Supplementary Materials

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Sequestration of defenses against predators drives specialized host plant associations in preadapted milkweed bugs (Heteroptera: Lygaeinae)

Short title: Predation as a driver of specialization

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Supplementary Methods

Origin of field-collected milkweed bugs for founding colonies and chemical analysis

Spilostethus saxatilis were collected on August 15th, 2015 in Nüstenbach (49°22'16.4"N 9°07'39.9"E) and on September 18th, 2015 in Berghausen (48°59'38.7"N 8°30'51.4"E), Baden-Württemberg, Germany, either from *Colchicum autumnale* (Colchicaceae) or feeding on other plants. *Horvathiolus superbus* was collected on August 15th and 16th, 2015 in a *Digitalis purpurea* (Plantaginaceae) habitat close to Eberbach (49°28'11.8"N 9°01'33.8"E), Baden-Württemberg, Germany. We collected the same species feeding on *Erysimum crepidifolium* (Brassicaceae) in a *Digitalis*-free habitat on June 12th and 13th 2018 in Schloßböckelheim (49°48'02.6"N 7°44'51.6"E), Rheinland-Pfalz, Germany. *Lygaeus equestris* was collected in the nature reserve 'Oderhänge Mallnow' north of Lebus (52°27'46.9"N 14°30'00.5"E), Brandenburg, Germany on April 19th, 2016 during the blooming of *Adonis vernalis* (Ranunculaceae). *Spilostethus pandurus* was collected from infructescences of *Urginea maritima* (Asparagaceae) in 'Parque Natural Sierra de Aracena y Picos de Aroche' (37°55'44.8"N 6°33'56.4"W), Aracena, Spain, in early November 2016. Another strain of *S. pandurus* which we used for predation and injection experiments was collected in Portugal in 2016 (40°38'46.8"N 8°39'01.2"W). *Pyrrhocoris apterus* for injection assays were collected in Giessen (50°34'25.5"N 8°40'03.2"E), Hesse, Germany.

Modelling of milkweed bug growth in seed mixture experiments

In addition to comparing final body mass of milkweed bug larvae after three weeks of feeding, we also modelled larval growth as a continuous process using the four sequential body mass measurements recorded during the experiment (initial mass, mass at weeks 1, 2, and 3). Log-transformed body masses were modelled using an asymptotic regression model (Paine et al. 2012; Züst et al. 2015), implemented as the *SSasymp* function in the *nlme* package for the statistical software R. Body mass was thus modelled as a function of the initial mass, a rate of increase, and an asymptotic mass at the end of the experiment. Effects of seed mixture were included for the rate of increase and asymptote parameters, while individual bugs (i.e. body mass means per Petri dish) were treated as random effects to account for repeated measures. To compare growth between seed mixture treatments, the absolute growth rate of bugs (AGR, mass gain per day) was calculated for the final day of the experiment (Züst et al. 2015).

Maintenance of milkweed bugs on sunflower seeds to purge toxins from guts

After the mixed seed feeding experiments, bugs were transferred to fresh sunflower seeds for 14 days to clean guts from residual dietary toxins that would bias the estimate of compounds sequestered into the body tissues. Since several true bug species, including *Oncopeltus fasciatus* are known to have a discontinuous digestive tract until the adult stage (Miles 1958), we cannot rule out that larvae had toxins remaining in their guts at the time of analysis (see Supplemental Table 2 for the number of days individual specimens had for purging during the adult stage). As an estimate, we quantified colchicum alkaloids in the gut of last instar larvae of *S. saxatilis* (extracted in 2 x 1 ml methanol, resuspended in 100 µl methanol and analyzed as described above) that were raised on *C. autumnale* seeds from the second larval instar and found them to contain only 1.44 µg alkaloids per gut (n = 4, SE = 0.76). Similarly, other authors found that the amount of cardenolides in filled guts of adult *O. fasciatus* raised on milkweed seeds was below a detection limit of 10 µg (Moore and Scudder 1985). Consequently, it seems unlikely that

substantial amounts of non-sequestered toxins remaining in the larval gut would have biased our results.

Comparison of colchicum alkaloids in hemolymph and defensive secretion of S. saxatilis

To compare concentrations of sequestered colchicum alkaloids between hemolymph and defensive secretion (i.e. clear droplets released at the integument upon attack), we used adults of *S. saxatilis* collected in the field (Berghausen, August 2016). Before collecting samples, we maintained bugs on pure *Colchicum* seeds (supplied with water) for 13 days under ambient conditions. To obtain defensive secretion, we anaesthetized bugs with CO_2 and squeezed them between the blades of broad tweezers. We collected emerging droplets of clear defensive fluid using 0.5 µl glass capillaries. Next, we cut off a hind-leg at the femur to collect hemolymph. We determined the collected volume based on the filling level of the capillaries. In total, we obtained 13 samples of hemolymph and seven samples of defensive secretion (including six paired samples, i.e. from the same individual). One hemolymph sample was excluded since the colchicum alkaloid peaks were too small for automatic integration. Before HPLC-analysis, filled capillaries were stored at -80°C until extraction. To analyze alkaloids, whole capillaries including liquid samples were homogenized and extracted with methanol as described in the HPLC-methods section of the main manuscript. Statistical comparison of colchicum alkaloid concentrations between *S. saxatilis* defensive secretion and hemolymph was carried out using matched pair analysis based on six paired samples (i.e., six samples of hemolymph and six samples of secretions from the same individuals).

HPLC analysis of museum specimens

To test for the occurrence of colchicum alkaloids and cardiac glycosides in museum specimens of *S. saxatilis*, we incubated dry insects in 1 ml of methanol for at least one day and collected supernatants in fresh vials. After repeating this procedure twice (i.e., 3 ml methanol in total), pooled supernatants were evaporated under N_2 and dissolved in 100 µl of methanol using a FastPrep homogenizer (6.5 m/s, 45 sec.). Before analyzing samples for colchicum alkaloids or cardenolides via HPLC using the respective methods (see above), samples were centrifuged (16,100 x g, 3 min) and filtered as described above.

Preparing seeds for HPLC

To compare toxins from host plant seeds to toxins sequestered by the bugs, seeds of *A. vernalis* (10.51 – 14.47 mg, commercial source), *C. autumnale* (5.91 – 10.03 mg, Berghausen, Germany, 2016), *D. purpurea* (59.44 – 63.72 mg, Eberbach, Germany, 2017), *E. crepidifolium* (10.19 – 10.65 mg, Schloßböckelheim, Germany, 2018), and *U. maritima* (2.89 – 7.66 mg, Aracena, Spain, 2016) were weighed and extracted with 0.5 ml (*A. vernalis*, *C. autumnale, U. maritima*) or 1 ml (*D. purpurea*, *E. crepidifolium*) methanol containing 0.01 mg/ml digitoxin (*A. vernalis*) or oleandrin (*D. purpurea*) as an internal standard (no internal standard for *C. autumnale, E. crepidifolium,* and *U. maritima*). For homogenization in a FastPrep homogenizer (2 x 6.5 m/s, 45 s), we used either zirconia beads for *D. purpurea* and *E. crepidifolium* or lysing matrix A (MP Biomedicals) for *A. vernalis*, *C. autumnale* and *U. maritima*. After grinding, samples were centrifuged (16,100 x g, 3 min) and supernatants were transferred to fresh vials. This procedure was repeated once for *D. purpurea* and *E. crepidifolium* seeds and twice for *A. vernalis*, *C. autumnale*, and *U. maritima* seeds. Supernatants of individual samples were pooled and evaporated under N_2. Before HPLC-analyses, dried residues were dissolved in 500 (*C. autumnale*, *U. maritima*), 200 (*D. purpurea*, *E. crepidifolium*) or 100 µl methanol (*A. vernalis*) and analyzed as described above.

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Evaluation of chromatograms

For chromatograms obtained from extracts of field-collected *H. superbus* from *D. purpurea*, *L. equestris*, *S. pandurus*, and *S. saxatilis* we considered all peaks of a sample showing compound specific absorption spectra (i.e. cardenolides, bufadienolides, and colchicum alkaloids) unless peaks were not automatically recognized by the software or had a signal to noise ratio < 2:1. For eggs of *L. equestris* and *S. saxatilis* as well as for the comparison between *S. saxatilis* secretion and hemolymph, the same approach was used. For *S. saxatilis* samples obtained during seed mixture assays, we followed the procedure described above. Peaks in chromatograms from extracts of *H. superbus* and *L. equestris* were only considered if they were present in at least 70% or 60% of samples, respectively. The same approach was used for *H. superbus* and *L. equestris* larvae obtained from the feeding experiments with lacewings. For larvae of *S. saxatilis* and *S. pandurus* peaks were included that were present in at least 80% or 70% of samples, respectively. To evaluate field-collected *H. superbus* from *E. crepidifolium* we included all peaks that occurred in at least in 65% of samples. All datasets that were compared statistically were evaluated based on identical criteria.

Structural identification via reference compounds and liquid chromatography – mass spectrometry

To verify structural identity of selected cardenolides and colchicum alkaloids we compared HPLC retention times of chromatographic peaks obtained from field-collected *L. equestris* samples with authentic standards of k-strophanthoside (Roth, Germany), strophanthidin (PhytoLab, Germany) and cymarin (PhytoLab, Germany), cardenolides which are known to occur in *A. vernalis* (Junior and Wichtl 1980). For the same purpose, extracts from *H. superbus* were compared to authentic digitoxigenin (Sigma-Aldrich, Germany), digitoxin (Sigma-Aldrich, Germany), digoxigenin (Sigma-Aldrich, Germany), digoxin (Sigma-Aldrich, Germany), gitoxigenin (Santa Cruz Biotechnology, USA), gitoxin (EDQM, France), lanatoside C (Sigma-Aldrich, Germany), purpurea glycoside A (EDQM, France), and purpurea glycoside B (EDQM, France) which are known to occur in *D. purpurea* (Luckner and Wichtl 2000), and erysimoside (Latoxan, France), known to occur in *E. crepidifolium* (Makarevich et al. 1974). Similarly, we screened extracts of *S. pandurus* collected from *U. maritima* for the *Urginea* bufadienolides proscillaridin A (PhytoLab, Germany) and scillaren A (Sigma-Aldrich, Germany) (Kopp et al. 1996). Last, we compared chromatograms from field-collected *S. saxatilis* to the authentic colchicum alkaloids colchicoside, 2-demethylcolchicine, 3-demethylcolchicine (Toronto Research Chemicals, Canada), and colchicine (Roth, Germany). In addition to the comparison of HPLC retention times, we compared mass spectra of the colchicum alkaloid compounds in the bug extracts to spectra of authentic alkaloid standards. Mass spectral analyses were performed on a Bruker micrOTOF-Q II mass spectrometer equipped with a Dionex Ultimate 3000 UHPLC and a C18 HPLC column (Kinetex C18, 2.6µ, 100A, 150 x 2.1 mm; Phenomenex). Compounds were separated by gradient elution with a flow rate of 150 mL min^{-1} and the following gradient of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile): 0 to 2 min 16% B, 25 min 70% B, 30 min 95%. The column was eluted with 95% B solvent for an additional 10 min and re-equilibrated at the starting condition for 5 min. Sodium formate infusions at the beginning of each sample run were used for the mass calibration.

Effect of sequestered toxins on consumption of milkweed bug larvae by lacewing larvae

Milkweed bug and lacewing larvae were weighed before the trial and after exposure of bugs to lacewing larvae (after 12 hours for *H. superbus* and 8 hours for the other species) to assess consumption of milkweed bug larvae by lacewings. For statistical analysis, only dead bugs and corresponding lacewing larvae were included to ensure that milkweed bug larvae had been actually attacked by the lacewings. The aim of this experiment was to evaluate potential deterrence of sequestered plant compounds on lacewing feeding.

To assess the effect of sequestered toxins on remaining body mass of milkweed bug larvae after partial consumption by lacewings, remaining body mass was $\log_{10}$-transformed. We tested potential differences between dietary treatments using ANCOVA in JMP and included the initial mass of the intact milkweed bug larvae (i.e. before the experiment) as well as the initial mass of the lacewing larvae (as an estimate for body size) in our model. Furthermore, we tested for an interaction between the dietary treatment and the initial mass of the bugs. Since we carried out two experimental rounds for *L. equestris*, we included 'experiment' as a blocking term. For the remains of one out of 52 *L. equestris* individuals and four out of 40 *S. saxatilis* individuals, body mass was zero (i.e. the mass was below the detection limit of the balance used), thus these data were removed before $\log_{10}$-transformation.

For the comparison of lacewing weights after feeding on milkweed bug larvae, we used the same model as for milkweed bugs using untransformed data with final mass of the lacewings as the main effect. We included the initial weight of bugs and lacewings as model effects. Due to the two experimental rounds for *L. equestris*, we again included 'experiment' as a blocking term.

Hand rearing juvenile birds for predation assays

Juvenile great tits were obtained from a population breeding in nest-boxes in mixed woods at the outskirts of Prague (50.0755˚ N, 14.4378˚ E). The juveniles were taken from nest-boxes when 12–15 days old, and hand reared in the laboratory. This way, they were naive with regard to experience with any kind of unpalatable, toxin-containing or warningly colored prey. The birds were kept in artificial nests until fledging and then housed in groups of three or four in indoor cages (60 x 50 x 50 cm) under illumination and temperature regimes simulating natural conditions. Their diet consisted of mealworms (*Tenebrio molitor* larvae) and commercial mixtures for hand-rearing passerine birds (Handmix, NutriBird, Gold Patee and Uni Patee Premium, Orlux). The birds participated in predation assays when at least 35-days old and fully independent. They were ringed individually and released into the locality of capture within a few days after experimentation. We obtained permissions from the Environmental Department of Municipality of Prague (S-MHMP-83637/2014/OZP-VII-3/R-8/F), Ministry of Agriculture (13060/2014-MZE-17214), and Ministry of the Environment of the Czech Republic (42521/ENV/14-2268/630/14) to hand rear the birds and carry out the experiments.

Attack latencies and duration of discomfort-indicating behavior in avian predators

In each trial of behavioral assays with great tits as predators, we recorded latency of the first attack (seizing or pecking the prey) and duration of discomfort-indicating behavior observed in birds (beak wiping and head shaking). Data were $\log_{10}$-transformed to meet the assumptions of normality and homogeneous variance and analyzed in the lme4 (Bates et al. 2015) and geepack (Højsgaard et al. 2006) packages in R (RCoreTeam 2018).

Attack latencies in the first trial were analyzed using a general linear model (ANOVA) with bug species and host plant toxicity entered as fixed effects. Latencies of the first attacks were also compared between bugs and control crickets by paired t-test. Changes in attack

latencies over the three trials were analyzed using a generalized estimating equation model (GEE) in a subset of data including only the birds that attacked all three bugs offered. Trial number, bug species and host plant toxicity were entered as fixed effects and bird individual (id) as a random effect.

Durations of discomfort-indicating behavior recorded during the first trial were analyzed using a general linear model (ANOVA) with bug species and host plant toxicity entered as fixed effects. To evaluate whether the general effect of host plant toxicity on discomfort-indicating reactions of birds also holds for each of the milkweed bug species studied, similar models were run separately for individual bug species.

Survival of milkweed bugs compared to control palatable prey

We used GEE models (package geepack (Højsgaard et al. 2006) in R (RCoreTeam 2018)) to compare survival probabilities of milkweed bugs raised on sunflower with survival probabilities of control crickets. The data included only the birds that attacked all three bugs offered and were analyzed for each milkweed bug species separately. Prey (cricket versus bug) and trial number were entered as fixed effects and bird individual (id) as a random effect.

Analysis of bugs eaten by avian predators

Following the observations of birds consuming the bugs in particular trials, we examined the remaining parts of the bugs using a stereomicroscope to determine what body parts the birds were able to consume. In a subset of data from first trials including only the cases when the bugs were killed, we analyzed an effect of host plant on the probability that at least part of the bug would be consumed. The data were analyzed separately for each milkweed bug species by generalized linear models (GLM) with binomial errors using the lme4 package (Bates et al. 2015) in R (RCoreTeam 2018), and the host plant was entered as a fixed effect. In addition, we compared the frequency of different body parts of bugs consumed by the birds using Fisher's exact test.

Supplementary Results

Sequestration on toxic seeds across different diets

On seed mixtures containing low amounts of toxic seeds of either *Digitalis*, *Adonis*, or *Colchicum*, sequestration of plant toxins was reduced by approximately 50 % in *H. superbus* (Welch's test: $F_{1,19} = 18.863$, $p < 0.001$) and *S. saxatilis* (Welch's test: $F_{3,20} = 83.568$, $p < 0.001$; Games-Howell post-hoc test significant at $p < 0.05$ for all comparisons except of pure sunflower seeds and the seed mixture without *C. autumnale* seeds) while *L. equestris* accumulated only about 10 % of the amount of cardenolides it contained on the pure toxic diet (Welch's test: $F_{1,10} = 66.206$, $p < 0.001$, (Figure 2d-f). Notably, we found toxins in all (n = 32) specimens sampled from the seed mixture treatments including *D. purpurea*, *A. vernalis*, or *C. autumnale* seeds, showing that toxic seeds were always accessed by the bugs, even if only available in small numbers within mixtures.

Structural identification of sequestered compounds and comparison to seed extracts

We were not able to identify individual compounds by comparing insect extracts to nine cardenolide reference compounds known to occur in *D. purpurea* (Luckner and Wichtl 2000) (Supplemental Figure 6a). Similarly, the cardenolide profile from *D. purpurea* seeds revealed only little putative overlap with cardenolides sequestered by the insects (Supplemental Figure 5a) indicating extensive metabolic transformation by the insect. In contrast, comparison of *H.*

superbus collected from *E. crepidifolium* revealed clear overlap with seed extracts (Supplemental Figure 5d) and we identified one substance as erysimoside based on the retention time of a commercial reference compound (Supplemental Figure 6d). The comparison of seed extracts from *A. vernalis* to extracts of *L. equestris* revealed no clear similarity (Supplemental Figure 5b). Three cardenolides sequestered by *L. equestris* were putatively identified as k-strophanthosid, strophanthidin and cymarin based on authentic reference standards (Supplemental Figure 6b). The comparison of the HPLC profiles between insect extracts and *Urginea* seeds suggested that at least the dominant bufadienolides found in the insect are identical to compounds found in the seeds (Supplemental Figure 5c). Nevertheless, we did not detect the bufadienolides scillaren A and proscillaridin A (Supplemental Figure 6e) that are reported to occur in bulbs of *U. maritima* (Kopp et al. 1996). In *S. saxatilis* from Berghausen we identified 12 peaks with absorption spectra similar to colchicine. Three of the four dominant peaks present in all extracts and accounting for > 90 % of the observed alkaloids were identified as colchicine (34.6 %), 2-demethyl-colchicine (16.2 %), and 3-demethyl-colchicine (20.1 %) based on retention time and molecular mass using authentic standards (Supplemental Figure 6c). We furthermore identified the colchicine glycoside colchicoside as a minor compound. In specimens obtained from Nüstenbach, we detected only nine peaks eight of which were identical with the ones observed in *S. saxatilis* from Berghausen including colchicine, 2 and 3-demethyl-colchicine, and colchicoside. Seeds of *C. autumnale* contained colchicine and colchicoside but none of the related alkaloids found in the bugs (Supplemental Figure 5e).

Effects of sequestered Digitalis-cardenolides on consumption of H. superbus by lacewing larvae

Carcasses from sunflower-raised bugs (n = 11) were lighter after attack compared to carcasses from *Digitalis* raised bugs (n = 12; $F_{1,18} = 39.205$, $p < 0.001$, Supplemental Figure 8a). The body mass of bugs prior to lacewing feeding ('initial mass') affected the remaining mass ($F_{1,18} = 23.825$, $p < 0.001$), while initial mass of lacewings (i.e. before feeding on a milkweed bug larva) had no effect ($F_{1,18} = 2.636$, $p = 0.122$). There was no interaction between initial mass of bugs and treatment ($F_{1,18} = 0.269$, $p = 0.611$) on remaining mass. In accordance with lower consumption of *Digitalis*-raised bugs, lacewing larvae gained more body mass when feeding on sunflower raised-bugs compared to feeding on *Digitalis*-raised bugs ($F_{1,18} = 48.833$, $p < 0.001$, Supplemental Figure 8e). The initial masses of bugs and lacewings influenced the final lacewing mass ($F_{1,18} = 9.091$, $p = 0.007$; $F_{1,18} = 306.9$, $p < 0.001$). In addition, final mass of lacewings was affected by an interaction between the initial mass of bugs and their diet ($F_{1,18} = 7.258$, $p = 0.015$). Across all host plant and milkweed bug species combinations, the time until attack did not differ between sunflower-raised and toxic seed-raised bugs (see archived data).

Effects of sequestered Adonis-cardenolides on consumption of L. equestris by lacewing larvae

Consumption of milkweed bugs by lacewing larvae was higher on sunflower-raised bugs (n = 21) compared to bugs raised on *Adonis* (n = 30; $F_{1,45} = 9.170$, $p = 0.004$, Supplemental Figure 8b). Again, the remaining mass of bugs was affected by their initial mass ($F_{1,45} = 23.854$, $p < 0.001$) and there was no interaction between initial mass and treatment ($F_{1,45} = 0.235$, $p = 0.630$). The mass of lacewing larvae before the trial (n = 22 for lacewings feeding on sunflower and n = 30 for lacewings feeding on *A. vernalis*-raised bugs) predicted how much body mass of the bugs was consumed ($F_{1,49} = 6.286$, $p = 0.016$). In accordance with the observed loss of body mass in the bugs, lacewings gained more body mass when feeding on sunflower-raised bugs compared to *Adonis*-raised bugs ($F_{1,46} = 8.02$, $p = 0.007$, Supplemental Figure 8f). The initial masses of bugs and lacewings determined the final body mass of the lacewing larvae ($F_{1,46} = 6.286$, $p = 0.016$;

$F_{1,46} = 98.245$, $p < 0.001$). While the interaction between diet and initial mass of the bugs had an effect on lacewing final mass the round of experiment had no influence ($F_{1,46} = 4.3$, $p = 0.044$; $F_{1,46} = 0.045$, $p = 0.833$).

Effects of sequestered Urginea-bufadienolides on consumption of S. pandurus by lacewing larvae. In contrast to the other species, we only found a marginal effect of the diet on remaining milkweed bug mass after the lacewing attack ($F_{1,33} = 3.79$, $p = 0.06$; n = 19 for sunflower and *Urginea*-raised bugs, Supplemental Figure 8c). The initial mass of the bugs affected the remaining mass after the experiment ($F_{1,33} = 21.202$, $p < 0.001$) and there was a significant interaction between diet and initial mass affecting remaining body mass ($F_{1,33} = 5.602$, $p = 0.024$). The initial mass of lacewing larvae had no effect on the remaining mass of the bugs after consumption ($F_{1,33} = 1.199$, $p = 0.282$). Nevertheless, lacewing larvae were heavier after feeding on sunflower raised *S. pandurus* larvae compared to feeding on bugs raised on *Urginea* seeds ($F_{1,33} = 195.969$, $p < 0.001$, Supplemental Figure 8g). Besides the diet, the initial mass of bugs and lacewings as well as the interaction between initial mass of bugs and diet affected body mass of lacewings after the trial ($F_{1,33} = 44.982$, $p < 0.001$; $F_{1,33} = 174.744$, $p < 0.001$; $F_{1,33} = 20.639$, $p < 0.001$).

Effects of sequestered Colchicum-alkaloids on consumption of S. saxatilis by lacewing larvae Body mass of *S. saxatilis* after lacewing predation was higher than body mass of *Oncopeltus*, Supplemental Figure 8d). The initial masses of bugs (n = 16 for *Oncopeltus* and n = 20 for *S. saxatilis*) and lacewings did not affect remaining mass of bugs after attack ($F_{1,31} = 2.91$, $p = 0.098$ and $F_{1,31} = 0.012$, $p = 0.914$) and there was no interaction between the initial mass of bugs and treatment affecting remaining body mass after attack ($F_{1,31} = 0.143$, $p = 0.708$). Lacewing larvae assigned to *O. fasciatus* larvae (n = 20) were heavier compared to lacewing larvae assigned to larvae of *S. saxatilis* (n = 20) after the trial (2-fold vs. 1.75-fold, $F_{1,35} = 129.881$, $p < 0.001$, Supplemental Figure 8h). The initial masses of bugs and lacewings affected the final mass of lacewings ($F_{1,35} = 12.225$, $p = 0.001$; $F_{1,35} = 801.952$, $p < 0.001$) and there was an interaction between the initial mass of bugs and treatment affecting lacewing final mass ($F_{1,35} = 41.971$, $p < 0.001$).

Attack latencies and duration of discomfort-indicating behavior in avian predators
In first encounters with novel prey, birds did not hesitate longer before attacking milkweed bugs than before attacking control crickets (paired t-test, $t = 0.173$, $df = 99$, $p = 0.863$; Supplemental Figure 9a). Furthermore, attack latencies were influenced neither by milkweed bug species (ANOVA, $F_{2,96} = 1.248$, $p = 0.292$) nor by host plant toxicity (ANOVA, $F_{1,96} = 1.871$, $p = 0.113$). Thus, we have not found any evidence of an innate bias against warningly colored milkweed bugs or for before-attack defensive effects of sequestered host plant toxins. Nevertheless, birds that attacked all three bugs in a row hesitated longer before attacking the bugs raised on toxic host plants upon repeated encounters (GEE, trial, $\chi^2_1 = 2.197$, $p = 0.138$; host plant, $\chi^2_1 = 27.938$, $p < 0.001$; trial: host plant interaction, $\chi^2_1 = 13.866$, $p < 0.001$; Supplemental Figure 9b).

When handling the bugs, birds often responded by discomfort-indicating behavior (head shaking and beak wiping). Durations of this behavior in the first trial were similar across all bug species tested (ANOVA, $F_{2,94} = 1.929$, $p = 0.151$). However, the birds tested with bugs raised on toxic host plants spent more time by discomfort-indicating behavior than the birds tested with bugs from control sunflower (ANOVA, $F_{1,94} = 37.989$, $p < 0.001$; Supplemental Figure 10). There was no interaction between the two factors (ANOVA, $F_{2,94} = 0.987$, $p = 0.376$). When analyzed separately for each species, birds spent longer time by discomfort-indicating behavior

when attacking and handling bugs raised on toxic host plants than when handling bugs from sunflower (ANOVA, *S. saxatilis*: $F_{1,38} = 11.271$, $p = 0.002$, *L. equestris*: $F_{1,38} = 20.962$, $p < 0.001$, *H. superbus*: $F_{1,18} = 6.177$, $p = 0.023$; Supplemental Figure 10a-c). This result indicates that chemicals sequestered from host plants cause stronger aversive reactions upon direct contact than the secretion of metathoracic glands alone.

Survival of milkweed bugs compared to control palatable prey

In all three species of milkweed bugs, the probability to survive repeated attacks by avian predators was higher for the sunflower-raised bugs than for control crickets (GEE, *S. saxatilis*: $\chi^2_1 = 6.235$, $p = 0.012$; *L. equestris*: $\chi^2_1 = 6.534$, $p = 0.011$; *H. superbus*: $\chi^2_1 = 3.899$, $p = 0.048$). These results indicate that the line of defense based on the secretion of metathoracic scent glands is effective by itself, even though its sole effect is considerably smaller compared to when it is combined with sequestration of host plant chemicals.

Consumption of bugs by avian predators

In all three milkweed bug species tested, host plant affected the probability that the birds would eat at least a part of the bug attacked and killed in the first trial (GLM, *S. saxatilis*: $\chi^2_{1,24} = 13.214$, $P < 0.001$; *L. equestris*: $\chi^2_{1,27} = 5.849$, $P = 0.016$; *H. superbus*: $\chi^2_{1,15} = 7.945$, $P = 0.005$). Whereas the birds frequently ate at least some parts of sunflower-raised bugs, consumption of bugs raised on toxic host plants was exceptionally rare. Out of 20 birds tested with *S. saxatilis* and *L. equestris*, only two and four birds, respectively, ate some parts of *Colchicum*- and *Adonis*-raised bugs, and in all cases, it was only a small part of the abdomen (fat body). Likewise, out of 10 birds tested with *H. superbus*, only one bird consumed a small part of the abdomen of a *Digitalis*-raised bug. Some of the birds tested with bugs from non-toxic host plants consumed whole bugs or left only few fragments of cuticle, but they nevertheless consumed parts of the abdomen (usually the fat body) significantly more frequently than other parts of the bug (two tailed Fisher's exact test, *S. saxatilis:* $P < 0.001$; *L. equestris*: $P < 0.001$; *H. superbus*: $P < 0.021$).

Injection experiments with ouabain and colchicine

P. apterus showed clear signs of intoxication in a dose-dependent manner for both toxins, ouabain (Cochrane-Armitage trend test, $Z = -4.006$, $p < 0.001$) and colchicine (Cochrane-Armitage trend test, $Z = -4.887$, $p < 0.001$, Supplemental Figure 11a,d). While unaffected by ouabain (Figure 4), *O. fasciatus* responded to colchicine in a dose-dependent manner (Cochrane-Armitage trend test, $Z = -3.453$, $p < 0.001$, Supplemental Figure 11b). At a dose of 5 µg per animal, 100% of individuals were affected while *S. saxatilis* tolerated up to 30 µg which was the highest dose tested ($p = 0.467$ compared to specimens injected with 5 µg ouabain that were all surviving; two tailed Fisher's exact test, Supplemental Figure 11e). *S. pandurus*, although being a congener of *S. saxatilis*, was unable to tolerate colchicine and was affected in a dose dependent fashion (Cochrane-Armitage trend test, $Z = -3.933$, $p < 0.001$, Supplemental Figure 11c).

Natural history remarks

When collecting milkweed bugs in the field we also observed feeding behavior and host plant use. In general, feeding was mainly restricted to fruits or flowers. *H. superbus* was only observed on *D. purpurea* plants (Supplemental Figure 1) and substrates such as bark and stumps but never on other plants. Early in the season, we found the bugs walking on *Digitalis*-leaves and stems or feeding on flowers. Later in the season, we typically found *H. superbus* in the opened ripe *Digitalis*-pods (adults and larvae). In a *Digitalis*-free habitat, we found *H. superbus* exclusively on seedpods of *E. crepidifolium*. In this habitat, the insects may also suck on the fleshy leaves of

Sedum spec., a cushion plant that they may use as a refuge. The feeding ecology of *L. equestris* has been described elsewhere (Solbreck and Kugelberg 1972). Our own observations confirm that in *A. vernalis* habitats, *Adonis* is the primary host plant early in the season. We and others recorded *S. saxatilis* feeding on more than 40 plant species from more than 15 botanical families (Supplemental Table 3). *S. saxatilis* oviposits into *C. autumnale* seedpods and early larval stages of *S. saxatilis* were only observed in ripe fruits of *C. autumnale* (Supplemental Figure 1).

Supplemental Table 1. Plant species used for seed mixture experiments. Botanical families in parentheses.

Plant species used for *S. saxatilis*	Plant species used for *H. superbus*	Plant species used for *L. equestris*
Achillea millefolium (Asteraceae)	*Achillea millefolium* (Asteraceae)	*Achillea millefolium* (Asteraceae)
Bupleurum falcatum (Apiaceae)	*Bupleurum falcatum* (Apiaceae)	*Angelica archangelica* (Apiaceae)
Centaurea jacea (Asteraceae)	*Centaurea jacea* (Asteraceae)	*Centaurea scabiosa* (Asteraceae)
Cichorium intybus (Asteraceae)	*Cichorium intybus* (Asteraceae)	*Cichorum intybus* (Asteraceae)
Daucus carota (Apiaceae)	*Daucus carota* (Apiaceae)	*Cirsium arvense* (Asteraceae)
Hieracium pilosella (Asteraceae)	*Hieracium pilosella* (Asteraceae)	*Dactylis glomerata* (Poaceae)
Origanum vulgare (Lamiaceae)	*Origanum vulgare* (Lamiaceae)	*Daucus carota* (Apiaceae)
Plantago major (Plantaginaceae)	*Plantago major* (Plantaginaceae)	*Hieracium pilosella* (Asteraceae)
Tanacetum vulgare (Asteraceae)	*Tanacetum vulgare* (Asteraceae)	*Pimpinella saxifraga* (Apiaceae)
Taraxacum officinale (Asteraceae)	*Taraxacum officinale* (Asteraceae)	*Plantago major* (Plantaginaceae)
Tragopogon pratensis (Asteraceae)	*Tragopogon pratensis* (Asteraceae)	*Taraxacum officinale* (Asteraceae)
		Thymus serpyllum (Lamiaceae)
		Tragopogon pratensis (Asteraceae)
		Urtica dioica (Urticaceae)
		Vincetoxicum hirundinaria (Apocynaceae)

Note.- For *S. saxatilis* and *H. superbus* mean weights of seeds per plant species ranged from 12 - 13 mg (with 91 % of plant species being close to 12 mg) and from 9 - 13 mg for *L. equestris*, with 87 % of host seed species being close to 9 mg per Petri dish. Deviations from 9 mg are due to the extensive seed sizes of certain species (*T. pratensis*) i.e. individual seeds exceeded 9 mg.

Supplemental Table 2. Number of days the bugs were spending on sunflower seeds as adults (i.e. with a continuous gut lumen) for purging digestive tracts from potential residues of dietary toxins (cardenolides or colchicum alkaloids).

S. saxatilis		*L. equestris*		*H. superbus*	
mix with *Colchicum* (n = 11)	pure *Colchicum* (n = 11)	mix with *Adonis* (n = 10)	pure *Adonis* (n = 11)	mix with *Digitalis* (n = 11)	pure *Digitalis* (n = 10)
0	0	12	14	0	7
3	0	14	3	10	7
1	0	14	7	11	11
1	0	7	0	8	8
3	0	8	8	10	0
3	0	14	0	11	4
3	1	14	0	4	3
6	0	14	6	7	10
3	0	11	6	8	6
1	0	14	0	11	11
4	0		6	7	

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Supplemental Table 3. Host plants recorded for *S. saxatilis* in the field.

Plant species	Location	Plant family
Bupleurum falcatum	Germany, Nüstenbach*,[1]	Apiaceae
Daucus carota	Germany, Nüstenbach*,[1]	Apiaceae
Heracleum spec.	Germany[2]	Apiaceae
Asclepias syriaca	'Europe orientale'[2]	Apocynaceae
Vincetoxicum hirundinaria	'Europe orientale'[2], Czech Republic, South Moravia*,[3]	Apocynaceae
Vincetoxicum stepposum	'Europe orientale'[2]	Apocynaceae
Achillea millefolium	Czech Republic, South Moravia*,[3]	Asteraceae
Carduus acanthoides	Czech Republic, South Moravia*,[3]	Asteraceae
Centaurea scabiosa	Czech Republic, South Moravia*,[3]	Asteraceae
Centaurea spec.	Germany, Nüstenbach*,[1]	Asteraceae
Cichorium intybus	Germany, Nüstenbach(*),[1], Czech Republic, South Moravia*,[3]	Asteraceae
Cirsium pannonicum	Czech Republic, South Moravia*,[3]	Asteraceae
Cirsium spec.	Germany, Nüstenbach[1]	Asteraceae
Cirsium vulgare	Czech Republic, South Moravia*,[3]	Asteraceae
Crepis biennis	Germany, Berghausen*,[1]	Asteraceae
Hieracium spec.	Germany[2]	Asteraceae
Inula conyzae	Germany, Nüstenbach*,[1]	Asteraceae
Inula hirta	Czech Republic, South Moravia*,[3]	Asteraceae
Leontodon hispidus	Germany, Berghausen(*),[1]	Asteraceae
Leucanthemum vulgare	Czech Republic, South Moravia*,[3]	Asteraceae
Senecio cf. *vulgaris*[1]	Germany, Berghausen*,[1]	Asteraceae
Senecio jacobaea	Czech Republic, South Moravia*,[3]	Asteraceae
Solidago virgaurea	Germany, Nüstenbach(*),[1]	Asteraceae
Tanacetum vulgare	Czech Republic, South Moravia*,[3]	Asteraceae
Taraxacum sect. Ruderalia	Czech Republic, South Moravia*,[3]	Asteraceae
Tragopogon spec.	Germany[2]	Asteraceae
Colchicum autumnale	Germany, Berghausen*,[1], Nüstenbach*,[1], Austria[1,2], Czech Republic, South Moravia*,[3]	Colchicaceae
Knautia arvensis	Germany, Berghausen(*),[1]	Dipsacaceae
Euphorbia cyparissias	Czech Republic, South Moravia*,[3]	Euphorbiaceae
Ononis spinosa	Czech Republic, South Moravia*,[3]	Fabaceae
Trifolium repens	Czech Republic, South Moravia*,[3]	Fabaceae

Hypericum perforatum	Czech Republic, South Moravia*[,3]	Hypericaceae
Mentha longifolia	Italy[2], Eastern Europe[2]	Lamiaceae
Origanum vulgare	Germany, Nüstenbach*[,1]	Lamiaceae
Salvia pratensis	Czech Republic, South Moravia*[,3]	Lamiaceae
Linaria vulgaris	Czech Republic, South Moravia*[,3]	Plantaginaceae
Plantago lanceolata	Germany, Berghausen*[,1], Czech Republic, South Moravia*[,3]	Plantaginaceae
Holcus spec.	Germany, Berghausen(*)[,1]	Poaceae
Rumex crispus	Czech Republic, South Moravia*[,3]	Polygonaceae
Ranunculus polyanthemus	South Moravia*[,3]	Ranunculaceae
Ranunculus spec.	Germany, Berghausen[1]	Ranunculaceae
Agrimonia eupatoria	Germany, Berghausen*[,1]	Rosaceae
Geum urbanum	Germany, Berghausen*[,1]	Rosaceae
Potentilla tabernaemontani	Czech Republic, South Moravia*[,3]	Rosaceae
Rubus spec. (stem, leaf)	Germany, Berghausen*[,1]	Rosaceae
Galium spec.	Germany, Berghausen*[,1]	Rubiaceae
Galium verum	Czech Republic, South Moravia*[,3]	Rubiaceae
Salix alba	Czech Republic, South Moravia*[,3]	Salicaceae
Verbascum phoeniceum	Czech Republic, South Moravia*[,3]	Scrophulariaceae

Note.- Superscript numbers indicate sources of host plant data: [1] = own observation, [2] = Péricart (1998), [3] = Banar (2003). Asterisks indicate actual feeding observations.

Supplemental Figure 1. Natural history observations on milkweed bug species. (a) Eggs of *S. saxatilis* (white arrow) in a *C. autumnale* infructescence (Germany, Baden-Württemberg, Berghausen, June 1st, 2016). (b) Early instar larvae of *S. saxatilis* sitting on a dry *Colchicum* seedpod (Germany, Baden-Württemberg, Berghausen, June 15th, 2016). (c) Adults of *S. saxatilis* feeding on a *C. autumnale* flower (Germany, Baden-Württemberg, Berghausen, September 18th, 2015). (d) *H. superbus* on *D. purpurea* (Germany, Baden-Württemberg, Eberbach, June 5th, 2016). (e) *S. pandurus* on *U. maritima* (Spain, Sierra de Aracena and Picos de Aroche Natural Park, Cañaveral de León,

August 20th, 2014). (f) *H. superbus* on *E. crepidifolium* (Germany, Rheinland-Pfalz, Schloßböckelheim, June 13th, 2016). (g) *L. equestris* on *A. vernalis* (Germany, Brandenburg, Mallnow, April 19th, 2016.

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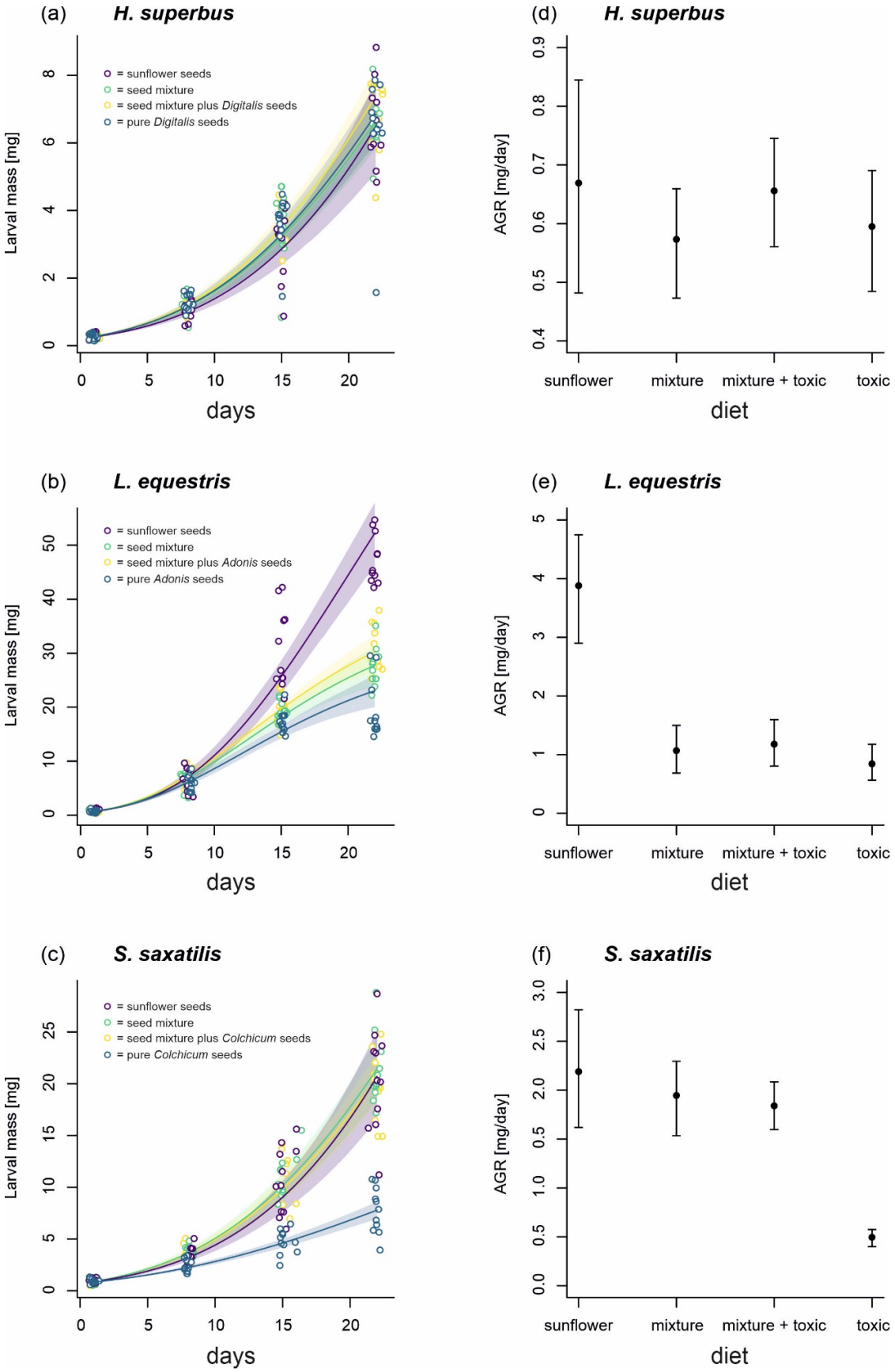

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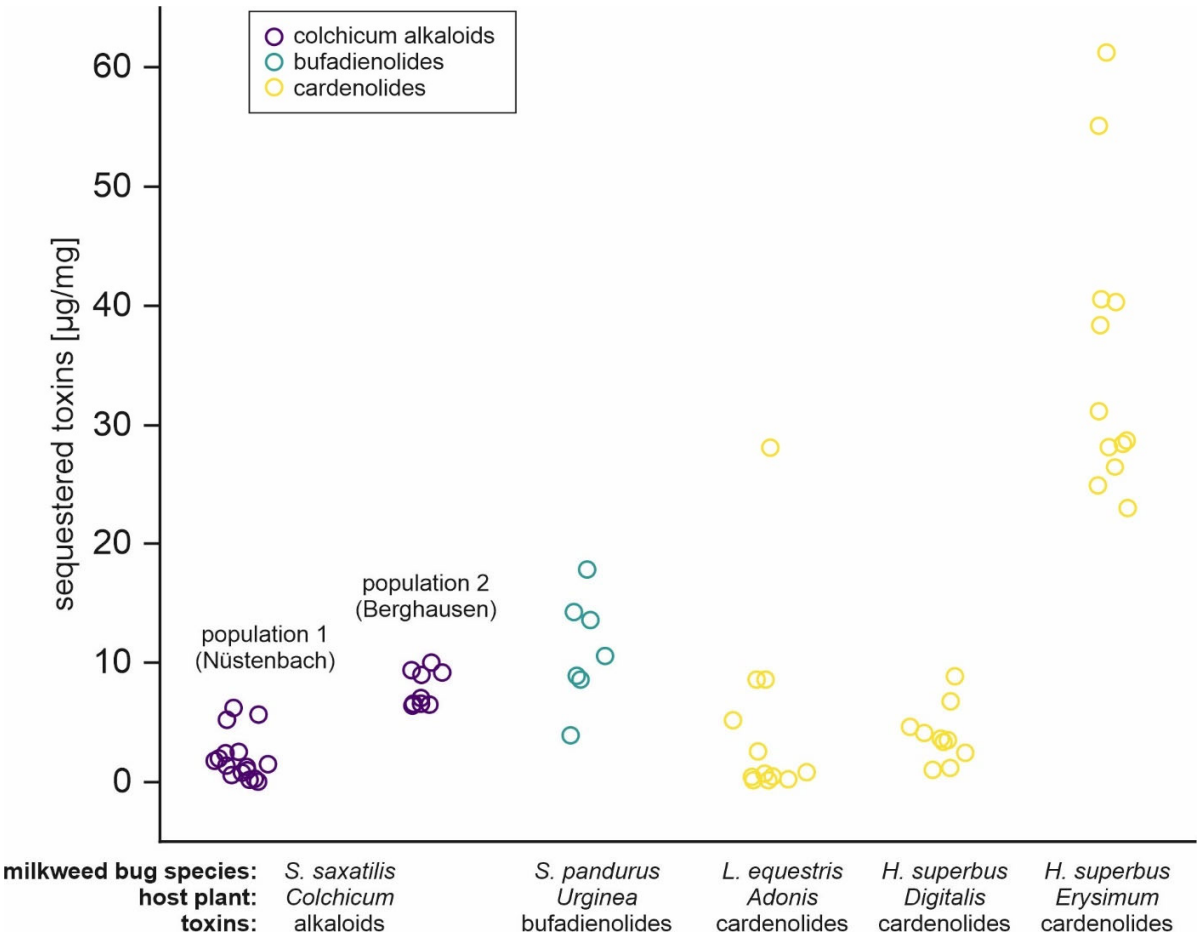
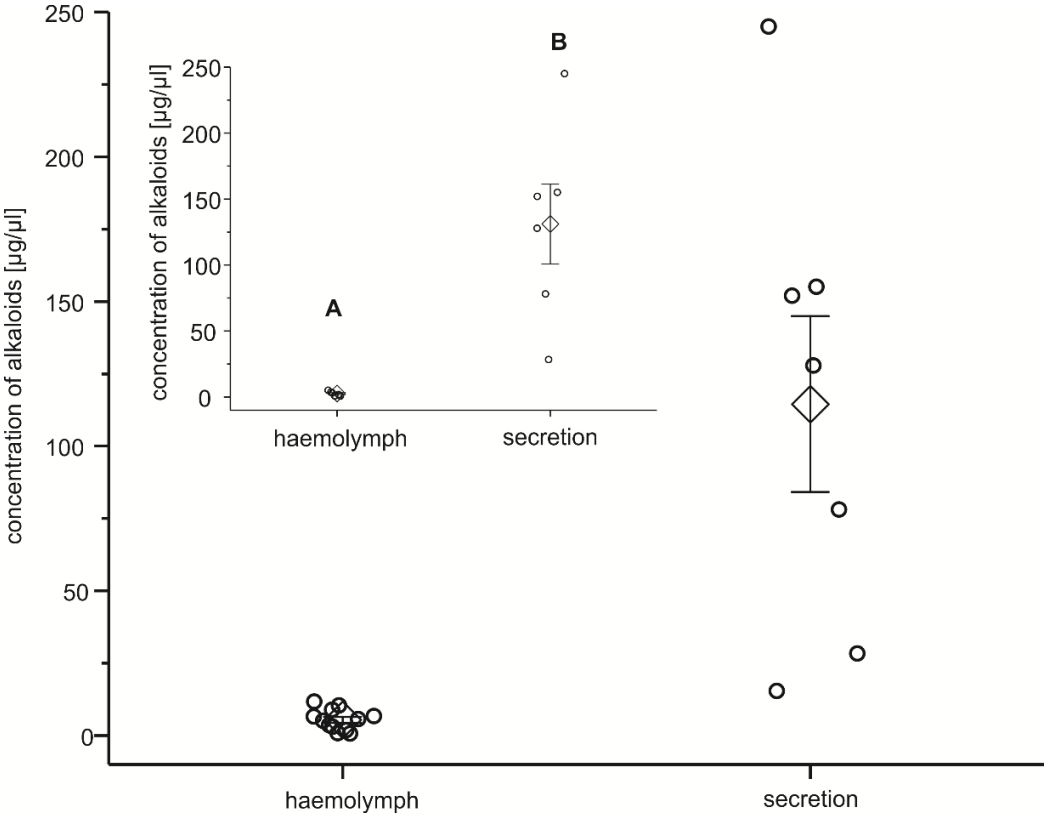
Supplemental Figure 2. Growth of milkweed bug larvae on four different seed diets. (a-c) Weight gain of *H. superbus*, *L. equestris*, and *S. saxatilis* larvae feeding on sunflower seeds (control, purple), a non-toxic seed mixture (green), a seed mixture with toxic seeds (yellow), or toxic seeds only (blue). Points are average weights of 1-3 larvae per Petri dish (replicates), measured sequentially over three weeks. Growth was modelled as an asymptotic process using a non-linear mixed effects model (function *nlme* with *SSasymp* in R) with log-weight as the response, a random effect of Petri dish to account for repeated measures, and fixed effects of species and treatment on individual model parameters (*K*: species × treatment, $F_{6,374} = 165.4$, $p < 0.001$; M_0: species, $F_{2,374} = 202.7$, $p < 0.001$; *log-rate constant*: species, $F_{2,374} = 83.7$, $p < 0.001$; treatment, $F_{3,374} = 4.9$, $p = 0.002$). Solid lines are model predictions, and shaded areas are 95% population prediction intervals, generated by drawing random values from the estimated sampling distribution of each regression parameter. (d-e) Absolute growth rates for *H. superbus*, *L. equestris*, and *S. saxatilis* larvae feeding on different seed diets. AGR values were calculated for growth on day 22 (final day of the experiment), using the model parameters from the asymptotic regression model displayed in panels a-c. Error bars are 95% population prediction intervals.



Supplemental Figure 3. Concentrations of plant toxins sequestered by milkweed bugs collected in the field. Shown are raw data, each circle represents a milkweed bug individual.



Supplemental Figure 4. Concentration of colchicum alkaloids in *Spilostethus saxatilis* hemolymph (n = 12) and defensive secretion (n = 7). Diamonds are means ± SE, circles represent jittered raw data. Subset of six paired samples obtained from the same individuals used for statistical comparison (inset).

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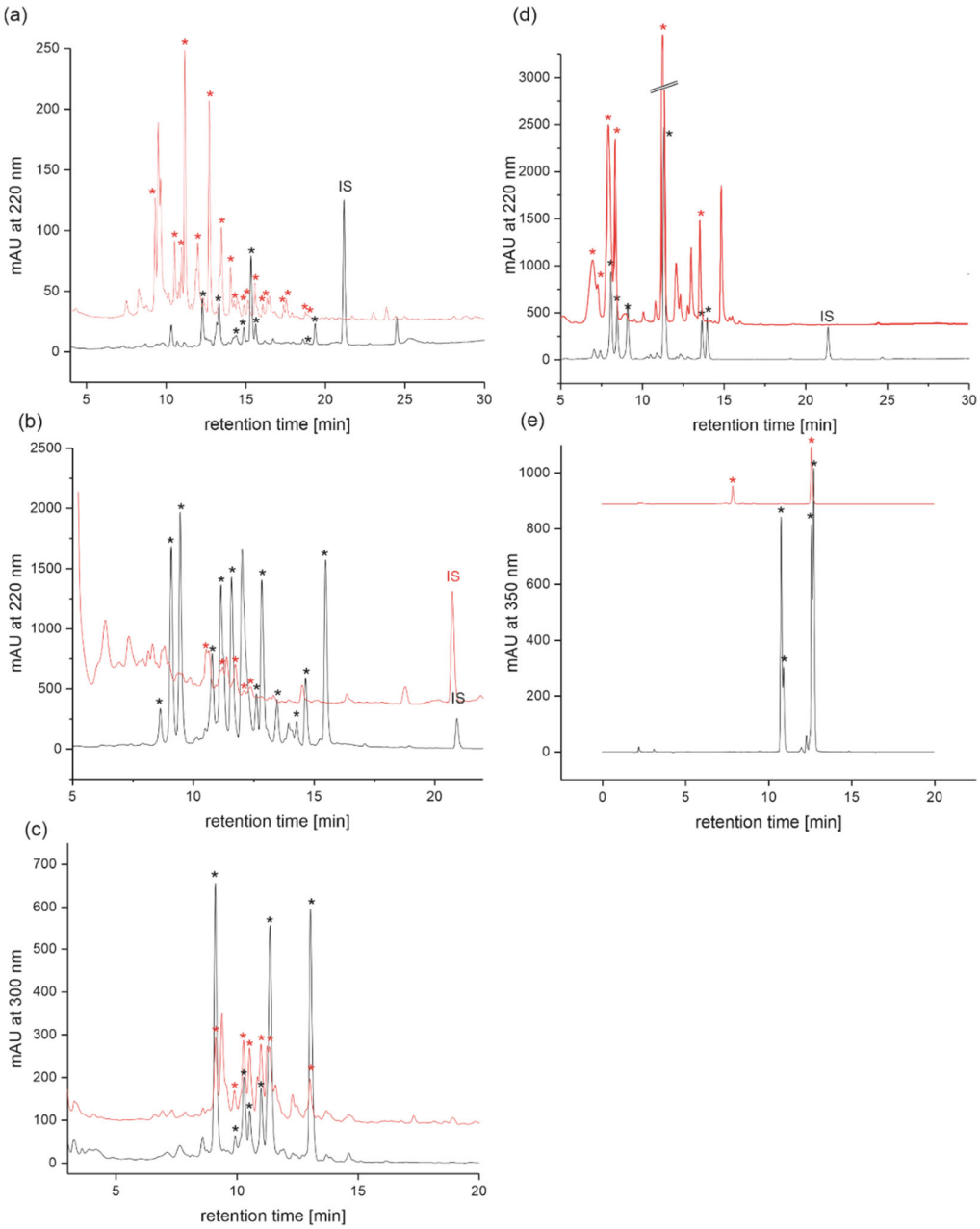

Supplemental Figure 5. Comparison of milkweed bug and host plant seed extracts with HPLC-DAD. (a) *H. superbus* vs. *D. purpurea* seeds, (b) *L. equestris* vs. *A. vernalis* seeds, (c) *S. pandurus* vs. *U. maritima* seeds, (d) *H. superbus* vs. seeds of *E. crepidifolium*, (e) *S. saxatilis* vs. *C. autumnale* seeds. Top chromatograms (red) always represent seed; bottom chromatograms (black) always represent insect extracts. Asterisks indicate the most prominent cardiac glycoside or colchicum alkaloid peaks, respectively.

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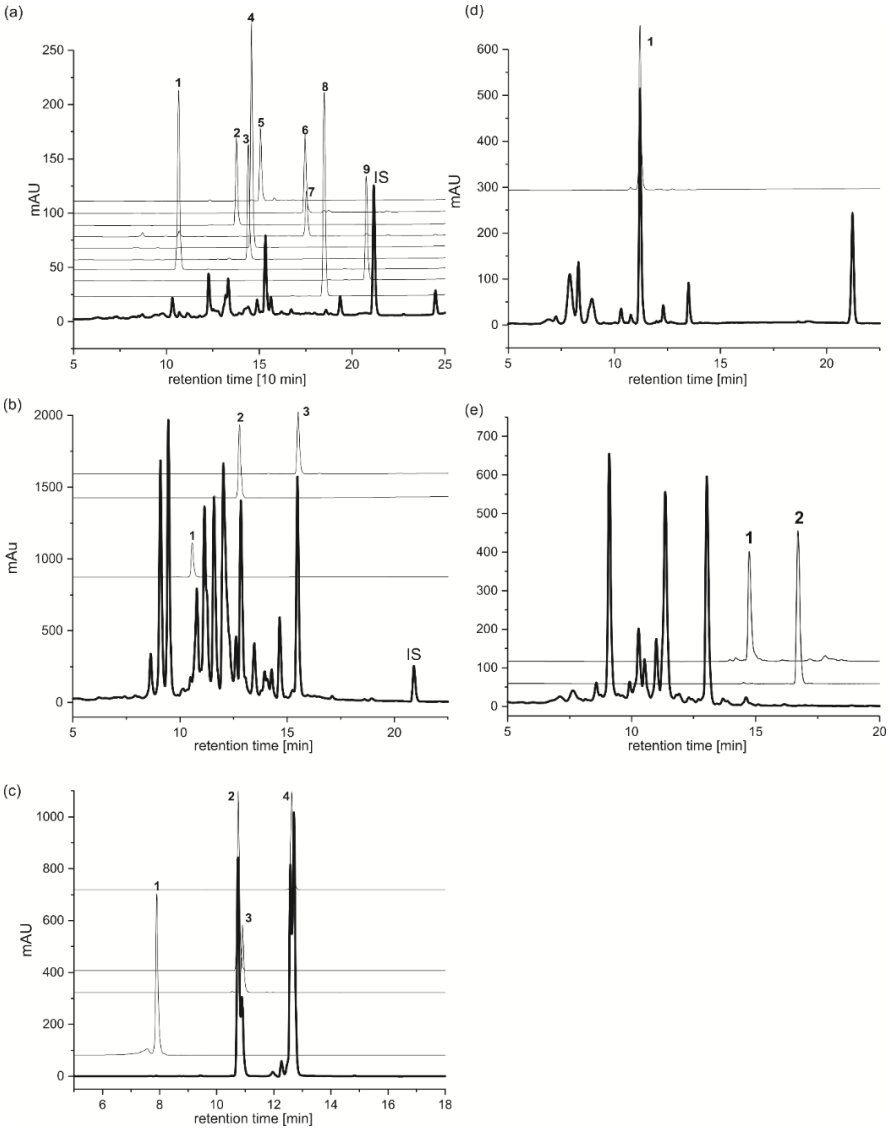

Supplemental Figure 6. Comparison of HPLC-chromatograms obtained from field-collected milkweed bugs with authentic reference compounds. (a) *H. superbus* from *D. purpurea* compared to compounds reported from *D. purpurea*: 1 = digoxigenin, 2 = lanatoside C, 3 = digoxin, 4 = gitoxigenin, 5 = purpurea glycoside B, 6 = purpurea glycoside A, 7 = gitoxin, 8 = digitoxigenin, 9 = digitoxin. (b) *L. equestris* collected from *A. vernalis* compared to 1 = k-strophanthosid, 2 = strophanthidin and 3 = cymarin that all occur in *A. vernalis*. (c) *S. saxatilis* from *C. autumnale* compared to authentic colchicum alkaloid standards: 1 = colchicoside, 2 = 3-demethyl colchicine, 3 = 2-demethyl colchicine, and 4 = colchicine. (d) *H. superbus* from *E. crepidifolium* compared to erysimoside (1). (e) *S. pandurus* from *U. maritima* compared to the *Urginea* bufadienolides 1 = scillaren A, 2 = proscillaridin A.

Supplemental Figure 7. Origin of *S. saxatilis* museum specimens used for chemical analysis. Numbers in parentheses indicate numbers of specimens sampled per location (MFNB: Museum für Naturkunde, Berlin, Germany; SMNK: Staatliches Museum für Naturkunde Karlsruhe, Germany; SDEI: Senckenberg Deutsches Entomologisches Institut, Müncheberg, Germany). Countries on labels were translated. 1: Germany, Odenwald, Dreieichenhain, September 15th 1925, MFNB (1); 2: Germany, Bamberg, Hallstadt, Börstig, August 3rd 1940, leg. Schneid, MFNB (1); 3: Germany, Baden, Bergstraße, Weinheim, May 1952, leg. H. Nowotny, SMNK (1); 4: Germany, Bienwald, Büchelberg, September 10th 1987, leg. Roesler, SMNK (2); 5: Germany, Karlsruhe, Maxau, August 22nd 1959, leg. Kormann; Germany, Karlsruhe, Maxau, August 1947, leg. Nowotny, SMNK (2); 6: Germany, Baden, Kaiserstuhl, June 21st 1953, leg. H. Nowotny, SMNK (1); 7: Germany, Baden, Hegau, August 8th-20th 1935, leg. Leininger, SMNK (2); 8: Germany, Bodensee, Allensbach, September 1919, leg. W. Ramme, MFNB (2); 9: Germany, Wollmatingen, August 10th 1928, leg. Leininger, SMNK (2); 10: Germany, Berchtesgaden, Bischofswiesen, August 21st-28th 1958, leg. Papperitz, SDEI (1); 11: Switzerland, Glarisegg, May 6th-10th 1906, SDEI (1); 12: Hungary, Mecsek-Gebirge, Mánfa, May 30th 1977, leg. U. Göllner, MFNB (1); 13: Romania, Brașov, August 13th 1905, leg. E.J. Lehmann, MFNB (1); 14: Italy, Alpi Marittime Natural Park, Juniperus phoenicea Riserva Naturale, August 10th 2013, leg. J. Deckert, MFNB (2); 15: Bosnia and Herzegovina, Bjelašnica, July 21st and 23rd 1909, leg. F. Schumacher, MFNB (2); 16: France, Riez, July 1985, leg. J. Haupt, MFNB (1); 17: Croatia, Dubrovnik, April 13th and May 1st 1938, leg. Dr. Feige, SDEI (3); 18: Albania, Dajti Südhang, June 30th 1961, leg. Expedition DEI, SDEI (1); 19: Republic of Macedonia, Hügel bei Stari Dojran, August 3rd 1975, leg. U. Göllner, MFNB (1); 20: France, Corsica, Bocognano, 1905, leg. O. Leonhard, SDEI (1); 21: Kingdom of Morocco, Taza, MFNB (1).

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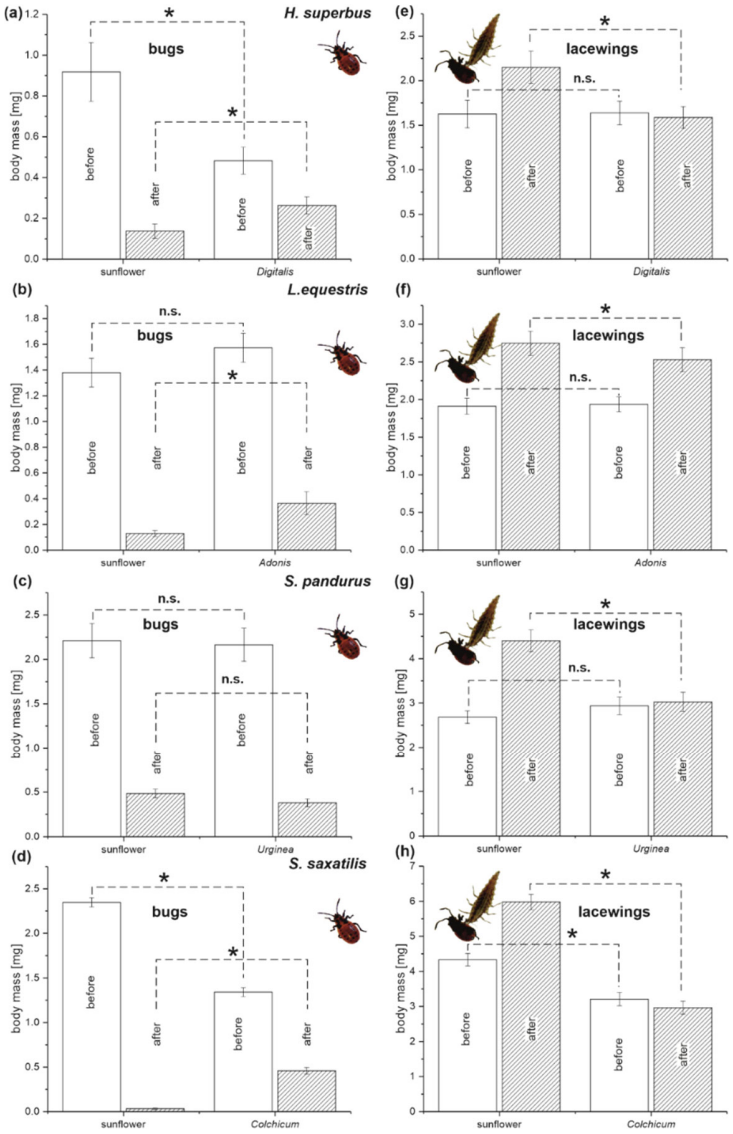
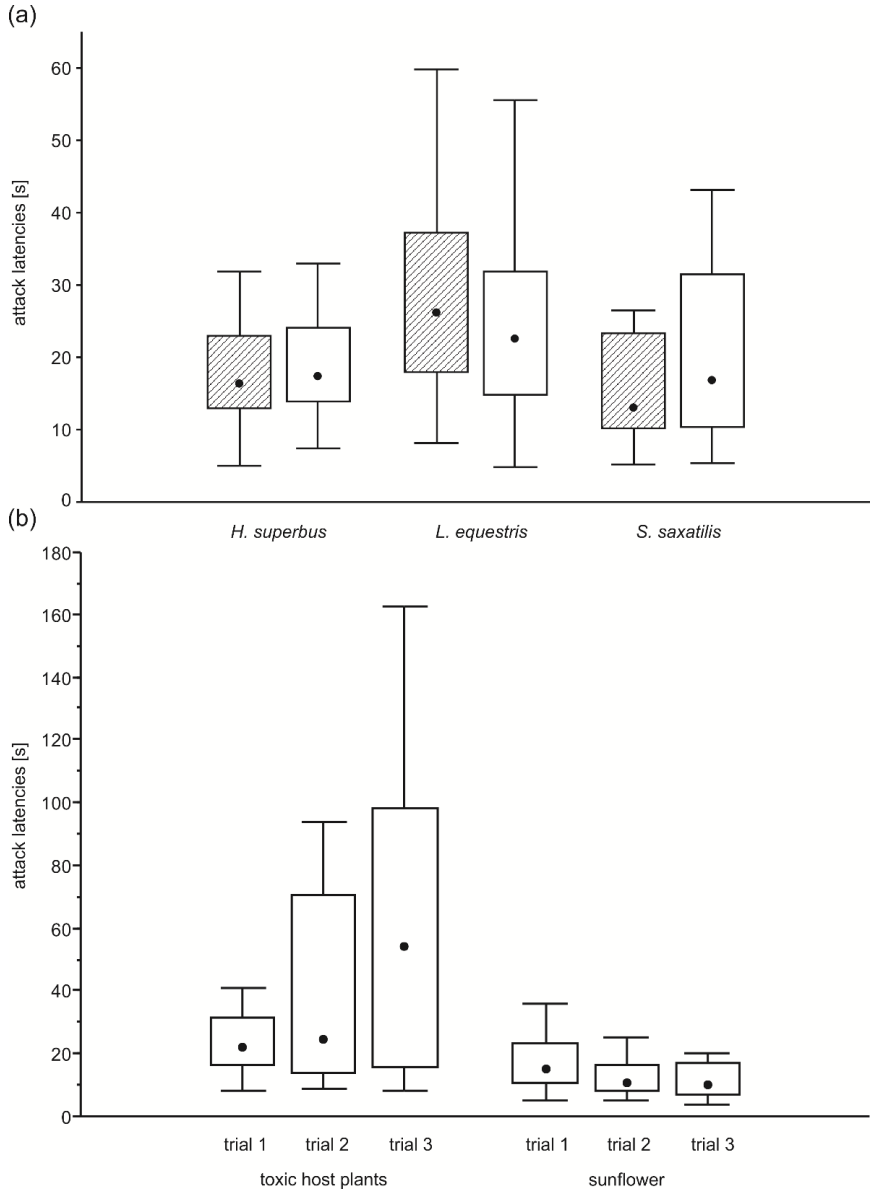
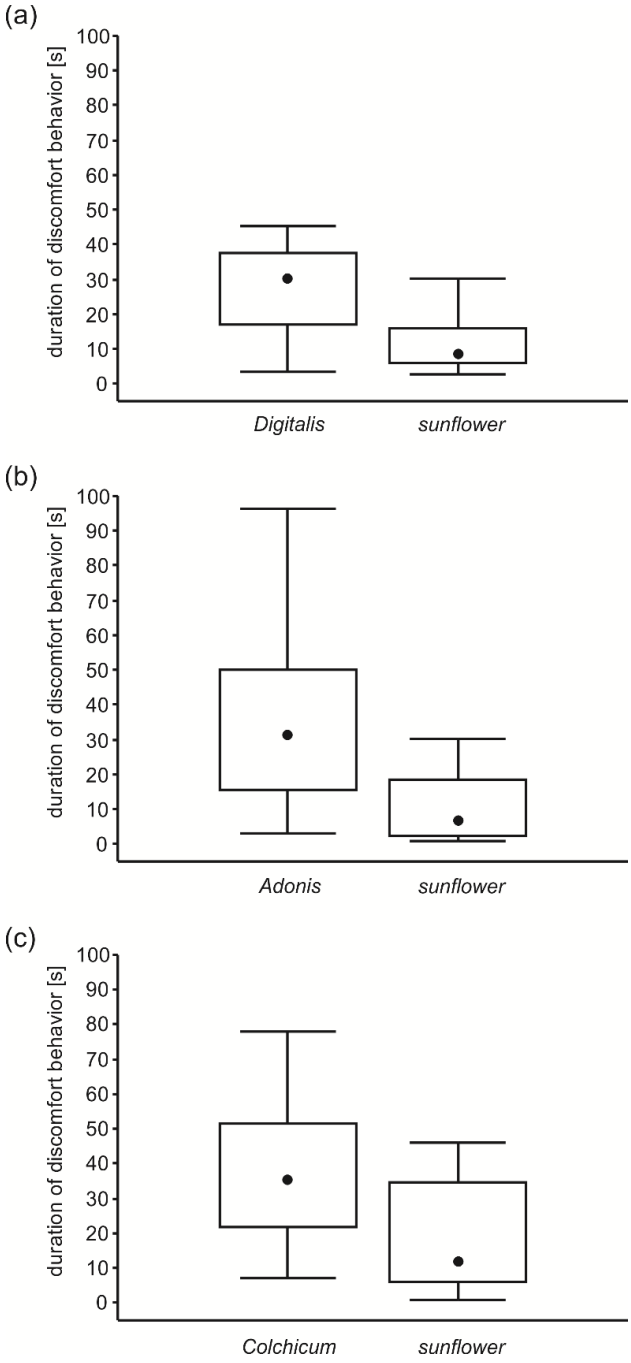


Supplemental Figure 8. Consumption of four species of milkweed bugs by lacewing larvae (*C. carnea*). Early instar larvae of milkweed bugs were raised either on sunflower seeds (controls) or on seeds of plant species containing toxins for sequestration. Left panel: Body mass of milkweed bug larvae before (open bars) and after lacewing attacks (hatched bars). (a) *H. superbus* on *D. purpurea*, (b) *L. equestris* on *A. vernalis*, (c) *S. pandurus* on *U. maritima*, (d) S. *saxatilis* on *C. autumnale*. Right panel: Body mass of lacewing larvae before (open bars) and after feeding on milkweed bug larvae (hatched bars). (e) *H. superbus* on *D. purpurea*, (f) *L. equestris* on *A. vernalis*, (g) *S. pandurus* on *U. maritima*, (h) S. *saxatilis* on *C. autumnale*. Shown are means ± SE. Asterisks indicate significant differences at p = 0.05, n.s. = not significant.

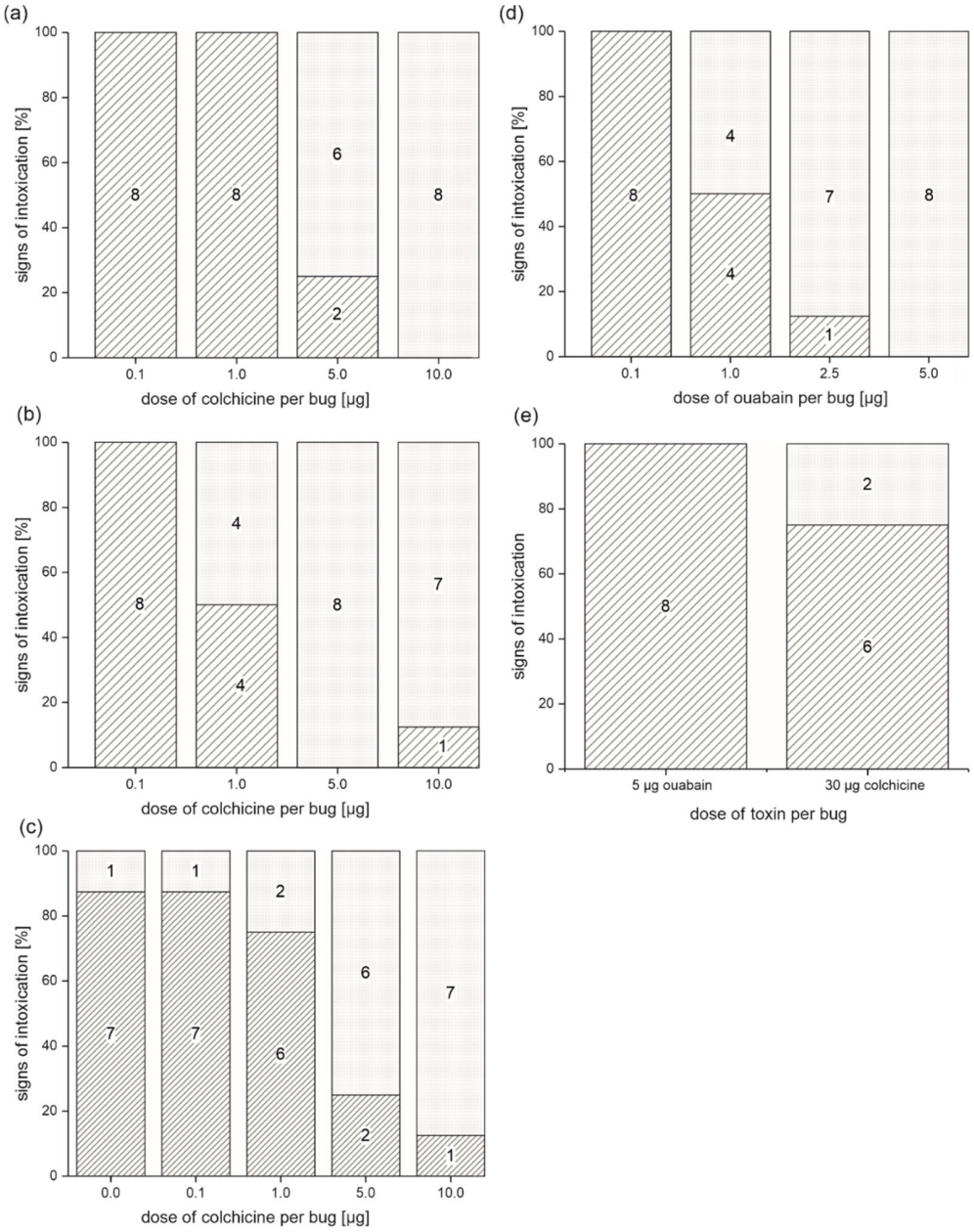


Supplemental Figure 9. Attack latencies of juvenile great tits (*Parus major*) tested with milkweed bugs and control palatable prey. (a) Attack latencies in first encounters with novel palatable prey (crickets; hatched rectangles) and milkweed bugs (*H. superbus*, *L. equestris*, and *S. saxatilis*; open rectangles). (b) Changes in attack latencies across three successive trials with milkweed bugs raised either on seeds from toxic host plants or sunflower seeds as a control (data from *H. superbus*, *L. equestris*, and *S. saxatilis* pooled). Only the data from bugs that were attacked by birds in the respective trials are included. Boxes represent median (points), quartiles (rectangles) and range (whiskers).



Supplemental Figure 10. Durations of discomfort-indicating behavior (beak wiping, head shaking) exhibited by juvenile great tits (*Parus major*) following their first contact (attack and handling) with bugs raised either on seeds of toxic host plants or sunflower as a control. Birds tested with *H. superbus* (a), *L. equestris* (b), and *S. saxatilis* (c). Boxes represent median (points), quartiles (rectangles) and range (whiskers).

Supplemental Material (not copyedited or formatted) for: Georg Petschenka, Rayko Halitschke, Tobias Zust, Anna Roth, Sabrina Stiehler, Linda Tenbusch, Christoph Hartwig, Juan Francisco Moreno Gamez, Robert Trusch, Jurgen Deckert, Katerina Chalusova, Andreas Vilcinskas, Alice Exnerovova. 2022. "Sequestration of Defenses against Predators Drives Specialized Host Plant Associations in Preadapted Milkweed Bugs (Heteroptera: Lygaeinae)."
The American Naturalist 199(6). DOI: https://doi.org/10.1086/719196.



Supplemental Figure 11. Resistance of *P. apterus*, *O. fasciatus*, *S. saxatilis*, and *S. pandurus* to injected ouabain and colchicine. (a) Effect of increasing doses of injected colchicine and ouabain (d) on *P. apterus*, (b) Effect of increasing doses of injected colchicine on *O. fasciatus*, (c) Effect of increasing doses of injected colchicine on *S. pandurus*. (e) Effect of injected ouabain and a high dose of colchicine on *S. saxatilis*. Bars show proportions of individuals that either showed signs of intoxication (open) or showed no signs of intoxication (hatched). Numbers in stacked bars represent the actual number of specimens.