Rootworm management: status of GM traits, insecticides and potential new tools

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Abstract

Western corn rootworm (*Diabrotica virgifera virgifera* LeConte) and northern corn rootworm (*Diabrotica barberi* (Smith and Lawrence)) are major pests of maize in the USA. These pests have been managed with a variety of tactics over the last century. Both *Diabrotica* spp. have adapted to crop rotation in different ways in certain regions of the USA as well as to some of the insecticides targeted at them. *D. v. virgifera* has adapted to more of the chemical control measures and transgenic control methods. Discussed in this review are the challenges associated with managing both species, and how current management strategies might be combined and implemented to help manage damage from these species. Also, we discuss the potential for new technologies, such as RNA interference, to be used in the future.

Review Methodology: Literature was selected based on its relevance to adaptations with western and northern corn rootworms (*Diabrotica* spp.). Recent reviews on ecology, behavior, population dynamics, genetics, and Bt maize resistance have been already written. We have directed our focus towards updating readers on new findings in areas as they relate to Bt maize and *Diabrotica* spp., but have done this from a historical perspective including the history of these pests, in general. We hypothesize why refuges did not delay resistance to Bt maize targeting rootworm. Lastly, we review potential new techniques which may be used to help manage *Diabrotica* spp. or combat Bt resistance.

Background/Introduction

Since 1909, western corn rootworm (*Diabrotica virgifera virgifera* LeConte) has been known to attack the roots of maize, *Zea mays* L. [1–5]. Over the last century, the range of *D. v. virgifera* as a pest of maize has expanded beyond Fort Collins, Colorado to cover 39 states in the USA, Mexico, two provinces of Canada, three countries in Central America and 29 countries in Europe [6, 7]. There is some evidence that the pest followed the migration of maize northward from Mexico thousands of years ago [2].

Economic losses caused by *D. v. virgifera* and *Diabrotica barberi* have been estimated between one and two billion dollars [8, 9]. These estimates include yield losses through direct and indirect routes, and include management costs. *D. v. virgifera* eggs have a developmental threshold of 11.1°C and hatch in the soil after accumulating the needed number of degree days [10, 11], which depends somewhat on the location and temperature fluctuations. Upon hatch, neonate larvae find respiring plant roots by orienting to carbon dioxide [12–14]. Using behavioural bioassays, it is possible to distinguish hosts from nonhosts [15]. Host recognition cues involved in this assay include monogalactosyldiacylglycerols (MGDGs) and other cues, which were recently isolated identified from corn root extracts [16] based on the same behavioural bioassays. Long-chain free fatty acids and short-chain sugars were similarly isolated and identified as *D. v. virgifera* larval feeding stimulants [17]. Maize is its primary host, but larvae can develop at least partially on many grass species and can develop to the adult stage on a number of these plants [18, 19]. Larvae continue to develop and consume root tissue until the insect reaches the pupal stage, at which time it creates an earthen cell. Adults will begin to emerge approximately 10 days later, with males

emerging first due to protandry [20]. Overall timing of adult emergence (June–August) will vary depending on accumulated degree days [10, 11].

Larval feeding can have an array of impacts on the maize plant's development. Root regrowth is often triggered, depending on maize genotype, by root damage [21]. If root damage is minor, then the plant may be better off than had its roots not been damaged at all, especially under dry conditions [22, 23]. At higher densities, the root feeding may cause severe and permanent damage to the root system. Such severe root damage can limit the ability of the plant to uptake moisture and nutrients from the soil, which then impacts yield [24–27]. Often, the ability of the plant to stand upright is impacted, so an additional yield loss component is due to unharvested grain [28]. Adult feeding typically does not have an impact on the plant's yield unless densities are very high prior to anthesis [29–30].

Rearing and handling techniques for *D. v. virgifera* on a large scale have been well established for decades [31]. Non-diapausing colonies of *D. v. virgifera* populations, allow some research to be expedited [32]. Unfortunately, research with *D. barberi* is lacking because adult handling techniques have not been able to produce sufficient numbers of eggs for large experiments. Additionally, a non-diapausing strain of *D. barberi* does not exist, hampering research in many aspects with this species. The non-diapausing trait has been documented in varying degrees [33, 34] and may make a non-diapausing strain a real possibility.

A Historical Perspective on *Diabrotica* Management, Research and Resistance Development

Crop rotation

D. v. virgifera has been a difficult pest to manage in the USA. Crop rotation, where a non-host is planted following a host, was initially recommended [5]. This tactic was the only control tactic for the first half of the twentieth century. Management with this tactic is still by far the most effective management tactic in most areas against both species. Unfortunately, scientists in Illinois discovered a strain of *D. v. Virgifera*, which had lost its fidelity to lay eggs only in the maize fields [35]. Instead, *D. v. virgifera* females began to lay eggs in both maize and soybean fields in this region. The strain began near Urbana, Illinois, and has since spread to larger portions of Illinois, Indiana, and to a lesser extent in surrounding states [36].

Larval gut tissue of *D. v. virgifera* has a diverse microbial community [37]. In *D. v. virgifera*, a shift in adult gut microbiota enterotype was associated with increased resistance to soybean defence compounds, and likely contributed to the development of resistance to crop rotation [38]. Comparison of gut microbiota between rotation resistant *D. v. virgifera* populations and wild-type *D. v. virgifera* populations revealed shifts in the microbial community composition upon adaptation to soybean tissue diet in adult *D. v. virgifera*. Note that *D. v. virgifera* larvae cannot survive on soybean tissues. Manipulation of the gut microbiota through the use of antibiotics reduced the resistance to soybean defence compounds to a level similar to that of wild-type *D. v. virgifera* [38].

Similar to *D. v. virgifera*, *D. barberi* has developed a mechanism to circumvent the effectiveness of crop rotation. 'Extended diapause' means some eggs hatch two or more winters after being laid in the soil [39]. Because larvae die if eggs hatch when corn is absent, extended diapause allows *D. barberi* to selectively adapt to local crop rotations, putting all corn at risk. Extended diapause had been a problem in parts of Minnesota, Wisconsin, South Dakota and Iowa prior to the population crashes of *D. barberi* when Bt corn targeting rootworm started to dominate the landscape. Areas formerly dominated by *D. barberi* (parts of Minnesota, Wisconsin, South Dakota and Iowa) had a drastic reversal in the dominant *Diabrotica* species, with *D. v. virgifera* becoming the predominant species. More recently, *D. barberi* populations have recovered in some of these areas as documented by the Wisconsin Department of Agriculture [40]. *D. barberi* populations spiked dramatically in 2015, nearly a decade after the populations crashed. Scattered extended diapause problems have just begun to resurface in rotated corn fields in Minnesota (Ken Ostlie, personal communication). In Missouri, where extended diapause has not been documented, populations of *D. barberi* were also found in large numbers in first year corn during the 2016 growing season [41]. These developments suggest changes are underway within *D. barberi* populations.

Chemical control

Near the middle of the twentieth century, soil applied insecticides became available for *Diabrotica* spp. management [8]. Cyclodienes were broadcast over the entire field, thereby exposing all larvae to the pesticide. This widespread exposure likely hastened the development of resistance by *D. v. Virgifera*, which was documented within just a few years [42]. Cyclodiene resistance has persisted decades beyond the ban of this pesticide class [43, 44]. Insecticides applied directly over rows of maize were referred to as banded insecticides. Two insecticide classes replaced the cyclodienes (carbamates and organophosphates) but were more expensive, so these pesticides were only applied over the row. No resistance has developed using this application method. Current theory suggests that roots outside the insecticidal zone provide a built-in 'refuge' to produce susceptible adults [45]. Refuges will be discussed below in reference to transgenic corn targeting *D. v. virgifera*.

A practice sometimes referred to as 'beetle bombing' uses foliar applications of the insecticide to prevent gravid females from laying eggs in maize fields, thus reducing the insect's impact on next year's crop [46]. Beetle bombing with organophosphates and carbamates resulted in *D. v. virgifera* adults developing resistance to insecticides within these two classes in the same Nebraska region where resistance to cyclodienes evolved [47]. This resistance also significantly impacted the larval susceptibility to organophosphates and carbamates [48].

More recently, a newer class of insecticide, pyrethroids, has been used to control *D. v. virgifera* adults. Interestingly, foliar applications of this insecticide are also used to control other pests, such as the two spotted spider mite (*Tetranychus urticae* Koch) or western bean cutworm (*Striacosta albicosta* (Smith)). While the application of the insecticide may be used to control other pests, *D. v. virgifera* adults are likely to be exposed and experience the selection pressure. Multiple applications within a season and non-target effects have likely contributed to the development of bifenthrin resistance for *D. v. virgifera* [45].

Biological control agents

Currently, there are several different options being explored in the biological control area, all of which are entomopathogenic organisms. Two genera of entomopathogenic fungi, *Metarhizium* and *Beauvaria*, have been investigated for their potential as a biological control agent of *D. v. virgifera* [49–54]. No commercial products are available as a result of the entomopathogenic fungus work. A great deal of research has gone into studying entomopathogenic nematodes and their interactions with *D. v. virgifera* [53–57]. *Heterorhabditis* and *Steinernema* spp. have been the two genera of focus in recent studies involving nematode–rootworm interactions. Nematodes are available for management of *D. v. virgifera* in parts of Europe [58]. Again, little work with these biological control agents has been done with *D. barberi*, likely due to a shortfall in available eggs for studies.

Bt Maize, Mortality and Implications for Resistance Management

Refuges, theory and concerns of resistance development

Transgenic maize hybrids expressing crystalline proteins with insecticidal activity derived from a soil-dwelling bacterium, *Bacillus thuringiensis* Berliner (Bt), have been available since 1996 [59]. Since organic growers used Bt to control pests, concern over the possibility of Bt resistance development were heightened more than for insecticides and contributed to the US Environmental Protection

Table 1 Effects of various hypothetical efficacies with a 20% block refuge on the total number of insects produced from refuge plants versus Bt plants

Hypothetical efficacy (%)	Ratio of insects from refuge versus transgenic[1]
90	2.5:1
99	25:1
99.9	250:1
99.99	2500:1
99.999	25 000:1

[1]Ratio calculated through use of this formula:

$$\frac{(1 \times \text{percent refuge size})}{(\text{corrected survival} \times \text{percent Bt size})}.$$

Agency's (EPA) mandate for insect resistance management (IRM) plans to be in place to slow the development of Bt resistance. Products were registered with a structured refuge in hopes of slowing resistance development [60]. The IRM plans for the first Bt crops in the USA implemented a 20% structured refuge. More recently, refuge requirements for seed blends of single events were set at 10 and 5% for pyramided products in the USA. The structured refuge strategy is optimal if toxin mortality is very high, initial frequency of alleles bestowing resistance is low, fitness costs of resistance are present, targeted insects mate randomly in the field and resistance to the Bt toxin is recessive [61, 62]. The mortality caused by a Bt product is perhaps the most important because this can lead to a landscape in which resistant alleles from the Bt crop are vastly overwhelmed by susceptible alleles from the refuge.

The mathematics on why the level of toxicity is so important in the effectiveness of refuge is clarified in Table 1. If a toxin kills 90% of susceptible larvae, survivorship from a perfect 20% block refuge compared with an 80% Bt field is only 2.5:1 ((0.2 proportion of field × 1.0 survivors)/(0.1 survivors × 0.8 proportion of the field)). The minimum definition of 'high dose' in the field is that 99.99% of susceptible larvae die following exposure to a transgenic plant [63]. The ratio of insects produced from a perfect 20% block refuge with a hypothetical efficacy of 99.99% would be 2500:1 ((0.2 proportion of field × 1.0 survivors)/(0.0001 survivors × 0.8 proportion of the field)). This ratio is considered a minimum: 'Think in terms of thousands to one or millions to one.' said Bruce Tabashnik during his talk at the Entomological Society of America in 2014 when referring to these ratios. The refuge program has been quite successful for some products, especially those which are truly high dose [64]. Even growers who do not plant Bt crops sometimes benefit from those who do plant Bt crops [65]. The structured refuge program has been less successful in instances in which the Bt crop is not high dose [64]. There are some that believe that the era of the Cry toxin is ending [66].

Table 2 Efficacies of Bt products with current events targeting *Diabrotica* spp. and the effect they have on the number of insects produced from refuge plants versus Bt plants

Protein in current or former commercial events (Scenario)[1]	*D. v. virgifera* efficacy (%)[2]	Calculated ratio[3]	*D. barberi* efficacy (%)[2]	Calculated ratio[3]
Cry3Bb1 (Field)[5]	98.49	16.56:1	86.39	1.84:1
Cry34/35Ab1 (Field)[7,8]	97.3	9.26:1	78.94	1.19:1
mCry3A (Field)[8]	94.88	4.88:1	86.68	1.88:1
eCry3.1Ab (Field)[9, 10, 11]	99.79	119.05:1	95.92	6.13:1
Cry3Bb1 + Cry34/35Ab1, pure (Field)[12]	99.14	6.12:1	97.71	2.30:1
mCry3A + eCry3.1Ab, pure (Field)	99.9[11]	58.48:1	96.26[13]	1.41:1

[1]Refuge size assumed to be 20% block refuge for single toxin products and 5% for pyramided toxin products sold as refuge-in-bag; Refuge-in-bag products have a smaller requirement than block refuges for single toxin products.
[2]Highest efficacy data used.
[3]Ratio calculated through use of this formula: $\dfrac{(1 \times \text{percent refuge size})}{(\text{corrected survival} \times \text{percent Bt size})}$.
[4]Data from [72].
[5]Data from [99].
[6]Data from [100].
[7]*D. v. virgifera* data from [101], *D. barberi* data from Table 7 of [102].
[8]Data from [103].
[9]Commercial hybrids express mCry3A and eCry3.1Ab proteins.
[10]This treatment is not commercially available but was used to evaluate the likelihood of resistance development.
[11]Data from Hibbard *et al.* (2011) [78].
[12]Data from Tables 4 and 8 of [102].
[13]Data from [104].

Transgenic maize targeting Diabrotica spp.

In 2003, the first hybrids expressing a Bt-derived insecticidal protein (Cry3Bb1, event MON863) active against *Diabrotica* spp. was registered for commercial use [67]. Over the next decade, four more proteins (Cry34Ab1/Cry35Ab1, mCry3A and eCry3.1Ab) and four additional events (DAS-59122-7, MIR604, MON88017 and 5307) were registered for commercial sale [68–70]. Event MON863 with an antibiotic marker was replaced by event MON88017, which also expressed Cry3Bb1, but came with resistance to glyphosate as a selectable marker. Expression of both Cry34Ab1 and Cry35Ab1 proteins are required for activity against *Diabrotica* spp. Some of the genes responsible for the expression of these Bt proteins have been stacked in maize hybrids resulting in pyramid Bt products. Pyramided products are designed to extend the life of both proteins by improving efficacy and adding multiple modes of action [71]. Maize products expressing the eCry3.1Ab protein are only sold in hybrids that also express the mCry3A simultaneously under the product name Agrisure Duracade®.

Concerns of resistance development led to laboratory selection experiments with *D. v. virgifera*. Within three generations of selection, colonies of *D. v. virgifera* developed nearly complete resistance to Cry3Bb1 [72]. Nearly complete resistance to maize expressing mCry3A or eCry3.1Ab singly was also selected for within a few generations [73, 74]. Each laboratory selection attempt for Cry3Bb1, mCry3A and eCry3.1Ab has been successful [72–75]. Maize expressing Cry34/35Ab1 has been much more difficult to develop resistance to and complete resistance has not yet been achieved after more than 20 generations of selection. For example, after 10 generations of selection, survival of *D. v. virgifera* on Cry34/35Ab1-expressing maize was only 20% relative to a near-isoline [76]. Deitloff *et al.* [77] evaluated refuge scenarios by selecting *D. v. virgifera* on Cry34/35Ab1 in a laboratory setting. A seed mix scenario failed to delay the development of resistance after 10 generations.

The highest published estimate for mortality was for the combination of eCry3.1Ab + mCry3A with an efficacy of 99.91% [78]. However, this efficacy with a 5% refuge only provides a ratio of 58 susceptible insects for each adult from the Bt portion of the maize field (Table 2). Cry3Bb1 (Event MON863) registration initially required a 20% block refuge. The ratio of insects from refuge to Bt was 16.56:1, and for mCry3A, it is only 4.88:1 (Table 2). Since these ratios are not remotely close 'thousands to one or millions to one' as for truly high dose products, it is not surprising that resistance developed quite quickly to these events in the field [79–81], especially since the only instance in which the refuge concept was tested with lower dose events, it did not delay resistance when deployed in the manner currently dominating the market [77]. As with other strategies, Bt maize has not remained as effective after its popularity as a management strategy has increased. Since the first report of field-evolved resistance to Cry3Bb1, other states have documented *D. v. virgifera* populations with Cry3Bb1 resistance [81–83]. Unfortunately, some level of resistance to Cry3Bb1 confers cross-resistance to both mCry3A and eCry3.1Ab [81]. One publication has documented incomplete resistance to Cry34/35Ab1 [84]. Single-gene products are just now beginning to be phased out of the market. Unfortunately, all current commercial transgenic products including pyramids targeting *Diabrotica* spp. also are not considered 'high-dose' so refuges are likely to do little, if anything as currently implemented [77].

While the Bt proteins expressed by transgenic maize hybrids do add protection against *Diabrotica* spp., limited studies have shown that the efficacy is less for *D. barberi* versus *D. v. virgifera* (Table 2). Given Bt's reduced efficacy against *D. barberi*, there were early concerns that populations of *D. barberi* might develop resistance to Bt before *D. v. virgifera*. This scenario did not manifest; instead, *D. barberi* populations crashed during the time frame when Bt corn targeting *Diabrotica* spp. became widely adopted. *D. v. virgifera* populations later blossomed from development of Bt resistance.

Why has Bt resistance not yet developed in *D. barberi*? It is tempting to infer reduced capacity for resistance, but a more likely explanation may lie in their resistance to crop rotation via extended diapause [39]. The extended diapause biotype predominates in the geographical region mentioned above. Thus, only one *D. barberi* generation every 2 years would delay resistance to Bt corn at least two fold. *D. v. virgifera* resistance to Bt took six years to develop (2003–2009). Given this length of time with *D. v. virgifera*, signs of *D. barberi* Bt resistance could be expected to occur soon, assuming similar mechanism of resistance and gene frequencies are present in both species.

Microbes and their implications for Bt resistance

Microbes have been documented to influence the susceptibility of lepidopteran insects targeted by Bt plants. Gut microbiota actually appear to be required for Bt susceptibility in lepidopteran pests [85–89]. Gut microbes also play a role in crop rotation resistance in *D. v. virgifera*, but the role of gut microbiota in Bt resistance and susceptibility is unknown [37]. Feeding of *D. v. virgifera* larvae on corn root tissue was shown to affect root rhizosphere microbiota composition, indicating complex, multitrophic interactions [90].

Changing guidelines for resistance management

Previously, registrants were required to conduct annual, random sampling programs to monitor susceptibility as a condition of registration [67–70]. In order to comply, registrants collected both random populations and targeted populations (fields with greater than expected damage) when possible (i.e. if notified before adults died, resources available for collection, etc.), and eggs produced were collected. These eggs were then overwintered, allowed to hatch and then tested in diet toxicity assays and possibly plant assays. When resistance developed to Cry3Bb1 and mCry3A, the US EPA convened a Scientific Advisory Panel (SAP) to discuss changes to resistance monitoring programs. After considering the recommendations of the SAP, the EPA altered the compliance requirements for registrants. Registrants are no longer required to conduct random sampling; instead, they are required to collect

adults from fields with performance issues, when possible, to test the offspring. Furthermore, registrants are now encouraged to conduct plant assays instead of diet toxicity assays [91].

Registrants and academic researchers can use a variety of assays to test *D. v. virgifera* populations for Bt resistance and product efficacy characterizations. Researchers tend to use just one assay to make characterizations [80, 82]. There are at least three different plant assays that could be used, each with the capability to estimate the survival rate and developmental parameters [81]. Developmental parameters are extremely valuable to characterize Bt resistance, but have not always been used. The first plant assay, a seedling assay, uses many maize seeds (Bt and non-Bt) and eggs or larvae in a relatively small, plastic container with a few dozen germinated seeds. The second and third assays, a single plant and greenhouse pot assay, are not much different. The single plant assay uses neonate larvae (<24 h old) on a V5 maize plant (Bt or non-Bt). Larvae are left to feed for 17 days before being extracted and data collected [79, 84]. Greenhouse pot assays may use larvae or eggs but still uses V5 maize plants like the single plant assay. While each assay is likely capable of detecting resistance, there may be one assay or one variable that is best able to discriminate between susceptible and resistant populations and this could also be toxin specific. Further research is needed to clarify optimal assays.

As discussed above, resistance monitoring programs by registrants were previously conducted using diet-toxicity assays. Data generated and submitted to the EPA for different proteins have not been comparable due to different proprietary artificial diet formulations used by each of the major companies. Since these diets are proprietary, academic researchers must obtain special permission to access to them. This issue may have influenced the EPA towards a shift away from diet toxicity assays in resistance monitoring programs. If a single, public and easy-to-use artificial diet can be generated for this purpose, then some of the problems will be addressed. Data from resistance monitoring programs could be compared for the different Bt proteins. Secondly, a public diet would allow academic researchers to conduct these assays with their own toxins or other toxins for which they are able to access.

Future concerns and efforts

Due to the very adaptive nature of *Diabrotica* spp., we propose areas of research that could be further improved upon or investigated for applications to rootworm management. Soil insecticides often do not significantly control *D. v. virgifera* population levels [92]. When feeding stimulants were added to thiamethoxam, the level of the toxin needed to kill 50% of the larvae was reduced by more than 100 000-fold [93]. This demonstrates that understanding the chemical ecology of this pest can improve

management strategies. Repellant properties of methyl anthranilate from corn root extract have been documented [94]. Perhaps by placing methyl anthranilate in furrow with non-Bt maize plants, larvae could be pushed to plants with insecticides or transgenic maize as part of a push-pull strategy analogous to other similar strategies utilized in other systems.

Earlier work on host location cues, specifically CO_2 [12–14], is now being utilized in experiments with an attract-and-kill strategy [95]. Through use of CO_2-emitting capsules, larvae were attracted to maize roots treated with the insecticide tethfluthrin. Similarly, use of a repellant may create a similar effect as the attract-and-kill strategy. As more research is conducted with *Diabrotica* spp. ecology and chemical ecology, additional management strategies may become available in the future.

Molecular biology, like chemical ecology, continues to give insight into finding additional management strategies. By understanding how cells work, different processes can be manipulated to control *D. v. virgifera*. Baum *et al.* [96] was the first to discuss the potential of RNA interference (RNAi) for the control of *D. v. virgifera*. Expression of specific double-stranded RNA fragments by plants elicits a defence mechanism where cells no longer transcribe targeted genes into proteins. Depending on how many redundancies are present in the insect genome, the insect may begin to deteriorate or even die if the protein plays a crucial role in the survival of the insect. Monsanto Company was the first to announce a maize product, which utilizes RNAi technology. This product, announced as SmartStax Pro, will express two Bt events (MON88017 and DAS-59122-7) targeting *Diabrotica* spp. and RNAi technology with one target gene (DvSnf7). SmartStax Pro is currently awaiting registration for commercial use by the US EPA. Since Monsanto's announcement, at least two other companies have announced plans to use RNAi in a product. DuPont Pioneer announced two *Diabrotica* spp. target genes that will be expressed simultaneously by maize plants. Syngenta has announced a product where dsRNA is applied as a soil treatment rather than through a transgenic plant.

Lastly, one recently published scientific breakthrough may help to overcome Bt resistance [97]. Phage-assisted continuous evolution takes advantage of naturally occurring processes to expedite Bt toxin evolution. These evolved Bt toxins have a high binding affinity for new receptors on the midgut tissue. This technique allowed the researchers to improve the efficacy of the Cry1Ac by 335-fold, even against Cry1Ac resistant insects. This tool might be effective for overcoming Bt resistance.

Concluding Remarks

Diabrotica spp. have a long history of adapting to management practices. Some practices have remained effective for several decades, while others begin to lose efficacy within just a few generation of selection. Until high dose transgenic maize hybrids targeting *Diabrotica* spp. are created, current refuge strategies are likely inadequate to significantly delay resistance development by these pests (Tables 1, 2). Multiple management tactics should be employed by growers, industry and regulatory agencies, when possible, to combat the adaptive nature of these pests [98]. While some new products are nearing the market, it is clear that *Diabrotica* spp. will continue to adapt. Continued research on all aspects of *Diabrotica* spp. is needed if maize growers are to have permanent success against *D. v. virgifera* and *D. barberi*. This needs to include adaptive IRM approaches and pro-active, integrated IRM-pest management strategies [98].

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