both experiments (survival and feeding) were carried out twice, since we initially assumed that the lack of an effect was due to the thick wall of the *A. vernalis* follicle rendering the seed inaccessible to small *L. equestris* larvae. Therefore, we repeated the experiments and maintained larvae on *A. vernalis* follicles chopped with a razor blade. Because of an overall lack of effect on lacewing behavior, both experiments were combined for analyses. Details of this experiment are described in the supplemental PDF.

#### *Behavioral Assays with Birds*

Adults of *S. saxatilis*, *L. equestris*, and *H. superbus* raised either on sunflower seeds (nontoxic control) or on toxic seeds were offered to great tits (*Parus major*; Passeriformes: Paridae) to test whether sequestered toxins protect bugs against avian predators. Great tits are generalist insectivorous birds that share the geographic range and habitats with the milkweed bug species tested and are therefore potential natural predators (Cramp et al. 1993). Since wild-caught great tits usually avoid aposematic prey, including milkweed bugs (Hotová Svádová et al. 2010), and this avoidance is largely based on individual learning (Hotová Svádová et al. 2013), we tested hand-reared juvenile birds, which were naive to the experience with any aposematic insect prey.

Details of the hand-rearing protocol are described in the supplemental PDF.

For this experiment, we raised milkweed bugs from the first or second instar to adults either on pure sunflower seeds or on a 1:1 mixture of sunflower seeds and toxic seeds (*C. autumnale* for *S. saxatilis*, *A. vernalis* for *L. equestris*, and *D. purpurea* for *H. superbus*) in plastic containers and supplied them with water as described above. Larvae were maintained in a growth chamber (Fitotron SGC 120, Weiss Technik, Loughborough, United Kingdom) with a 16L:8D photoperiod at 26°C–27°C and 60% humidity. Before the predation assay, we transferred adult bugs to pure sunflower seeds and kept them for at least 1 week under the conditions described above to purge their guts of potentially retained plant toxins.

Birds were tested individually in wood-frame cages (70 cm × 70 cm × 70 cm) with wire-mesh walls equipped with a perch, a water bowl, and a feeding tray. Cages were illuminated by daylight-simulating Osram Biolux 18-W/965 tubes. Prey was offered to birds in glass Petri dishes (diameter: 50 mm) placed on the light-colored wooden feeding tray. This way, all prey items appeared conspicuous against the background. Before the experiment, the birds were habituated to the experimental cage, trained to eat live mealworms from the tray, and deprived of food for 2 h. We observed the birds through the one-way mirror in the front wall of the cage and recorded their behavior using the Observer XT (Noldus, Wageningen, Netherlands) software.

We divided the birds into three experimental groups: 40 birds were tested with *S. saxatilis*, 40 birds were tested with *L. equestris*, and 20 birds were tested with *H. superbus*. Within each group, half of the birds were tested with bugs raised on seeds of their respective toxic host plants, and the other half were tested with bugs raised on sunflower seeds. To account for potential effects of prey novelty, we used Jamaican field crickets (*Gryllus assimilis*) of a size similar to the bugs as control palatable prey that would be unfamiliar to the birds. The experiment consisted of a sequence of 5-min trials following immediately one after another in which the birds were alternately offered a milkweed bug or a cricket, starting with the cricket. Each bird was offered three milkweed bugs and three crickets altogether. In each trial, we recorded whether the prey was attacked (pecked or seized), killed, and eaten (at least partly); the latency of the first attack; and the duration of discomfort-indicating behavior (beak wiping and head shaking) observed in birds. If the bug was attacked but alive at the end of the trial, it was provided with water and sunflower seeds and checked for survival the next day.

Bird predation data were analyzed using generalized linear models in R (R Core Team 2018). Attack probabilities and survival probabilities of the bugs were compared across the three trials using generalized estimating

equation (GEE) models (package *geepack*; Højsgaard et al. 2006) with binomial errors. We entered trial number, bug species, and host plant toxicity as fixed effects and bird individual as a random effect. The models initially included all possible two-way interactions and were simplified by comparing nested models using the quasi-information criterion. In the analysis of survival probabilities, only the data from bugs attacked by birds were included. To find out whether the general effect of host plant toxicity on reactions of birds also holds for each of the milkweed bug species studied, the abovementioned models were run separately for each bug species. Besides attack and survival probabilities, we analyzed attack latencies, durations of discomfort-indicating behavior of birds, whether the bugs were (at least partly) eaten, and survival of bugs compared with control crickets; see the supplemental PDF for details. In all models, an equivalent of the partial sums of squares method (type III ANOVA table) was used to estimate the effects.

#### *Injection Experiments to Assess Cardenolide and Colchicine Resistance*

We injected adults of field-collected *P. apterus* or sunflower-raised *O. fasciatus*, *S. saxatilis*, and *S. pandurus* with colchicine or the cardenolide ouabain (Sigma-Aldrich) to test for the ability to tolerate these toxins in the body cavity (i.e., to mimic sequestration). We injected 1  $\mu$ L of toxins (dissolved in phosphate-buffered saline [PBS], pH 7.4) or PBS as a control with glass capillary needles. Solutions were injected laterally between the penultimate and the last abdominal segment using a micromanipulator and a microsyringe pump injector (World Precision Instruments, Sarasota, FL) under a dissecting microscope ( $n = 8$  individuals per dose).

In total, we carried out three injection experiments. In experiment 1, *P. apterus*, *O. fasciatus*, and *S. saxatilis* were injected with a high dose of either ouabain (5 mg/mL) or colchicine (10 mg/mL). As we observed no effect of colchicine in *S. saxatilis* as opposed to the other species, we injected *S. saxatilis* with an even higher dose of colchicine (30 mg/mL) in a second experiment and again injected ouabain (5 mg/mL) into additional specimens for comparison. Since *P. apterus* responded to both toxins in the first trial, we injected colchicine at concentrations of 0.1, 1, 5, or 10 mg/mL or ouabain at concentrations of 0.1, 1, 2.5, or 5 mg/mL within the same attempt to address the extent of resistance quantitatively. *Oncopeltus fasciatus* that tolerated the high dose of ouabain (5 mg/mL) were injected only with increasing concentrations of colchicine (0.1, 1, 5, and 10 mg/mL). To keep the number of injected animals as low as possible, we omitted injections of PBS during experiment 2 (tolerance to injections per se was already apparent from

the first experiment). Last (experiment 3), we injected *S. pandurus*, a congener of *S. saxatilis*, with PBS and colchicine at concentrations of 0.1, 1, 5, or 10 mg/mL. After injection, we maintained bugs individually in Petri dishes with a water source (see above) and one sunflower seed under ambient conditions. On the next day, we assessed bugs for signs of paralysis (i.e., inability to walk, slowed movement of legs and antennae). These observations were not carried out blind to treatment, but the effects were very obvious and therefore unlikely to be affected by observer bias. Injection assays with one dose of a toxin were analyzed using Fisher's exact test in JMP with Bonferroni correction for multiple comparisons. For comparing dose-dependent effects, we used the Cochran-Armitage trend test in JMP. All data underlying our study have been deposited in the Dryad Digital Repository (<https://doi.org/10.5061/dryad.bk3j9kdc>; Petschenka et al. 2022).

## Results

### *Growth and Development of Milkweed Bugs on Different Diets*

We tested whether the availability of toxic seeds (*Digitalis purpurea* for *Horvathiolus superbus*, *Adonis vernalis* for *Lygaeus equestris*, and *Colchicum autumnale* for *Spilostethus saxatilis*; see fig. 1) influences larval development (i.e., growth and time until the adult stage). After 3 weeks, a diet of pure sunflower seeds resulted in maximal growth in all three insect species tested (fig. 2a–2c). We found a significant effect of seed diet on growth of *L. equestris* ( $F_{3,37} = 109.716$ ,  $P < .001$ ) and *S. saxatilis* ( $F_{3,39} = 56.631$ ,  $P < .001$ ) but not of *H. superbus* ( $F_{3,35} = 1.195$ ,  $P = .326$ ), which grew equally across all treatments. On diverse seed mixtures without toxic seeds, *S. saxatilis* grew as well as on sunflower seeds (least squares means [LSMeans] Tukey honest significant difference [HSD]:  $P = .883$ ), while *L. equestris* reached only about half of the body mass compared with the sunflower diet (LSMeans Tukey HSD:  $P < .001$ ). Inclusion of toxic seeds in these mixtures did not affect growth for either of the species (LSMeans Tukey HSD: *L. equestris*,  $P = .166$ ; *S. saxatilis*,  $P = .936$ ). For both species, diets consisting exclusively of toxic seeds resulted in lower body mass compared with seed mixtures (LSMeans Tukey HSD: *L. equestris*,  $P < .001$ ; *S. saxatilis*,  $P < .001$ ) and resulted in a reduction of >50% in body mass compared with the sunflower diet (LSMeans Tukey HSD: *L. equestris*,  $P < .001$ ; *S. saxatilis*,  $P < .001$ ). Initial body mass affected the final weight of *S. saxatilis* ( $F_{1,39} = 5.453$ ,  $P = .025$ ) but not of *L. equestris* ( $F_{1,37} = 1.114$ ,  $P = .298$ ) and *H. superbus* ( $F_{1,35} = 1.176$ ,  $P = .286$ ). The modeling of larval growth as a continuous process and comparison of absolute growth rates revealed similar results (fig. S2).

*Horvathiolus superbus* developed equally fast on all diets ( $F_{3,35} = 2.22$ ,  $P = .103$ ;  $n = 9$  or 10 for all treatments), while the dietary treatment affected developmental time in *L. equestris* ( $F_{3,40} = 7.168$ ,  $P < 0.001$ ;  $n = 11$  for all treatments). Larvae needed longer to reach the adult stage on pure toxic seeds compared with sunflower seeds and the toxic seed mixture (LSMeans Tukey HSD:  $P = .002$ ;  $P < .001$ ) but not compared with the nontoxic seed mixture (LSMeans Tukey HSD:  $P = .097$ ). Developmental speed between the other diets was not different (LSMeans Tukey HSD: nontoxic seed mixture vs. toxic seed mixture,  $P = .3$ ; nontoxic seed mixture vs. sunflower,  $P = .48$ ; sunflower vs. toxic seed mixture,  $P = .987$ ). Although we omitted formal statistical analysis for *S. saxatilis*, only one of 11 individuals from the pure toxic diet reached adulthood during the time of observation (as opposed to between six and 11 of 11 on the other diets). Similarly, mortality during the feeding experiment was highest on the pure toxic diet, with dead bugs occurring in six of 11 Petri dishes compared with one to three in the other treatments. In *L. equestris* and *H. superbus*, differences across diets were less pronounced (deaths occurred in two to three and three to five of 11 Petri dishes).

### *Sequestration in Milkweed Bugs on Different Diets*

In addition to growth, we quantified sequestration of cardenolides and colchicum alkaloids. We found substantial sequestration of cardenolides in *H. superbus* and in *L. equestris* raised on pure *Digitalis* or pure *Adonis* seeds and seed mixtures containing toxic seeds, respectively, while bugs raised on sunflower seeds or seed mixtures without toxic seeds were devoid of cardenolides (fig. 2d, 2e). Similarly, *S. saxatilis* raised on seeds of *Colchicum* and seed mixtures containing *Colchicum* seeds sequestered high amounts of colchicum alkaloids (fig. 2f). However, bugs from diet treatments lacking *Colchicum* seeds still contained low levels of these toxins, which are most likely derived from field-collected females by transfer via the egg. The amount of sequestered toxins was always highest for the diets composed of pure toxic seeds (Welch's test of diet effect: *H. superbus*,  $F_{1,19} = 18.863$ ,  $P < .001$ ; *L. equestris*,  $F_{1,10} = 66.206$ ,  $P < .001$ ; *S. saxatilis*,  $F_{3,20} = 83.568$ ,  $P < .001$ ).

### *Sequestration of Plant Toxins in Field-Collected Milkweed Bugs*

We collected adult milkweed bugs in the field to test for sequestration of plant toxins under natural conditions (figs. 1, S3). All specimens of *H. superbus* ( $n = 10$ ) from a habitat with *D. purpurea* contained cardenolides ranging from 1 to



8.9  $\mu\text{g}/\text{mg}$  dry mass (2.6–28.6  $\mu\text{g}/\text{individual}$ ;  $n = 10$ ). *Horvathiolus superbus* collected from a *Digitalis*-free habitat ( $n = 12$ ) that we observed feeding on cardenolide-rich pods of *E. crepidifolium* invariably contained high amounts of sequestered cardenolides ranging from 23 to 61.2  $\mu\text{g}/\text{mg}$  (41–147  $\mu\text{g}/\text{individual}$ ). All *L. equestris* obtained from the *A. vernalis* site had cardenolide concentrations ranging from 0.14 to 28.12  $\mu\text{g}/\text{mg}$  dry mass (4.7–459.6  $\mu\text{g}/\text{individual}$ ;  $n = 12$ ). Moreover, we detected cardenolides in eggs laid by field-collected females ( $0.23 \pm 0.01 \mu\text{g}/\text{mg}$  dry weight [mean  $\pm$  SE];  $n = 3$ ). *Spilostethus pandurus* collected from infructescences of *Urginea maritima* contained up to 17.8  $\mu\text{g}$  bufadienolides/ $\text{mg}$  dry mass (up to 808  $\mu\text{g}/\text{individual}$ ;  $n = 7$ ).

Adults of *S. saxatilis* obtained from two different populations consistently contained colchicum alkaloids ranging from 0.05 to 6.2  $\mu\text{g}/\text{mg}$  dry mass (1.55–113.7  $\mu\text{g}/\text{individual}$ ; Nüstenbach,  $n = 16$ ) and from 6.4 to 9.4  $\mu\text{g}/\text{mg}$  dry mass (134.3–214.3  $\mu\text{g}/\text{individual}$ ; Berghausen,  $n = 9$ ). Eggs from field-collected females contained colchicum alkaloids ( $0.94 \pm 0.26 \mu\text{g}/\text{mg}$  dry weight [mean  $\pm$  SE];  $n = 5$ ). Concentrations of colchicum alkaloids in *S. saxatilis* defensive secretion were more than 50 times higher compared with hemolymph (paired *t*-test on six paired samples:  $t = 4.234$ ,  $df = 5$ ,  $P = .008$ ; fig. S4). The comparison of sequestered toxins to toxins present in the host plant seeds revealed a clear overlap of individual compounds only for some bug–plant species pairs (fig. S5), indicating extensive metabolism or selective uptake and up-concentration by milkweed bugs (see the supplemental PDF for details on structural identification and comparison to seed extracts). Comparisons to authentic reference compounds (bufadienolides, cardenolides, and colchicum alkaloids) allowed for identification of individual compounds only in some cases (fig. S6).

#### Colchicum Alkaloids in Museum Specimens

We screened 30 museum specimens of *S. saxatilis* from 21 locations in 10 European countries and one location in North Africa (fig. S7). Although some of the specimens were more than 110 years old, we detected substantial amounts of colchicum alkaloids ranging from 0.8 to 182.5  $\mu\text{g}/\text{individual}$  ( $58 \pm 47.58$ , mean  $\pm$  SD) in all specimens. Remarkably, only two of the specimens tested contained trace amounts of putative cardenolides, suggesting that sequestration of cardenolides does not play a role for the defense of *S. saxatilis*.

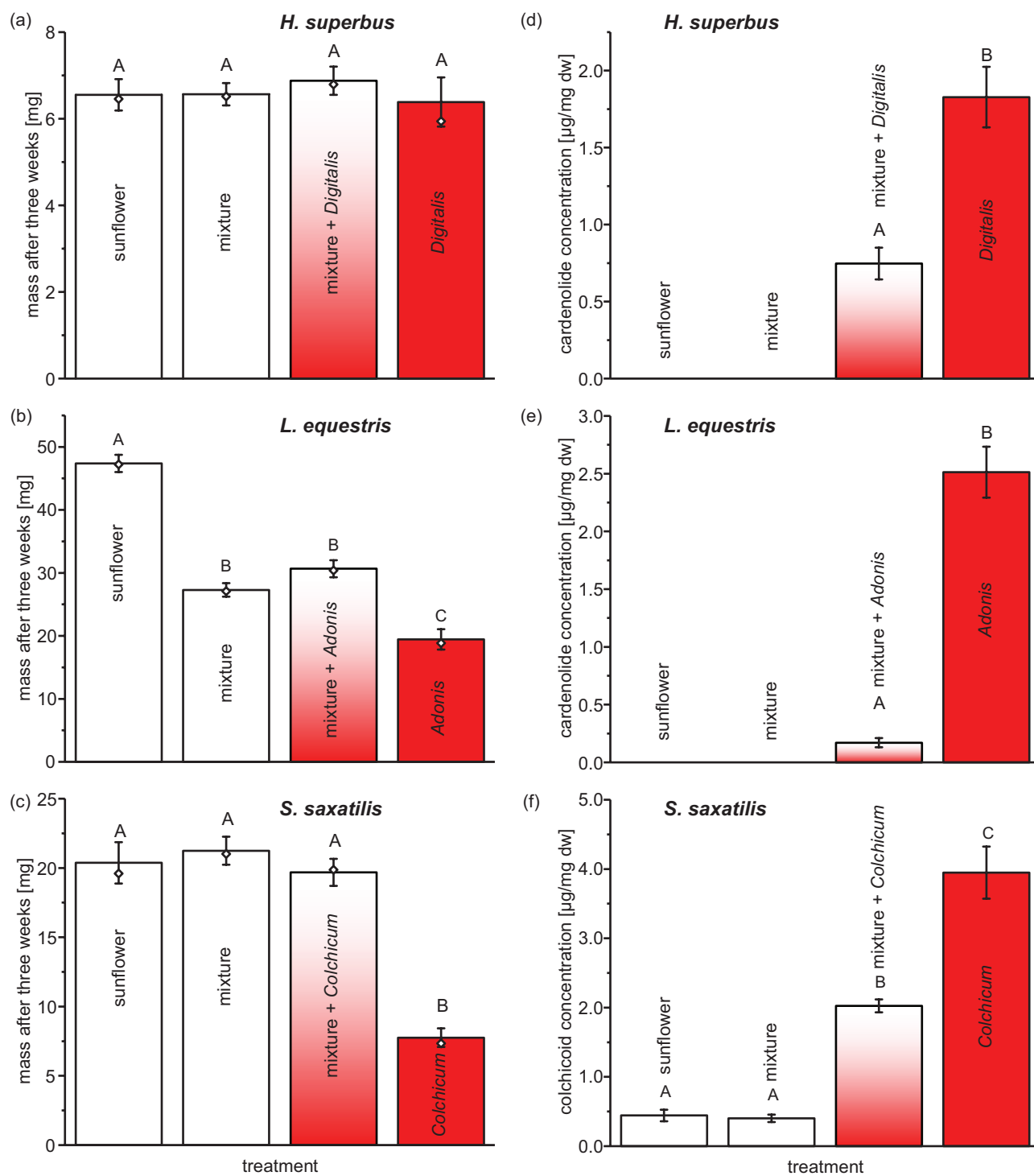
#### Effect of Sequestered Toxins on Lacewing Predation

We assessed the effect of sequestered plant toxins on lacewing predation in four species of milkweed bugs.

*Horvathiolus superbus* larvae raised on seeds of *Digitalis* contained  $3.15 \pm 0.99 \mu\text{g}$  cardenolides/ $\text{mg}$  dry weight (mean  $\pm$  SE;  $n = 7$ ) and survived lacewing attacks more often compared with sunflower-raised individuals (fig. 3a; two-tailed Fisher's exact test:  $P = .02$ ). However, even though *L. equestris* larvae raised on *Adonis* seeds contained higher cardenolide amounts (whole seeds:  $4.95 \pm 0.27 \mu\text{g}/\text{mg}$  [mean  $\pm$  SE],  $n = 8$ ; chopped seeds:  $5.01 \pm 0.45 \mu\text{g}/\text{mg}$ ,  $n = 5$ ), only one larva out of a total of 63 *L. equestris* larvae tested ( $n = 32$  for sunflower seeds,  $n = 31$  for *Adonis* seeds) survived the first attack by a lacewing larva (fig. 3b; two-tailed Fisher's exact test:  $P = 1$ ). Similarly, *S. pandurus* larvae raised on *Urginea* seeds contained  $16.11 \pm 4.39 \mu\text{g}/\text{mg}$  bufadienolides ( $n = 7$ ), yet only one larva of all 40 tested survived the first lacewing attack ( $n = 20$  for both treatments; two-tailed Fisher's exact test:  $P = 1$ ). In contrast, sequestration of colchicum alkaloids protected *S. saxatilis* from lacewing attacks. Thirteen of 20 larvae derived from eggs of field-collected females survived the first attack by a lacewing larva (fig. 3c), while there was no survival in sunflower-raised *Oncopeltus* larvae used as a control (two-tailed Fisher's exact test:  $P < .001$ ). Concentrations of colchicum alkaloids in *S. saxatilis* larvae were  $53.15 \pm 3.12 \mu\text{g}/\text{mg}$  [mean  $\pm$  SE],  $n = 10$ ). Even though only two of four systems tested showed an effect on survival, there was at least some evidence for a negative effect of sequestered compounds on consumption and weight gain by lacewing larvae in all four systems (fig. S8; see the supplemental PDF for details).

#### Effects of Sequestered Toxins on Defense against Avian Predators

We analyzed the effects of sequestered plant toxins on defense against avian predators in *L. equestris*, *S. saxatilis*, and *H. superbus*. Overall attack probabilities were similar for all bug species (GEE:  $\chi^2_2 = 3.736$ ,  $P = .154$ ) but significantly lower when the birds were tested with bugs from toxic host plants (GEE:  $\chi^2_1 = 5.596$ ,  $P = .018$ ). Attack probabilities decreased over successive trials (GEE:  $\chi^2_1 = 93.819$ ,  $P < .001$ ), and the decrease was affected by neither bug species (GEE: trial-species interaction,  $\chi^2_2 = 2.032$ ,  $P = .362$ ) nor host plant toxicity (GEE: trial–host plant interaction,  $\chi^2_1 = 0.815$ ,  $P = .367$ ). However, differences in the decrease of attack probabilities became apparent if species were analyzed separately. In *S. saxatilis*, attack probabilities decreased over trials (GEE:  $\chi^2_1 = 44.267$ ,  $P < .001$ ) and more so when the bugs were coming from *C. autumnale* than from sunflower (GEE: host plant,  $\chi^2_1 = 2.414$ ,  $P = .121$ ; trial–host plant interaction,  $\chi^2_1 = 6.009$ ,  $P = .014$ ). Likewise, attack probabilities toward *H. superbus* decreased over trials (GEE:  $\chi^2_1 = 25.319$ ,  $P < .001$ ), and the decrease was steeper for bugs raised on *D. purpurea* (GEE: host plant,



**Figure 2:** Growth of milkweed bugs and sequestration of plant toxins across different diets. Larvae of *Horvathiolus superbis* (a), *Lygaeus equestris* (b), and *Spilostethus saxatilis* (c) were raised on sunflower seeds; a seed mixture; the same mixture containing *Digitalis* (for *H. superbis*), *Adonis* (for *L. equestris*), or *Colchicum* (for *S. saxatilis*) seeds; or pure *Digitalis*, *Adonis*, or *Colchicum* seeds only. Larval mass was recorded over a period of 3 weeks. Bars represent means of body mass after 3 weeks  $\pm$  SEs. Diamonds represent retransformed model means of data that were  $\log_{10}$  transformed for statistical analysis. Sample sizes for the obtained body masses for *H. superbis* were  $n = 11$  for sunflower seeds,  $n = 10$  for seed mixture,  $n = 11$  for mixture plus *Digitalis*,  $n = 10$  for *Digitalis* seeds only; for *L. equestris* and *S. saxatilis*,  $n = 11$  for all diets. After the growth experiment, bugs were harvested for chemical analyses. The amount of sequestered cardiac glycosides

$\chi^2_1 = 1.249$ ,  $P = .412$ ; trial–host plant interaction,  $\chi^2_1 = 6.525$ ,  $P = .011$ ). In contrast, attack probabilities toward *L. equestris* decreased over trials irrespective of host plant toxicity (GEE:  $\chi^2_1 = 28.644$ ,  $P < .001$ ; host plant,  $\chi^2_1 = 0.056$ ,  $P = .813$ ; trial–host plant interaction,  $\chi^2_1 = 1.067$ ,  $P = .302$ ).

Overall survival probabilities following the attacks were higher in bugs from toxic host plants (GEE:  $\chi^2_1 = 24.287$ ,  $P < .001$ ) and increased significantly over successive trials (GEE:  $\chi^2_1 = 16.582$ ,  $P < .001$ ); this increase was steeper in the bugs from toxic host plants (GEE: trial–host plant interaction,  $\chi^2_2 = 9.341$ ,  $P = .002$ ; fig. 3*d–3f*). Bug species affected neither the overall survival (GEE:  $\chi^2_2 = 0.777$ ,  $P = .678$ ) nor its increase over the trials (GEE: trial–species interaction,  $\chi^2_1 = 3.096$ ,  $P = .213$ ). Separate analyses for each species revealed that survival probabilities were generally higher if milkweed bugs were raised on toxic host plants (GEE: *S. saxatilis*,  $\chi^2_1 = 12.471$ ,  $P < .001$ ; *L. equestris*,  $\chi^2_1 = 10.037$ ,  $P = .002$ ; *H. superbus*,  $\chi^2_1 = 5.086$ ,  $P = .024$ ), and the survival increased over the trials (GEE: *S. saxatilis*,  $\chi^2_1 = 5.004$ ,  $P = .025$ ; *L. equestris*,  $\chi^2_1 = 5.757$ ,  $P = .016$ ; *H. superbus*,  $\chi^2_1 = 5.992$ ,  $P = .014$ ; fig. 3*d–3f*). The increase was steeper in *S. saxatilis* and *L. equestris* coming from toxic host plants (GEE: trial–host plant interaction, *S. saxatilis*:  $\chi^2_1 = 6.097$ ,  $P = .014$ ; *L. equestris*:  $\chi^2_1 = 4.394$ ,  $P = .036$ ), but there was no significant difference in *H. superbus* (GEE:  $\chi^2_1 = 2.442$ ,  $P = .118$ ).

Sequestered plant toxins also increased attack latencies in repeated encounters (fig. S9), prolonged durations of discomfort-indicating behavior observed in birds (fig. S10), and decreased the chance that the birds would consume the bugs. Nevertheless, milkweed bugs devoid of sequestered toxins were not entirely undefended against the avian predators, indicating the defensive role of endogenous scent gland secretion (see the supplemental PDF for details).

#### *In Vivo Tolerance to Injected Ouabain and Colchicine*

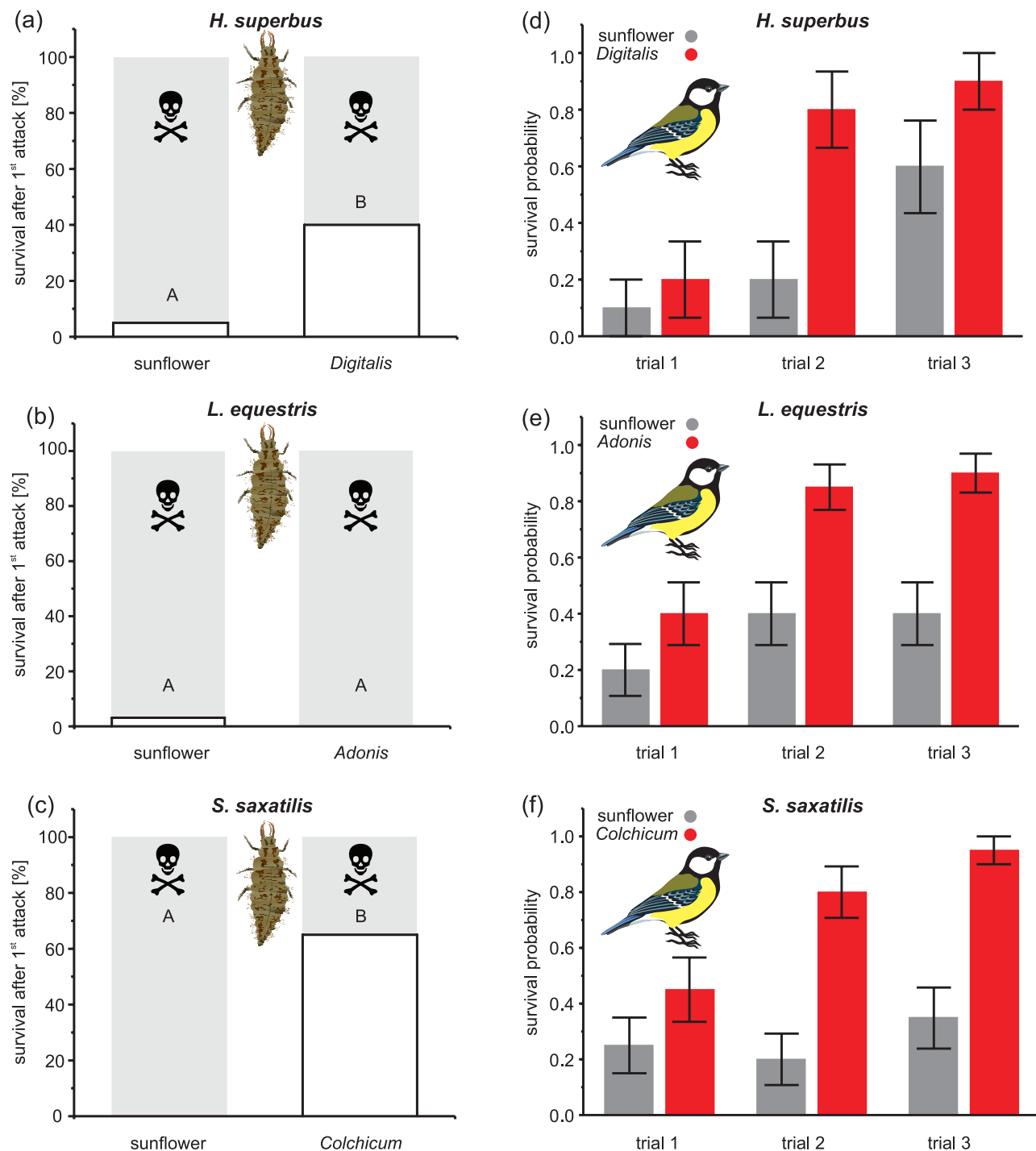
Because of the rare occurrence of colchicine in nature, which is restricted to *Colchicum* spp. and other Colchicaceae (Vinnersten and Larsson 2010), we predicted that *S. saxatilis* was not preadapted to this toxin but evolved novel resistance traits against colchicine. In addition, we predicted that this species would retain resistance against cardiac glycosides on the basis of its evolutionary history. To test for toxin re-

sistance and to mimic sequestration, we injected colchicine or ouabain directly into the body cavity of milkweed bugs. Besides *S. saxatilis*, we used *Pyrrhocoris apterus* (Pyrrhocoridae) as a nonadapted out-group, and the lygaeine *Onco-peltus fasciatus* as a cardenolide-sequestering milkweed specialist. None of the species tested was affected by blank injections of the solvent PBS (figs. 4, S11). As expected, *P. apterus* was unable to tolerate injections of either 5  $\mu\text{g}$  of ouabain or 10  $\mu\text{g}$  of colchicine (ouabain vs. PBS,  $P = .001$ ; colchicine vs. PBS,  $P = .001$ ; two-tailed Fisher's exact test at  $P = .025$  after Bonferroni correction). All *O. fasciatus* individuals tolerated an injection of 5  $\mu\text{g}$  of ouabain but were not able to tolerate 10  $\mu\text{g}$  of colchicine (two-tailed Fisher's exact test:  $P = .001$ ). As predicted, *S. saxatilis* tolerated injections with both classes of toxins (ouabain vs. PBS,  $P = 1$ ; colchicine vs. PBS,  $P = 1$ ; two-tailed Fisher's exact test at  $P = .025$  after Bonferroni correction) and even tolerated colchicine up to 30  $\mu\text{g}$  colchicine/individual as the highest dose tested (see the supplemental PDF for details). *Spilostethus pandurus*, a congener of *S. saxatilis*, was not able to tolerate colchicine injections. Moreover, we found that *O. fasciatus* and *P. apterus* responded in a dose-dependent manner to toxins for which they lack tolerance (fig. S11).

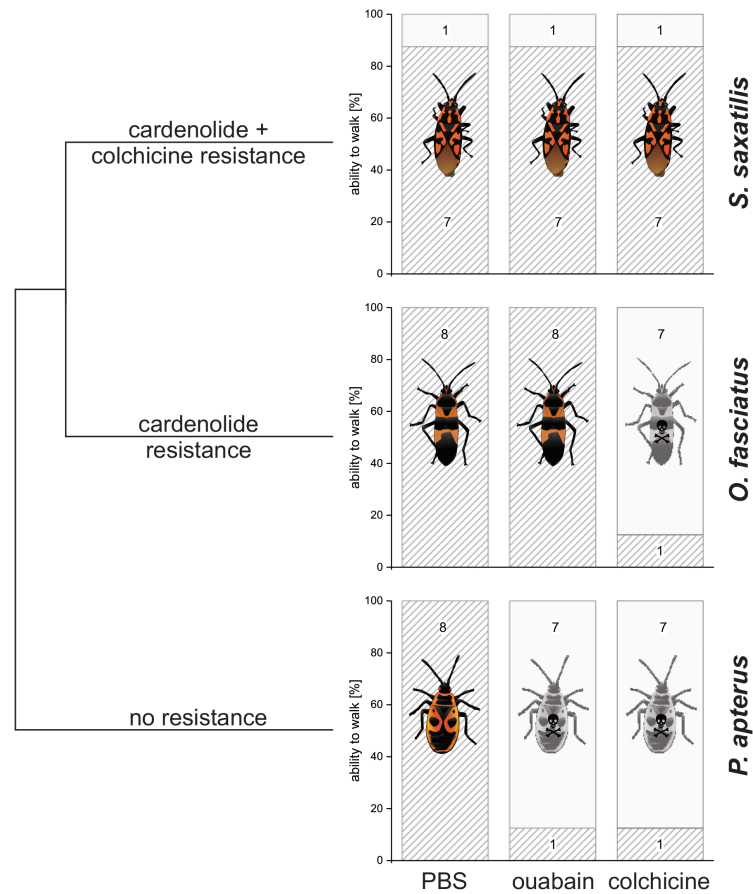
## Discussion

It is widely accepted that coevolution between insects and plants occurs in a multitrophic context. Nevertheless, our understanding of the evolutionary drivers that shape these interactions is still limited, especially with regard to the underlying mechanisms. Coevolutionary theory posits that occupation of novel dietary niches depends on insect resistance to host plant toxins and favors specialization, as increased resistance is expected to trade off with dietary breadth of the herbivore. Moreover, dietary specialization in herbivores is frequently associated with sequestration of host plant toxins, and it was recently shown that interactions with higher trophic levels (predators and parasitoids) likely select for specific resistance traits in insects (Petschenka and Agrawal 2015). However, the interplay between specific adaptations and acquisition of plant toxins for defense as a driver of host shifts (Murphy and Feeny 2006) and specialization has never been addressed. Here, we tested whether sequestration of plant toxins used for antipredator defense and associated physiological preadaptation in milkweed bugs

from *Digitalis* (*d*) and *Adonis* (*e*) seeds or colchicum alkaloids (*f*) was always highest on pure diets, but sequestration was also substantial in seed mixtures containing *Digitalis*, *Adonis*, or *Colchicum* seeds. Colchicum alkaloids found in bugs raised on sunflower seeds or seed mixtures lacking *Colchicum* seeds originate from maternal egg transfer. Bars represent mean concentrations of sequestered toxins  $\pm$  SEs. Sample sizes are identical to the ones mentioned above except  $n = 10$  for *L. equestris* on the seed mixture with *Adonis*. Different uppercase letters above bars indicate significant differences among treatments ( $P < .05$ , Tukey honest significant difference).



**Figure 3:** Predation by lacewing larvae (*Chrysoperla carnea*) and great tits (*Parus major*) on three species of milkweed bugs. Milkweed bugs were raised either on sunflower seeds (controls) or on seeds of the following plant species: *Digitalis purpurea* (for *Horvathiolus superbus*), *Adonis vernalis* (for *Lygaeus equestris*), or *Colchicum autumnale* (for *Spilostethus saxatilis*). Results for an additional experiment with larvae of *Spilostethus pandurus* raised on seeds of *Urginea maritima*, which was carried out with lacewing larvae only, are reported in the article. Please note that in the experiment with *S. saxatilis* and lacewing larvae, sunflower-raised larvae of *Oncopeltus fasciatus* were used as a control, since eggs from field-collected *S. saxatilis* contain colchicum alkaloids via maternal transfer. Since sunflower-raised larvae of all other species tested were palatable to lacewings, higher survival of *S. saxatilis* compared with *O. fasciatus* nymphs is likely due to sequestered colchicum alkaloids and not based on an intrinsic unpalatability of *S. saxatilis* compared with *O. fasciatus*. a–c, We assessed the proportion of milkweed bug larvae that survived the first lacewing attack: *H. superbus* ( $n = 20$  for both diets), *L. equestris* (sunflower:  $n = 32$ ,



**Figure 4:** Resistance of *Pyrrhocoris apterus*, *Oncopeltus fasciatus*, and *Spilostethus saxatilis* to injected toxins. Adult hemipteran specimens were injected with either phosphate-buffered saline (PBS; control), the cardenolide ouabain (5 mg/mL; i.e., 5  $\mu$ g/individual), or the alkaloid colchicine (10 mg/mL; i.e., 10  $\mu$ g/individual). On the left, we mapped resistance phenotypes on a scheme depicting the phylogenetic relationships of the species involved. Bar charts on the right show the percentage of individuals that showed no signs of intoxication on the next day after injecting toxins (hatched). Insect icons are intended to visualize either a toxic effect (gray bug with skull) or no toxic effect (colored bug). Numbers in stacked bars indicate the actual number of affected or unaffected individuals. Note that we also tested *Spilostethus pandurus*, a congener of *S. saxatilis*, and found it not to possess resistance to colchicine (see fig. S11).

mediated their specific associations with particular host plants.

In accordance with their global association with plants in the Apocynaceae, milkweed bugs (Lygaeinae) are preadapted to cardiac glycosides occurring in other host plants. We identified three milkweed bug species (*Lygaeus equestris*, *Horvathiolus superbus*, and *Spilostethus pandurus*) that independently colonized plants from the botanical families Asparagaceae, Brassicaceae, Plantaginaceae, and Ranunculaceae, which convergently produce cardiac glycosides. A fourth species, *Spilostethus saxatilis*, is obligatorily associ-

ated with the Colchicaceae *Colchicum autumnale* (fig. 1), producing a chemically unrelated but highly toxic defensive compound, colchicine. To test whether these host shifts were mediated by the benefit of exploiting a new food resource or getting access to a novel source of defensive toxins for sequestration, we carried out a set of experiments with *H. superbus* raised on *Digitalis purpurea* seeds, *L. equestris* raised on *Adonis vernalis* seeds, and *S. saxatilis* raised on *C. autumnale* seeds to compare growth, sequestration, and protection against predators on the novel host plants.

*A. vernalis*:  $n = 31$ ), and *S. saxatilis* ( $n = 20$  for both diets). Gray (skull) indicates the proportion of bugs that were killed; white indicates the proportion of bugs that survived the first attack by the lacewing larva. Different uppercase letters above bars indicate significant differences among treatments. *d-f*, Survival probabilities of adult milkweed bugs raised on sunflower seeds (gray bars) or on seeds of toxic host plants (red bars) across three successive encounters with juvenile great tits: *H. superbus*, *L. equestris*, and *S. saxatilis*. Only the data from bugs attacked by birds are included. Bars represent mean survival probabilities  $\pm$  SEs.

In opposition to the novel host plants representing valuable food resources for milkweed bugs, we found that the inclusion of toxic seeds in the diet had either negative effects on growth or, at most, neutral effects on growth. On pure diets of toxic *A. vernalis* and *C. autumnale* seeds, growth was reduced substantially, indicating that seeds from these plant species alone are not an optimal diet. Only *H. superbus* grew equally well on toxic *D. purpurea* seeds as on all other diets, suggesting a higher nutritional content of these seeds or a higher degree of dietary specialization in this milkweed bug species. Our results demonstrate that seeds from toxic host plants are not required for and may even impair successful development. This is in line with Kugelberg et al. (1974), who reported that *L. equestris* raised on a pure diet of *A. vernalis* had a shorter life span and oviposition period, produced fewer eggs, and had the lowest number of hatching nymphs per female compared with three other diets, including seed mixtures. While toxic host plants may provide nutritional resources temporarily and the ability to feed on additional host plants may bring some benefits even if costly, it seems rather unlikely that specific associations with these plants were evolutionarily driven by the advantage of occupying novel dietary niches.

In nature, both *L. equestris* and *S. saxatilis* use a great diversity of host plants, and while we lack information on *H. superbus*, it is unlikely to be dietarily restricted to *D. purpurea* alone. For *L. equestris*, 60 plant species from roughly 20 botanical families (Solbreck and Kugelberg 1972) have been recorded, and for *S. saxatilis*, more than 40 species from more than 15 families have been recorded (table S3). Consequently, both species should be considered generalists from a dietary perspective, making their frequent association with nutritionally inferior, rare toxic plants (e.g., *A. vernalis*) even more surprising. However, these associations can be explained by the fact that all milkweed bug species are able to sequester cardiac glycosides from their evolutionarily novel hosts. Moreover, *S. saxatilis* evolved the ability to sequester alkaloids from *C. autumnale*, likely using some of the same mechanisms for uptake, storage, and release that are used for cardiac glycoside sequestration.

Cardiac glycoside-resistant Na<sup>+</sup>/K<sup>+</sup>-ATPases and sequestration of cardiac glycosides apparently are synapomorphic traits of the Lygaeinae (Bramer et al. 2015). In addition, milkweed bugs concentrate cardiac glycosides far above hemolymph levels in specialized storage compartments (Scudder et al. 1986; Bramer et al. 2017) from which they are released upon predator attack. Remarkably, we found an identical suite of adaptations to colchicum alkaloids in *S. saxatilis*, with colchicine and related alkaloids being highly enriched in the defensive secretion compared with the hemolymph. To tolerate sequestration of these compounds, *S. saxatilis* appears to have evolved a novel re-

sistance trait against colchicine, which is not present in its congener *S. pandurus*.

Specialization of *S. saxatilis* to *C. autumnale* (and maybe other *Colchicum* species) is strongly evidenced by our screening of museum specimens. The presence of colchicum alkaloids in 30 randomly selected specimens from 11 countries in Europe and North Africa clearly shows that each individual accessed *Colchicum* during its lifetime. Since we detected cardiac glycosides in similarly old museum specimens of other milkweed bugs (G. Petschenka, unpublished data), the lack of cardenolides in *S. saxatilis* is likely not an extraction bias but rather suggests that this species completely shifted from the use of cardenolides to the novel defense. Oviposition into *Colchicum* seedpods and allocation of high amounts of alkaloids into the eggs finally supports a close association of *S. saxatilis* with *C. autumnale*. Remarkably, *S. saxatilis* still maintains resistance to cardiac glycosides and accumulated resistance traits against different classes of plant toxins over evolutionary time, even though target site insensitivity of Na<sup>+</sup>/K<sup>+</sup>-ATPase was suggested to incur a physiological cost in *O. fasciatus* (Dalla and Dobler 2016). However, maintenance of a cardiac glycoside-resistant Na<sup>+</sup>/K<sup>+</sup>-ATPase was also found in a milkweed bug species of the genus *Arocatus* that lost its association with cardiac glycoside-containing plants (Bramer et al. 2015).

The results of our predation assays revealed that feeding on either cardiac glycoside-containing seeds or colchicum alkaloid-containing seeds at least partially protects milkweed bugs against predators, in particular, lacewing larvae and passerine birds. We found higher survival after lacewing attacks for *H. superbus* raised on *D. purpurea* seeds and for *S. saxatilis* larvae that derived colchicum alkaloids from seeds of *C. autumnale*. In contrast, *L. equestris* raised on *A. vernalis* seeds and *S. pandurus* raised on *Urginea maritima* seeds were not protected against lacewings, suggesting that predator-prey interactions are likely affected by the source and quality (rather than quantity) of sequestered plant toxins, the sequestering insect species, or a combination of both. Despite these differences, our results agree with previous studies (Berenbaum and Miliczky 1984; Skow and Jakob 2005) in supporting the hypothesis that sequestration of plant toxins mediates effective defense of milkweed bugs against arthropod predators.

In experiments with avian predators, sequestration of host plant chemicals decreased attack probabilities and increased prey survival compared with sunflower-raised bugs. This effect was present in all three milkweed bug species, with colchicine having been equally effective as cardenolides. That the sequestered chemicals are aversive for birds was also evidenced by their discomfort-indicating behavior following the contact with bugs from toxic host plants. Higher effectiveness of sequestered than autogenous

chemicals against avian predators has also been found in other studied systems (e.g., leaf beetles [Zvereva et al. 2018] and lanternflies [Song et al. 2018]). Nevertheless, our results show that the autogenous scent gland secretion can by itself increase the bug survival compared with undefended prey. Our findings also indicate that besides being highly toxic, cardenolides (Brower and Fink 1985) and colchicine protect the bugs from avian predators because of a strongly aversive taste; this effect is enhanced by specialized storage compartments (Bramer et al. 2017), which allow the sequestered chemicals to be released immediately upon attack. Consequently, the bugs raised on toxic host plants frequently survived bird attacks and were almost never eaten, while sunflower-raised bugs were frequently killed and at least partly consumed.

The increased effectiveness of sequestered over autogenous defenses indicates that milkweed bugs represent an instance of automimicry (i.e., intraspecific variation in antipredator defense when less defended individuals gain protection by resembling better-defended conspecifics; Brower et al. 1967; Ruxton et al. 2004; Speed et al. 2012). Decreasing attack probabilities and increasing bug survival across trials suggest that birds combined decisions based on visual cues with taste sampling (Skelhorn and Rowe 2006; Sherratt 2011), which allows predators to discriminate between defended individuals and automimics (Guilford 1994; Gamberale-Stille and Guilford 2004). Nevertheless, different defense chemicals could still be equally effective against some predators (Tullberg et al. 2000; Chouteau et al. 2019), and unpredictability of defense may by itself increase predator avoidance (Sherratt et al. 2004; Skelhorn and Rowe 2005; Barnett et al. 2014). Therefore, it is possible that automimicry in milkweed bugs represents an evolutionary stable strategy (Speed et al. 2006; Svennungsen and Holen 2007) maintained by the trade-off between development and defense (Ruxton and Speed 2006). Because the milkweed bugs frequently participate in multispecies mimetic complexes (Hotová Svádová et al. 2010; Burdfield-Steel and Shuker 2014), intraspecific variation in their antipredator defense may affect their mimetic relationships with similarly colored species.

Sequestration of cardiac glycosides from *A. vernalis*, *D. purpurea*, *Erysimum crepidifolium*, and *U. maritima* was most likely facilitated by preadaptation, yet sequestration of colchicum alkaloids is an entirely novel ability. However, while colchicum alkaloids drastically differ from cardiac glycosides in their mode of action, the two types of compounds nonetheless share some similarities: both comprise small, chemically stable, mostly lipophilic molecules that do not require enzymatic activation to become highly toxic. Therefore, even though *S. saxatilis* likely lacked preadaptations for tolerance of the novel toxin, its aposematic coloration and sequestration

machinery for uptake, specialized storage, and release of toxins (Bramer et al. 2017) may well have facilitated the evolution of colchicum alkaloid sequestration. Our findings therefore demonstrate that host shifts of specialized insects can be mediated by preadaptation to specific toxins and convergent evolution of plant toxins in unrelated plant taxa. At the same time, we propose that suites of traits involved in sequestration of one type of chemical may similarly represent preadaptations facilitating shifts to entirely novel classes of chemically unrelated compounds, particularly if favored by large benefits (i.e., sequestration of highly toxic colchicine for antipredator defense).

In conclusion, host plant specialization in milkweed bugs is not an evolutionary dead end (Termonia et al. 2001), and evolutionary plasticity is maintained by several mechanisms, including preadaptation with regard to different traits of the same syndrome. Our findings demonstrate that it is insufficient to classify the degree of specialization in insects solely according to their trophic interactions. Species that classify as dietary generalists may still specialize to host plants serving as a source of sequestered toxins for antipredator defense. Interactions driven by the third trophic level (predators and parasitoids) can therefore direct specialization of bitrophic interactions between herbivores and their host plants.

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### Statement of Authorship

The study was designed by G.P. and A.E. Funding was acquired by G.P., A.E., and A.V. Methods and experimental designs were developed by G.P., A.E., R.H., and T.Z. Data were collected by A.E., A.R., G.P., J.D., J.F.M.G., K.C., L.T., and S.S. and analyzed by A.E., C.H., G.P., R.H., and T.Z. Data were visualized by A.E., G.P., L.T., and T.Z. Resources were provided by A.E., A.V., G.P., J.D., J.F.M.G., and R.T. The experimental work was supervised by A.E. and G.P. The original draft of the manuscript was written by G.P. and A.E. with great support from T.Z. All authors reviewed and edited the manuscript.

### Data and Code Availability

All data reported in this article have been archived in the Dryad Digital Repository (<https://doi.org/10.5061/dryad.bk3j9kdcc>; Petschenka et al. 2022).

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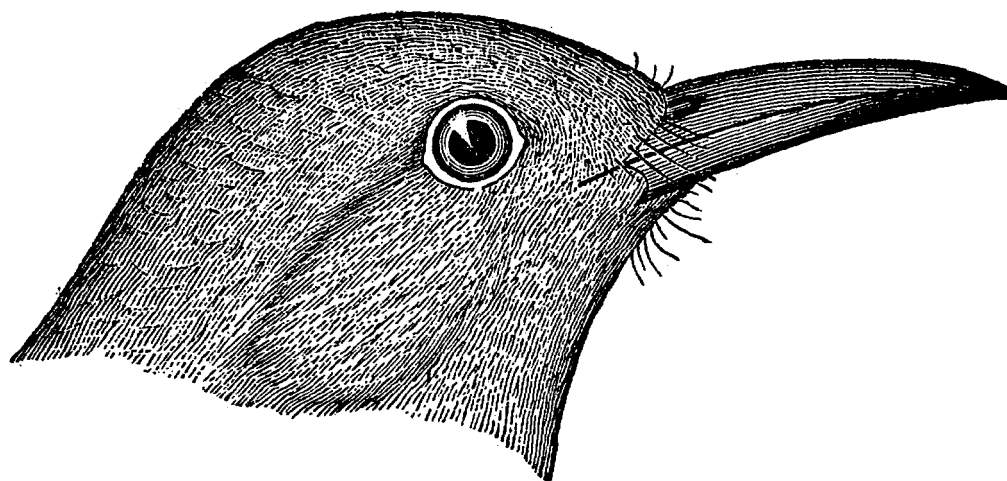
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“Soon after I received my first specimens of var. *Palmeri*, Lt. Bendire sent me a bird I could not make out at all. . . . I wrote to Lt. Bendire, who replied at once that the bird was an entirely distinct species, laying a very different egg, and having somewhat dissimilar habits. . . .” Figured: “Bendire’s Mocking-thrush.” From “Some United States Birds, New to Science, and Other Things Ornithological” by Elliott Coues (*The American Naturalist*, 1873, 7:321–331).