PLANT SCIENCE

Plant iron acquisition strategy exploited by an insect herbivore

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Insect herbivores depend on their host plants to acquire macro- and micronutrients. Here we asked how a specialist herbivore and damaging maize pest, the western corn rootworm, finds and accesses plant-derived micronutrients. We show that the root-feeding larvae use complexes between iron and benzoxazinoid secondary metabolites to identify maize as a host, to forage within the maize root system, and to increase their growth. Maize plants use these same benzoxazinoids for protection against generalist herbivores and, as shown here, for iron uptake. We identify an iron transporter that allows the corn rootworm to benefit from complexes between iron and benzoxazinoids. Thus, foraging for an essential plant-derived complex between a micronutrient and a secondary metabolite shapes the interaction between maize and a specialist herbivore.

Iron (Fe) can be a limiting micronutrient for plants and herbivores (*1*). Plants increase Fe availability by secreting reducing agents and Fe chelators, so-called phytosiderophores, into the rhizosphere (*2*). Benzoxazinoid secondary metabolites, which are produced and exuded by grasses such as maize, may act as phytosiderophores in addition to protecting plants against herbivores (*3*, *4*). The western corn rootworm (WCR), a worldwide maize pest, can tolerate and sequester benzoxazinoids (*5*). In addition, root-feeding WCR larvae prefer benzoxazinoid-producing maize plants and require benzoxazinoids to identify crown roots as the preferred feeding site (*6*). WCR crown root damage reduces plant growth (*6*).

Here, we investigate the potential role of benzoxazinoids as phytosiderophores and WCR foraging cues. To identify the chemical motif used by WCR, we evaluated WCR behavior on different maize benzoxazinoid mutants (Fig. 1 and fig. S1). WCR larvae preferred wild-type (WT) rather than benzoxazinoid-deficient *bx1* and *bx2* mutant plants. The larvae also preferred WT to *bx6* mutants, which overaccumulate DIBOA-Glc at the expense of 7-*O*–methylated and 8-*O*–methylated benzoxazinoids, suggesting that DIBOA-Glc is not a preferred benzoxazinoid. WCR larvae preferred *bx13* mutant plants deficient in 8-*O*–methylated benzoxazinoids, suggesting that 8-*O*–methylated benzoxazinoids are not preferred. To test the influence of *N*-*O*–methylation, we overexpressed the DIMBOA-Glc *O*-methyltransferase *ZmBx12* (*Bx12oe*) (fig. S1), resulting in plants with excess HDMBOA-Glc and reduced DIMBOA and DIMBOA-Glc (Fig. 1). WCR preferred WT over *ZmBx12*-overexpressing plants, suggesting that *N*-*O*–methylated benzoxazinoids are not preferred (Fig. 1). Thus, 7-*O*–methylated, *N*-hydroxylated benzoxazinoids such as DIMBOA and DIMBOA-Glc are associated with WCR feeding preference.

We next investigated the role of benzoxazinoids for within-plant feeding preferences. WCR no longer distinguished between crown and primary roots of *bx1*, *bx2*, *bx6*, and *Bx12oe* plants, while preference was intact in *bx13* plants (figs. S2 and S3). The correlation between plant preference and tissue preference suggests that the same benzoxazinoids may mediate plant preference and within-plant foraging. Closer inspection of crown and primary root benzoxazinoid profiles revealed a positive correlation between DIMBOA accumulation in crown roots relative to primary roots and WCR preference for crown roots relative to primary roots across the different maize mutants (R^2 = 0.56; P = 0.03, fig. S2).

Contrary to what was expected from the experiments with the benzoxazinoid mutant plants, purified DIMBOA and DIMBOA-Glc did not elicit WCR feeding preference (Fig. 2). The DIMBOA breakdown product MBOA, a potential volatile WCR attractant (*7*), was equally inactive (fig. S4).

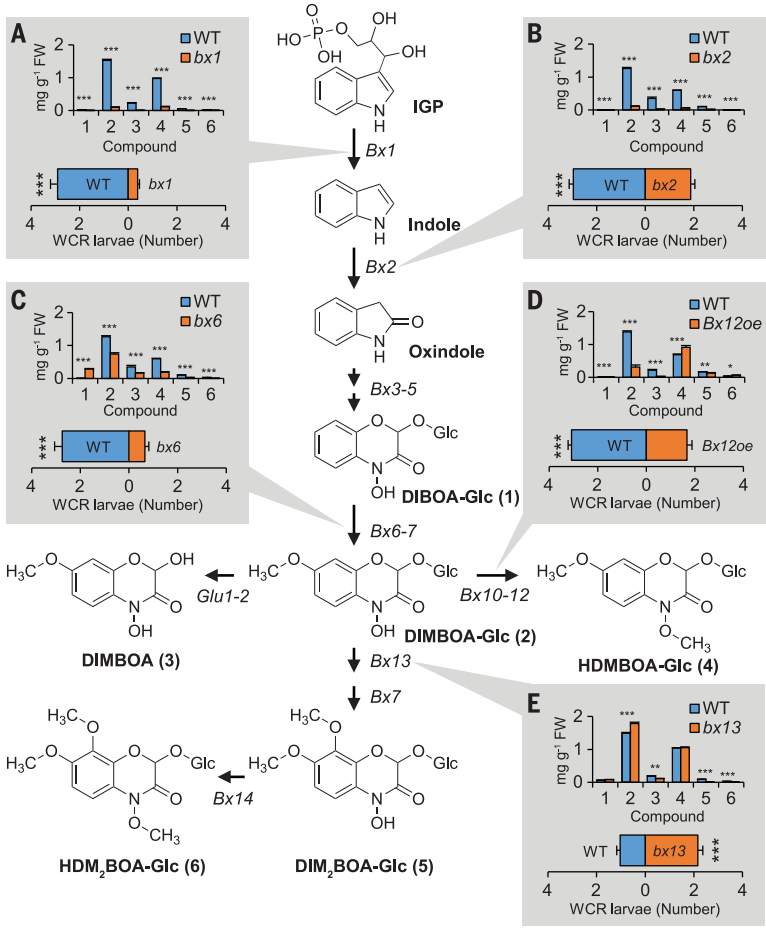

Fig. 1. Bioactivity-guided genetic pathway fractionation reveals that feeding preferences of WCR larvae are associated with 7-*O*–methylated, *N*-hydroxylated benzoxazinoid secondary metabolites in maize roots. (**A** to **E**) The benzoxazinoid biosynthesis pathway, including its major products (1 to 6), is shown. Gray boxes denote mutants and transgenic plants with altered activity of the corresponding enzymes and their respective WT lines. Benzoxazinoid levels (+SE, n = 6 to 10 biological replicates) of mutant and WT plants and WCR feeding preferences are shown (+SE, n = 12 to 20 choice situations with five larvae each). Full time courses are shown in fig. S1. FW, fresh weight. Asterisks indicate significant differences (*P < 0.05; **P < 0.01; ***P < 0.001).

Because *N*-hydroxylated benzoxazinoids such as DIMBOA and DIMBOA-Glc, but not *N*-*O*–methylated benzoxazinoids, can form stable complexes with Fe (*3*), we hypothesized that these complexes may serve as WCR foraging cues. Within the pH range of the maize rhizosphere (4.5 to 7.5) and given that DIMBOA is the main benzoxazinoid around maize roots (*8*), Fe complexes with two [referred to as Fe(III)(DIMBOA)$_2$] and three DIMBOA ligands [referred to as Fe(III)(DIMBOA)$_3$] are likely to accumulate at the root surface. Spectrophotometric measurements of synthetically prepared complexes (fig. S5A) confirmed the pH-dependent formation of Fe(III)(DIMBOA)$_2$ and Fe(III)(DIMBOA)$_3$ (collectively referred to as Fe-DIMBOA, fig. S5B). DIMBOA reacted preferentially with Fe, followed by aluminum (Al) and molybdenum (Mo) (fig. S6). Formation of Fe-DIMBOA was observed on WT crown roots, but not on *bx1* roots (fig. S7). At estimated physiological doses of 1.26×10^{-10} mol cm^{-2} (fig. S8), Fe(III)(DIMBOA)$_2$ and Fe(III)(DIMBOA)$_3$, but not Al-DIMBOA, Fe-DIMBOA-Glc, and Fe-EDTA, elicited WCR feeding preference (Fig. 2D and fig. S9). Fe(III)(DIMBOA)$_3$ increased WCR feeding across a range of physiologically relevant concentrations from 1.26×10^{-14} to 1.26×10^{-10} mol cm^{-2} (fig. S10). Preference for Fe(III)(DIMBOA)$_3$ was independent of larval stage, previous benzoxazinoid exposure, and the presence of maize roots (Fig. 2E and fig. S11). Fe(III)(DIMBOA)$_3$ rescued recruitment of WCR larvae to *bx1* mutants in soil (Fig. 2F) and to individual *bx1* roots (Fig. 2G). Primary roots of WT plants became more attractive upon Fe(III)(DIMBOA)$_3$ application and were as attractive as crown roots 6 hours after application (Fig. 2H and fig. S12). Host plant acceptance on *bx1* mutant roots was fully complemented by Fe(III)(DIMBOA)$_3$ (fig. S13). Fe(III)(DIMBOA)$_3$ application also increased WCR feeding on rice and barley, two non–host plant species (fig. S13). Thus, in addition to primary metabolites (*9*, *10*), Fe-DIMBOA mediates host recognition, acceptance, and within-plant foraging of WCR.

To investigate the impact of Fe-DIMBOA on WCR performance, we grew WT and *bx1* mutant plants in nutrient solutions. In the presence of free Fe that can form complexes with DIMBOA, WCR larvae grew better on WT than *bx1* plants (Fig. 3A). No difference was observed in the presence of Fe-EDTA or in the absence of Fe. Exogenous application of DIMBOA rescued larval growth on *bx1* mutants (Fig. 3B). Fe concentrations in WCR mirrored larval performance (Fig. 3C). Thus, the interaction between free Fe and DIMBOA increases WCR Fe supply and performance.

Fe supply is critical for plant performance. Leaf chlorosis, a typical sign of iron deficiency, was observed in *bx1*, *bx2*, and *bx6* mutant seedlings growing in nutrient solution and potting soil supplemented with Fe salts (Fig. 3E and fig. S14). No chlorosis was observed in *bx* mutants supplied with Fe-EDTA (fig. S14). DIMBOA supplementation rescued *bx1* mutants supplied with free Fe (fig. S14). Fe concentrations in the maize xylem mirrored chlorophyll patterns (Fig. 3, F and G). No differences in plant biomass were observed between genotypes (fig. S14F). Thus, the interaction between free Fe and DIMBOA increases maize Fe supply. As maize and WCR Fe contents are correlated across treatments and genotypes, the positive effect of Fe-DIMBOA on WCR may be due to direct or plant-mediated effects.

To better understand the connection between WCR performance and Fe availability, we manipulated the capacity of WCR to acquire Fe. We identified a WCR homolog of the human divalent metal transporter-1 (DMT1) (*11*), here named *DvIRT1* (fig. S15). In *Drosophila melanogaster*, the DMT1 homolog *Mlv* is required for Fe homeostasis and feeding decisions (*12*). *DvIRT1* rescued the growth of an Fe-transport–deficient yeast strain in the presence of free or complexed Fe, including Fe-DIMBOA (Fig. 4A). Silencing of *DvIRT1* in WCR (Fig. 4B) resulted in WCR Fe deficiency (Fig. 4C). *DvIRT1* silencing did not change WCR feeding preferences (Fig. 4D). However, *DvIRT1* was required for the benzoxazinoid-dependent increase in WCR Fe supply and performance (Fig. 4E). Thus, *DvIRT1* enables WCR to acquire Fe in various forms, including Fe-DIMBOA.

WCR may derive multiple benefits from Fe-DIMBOA. First, as Fe-DIMBOA is only produced by a few other plant species (*13*), it is a reliable host-recognition cue. Second, Fe-DIMBOA levels are highest for crown roots, which are a better food source for WCR than primary roots (*5*). Third, Fe-DIMBOA is accepted as a substrate by DvIRT1 and directly improves Fe homeostasis and WCR performance. Fourth, WCR larvae sequester the DIMBOA breakdown product MBOA for self-defense against entomopathogenic nematodes (*6*). Fe-DIMBOA may therefore provide Fe as well as DIMBOA as an immune precursor.

Grasses can use L-methionine–derived mugineic acids to chelate Fe (*2*). Here, we show that benzoxazinoids also contribute to the Fe supply of young maize plants. In addition, benzoxazinoids

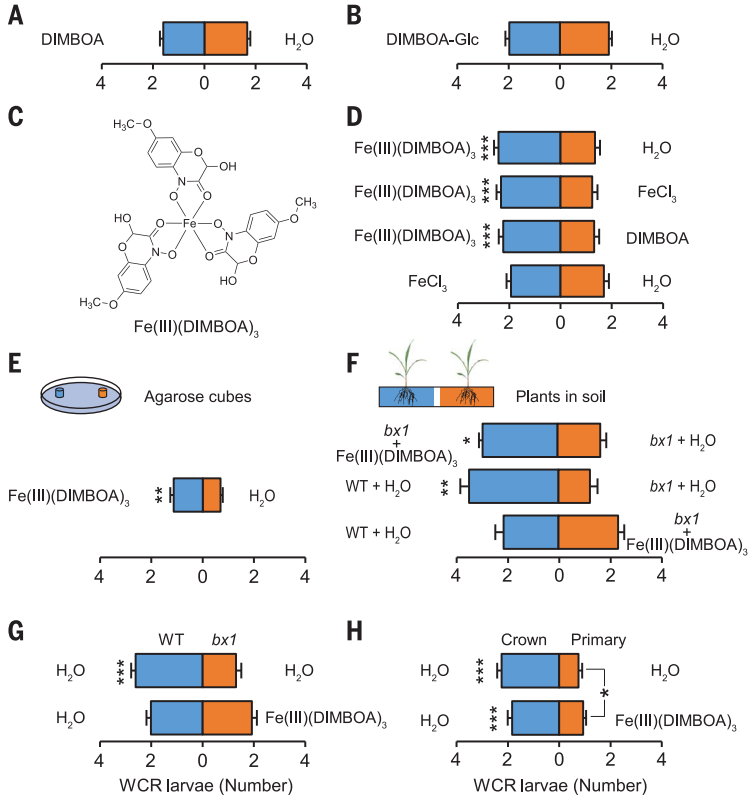

Fig. 2. Complexes between iron (Fe) and the benzoxazinoid DIMBOA mediate host recognition, acceptance, and within-plant foraging of WCR larvae. (**A** and **B**) Influence of pure DIMBOA and DIMBOA-Glc on WCR feeding preference on benzoxazinoid-deficient mutant roots. (**C**) Chemical structure of the Fe complex Fe(III)(DIMBOA)$_3$. (**D** and **E**) Effect of synthetic Fe(III)(DIMBOA)$_3$ on WCR feeding preference for roots (D) and agarose cubes (E). (**F**) WCR feeding preference for soil-grown WT and *bx1* mutant plants supplied with Fe(III)(DIMBOA)$_3$. (**G** and **H**) Influence of Fe(III)(DIMBOA)$_3$ complementation on within- and between-plant WCR feeding preferences. [+SE, n = 20, except (F), n = 10 to 11 choice situations with five larvae each]. Full time courses are shown in figs. S4, S9, and S12. Asterisks indicate significant differences (*P < 0.05; **P < 0.01; ***P < 0.001).

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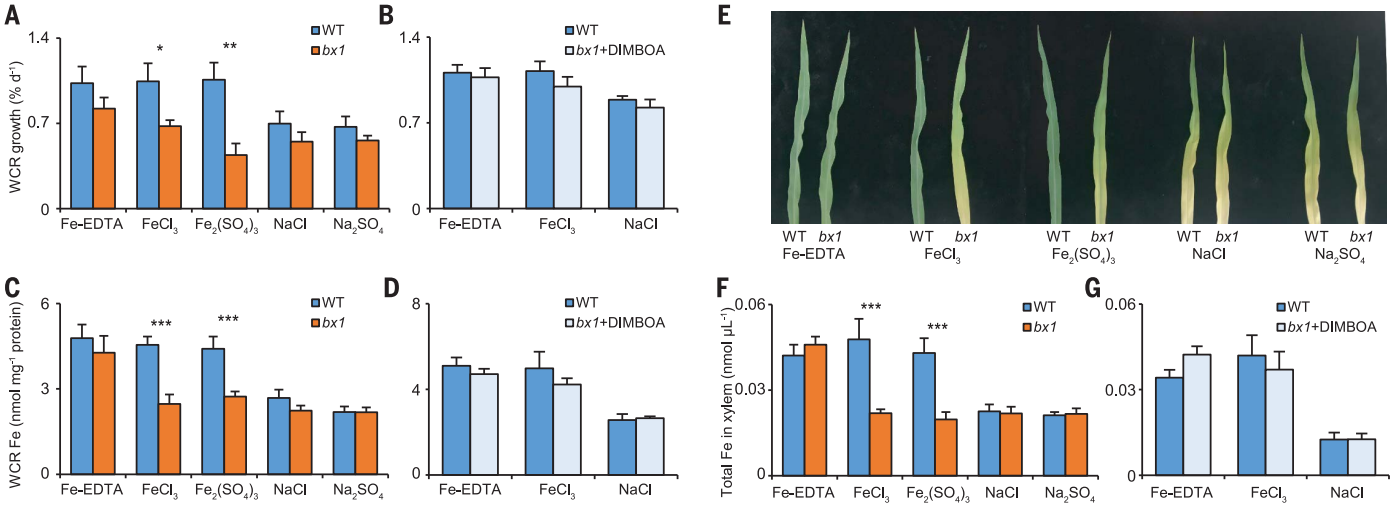

Fig. 3. Interactions between free Fe and the benzoxazinoid DIMBOA determine growth and Fe homeostasis of maize and WCR larvae. (**A** and **B**) Growth of WCR larvae feeding on WT or *bx1* roots supplied with different sources of Fe (+SE, n = 9 to 15 biological replicates) and pure DIMBOA (+SE, n = 15 to 20 biological replicates). (**C** and **D**) Corresponding Fe contents of WCR (+SE, n = 5 biological replicates, with 4 to 5 larvae pooled per replicate). (**E**) Representative photographs of leaves of WT and *bx1* maize plants grown in nutrient solutions with different sources of Fe. (**F** and **G**) Average Fe content in the maize xylem sap (+SE, n = 5 biological replicates, with sap of four plants pooled per replicate). Asterisks indicate significant differences (*P < 0.05; **P < 0.01; ***P < 0.001).

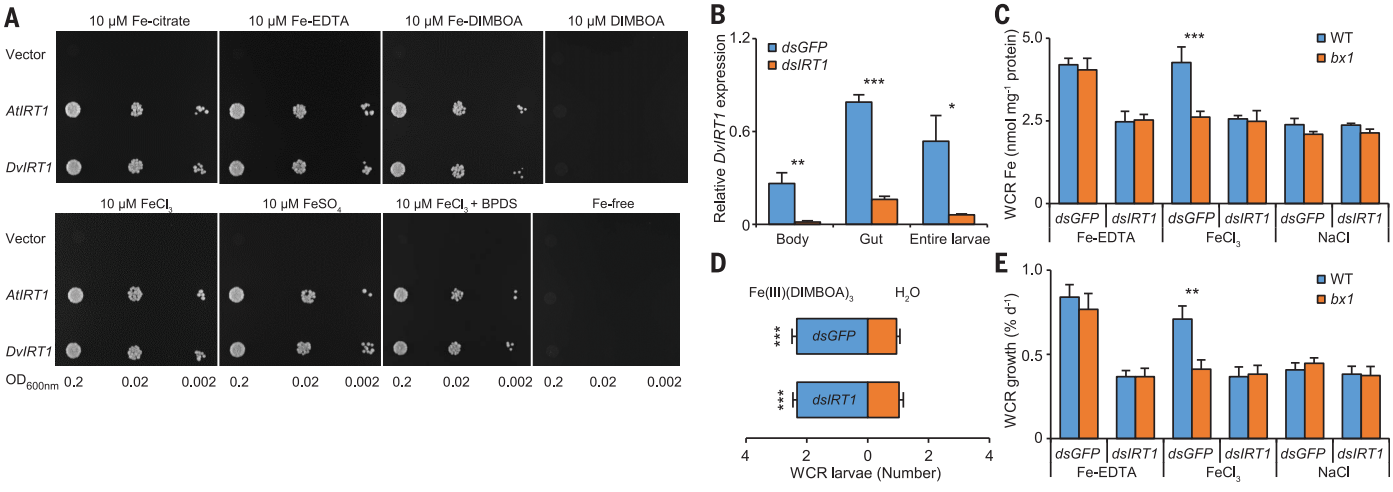

Fig. 4. The Fe transporter DvIRT1 is required for Fe-DIMBOA–dependent WCR performance. (**A**) Functional complementation of a yeast Fe-uptake mutant. Plasmids expressing *AtIRT1* (positive control, *DvIRT1*) or the empty vector pFL61 (negative control) were individually introduced into the Fe uptake–defective yeast DEY1453 strain, and the growth media were supplemented with different Fe sources. (**B**) Average expression levels of *DvIRT1* in WCR larvae after feeding with double-stranded RNA of *GFP* (*dsGFP*, negative control) or *DvIRT1* (*dsIRT1*, +SE, n = 3 biological replicates). (**D**) Feeding preference of *dsGFP*- and *dsIRT1*-fed WCR larvae (+SE, n = 19 to 20 choice situations with five larvae each). (**C** and **E**) Fe content (+SE, n = 4 biological replicates, with 3 to 5 larvae pooled per replicate) and growth (+SE, n = 20 biological replicates) of *dsGFP*- or *dsIRT1*-exposed larvae feeding on WT or *bx1* roots with different Fe source treatments. Full time courses are shown in fig. S16. Asterisks indicate significant differences (*P < 0.05; **P < 0.01; ***P < 0.001).

also act as resistance factors against different pests and diseases and shape the root microbiome (*8*, *14*) but, as shown here, also increase the performance of a specialist herbivore. The diverse costs and benefits of the benzoxazinoid pathway for maize represent an optimization problem for plant breeding that may have contributed to the persistence of WCR as a damaging maize pest.

Essential trace metals such as Fe influence herbivore performance and herbivore community composition (*15*–*17*). As trace metals are often present as complexes in plants (*2*, *18*), the ability of herbivores to detect and respond to these complexes may shape plant-herbivore interactions in agricultural and natural ecosystems.

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ACKNOWLEDGMENTS

We thank W. French and C. Nielson (USDA Brookings) for supplying WCR eggs, D. Eide and J. Taggart (University of Wisconsin–Madison) for sharing yeast strains and plasmids, and N. Ségaud and M. Albrecht (University of Bern) for help with Fe-DIMBOA characterization. **Funding:** The work of M.E. was supported by the Swiss National Science Foundation (grants 155781, 160786, 157884) and the University of Bern. **Author contributions:** Study design: M.E., C.A.M.R. Experimental design and supervision: L.H., M.E., C.A.M.R., T.G.K., J.G. Data collection and analysis: L.H., C.A.M.R., P.M., M.Y., X.Z., J.D.B., V.H., D.R., V.G., T.G.K., M.E. Initial draft: L.H., M.E., C.A.M.R. Final version: All authors. **Competing interests:** The authors declare that they have no competing interests. **Data and materials availability:** All data needed to evaluate the conclusions in the paper are present in the main text or the supplementary materials. The sequence of *DvIRT1* has been deposited at NCBI (accession number MH715476). Additional data related to this paper may be requested from the authors.

SUPPLEMENTARY MATERIALS

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Materials and Methods
Figs. S1 to S19
Table S1
Data File S1
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23 February 2018; accepted 19 June 2018
10.1126/science.aat4082

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Science **361** (6403), 694-697.
DOI: 10.1126/science.aat4082

Pest subverts host plant's foraging

Plants need iron as a micronutrient, and they extract it from the rhizosphere by secreting chelating agents. Insect pests, such as the western corn rootworm, which annually cause millions of dollars' worth of lost yield, need iron, too. Hu *et al.* show that the rootworm exploits the plant's own iron-foraging system to detect its host and to seize iron for itself (see the Perspective by Kliebenstein). Plants produce benzoxazinoid compounds not only as a defense against many insects but also as iron chelators. Rootworm larvae are not harmed by benzoxazinoids; instead, they take advantage of their presence as a signal that food is near and of their properties as an iron chelator.

Science, this issue p. 694; see also p. 642

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