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Current Biology

The polyvalent sequestration ability of an economically important beetle

Highlights

- Western corn rootworm beetles sequester multiple toxins from their host plants
- Females transfer these toxins into their eggs, which protects them against predators
- Eggs with a mixture of toxins are better protected than eggs with individual toxins
- They optimize offspring survival using the full diversity of plant defense chemistry

Authors

Carla C.M. Arce,
Ricardo A.R. Machado,
Marine Mamin, ..., Matthias Erb,
Christelle A.M. Robert,
Ted C.J. Turlings

Correspondence

carla.marques@unine.ch (C.C.M.A.),
ted.turlings@unine.ch (T.C.J.T.)

In brief

Arce et al. show that the polyphagous beetle *Diabrotica virgifera virgifera*, which is a devastating pest of maize crops, has the ability to sequester a variety of metabolites from different host plants, transferring them to its eggs. This enhances egg protection against predators, contributing to its success as an invasive pest.

Arce et al., 2024, Current Biology *34*, 5417–5428
December 2, 2024
https://doi.org/10.1016/j.cub.2024.10.005

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The polyvalent sequestration ability of an economically important beetle

Carla C.M. Arce,[1,7,*] Ricardo A.R. Machado,[2] Marine Mamin,[1] Gaétan Glauser,[3] Pamela Bruno,[1] Betty Benrey,[4] Matthias Erb,[5] Christelle A.M. Robert,[6] and Ted C.J. Turlings[1,*]

[1]University of Neuchâtel, Institute of Biology, FARCE Laboratory, 2000 Neuchâtel, Switzerland
[2]University of Neuchâtel, Institute of Biology, Experimental Biology, 2000 Neuchâtel, Switzerland
[3]University of Neuchâtel, Neuchâtel Platform of Analytical Chemistry, 2000 Neuchâtel, Switzerland
[4]University of Neuchâtel, Institute of Biology, E-vol Laboratory, 2000 Neuchâtel, Switzerland
[5]University of Bern, Institute of Plant Sciences, Biotic Interactions Group, 2013 Bern, Switzerland
[6]University of Bern, Institute of Plant Sciences, Chemical Ecology Group, 2013 Bern, Switzerland
[7]Lead contact
*Correspondence: carla.marques@unine.ch (C.C.M.A.), ted.turlings@unine.ch (T.C.J.T.)
https://doi.org/10.1016/j.cub.2024.10.005

SUMMARY

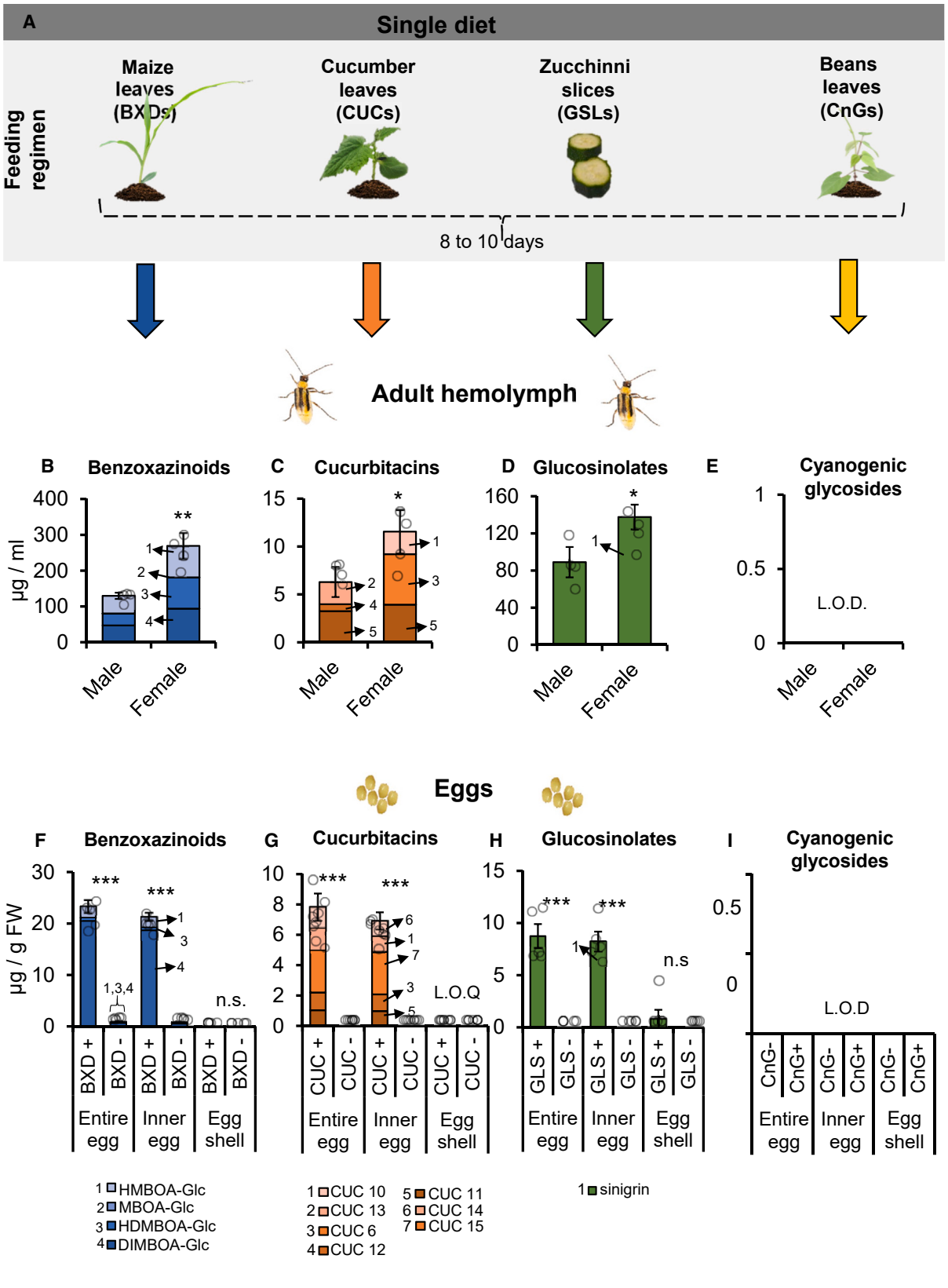
Many specialized herbivorous insects sequester single classes of toxic secondary metabolites from their host plants as protection against natural enemies. If and how herbivores can use multiple classes of plant toxins across the large chemical diversity of plants for self-protection is unknown. We show that the polyphagous adults of the beetle *Diabrotica virgifera* are capable of selectively accumulating benzoxazinoids, cucurbitacins, and glucosinolates but not cyanogenic glycosides. Female beetles transfer the sequestered defense metabolites into their eggs, protecting them against generalist predators. Eggs containing a mixture of toxins are better protected than eggs with individual toxins. This work shows how herbivores can exploit plant chemical diversity to their own benefit as a novel adaptive mechanism that contributes to the structuring of multitrophic interaction networks.

INTRODUCTION

Plants produce a large diversity of defense metabolites to ward off herbivores and survive in hostile and variable environments. Herbivores, in turn, have evolved the capacity to resist and tolerate plant defenses and thus to survive on toxic plants. Highly specialized insect herbivores can even selectively accumulate plant toxins and use them against their own enemies,[1–5] thus escalating the co-evolutionary arms race to the third trophic level.[6,7] A well-known example is the monarch butterfly, whose caterpillars exclusively feed on milkweed plants. They sequester highly toxic cardenolides from these plants, which are then stored in their hemolymph.[8,9] The cardenolides are transferred to the adult stage and their eggs, protecting them against predation.[10–12] Many other insects use this same strategy to safeguard themselves and their offspring from predation.[2,13] Plant defensive metabolites known to be sequestered by insects include cardiac glucosides, pyrrolizidine alkaloids, cucurbitacins, benzoxazinoids, cyanogenic, and iridoid glucosides and glucosinolates.[4,5] The specialized herbivores typically exploit single classes of defense compounds from their specific host plants or host plant families.[2,5]

The minority of insects are generalists that feed on multiple host plants and thus interact with many different types of plant defense metabolites.[4] Polyphagia is assumed to be a strategy to overcome toxicity of plant defense chemicals. By frequently switching among host plants, the ingestion of specific plant-derived toxins can be diluted.[14,15] In addition, this behavior may further maximize fitness by the acquisition of a more diverse spectrum of plant nutrients.[16–18] These two hypotheses are very challenging to test and to separate from each other.[16,19] To overcome the toxicity of plant defense chemicals, generalist herbivores can produce new products after metabolizing plant toxins,[2,4,5] albeit less efficiently than specialists. Passive sequestration appears to be the rule among generalist insects, but in theory, they could also actively accumulate plant defensive metabolites[20] to resist natural enemies.[2,5,21,22] In rare cases, herbivores have been reported to sequester multiple classes of plant toxins. For instance, *Grammia incorrupta*[21,23–25] and *Estigmene acrea*[22,26–30] sequester iridoid glycosides and alkaloids, and *Spilostethus saxatilis* accumulates alkaloids and cardenolides,[31] and some species of the genus *Athalia* do the same with iridoid glycosides and glucosinolates.[32] While these studies indicate that herbivores may have the potential to exploit diverse plant chemicals, the biological relevance of this phenomenon is unclear. It is also unknown to what extent herbivores may combine different plant toxins when feeding on different host plants. Answering these questions may well hold the key to a better understanding of the interaction between plant chemical diversity and multitrophic interaction networks.

The western corn rootworm (WCR) *Diabrotica virgifera* is one of the most destructive pests of maize. The larvae of this beetle are specialized to feed on maize roots and are thus well adapted to maize defensive chemistry. They use multiple volatile and exudate cues to locate host roots and navigate within the root system.[33–35] They furthermore accumulate high amounts of



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benzoxazinoids in their body, which convey resistance to entomopathogenic nematodes by acting as toxins and repellents[7,36] and their insect-killing bacterial symbionts.[6] In contrast to the larvae, the adult beetles are highly polyphagous. They feed on different plant species of the Poaceae, Fabaceae, Cucurbitaceae, Brassicaceae, and Asteraceae families and seem to mix diet sources.[37–41] The beetles are capable of tolerating different host plant defenses,[42] but whether they are capable of accumulating plant defense compounds to protect themselves and their eggs remains unknown.

Here we investigated if and how WCR adults accumulate different types of major defense metabolites as they feed on different host plants. Chemical analyses, after feeding beetles with wild and deficient mutant plants, revealed that adults selectively sequester, convert, and then transfer three chemically highly distinct classes of plant toxins to their eggs. In behavioral choice bioassays, the plant-derived toxins varied in their effectiveness as protection against insect predators and were more effective in combination than alone. Taken together, these results show that polyphagous insects can sequester several protective plant toxins simultaneously and use them in a synergistic manner to protect their progeny, thus demonstrating a mechanism by which plant chemical diversity can structure multitrophic interaction networks.

RESULTS

WCR adults sequester different plant defense compounds

To evaluate whether WCR adults sequester and transfer defense compounds from different host plants into their eggs, we fed adults with leaves of maize, cucumber, cabbage, or bean plants. These plant species were chosen since these insects are naturally from Mesoamerica, where these plants can be found growing very close or even together, as is the case for the traditional milpa systems.[43] We then measured the accumulation of the major defenses of each plant species (benzoxazinoids, cucurbitacins, glucosinolates, and cyanogenic glycosides, respectively) in the hemolymph and eggs of the beetles (Figure 1A). For each plant species, we also included mutant plants that produce no or very low levels of the defense compounds as food source (Figure 1A). Adult females fed on wild-type maize plants accumulated about 270 μg/mL of total benzoxazinoids in their hemolymph, whereas males accumulated about 130 μg/mL (Figure 1B). Females transferred about 25 μg/g fresh weight (FW) of the accumulated benzoxazinoids into their eggs, and only traces of benzoxazinoids were found on the egg surfaces (Figure 1F). Similar sequestration patterns were observed for cucurbitacins and glucosinolates. No sequestration of cyanogenic glucosides was observed. Adult females that were fed exclusively on cucurbitacin-containing cucumber plants accumulated up to 11.5 μg/mL of total cucurbitacin in their hemolymph, whereas males accumulated 6.3 μg/mL (Figure 1C; Table S1). Females transferred up to 7.8 μg/g FW of the accumulated cucurbitacins into their eggs, and none were found on the surface of the eggs (Figure 1G). Females also sequestered glucosinolates, although their performance was highly impaired when fed exclusively on cabbage plants, especially on the wild-type accession, which contains high levels of glucosinolates (Figures S1A and S1B). With this diet, we could only collect a few surviving adults but no eggs. The adults had up to 10.3 μg/mL of glucosinolates in their hemolymph (Figure S1C). Because of the poor survival on cabbage plants, we opted to feed sinigrin-spiked zucchini slices to the beetles to evaluate the protective effect of glucosinolates (Figure S1D). Sinigrin is one of the glucosinolates produced by wild *Brassica* accessions (Figure S1B). The zucchini experiment confirmed the ability of the adults to sequester this metabolite. Females accumulated up to 137 μg/mL and males up to 89 μg/mL of sinigrin (Figure 1D). Females transferred up to 8.74 μg/g FW of the accumulated sinigrin into their eggs, and no sinigrin was found on the surface of the eggs (Figure 1H). The feeding experiments with bean plants revealed that adults are unable to sequester the cyanogenic glucosides linamarin and lotaustralin from this plant (Figures 1E, 1I, and S2). Overall, the feeding assays demonstrate that WCR beetles accumulate different classes of plant defense compounds and transfer them to their eggs. Both sequestration and transfer are selective, as not all defense compounds are taken up, and the amounts and ratios of different individual defense compounds change significantly between males and females and the hemolymph and eggs.

WCR adults sequester multiple plant metabolites simultaneously

The WCR adults exhibited a remarkable ability to sequester various classes of plant metabolites simultaneously when we provided them with mixed diet regimens (Figures 2, 3, and S3). A short-term mixed diet regime (Figure S3A) revealed that, when WCR adults sequentially fed on different plant species, they accumulated plant metabolites from all plants, and, in all cases, females accumulated higher amounts than males (Figures S3B–S3D). They were found to also transfer these compounds selectively into their eggs (Figures S3E–S3G). The metabolite levels decreased over time, and compounds derived from the most recently consumed food source were in all cases found to be higher in the insect's hemolymph and

Figure 1. WCR adults individually sequestered and transferred several plant defensive metabolites into their eggs

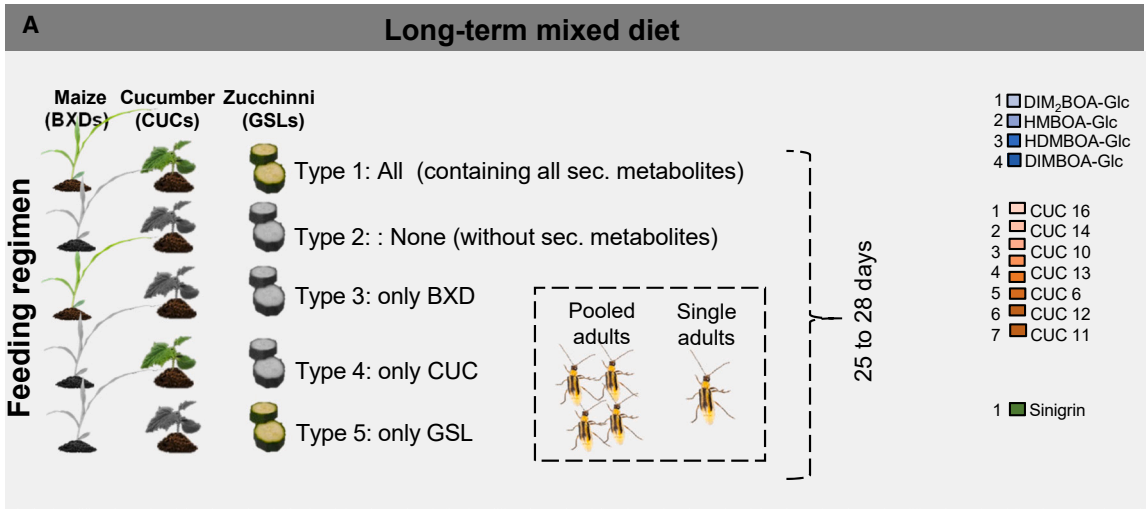
(A) Experimental design used to expose WCR adults to a single diet.

(B–E) Total amount of benzoxazinoids (BXDs; $n = 4$), cucurbitacins (CUCs; $n = 4$), glucosinolates (GSLs; $n = 4$), and cyanogenic glycosides (CnGs; $n = 4$) present in the hemolymph of WCR adults fed exclusively on maize leaves, cucumber leaves, sinigrin coated in slices of zucchini, or on leaves of bean plants for 8–10 days.

(F–I) Total amount of BXDs ($n = 5$), CUCs ($n = 8$), GSLs ($n = 5$), and CnGs ($n = 5$) present in entire eggs, inner eggs, or on eggshells of WCR fed separately on the previously mentioned plants.

Bars represent average (±SE). The positive (+) and negative (−) symbols on the x axis indicate diet plants that contained the metabolites or did not contain the metabolites, respectively. Each number indicated by arrow represents different metabolites found. Asterisks indicate significant differences in the amounts of metabolites (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; linear model followed by pairwise comparisons of least squares means). L.O.D., limit of detection; L.O.Q., limit of quantification.

See also Figures S1, S2, S4, S8, and S9; Table S1; and Data S1, S3, S4, S6, S7, S9, and S10.



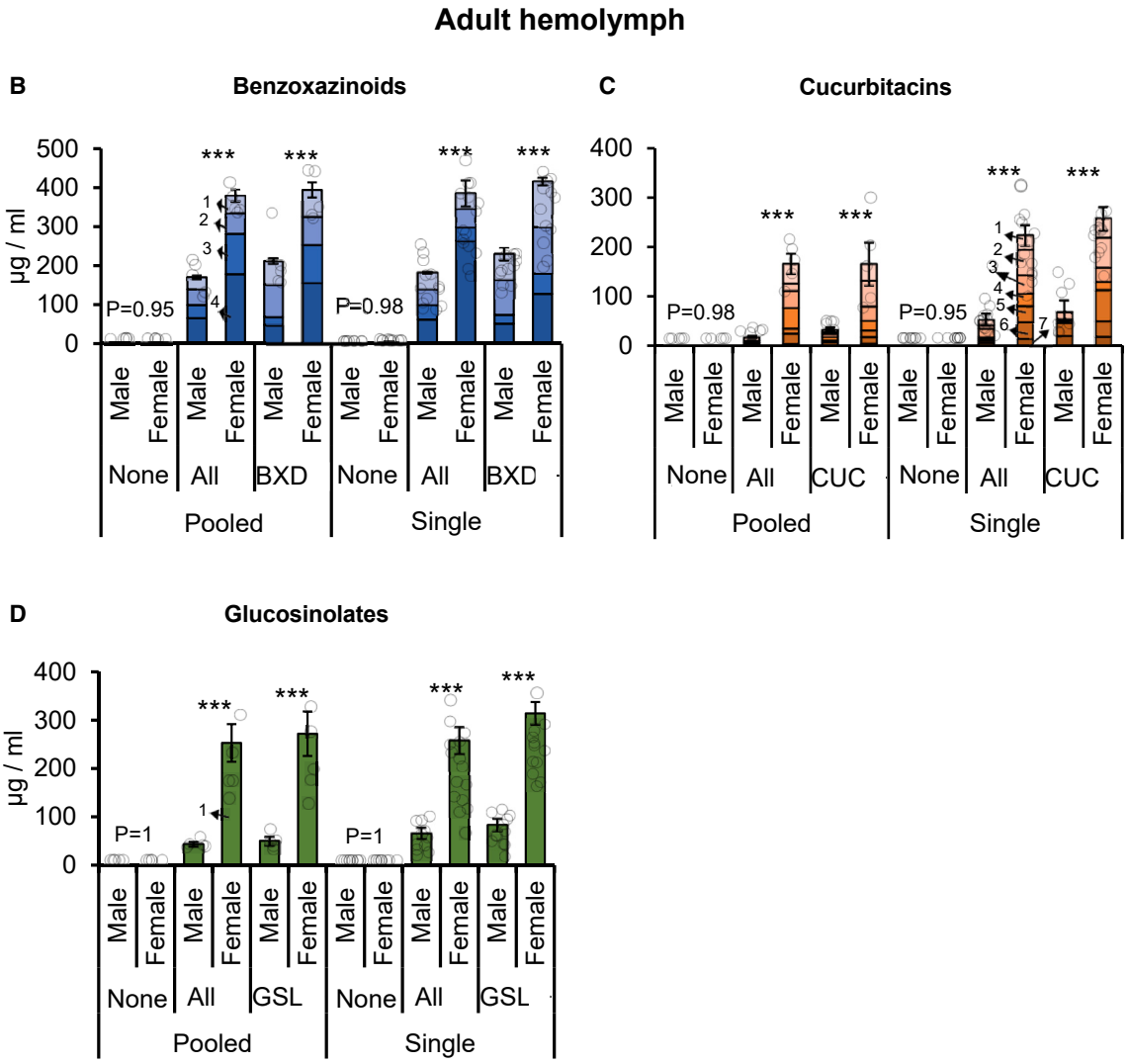


Figure 2. WCR adults simultaneously sequestered several plant defensive metabolites in their hemolymph

(A) Experimental design used to expose WCR adults to a long-term mixed diet. After feeding the adults on different food regimens, we took hemolymph samples from either a single adult (single) or 4 adults that were pooled together (pooled).

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eggs (Figures S3B–S3G). Similar sequestration patterns were observed in long-term mixed diet experiments (Figures 2 and 3). Adults accumulated all classes of secondary metabolites in their hemolymph (Figure 2), and the females transferred them to their eggs (Figure 3). Again, females sequestered higher levels of secondary metabolites than males (Figures 2B–2D; Tables S2–S11). Among the metabolites, benzoxazinoids were accumulated at higher levels, followed by glucosinolates and cucurbitacins (Figure 3). This observation suggests a discernible affinity of females for benzoxazinoids over other metabolite classes. Evidently, the adults are able to sequester all three of the secondary metabolite compounds, and the females transferred them to their eggs simultaneously, as the hemolymph of individual adults and the egg masses of single females (Figure S5) contained all the classes of secondary metabolites (Figures 2 and 3; Tables S2–S11). The levels observed when the insects were offered a combination of plant species or a single plant species were similar, indicating that there are no antagonistic effects of any of these metabolites on each other or trade-offs in sequestration strengths. Additionally, the feeding pattern was recorded, and no differences were observed among the food diet eaten (Figures S6 and S7). Taken together, the results reveal that WCR adults can sequester multiple classes of secondary metabolites simultaneously and transfer them to their eggs.

Close inspection of the concentrations and chemical identities of benzoxazinoids, cucurbitacins, and glucosinolates present in plant tissues, insect hemolymph, and eggs revealed the ability of WCR adults to metabolize some of them (Figures 1, 2, 3, and S1–S4; Tables S2–S11). More specifically, MBOA-Glc was found only in hemolymph of adults and not in maize plants (Figures 1, 2, 3, S2–S4, and S8). In the assays with cucumber leaves, a total of 16 distinct cucurbitacins were observed in plants, adult hemolymph, and eggs (Figures 1, 2, 3, S2–S4, and S8; Table S1). Among them, only cucurbitacin 15 was exclusively present, in minor quantities, in the eggs, while the remaining compounds were detected in both plants and beetle hemolymph (Figures 1, 2, 3, S2–S4, and S8; Table S1). Similarly, out of the eleven different glucosinolates found in wild cabbage plants and hemolymph, two were exclusively found in the hemolymph (Figures S1B, S1C, and S8). As no eggs were produced by the few adults that survived on wild cabbage plants, we were unable to assess if any of them were transferred to the eggs (but see the sinigrin experiments; Figures 1, 2, and 3).

Sequestered plant defense compounds deter predators

To assess the protective role of sequestered plant metabolites against natural enemies, we evaluated the feeding preferences and survival rates of two generalist egg predators, the rove beetle *Dalotia coriaria* and the minute pirate bug *Orius laevigatus*. They can be found in maize fields and are known to consume different life stages of the WCR.[44] To obtain eggs with and without plant secondary metabolites, WCR females were fed on plants containing or not the different plant metabolites in single diet regimes (Figure 1A). We observed that, in choice experiments, benzoxazinoids, cucurbitacins, and glucosinolates present in the eggs significantly deterred both egg predator species (Figures 4A and 4B; Videos S1 and S2). To assess the potential additive or synergistic effects of the different plant secondary metabolite classes, we evaluated predator preferences using eggs containing none, single (BXD, CUC, and GSL), or all three secondary metabolite classes (Figures 3A, 4C, and 4D). Both predators preferred to feed on eggs containing just a single compound class than to feed on eggs containing all the three compound classes together (Figures 4C and 4D). In addition, the deterrent effects observed when all compounds were present in the eggs were significantly stronger than the sum of the deterrent effects of individual compounds for *O. laevigatus* but not that strong for *D. coriaria*, indicating that these secondary metabolites can act synergistically as deterrents for some but not all predators (Figures 4E and 4F).

The toxicity of each individual metabolite class in the eggs was assessed through survival experiments. Combination measurements were not possible for logistical reasons. High mortality rates were observed for predators that had fed on eggs containing benzoxazinoids (Figures 5A and 5D). The mortality of *D. coriaria* adults that consumed eggs with benzoxazinoids reached 60% after 25 days (Figure 5A), while approximately 90% of *O. laevigatus* died within 7 days (Figure 5D). Sinigrin was about as toxic as benzoxazinoids (Figures 5C and 5F). More specifically, higher mortality rates of *D. coriaria* adults were observed when these predators were fed 25 days exclusively with eggs containing sinigrin than when fed with sinigrin-free eggs (Figure 5C). The pirate bug, *O. laevigatus*, showed 90% mortality after 7 days of feeding on sinigrin-containing eggs (Figure 5F). No toxic effect of cucurbitacins was observed for either predator (Figures 5B and 5E).

DISCUSSION

Generalist herbivores feed on multiple plant species and are therefore exposed to various classes of defense metabolites. While there is considerable knowledge on the defensive strategies employed by plants and specialist herbivores, the mechanisms by which generalist herbivores utilize this chemical diversity to protect their progeny against predators are not well understood. Here, we show that a polyphagous beetle can sequester and distill different classes of defense metabolites from different host plants into protective cocktails that they transfer to their eggs. The ability to use different plant defense compounds individually and in combination likely increases the chances of survival of this insect herbivore across diverse and dynamic agroecosystems and must have contributed to its success as an invasive pest.

(B–D) Total amount of benzoxazinoids (pooled $n = 5$; single $n = 9$–15), cucurbitacins (pooled $n = 5$; single $n = 9$–15), and glucosinolate (pooled $n = 5$; single $n = 9$–15) present in the hemolymph of WCR adults after 28 days of feeding on each regimen type.

Bars represent the average (±SE). Each number indicated by an arrow represents a different metabolite that was found. Asterisks indicate significant differences in the amounts of metabolites (***$p < 0.001$; **$p < 0.01$), and different letters represent significant differences among treatments. Linear model (family, Gaussian) followed by pairwise comparisons of least squares means.

See also Figures S3, S5, S7, and S9; Tables S1–S11; and Data S2 and S8.

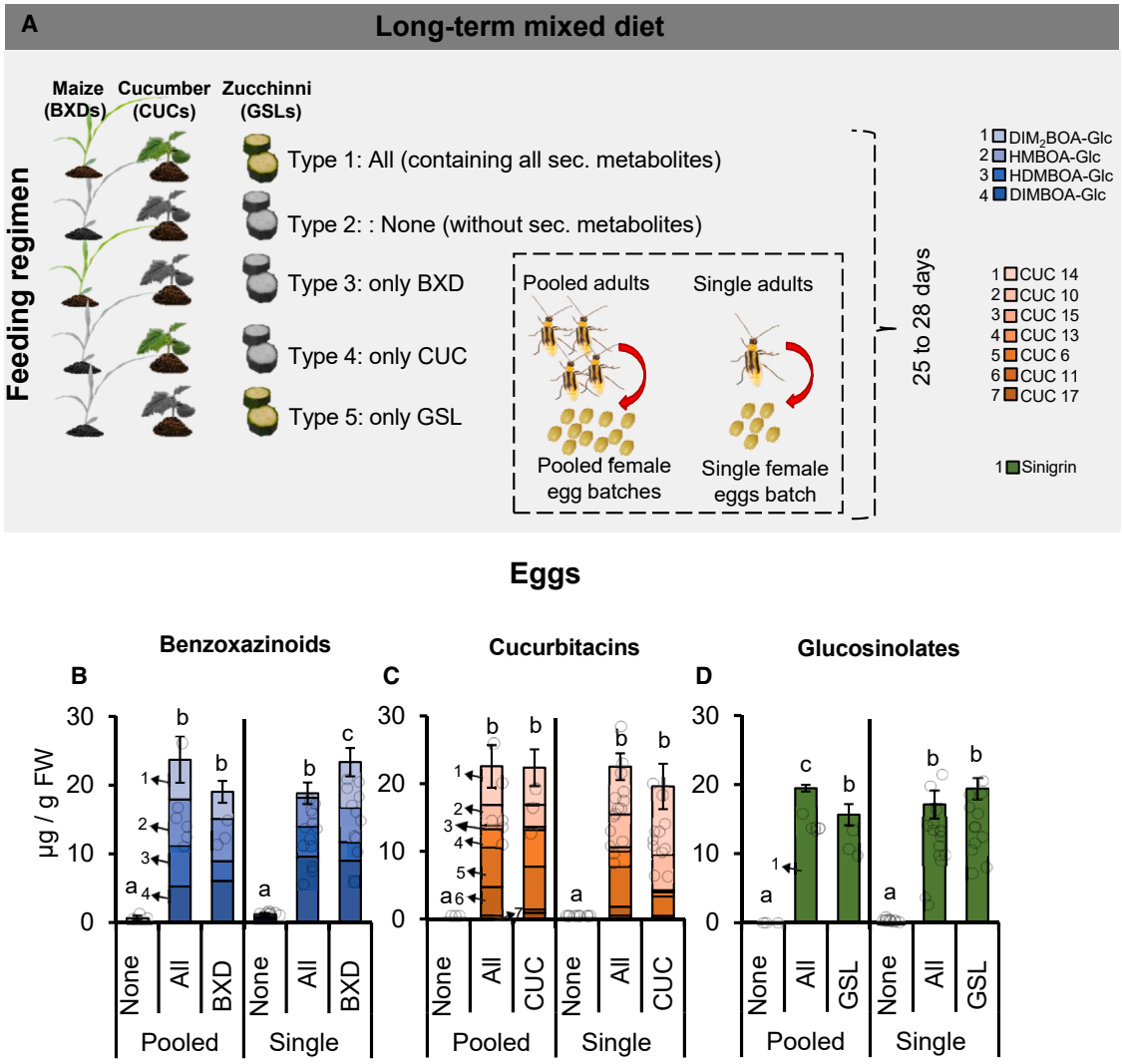

Figure 3. WCR adults simultaneously transfer several plant defensive metabolites into their eggs

(A) Experimental design used to expose WCR adults to a long-term mixed diet. After feeding the adults on different food regimens, the egg samples were collected either from several females that laid them together (pooled samples) or eggs that were collected from single females by dissecting their abdomen (single samples).

(B–D) Total amount of benzoxazinoids (pooled $n = 3–6$; single $n = 10–13$), cucurbitacins (pooled $n = 3–6$; single $n = 10–15$), and glucosinolates (pooled $n = 3–6$; single $n = 10–15$) present in the eggs of WCR adults after 28 days of feeding on each regimen type.

Bars represent the average (±SE). Each number indicated by arrow represents a different metabolite that was found. Different letters indicate significant differences within treatments. Linear model (family, Gaussian) followed by pairwise comparisons of least squares means.

See also Figures S3, S5, S7, and S9; Tables S1–S11; and Data S8.

Generalist herbivores are assumed to have multifunctional detoxification mechanisms to deal with a multitude of plant toxins.[4] They may also cope with toxic diets by frequently switching between different plant species to dilute toxins and thus reduce their toxicity.[18,21] This diet switching by generalists has been hypothesized to preclude the possibility of sequestration, which is typically seen as a trait of specialist herbivores.[45] We demonstrate here that the polyphagous WCR beetles can sequester at least three plant-derived metabolite groups (benzoxazinoids, cucurbitacins, and glucosinolates) under mixed diet feeding regimens and transfer each of these metabolites into their eggs simultaneously. The adult beetles are expected to draw considerable advantages from sequestering this variety of toxic compounds, as it increases the defensive arsenal against their natural enemies. By mixing their diet, the beetles can ensure protection against a broad spectrum of natural enemies and can strongly reduce the possibility that these enemies develop resistance against any specific defense. Indeed, we show that benzoxazinoids, cucurbitacins, and glucosinolates transferred to eggs deter attacks by generalist predators, displaying both individual toxicity and synergistic effects. Our results indicated no trade-offs among the sequestration of all three plant toxin families (no differences in adult mortality were observed), which is consistent with observations reported by

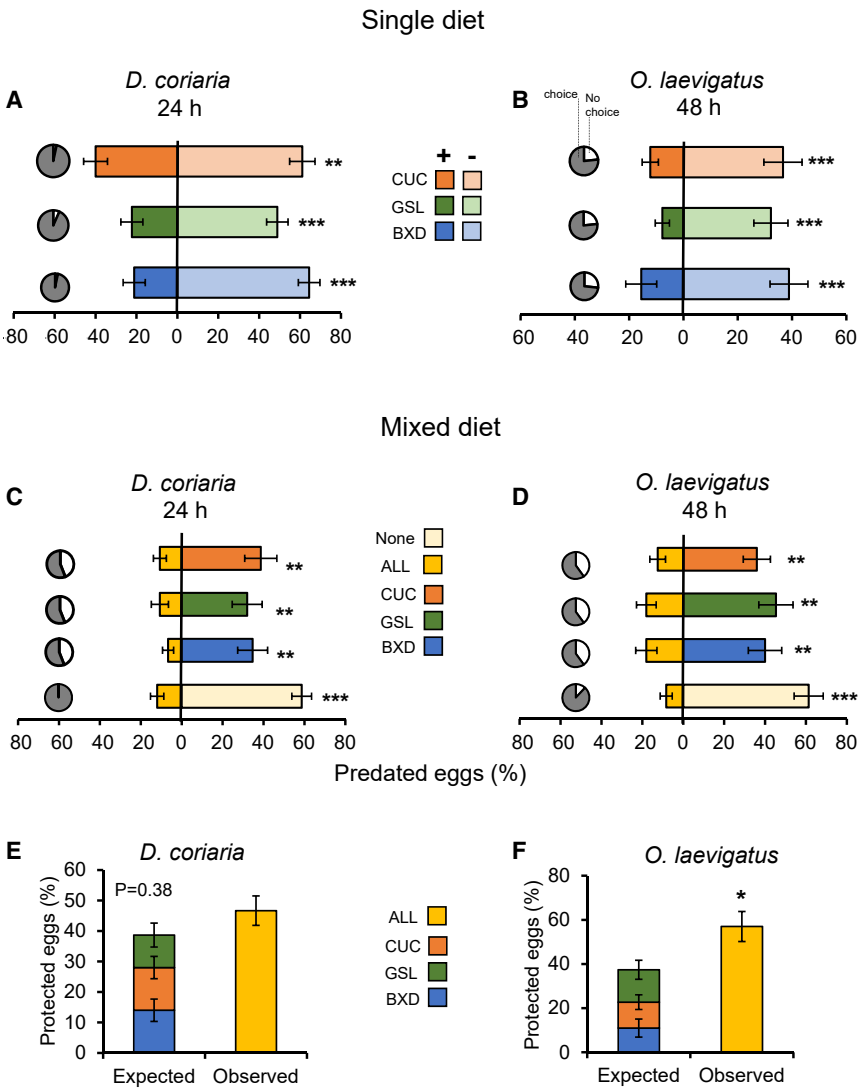

Figure 4. Plant defensive metabolites present in the eggs may deter predators and can have synergistic effects

(A–D) Choice experiments with WCR eggs either free of or containing plant defensive metabolites.

(A and B) *D. coriaria* and *O. laevigatus* preferences for WCR eggs either free of or containing plant metabolites were evaluated after 24 and 48 h, respectively. The eggs were obtained from adults that fed on a single diet over 15 days, eggs with (BXD+) and without (BXD−) benzoxazinoids (n = 30), containing (CUC+) or not (CUC−) cucurbitacins (n = 30), and with (GSL+) and without (GSL−) glucosinolates (n = 30) were used.

(C and D) Predation preference of *D. coriaria* and *O. laevigatus* on WCR eggs from adults that were fed either on a mixed diet (none, without sec. metabolites; all, presence of BXDs, CUCs, and GLSs metabolites together) or single diets with benzoxazinoids (BXD, n = 25), containing cucurbitacin (CUC, n = 25), and containing glucosinolate (GSL, n = 25) were evaluated after 24 and 48 h.

(E and F) Sum of the expected deterrent effects of individual metabolites and observed deterrent effects of all the metabolites combined.

Bars represent the percentages of protected eggs (±SE). Pie charts indicate the proportion of predators that ate (choice in gray) or not (no choice in white). Asterisks indicate significant differences in the predation rates: ***p < 0.001; **p < 0.01; *p < 0.05; false discovery rate (FDR) corrected (generalized linear model [family, Binomia]), followed by pairwise comparisons of least-squares means (LSM).

See also Data S3 and S4 and Videos S1 and S2.

other researchers (revised in Petschenka and Agrawal[20]). Our results indicate that the beetles are not able to sequester cyanogenic glucosides, contrary to certain lepidopteran species, which are able to sequester these compounds but at a cost.[46] Among the sequestered plant toxins, the benzoxazinoids seem to be accumulated by WCR in higher amounts than cucurbitacin and glucosinolate. This could indicate that they could have evolved a highly specialized adaptation to these plant toxins, which is also evident from the fact that WCR larvae are specialists on maize roots and therefore are exposed to benzoxazinoids during their entire immature life cycle phase.

Many lepidopteran, coleopteran, and hemipteran species are known to transfer a single plant toxin to their eggs, e.g., cardiac glucosides, pyrrolizidine alkaloids, iridoid glucosides, prenylated aromatics, cycasin, cyanogenic glucosides, and salicin, but their defensive role against parasitism and predation has received limited attention.[2,5,47,48] Some species of *Diabrotica* are known to transfer sequestered cucurbitacins into their eggs, which may defend them against entomopathogenic fungi,[49–51] but cucurbitacins in the eggs do not provide any effective protection against predators as was found for *D. undecimpunctata*.[52] We show the same here for *D. virgifera* and in a previous study for *D. balteata* larvae.[53] Glucosinolates have been reported in the eggs of a stink bug,[2,54] but their protective role in the eggs is unknown.[2,5] Yet that they provide protection to other life stages has been demonstrated; larvae of turnip sawfly are better defended against predatory ants and parasitoids wasps when they have accumulated glucosinolates in their hemolymph.[55] In a few cases, the sequestration and transfer of toxic metabolites into the eggs has also been shown to occur in vertebrates such as reptiles and birds, which provide protection against mammalian and avian predators.[56,57] Synergistic protective effects of chemical metabolites from host plants and herbivores are well documented in the literature (see above). However, to the best of our knowledge, no studies have reported such effects for sequestered toxins transferred to the progeny.[18,58] Benzoxazinoids, cucurbitacins, and glucosinolate transferred into WCR eggs may confer complementary toxicity, enhanced toxic potency, increase the defense spectrum, and inhibit detoxification by predators, but each of these potential effects remains to be tested.

WCR females were found to accumulate higher amounts of sequestered metabolites in their bodies than males, further supporting the notion that the sequestration is particularly important

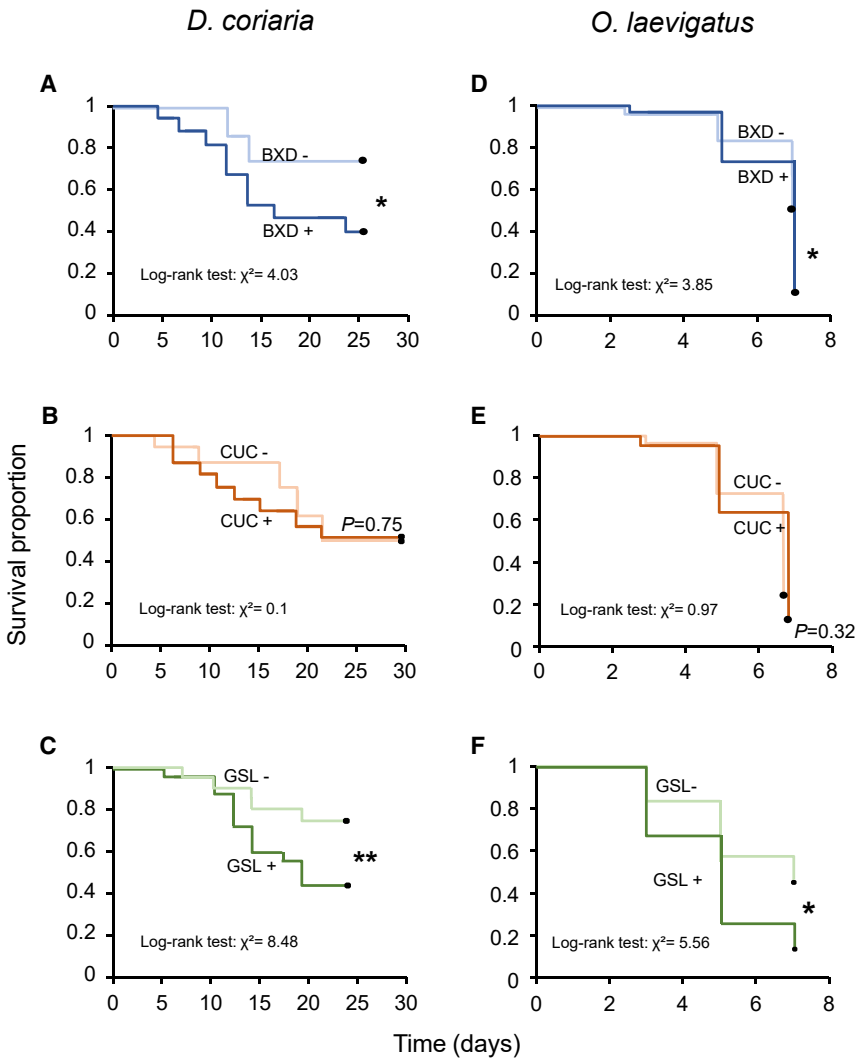

Figure 5. Benzoxazinoids and glucosinolates transferred into WCR eggs are toxic to predators, but cucurbitacins are not

(A–F) Survival curves of predators fed exclusively with WCR eggs either free of or containing plant defensive metabolites.

(A–C) *D. coriaria* fed exclusively with WCR eggs containing (BXD+) or not (BXD−) benzoxazinoids (A, $n = 20$), with (CUC+) and without (CUC−) cucurbitacins (B, $n = 20$), and containing (GSL+) or not (GSL−) glucosinolates (C, $n = 20$).

(D–F) *O. laevigatus* fed exclusively with WCR eggs containing (BXD+) or not (BXD−) benzoxazinoids (D, $n = 25$), with (CUC+) and without (CUC−) cucurbitacins (E, $n = 25$), and containing (GSL+) or not (GSL−) glucosinolates (F, $n = 25$).

Survival curves were calculated by Kaplan-Meier estimator. $^{**}p < 0.01$; $^{*}p < 0.05$.

See also Data S5.

for egg protection. Males may also contribute to this by transferring sequestered metabolites to females during mating, acting as a "nuptial gift,"[59] as observed in *D. balteata* where cucurbitacins are transferred through spermatophores,[49] potentially enhancing reproductive fitness by protecting their progeny.[2,5] Our findings strongly indicate that this is also the case for the WCR. This makes this insect an ideal model to study the evolutionary relevance of the indirect contribution by males to progeny protection, which should also play an important role in mate choice by the females, as is the case for other insects reviewed in Lewis and South.[60]

In plants, defense metabolites are primarily stored in their inactive glucosylated forms (protoxins), physically separated from activating enzymes like β-glucosidases. This two-component defense strategy is observed in plants that produce alkaloids, benzoxazinoids, cyanogenic glucosides, iridoid glucosides, glucosinolates, and cucurbitacins.[61,62] When herbivores attack and disrupt plant tissue, the protoxins come in contact with β-glucosidases that hydrolyze the glucosyl group, releasing active toxins known as aglucones.[61] WCR beetles were found to accumulate the defense metabolites in their hemolymph and transfer them into their eggs, mostly in the glucosylated form. How insects convert the aglucone form into a glucosylated, non-toxic form is still poorly understood.[63] In a few cases, it is known that this involves a process that attaches a sugar molecule to the toxic aglucone, which is commonly catalyzed by UDP-glucosyltransferases (UGTs).[61,64] Several caterpillars, like the rice armyworm, the Asian corn borer, the fall armyworm, and the Egyptian cotton leafworm, use UGTs to reglucosylate DIMBOA, reducing its toxicity.[65–69] However, Robert et al.[36] found that WCR larvae accumulate HDMBOA-Glc through mechanisms other than hydrolysis or reglucosylation of free HDMBOA, suggesting rapid transport or temporary stabilization via chemical modifications.[70] Similarly, monarch butterflies preferentially sequester cardenolides with a single glucoside moiety and seem to also employ a two-component defense strategy driven by β-glucosidases and UGTs.[71]

UGTs are not the only enzymes responsible for detoxification in insects. Cytochrome P450s, carboxyl/cholinesterases, and glutathione S-transferases are also widely used by insects for detoxification.[4] Importantly, gene expression of cytochrome P450 and of a cathepsin protease is upregulated in WCR larvae when fed on a diet that contains benzoxazinoids.[72] In addition, some beetles of the genus *Diabrotica* have been found to transform cucurbitacins ingested from *Cucurbita* plants by not only reglucosylation but also by acetylation, hydrogenation, and desaturation.[51,53,62,73,74] We also observed that, after feeding on cucumber leaves, WCR adults and their eggs contained deacetylated glucosidic cucurbitacins, which are not present in the plants. The molecular mechanism involved in this process remains to be determined.

The glucosinolates sequestered by WCR adults were accumulated in the hemolymph and mostly in their intact forms.

The turnip sawfly and the mustard aphid are able to do this too, but how these insects accumulate glucosinolates without metabolizing them also remains unknown.[55,75] We speculate that the rapid absorption of ingested glucosinolates across the gut epithelium and the suppression of plant myrosinase, the enzyme that hydrolyzes glucosinolates into toxins, may be behind the sequestration by WCR adults. For instance, flea beetles are known to use this strategy and having different enzymes responsible for myrosinases activities.[76,77] The diamondback moth, another crucifer specialist, uses a glucosinolate sulfatase in the gut to hydrolyze the sulfate group from the glucosinolates, making them unsusceptible to myrosinases and allowing their excretion without activation.[78] The generalist desert locust uses yet another strategy; it can feed on glucosinolate-containing plants by inducing the activity of the glucosinolate sulfatase enzymes.[79] All three of the toxin types sequestered by the WCR are moderately polar, which makes a passive diffusion through the gut membrane very unlikely reviewed in Opitz and Müller and Beran and Petschenka.[2,5] This implies an active uptake mechanism that involves specific transporters[5] and the beetles capacity to transform multiple toxic plant-derived compounds suggests that they possess an intricate array of detoxification and storage mechanisms.[80] Our data further suggest that even though the WCR has a superbly broad ability to sequester a diversity of plant secondary metabolites, it has a mechanism that specifically facilitates or has more affinity toward benzoxazinoids accumulation (Figure 3). This agrees with the fact that during the immature life stages, the insect is specialized to feed on maize roots.

Of the four types of plant toxins that we tested only cyanogenic glucosides were not sequestered by the WCR beetles. The sequestration of cyanogenic glucosides has only been reported in lepidopteran insects.[2,5] Larvae of *Zygaena filipendulae* and *Z. trifolii* are able to sequester linamarin and lotaustralin.[81] These compounds are present in both larvae and adults, and the larvae reglucosylate them after hydrolysis to again obtain the intact glucoside. In addition, many specialist and generalist insects tolerate cyanogenic glucosides by either detoxification or by excretion of their intact forms.[82,83]

Taken together, our findings reveal that WCR beetles have an exceptional ability to sequester multiple plant-derived defense metabolites that ensure their own protection as well as that of their progeny. WCR beetles produce over-wintering diapausing eggs, and their survival during this exceedingly long period in the soil may greatly depend on the multifarious ability of their parents to sequester and transfer plant-derived toxins. This ability may explain, at least in part, why WCR is such an important invasive pest.[84] Our findings suggest that the diversity of plant defensive chemistry can be mirrored in polyphagous insect herbivores and thereby structures multitrophic interaction networks.

RESOURCE AVAILABILITY

Lead contact

Further information should be directed to and will be provided by the lead contact, Carla C.M. Arce (carla.marques@unine.ch).

Materials availability

This study did not generate new unique reagents or materials.

Data and code availability

- Raw data are provided in the supplemental information and [https://doi.org/10.5061/dryad.v15dv425x].
- This study does not report original code.
- Any additional information required is available from the lead contact upon request.

ACKNOWLEDGMENTS

We would like to thank Stefan Toepfer (CABI, Hungary) and Bruce Hibbard (USDA, USA) for providing WCR eggs. All the technical support from Gaia Besomi, Aurélie Rosset, and Eloïse Ansermin. We thank Thomas Degen for the critical revision of the manuscript. We thank Paulo F. Cristaldo for supporting the survival statistical analysis. This study was supported by the Swiss National Science Foundation grant CRSII3_160786 (C.C.M.A., P.B., C.A.M.R., M.E., and T.C.J.T.) and grant PZ00P3_186094 (R.A.R.M.).

AUTHOR CONTRIBUTIONS

Conceptualization, C.C.M.A. and T.C.J.T.; funding acquisition, T.C.J.T. and M.E.; methodology, C.C.M.A., R.A.R.M., G.G., P.B., B.B., M.E., and C.A.M.R.; investigation, C.C.M.A., R.A.R.M., M.M., and P.B.; visualization, C.C.M.A. and R.A.R.M.; supervision, C.C.M.A. and T.C.J.T.; writing – original draft, C.C.M.A., R.A.R.M., and T.C.J.T.; writing – review and editing, C.C.M.A., R.A.R.M., T.C.J.T., M.M., B.B., P.B., G.G., M.E., and C.A.M.R.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2024.10.005.

Received: August 6, 2024
Revised: September 30, 2024
Accepted: October 1, 2024
Published: November 5, 2024

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
Analyzed data	This paper	https://doi.org/10.5061/dryad.v15dv425x
Raw data	This paper	www.ebi.ac.uk/metabolights/MTBLS11114
Experimental models: Organisms/strains		
The following insect species were used in this study: *Orius laevigatus*, *Dalotia coriaria*, *Diabrotica virgifera* and eggs of *Ephestia kuehniella* Z.	Commercial or in-house rearing	N/A
Maize plants (*Zea mays* var. Delprim); cucumber plants (*Cucumis sativus* L.); cabbage plants (*Brassica rapa rapa* L.).	Commercial or in-house rearing	N/A

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Plants

Maize seeds (*Zea mays* L.) of the variety B73 were provided by Delley Semences et Plantes SA (Delley, CH). The near-isogenic benzoxazinoid deficient bx1 mutant line in a B73 background was obtained by backcrossing the original bx1 mutant five times into B73.[36] Two varieties of cucumber (*Cucumis sativus* L.) were used: one that produces cucurbitacins (Hokus) and one that does not produce cucurbitacins (Tanja).[53] Seeds were purchased from Sluis Garden (NL) and Zollinger (CH), respectively. Two varieties of cabbage plants (Brassica rapa subsp. rapa) were used: one that produces high amounts of glucosinolates (Brassica rapa rapa L.; wild population) and one that produces less glucosinolates (*Brassica rapa rapa* L. var. pekinensis, Chinese cabbage).[85] The seeds of the cultivated plants were purchased from Samen-Mauser (CH), and those of the wild population were kindly provided by Dr. Tom de Jong from Leiden University (NL). As we observed high mortality rates and that females did not lay eggs when fed on wild cabbage plants that contain high amounts of glucosinolates, we eventually decided to evaluate the effects of glucosinolates by feeding the WCR adults with zucchini slices spiked with sinigrin (Figure S1; Data S6). A solution of sinigrin (CAS: 3952-98-5, Sigma Aldrich, CH) in water was applied to the slices to reach a sinigrin concentrations of 200 μg/g of zucchini, which is about the concentration of total glucosinolates found in leaves of the wild genotype of cabbage. Slices without sinigrin were fed to control beetles. Lastly, we used two species of bean plants, one that produces cyanogenic glucosides (*Phaseolus lunatus*) and one that does not produce cyanogenic glucosides (*Phaseolus vulgaris* L.). Seeds were purchased from Fordhook (Bush, US).[86,87]

All plants were grown under greenhouse conditions (24 ± 2°C, 60% relative humidity, 16:8 h L/D). Fertilizer was added every week after plant emergence (Capito, CH). Maize plants were used two weeks after germination, and cucumber, cabbage and bean plants were used 3-4 weeks after germination, all of them being in a vegetative stage.

Insects

We established our *in-house* colony with eggs of the western corn rootworm (WCR, *Diabrotica virgifera virgifera* (LeConte)) that were kindly supplied by USDA-ARS-NCARL (Brookings, US). Neonate larvae were maintained in soil, fed on four-day-old maize seedlings (Hybrid DFI 45321, DEFI genetics AG, CH), and kept at 25 ± 2°C, 60% relative humidity, and 16:8 h L/D cycles in quarantine facilities. Emerging adults were collected regularly, transferred to mesh cages, and were fed with water and one of the above-mentioned plant species. A Petri dish bottom filled with autoclaved soil was provided as oviposition substrate. Eggs were collected every four days and used in the experiments within three days. The predatory rove beetle *Dalotia coriaria* Kraatz (Coleoptera: Staphylinidae) and the minute pirate bug *Orius laevigatus* (Hemiptera: Anthocoridae) were purchased from Andermatt Biocontrol AG, CH. The adults of both predators were used for experiments within five days after arrival. They were fed with eggs of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae), also purchased from Andermatt Biocontrol AG. Insects were kept under controlled conditions (22 ± 2°C, 60% relative humidity, 16:8 h L/D cycles).

METHOD DETAILS

Single diet experiments

To evaluate whether WCR adults sequester and transfer plant defensive metabolites into their eggs, we fed adults with leaves of one of the plant species mentioned above, i.e. maize, cucumber, cabbage, or bean. With each food plant, two varieties with contrasting

capacities to produce secondary metabolites were used for the feeding experiments. Namely, benzoxazinoid-producing maize (BXD+) and benzoxazinoid-deficient maize (BXD-), cucurbitacin-producing cucumber (CUC+) and cucurbitacin-deficient cucumber (CUC-), bean producing cyanogenic glucosides (CnG+), bean deficient in cyanogenic glucosides (CnG-), glucosinolate-producing cabbage (GSL+), glucosinolate-deficient cabbage (GSL-). Newly emerged adults were fed on the different plants for 10–15 days, after which all adults were collected and their hemolymph sampled. Each sample consisted of 10 μl of hemolymph collected from 5 adults and a total of five independent samples were analyzed (*N*=5). A subset of female adults was left unharmed and allowed to oviposit for three days and their eggs were used to quantify the amounts of plant metabolites that were transferred. A total of 10-30 mg of eggs per sample and four independent samples were analyzed (*N*=4).

Mixed diet experiments

Short-term

To determine whether WCR adults sequester multiple plant toxins simultaneously and the persistence of these metabolites in the insect hemolymph under a mixed diet (Figure 2A), we quantified plant metabolites from insects that were fed with the different plant species sequentially as follows. First, we established three independent insect cages that initially contained either maize plants (BXD+), cucumber plants (CUC+), or glucosinolates-coated zucchini slices (GSL+). We then released 120 five-day-old adults (50 males and 70 females) into each cage. The adults were allowed to feed for three days, after which a second plant species was provided and the first plant species was removed. Again, the adults were allowed to feed for three days, after which a third plant species was provided in each cage, the second plant species was removed, and the insects were allowed to feed for three additional days. In all cases, plants were replaced daily and the degree of consumption was recorded (Figure S6). During the last 3 days of the experiment (days 7 to 9), Petri dish plates filled with autoclaved soil were offered as oviposition substrate. At day 10, eggs and adult hemolymph samples were collected for metabolite analyses. Three egg samples, each containing 15mg of eggs, four hemolymph samples obtained from males, and four hemolymph samples obtained from females were analyzed to determine the presence and quantities of plant-derived defense metabolites.

Long-Term

To determine whether WCR adults sequester different plant toxins simultaneously, we quantified plant metabolites from insects that were fed with each of the different plant species either alone or in combination (Figures 3A and S5). To this end, we established five independent insect cages: type 1 where insects encountered all the three secondary metabolites (BXDs+, CUCs+ and GSLs+), type 2 where insects encountered none of the secondary metabolites (BXDs-, CUCs- and GSLs-), type 3 where insects encountered only benzoxazinoids (BXDs+, CUCs- and GSLs-), type 4 where insects encountered only cucurbitacins (BXDs-, CUCs+ and GSLs-) and type 5 where insects encountered only glucosinolates-coated zucchini slices (BXDs-, CUCs- and GSLs+) (see Figure S5). We then released 170 seven-day-old adults (70 males and 100 females) into each cage. The adults were allowed to feed for 28 days. In all cases, plants were replaced daily and insect damage was recorded (Figure S7). During the last 5 days of the experiment, Petri plates filled with autoclaved soil were offered as oviposition substrate. Then, adult hemolymph samples and eggs were collected. Adult hemolymph was collected from one single adult (single adult) or from 4-5 adults and then pooled together (multiple adults). By collecting from individual adults, we could further test the ability of WCR to sequester each plant toxin family separately and evaluate their intrinsic variability. Similarly, eggs were collected from a single female after dissection (single female eggs, Figure S5) or from the Petri dishes (multiple female eggs).

Extraction of defense compounds from plant tissues

To quantify the secondary metabolites in the leaves, undamaged and WCR-damaged leaves from maize (*N*= 5), bean (*N*= 5), cabbage (*N*= 5), cucumber (*N*= 10) plants, and zucchini slices were used (*N*= *5*). The damaged plants were harvested after exposing them for 24 hours to ten WCR adults. Plant tissues were harvested and immediately flash frozen and ground to a fine powder in liquid nitrogen. Then, one ml of extraction buffer was added to fifty mg of ground plant material. The following buffers were used: MeOH:H_2O:formic acid (50:49.5:0.5) for benzoxazinoids, 100% MeOH for cucurbitacins, MeOH:H_2O:formic acid (70:29.5:0.5) for glucosinolates, and MeOH:H_2O (70:30) for cyanogenic glucosides.[36,53,85,87] Then, all samples, except for those from bean plant, were vortexed and centrifuged at 4°C (10 min, 9000 rpm). Bean plant samples were first heated at 90 °C for 10 min, sonicated for 5 min on ice and then centrifuged. Supernatants were collected in clean reaction tubes and analyzed for secondary metabolites by liquid chromatography-mass spectrometry as described below.

Hemolymph collection and extraction of metabolites

To collect insect hemolymph, WCR adults were placed on ice-cooled Petri dish plates for 10 min prior the hemolymph collection. Then, the second leg pair was excised using surgical scissors. Two μl of hemolymph per adult were collected from the wound. Hemolymph from 4-5 adults was pooled together and mixed with 10 μl of an anticoagulant buffer (98 mM of NaOH, 186 mM of NaCl, 17 mM of Na2EDTA, 41 mM of citric acid at pH 4.5). To extract metabolites from the hemolymph, 250 μl of extraction buffer per sample were added. The following buffers were used: MeOH:H_2O:formic acid (50:49.5:0.5) for benzoxazinoids, 100% MeOH for cucurbitacins, MeOH:H_2O:formic acid (70:29.5:0.5) for glucosinolates, and MeOH:H_2O (70:30) for cyanogenic glucosides. Samples were then centrifuged (4°C, 10 min, 9000 rpm), and supernatants were collected and filtered (13 mm Syringe filter, PTFE hydrophilic, 0.22 μm, BGB, CHE) prior to metabolite quantification.

Metabolite extraction from eggs

To extract metabolites from the surface of WCR eggs, we immersed 10-30mg of eggs in 250 μl of extraction buffer for 2 min. This procedure does not damage eggshells (confirmed by evaluating egg hatching rates). The same buffers as for the hemolymph and plant tissue extractions were added. Supernatants were collected and stored at -80 °C until analysis. To extract the metabolites present inside of WCR eggs, we added 250 μl of extraction buffer to the remaining eggs (from the step before) and ground them using a plastic pestle. The resulting egg suspension was then centrifuged at 4 °C for 10 min and 9000 rpm. Supernatants were collected and used for further analysis. To extract metabolites from entire eggs, we added 250 μl of extraction buffer to the eggs and ground them using a plastic pestle. The resulting egg suspension was then centrifuged at 4 °C for 10 min and 9000 rpm. Supernatants were collected and used for further analysis. All the supernatants were filtered (13 mm Syringe filter, PTFE hydrophilic, 0.22 μm, BGB, CH) before analyzing them by liquid chromatography as detailed below.

Predation bioassays

To evaluate the protective effects of the different sequestered plant metabolites, we evaluated predation preferences of adult *D. coriaria* and *O. laevigatus* insects. To this end, two clusters of three eggs each, one that contained plant metabolites and one that did not, were presented to either *D. coriaria* or to *O. laevigatus* adults. The number of predated eggs were recorded after 24h (*D. coriaria*) or for 48h (*O. laevigatus*). The predated eggs after damaged by each predator were either with part of the eggs shell removed (*D. coriaria*) or visibly shriveled (*O. laevigatus*). Eggs from females that had fed on single diets that contained or lack the various secondary metabolites were used (for details see section above "Single diet experiments") and to obtained eggs that either contained or not all the secondary metabolites were used from adults fed on a mixed diet (for details see section above "Mixed diet experiments -> Long-Term"). Egg clusters were placed onto a moistened filter paper inside a red Petri dish (Ø 92mm, Sarstedt Ag, DE). The two egg clusters were placed seven cm from each other. One predator was released per dish and thirty independent dishes were evaluated (*N*=30). The dishes were sealed with parafilm and incubated in darkness. We chose to work with red Petri dishes to decrease light exposure during evaluation. To further evaluate possible additive and/or synergistic protective effects of the plant toxins present in the eggs against the predators we used the Bliss Independence Model. It assumes that the effects of two or more toxins are independent of each other, and the combined effect can be predicted based on the probability of their individual effects. The model compares the predicted combined effect to the actual observed effect and if the observed effect is greater than the predicted effect, the combination is considered synergistic.

Predator survival

To determine the toxicity of the different plant metabolites for egg predators, we evaluated survival of *D. coriaria* and *O. laevigatus* adults that were fed *ad libitum* on eggs laid by WCR females fed on single diets of the different plant species. To this end, WCR eggs (10 for *O. laevigatus* or 20 for *D. coriaria*) were placed onto a humid filter paper inside a red Petri dish. One adult predator was released per dish. Experimental units were inspected daily to determine the number of alive predators and to replace the predated eggs. The predators that did not consume any egg were not included in the analyses. In the case of *D. coriaria*, a total of 20 independent dishes were evaluated, and in the case of *O. laevigatus*, 25 independent dishes.

QUANTIFICATION AND STATISTICAL ANALYSIS

Metabolites quantification

The quantification of benzoxazinoids,[88] cucurbitacins,[53] glucosinolates[89] and cyanogenic glucosides[87] was performed by using UHPLC-qTOF-MS instrument (Waters) made up of Acquity UPLC coupled to Synapt G2 high-resolution mass spectrometer. The column used to separate benzoxazinoids, cucurbitacins and cyanogenic glucosides in the chromatograph was an Acquity UPLC BEH C18 (50 x 2.1 mm, 1.7μm, Waters) and that for glucosinolates was an Acquity CSH C18 column (100 x 2.1 mm, 1.7 μm, Waters). The temperature was maintained at 25 °C. Two eluants were used, H_2O + formic acid 0.05% and acetonitrile + formic acid 0.05% as phases A and B, respectively. The injection volume was 2.5 μl. The mass spectrometer was operated in electrospray negative ionization for the acquisition and data processing we used the softwares MassLynx 4.1 and TargetLynx, respectively (Waters). Putative identifications of cucurbitacins were based on their retention times, exact masses and relative mass defects (allowing for molecular formula determination) and comparisons with those of the standard Cucurbitacin B as well as with available databases such as the Dictionary of Natural Product (CRC Press). In some cases, the presence of several possible cucurbitacin isomers prevented full identification. Peaks corresponding to known cucurbitacins were automatically integrated using TargetLynx with a 0.1 min chromatographic window centered on the retention time of each component and a 0.02 Da mass window centered on the $(M+HCOO)^-$ ion. Quantification of all cucurbitacins was done by external calibration using cucurbitacin B as standard (Cucurbitacin B is a natural triterpene present in plants of the Cucurbitaceae family among other type of cucurbitacins). Glucosinolate quantification was done with sinigrin as standard. Quantification of linamarin and lotaustralin was based on calibration curves prepared from pure linamarin and lotaustralin standards (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and that of benzoxazinoids using DIMBOA-Glc, HMBOA-Glc, HDMBOA-Glc, DIMBOA purified from plant extracts as well as a certified MBOA standard (Sigma-Aldrich). All chemical structures of the quantified molecules are presented in Figure S9.

Statistical analysis

Statistical tests were carried out in R statistical software (v. 4.0.0; R Development Core Team, 2020) using ANOVA, followed by residual analysis to verify the suitability of distributions of the tested models. Generalized Linear Models (GLM) with a Gaussian distribution followed by Least Squares Means (*LSMeans*) were used to test for differences in secondary metabolites (BXD, CUC, CnG and GLU) in plants, hemolymph and eggs. Partial least squares discriminant analysis (PLS-DA) was carried out using MetaboAnalyst 5.0 to evaluate quantitative and qualitative differences in metabolite levels between plants, WCR adult hemolymph and eggs. Generalized Linear Mixed Models (GLMMs) with a binomial distribution followed by FDR-corrected post-hoc tests were used to evaluate the effect of the different metabolites on predator feeding preferences. The effects of the different metabolites on predator mortality were analysed using Kaplan-Meier estimator by log-rank method in the Sigma Plot software (v. 11; Systat Software Inc., San Jose, CA, USA).