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Western Corn Rootworm, Cucurbits and Cucurbitacins

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For more than a century, researchers have noted the curious attraction of adult luperine chrysomelids in the subtribes Diabroticina and Aulacophorina to cucurbit species rich in the bitter compounds collectively called cucurbitacins (Webster, 1895; Contardi, 1939; Metcalf *et al*., 1980). The attraction is curious, not because these beetles can locate cucurbits over long distances by tracking flower and wound volatiles, and not because cucurbitacins are phagostimulants for Diabroticites that, despite their noxious effects on other insects (Nielsen *et al*., 1977; Tallamy *et al*., 1997a), cause them to eat anything containing these compounds (Sinha and Krishna, 1970; Metcalf *et al*., 1980). Such behaviours characterize most, if not all, phytophagous insects that specialize on a particular group of plants (Rosenthal and Janzen, 1979). What is curious is that most luperines that are attracted to cucurbits are not cucurbit specialists. Instead, they develop to maturity on plants from several families, such as *Poaceae*, *Solanaceae*, *Convolvulaceae*, *Fabaceae* and *Asclepiadaceae*.

The western corn rootworm (WCR), *Diabrotica virgifera virgifera*, is no exception. As a member of the *virgifera* subdivision of the large genus *Diabrotica* (Wilcox, 1972a), it is a specialist on the *Poaceae* and ancestrally probably reached maturity only on grasses, such as *Zea* spp., *Setaria* spp. and couch grasses in several genera (Smith, 1966; Clark and Hibbard, 2004; Oyediran *et al*., 2004; Moeser and Hibbard, Chapter 3, this volume). It is no surprise, then, that WCR has so successfully adopted the comparatively nutritious and toxin-free *Zea mays* for growth and reproduction. Yet WCR is the quintessential example of a luperine that, upon reaching adulthood, leaves its nutritionally complete grass host in search of cucurbits embittered with cucurbitacins. This chapter will explore the phylogenetic, evolutionary, ecological and applied implications of WCR's intriguing affinity for cucurbits.

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Taxonomy of Western Corn Rootworm

WCR (Coleoptera: Chrysomelidae), *D. v. virgifera* LeConte, belongs to the largest tribe within the Galerucinae, the Luperini (Wilcox, 1965, 1972a). Historically, the division of Galerucinae into tribes was in a state of flux. Several authors devised numerous classification systems, resulting in taxonomic chaos until Wilcox (1965) provided a revision based upon male characters that stabilized classification at this level. Wilcox (1965) and Seeno and Wilcox (1982) divided the Luperini into three subtribes: Aulacophorina (formerly known as Monleptina), Luperina and Diabroticina, with the latter containing *D. virgifera* and being strictly New World. The Diabroticina are divided further into four sections: Diabroticites, Ceratomites, Phyllecthrites and Trachyscelidites, with the genus *Diabrotica* being placed within the Diabroticites (Wilcox, 1972a). *Diabrotica* is divided into three species subgroups: *fucata*, *signifera* and *virgifera*, and consists of 333 valid species (Wilcox, 1972a).

The division of *Diabrotica* into subgroups was devised by Smith and Lawrence (1967) using external characters and has since been confirmed using allozyme markers (Krysan *et al.*, 1989) and phylogenetic analysis of nuclear and mitochondrial genes (Clark *et al.*, 2001). *D. virgifera* is classified within the *virgifera* species group, with LeConte providing the first formal description of the species in 1868 from beetles collected near Fort Wallace, Kansas, USA, in 1867 (Smith and Lawrence, 1967). While specimens in LeConte's collection are considered to be the original type series, Smith and Lawrence (1967) contend that *D. virgifera* was also part of Say's original *Diabrotica longicornis* (Say) mixed type series collected 21 km west of present-day Pueblo, Colourado, USA, in 1820. Their contention was based upon the original description and collection records from the same area in 1965. To prevent further taxonomic confusion within this group (*D. v. virgifera* would have had to become *D. longicornis*), Smith and Lawrence chose to keep the original names. Until 1980, WCR, as described by LeConte and known as a pest throughout the north central USA, was considered to be the only variation of the species; however, evidence based on mating compatibility (Krysan and Branson, 1977), egg diapause intensity (Krysan and Branson, 1977; Krysan *et al.*, 1977), behaviour (Krysan *et al.*, 1980) and morphology (Krysan *et al.*, 1980) confirmed that *D. virgifera* has two subspecies, with WCR becoming *D. v. virgifera* and Mexican corn rootworm becoming *D. v. zeae* Krysan and Smith (Krysan *et al.*, 1980).

Purported Origin of Western Corn Rootworm

The geographical distribution of WCR is primarily constrained by the presence of suitable larval hosts (Branson and Krysan, 1981). For populations to exist, there must be phenological synchrony between egg hatch and the availability of host roots for larvae to feed upon. One mechanism that allows this synchrony to occur for many *virgifera* group taxa is an egg diapause that breaks as conditions become favourable (Branson and Krysan, 1981; Krysan, 1982). Branson and Krysan (1981) argued that this adaptation allows *virgifera* species to specialize on perennial grasses that become available during the rainy season in the tropics or during the summer months in more temperate regions. Their argument is supported by the presence of *virgifera* group taxa such as *Diabrotica lemniscata* LeConte, *Diabrotica cristata* (Harris) and *D. longicornis* (Say) in temperate regions of the USA where perennial grasses are a permanent feature in the landscape; however, most of the larval host plants are unknown, especially for non-economically important species. The planting of maize on an annual basis in temperate regions within the USA provides a perennial resource for WCR to exploit. Thus, the distribution of species in the *virgifera* group apparently is not directly related to climate, as most species in the group are capable of withstanding environmental extremes such as freezing and drought in the egg stage (Krysan and Smith, 1987). Instead, species in this group are limited to the distribution of their obligatory larval host plants. Furthermore, Krysan and Smith (1987) hypothesized that the progenitor of the *virgifera* group was probably a specialist on grasses in a region that typically had alternating wet and dry seasons, much like what occurs in parts of Mexico and Guatemala, the region that contains the greatest diversity of taxa for the *virgifera* species group.

Several studies provide evidence that many grasses other than maize fall within the larval host range of WCR (Branson and Ortman, 1967, 1970; Clark and Hibbard, 2004; Oyediran *et al.*, 2004). Furthermore, there is evidence that WCR and its subspecies, Mexican corn rootworm, continue to reproduce on plants other than maize in environments near maize production systems (Hill and Mayo, 1980; Branson *et al.*, 1982). It is difficult to infer origin of the species based on these works. Branson and Krysan (1981) provided the most plausible explanation regarding the origin of WCR. They hypothesized that *D. virgifera* (both subspecies) initially included progenitorial maize as an incidental host prior to its transformation to a food crop by indigenous peoples. As maize was developed in what is now present-day Guatemala and Mexico, it is thought that *D. virgifera* remained associated with it as its range expanded northward through trade and the expansion of prehistoric agriculture (Smith, 1966; Galinat, 1977; Branson and Krysan, 1981). While both rootworm subspecies were most probably associated with maize as it was cultivated by these early indigenous peoples, it is likely that neither subspecies reached pest status because the type of agriculture practised by those ancient peoples did not promote a build-up of rootworm populations (Smith, 1966; Mangelsdorf, 1974). Population expansion did not occur until the Spanish introduction of European-style maize monocultures, which created favourable environments for massive build-ups of *D. virgifera* populations in regions where these monocultures exist. A classic example of this occurred when the westward expansion of maize monoculture in the north central USA reached the eastern limits of the WCR

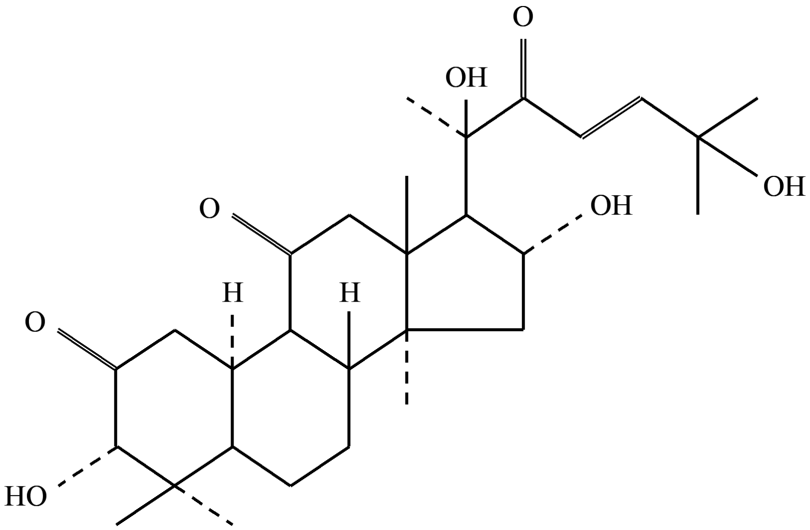

Fig. 4.1. Cucurbitacin B.

geographical range, presumably sometime in the 1940s in western Kansas or Nebraska and finally the east coast of the USA by 1983 (Chiang, 1973; Krysan and Smith, 1987). While this evidence suggests a strong WCR association with maize, it should be noted the that LeConte's original type series collected in 1868 and Say's original mixed type series of *D. longicornis* collected in 1820 (which most probably contained WCR) were both collected from *Cucurbita foetidissima* Humboldt, Bonpland and Kunth in geographical regions that had a high probability of being devoid of maize at the time (Smith and Lawrence, 1967). Therefore, it is likely that WCR had an association with grasses beyond maize prior to its well-documented eastward expansion in the north central USA.

WCR–Cucurbitacin Interactions

The cucurbitacins are a group of non-volatile, highly oxygenated tetracyclic triterpenes with a unique $19(10\rightarrow9\beta)$*abeo*-10-α-lanostane (cucurbitane) skeleton (Fig. 4.1). The biological activity of this group of compounds has been recognized for centuries and cucurbitacin has been used as a laxative and emetic and in the treatment of malaria, dysentery and dysmenorrhoea (Lavie and Glotter, 1971; Halaweish, 1987; Miro, 1995). More recently, cucurbitacins have received a great deal of attention because of their antitumour properties, differential cytotoxicity towards renal, brain tumour and melanoma cell lines (Cardellina *et al.*, 1990; Fuller *et al.*, 1994); their inhibition of cell adhesion (Musza *et al.*, 1994) and possible antifungal effects (Bar-Nun and Mayer, 1989).

Cucurbitacins are produced in at least some tissues of all members of the *Cucurbitaceae* (Gibbs, 1974; Guha and Sen, 1975; Jeffrey, 1980) and a few species in other plant families (Curtis and Meade, 1971; Pohlman, 1975; Dryer and Trousdale, 1978; Thorne, 1981). In most species they are concentrated in roots and fruits, with lesser amounts in stems and leaves. Because of their extreme bitterness, cucurbitacins are thought to be involved in plant protection against herbivores (Metcalf, 1985; Tallamy and Krischik, 1989). Nevertheless, cucurbitacins are phagostimulants for both adults (Metcalf *et al.*, 1980) and larvae (DeHeer and Tallamy, 1991) of several luperine species in the subtribes Aulacophorina and Diabroticina (Table 4.1) and can have important ecological consequences for plants that possess them (Tallamy and Krischik, 1989). Adult luperines can detect cucurbitacins in nanogram quantities and readily devour bitter plant material (Metcalf, 1994; Tallamy *et al.*, 1998). In addition to WCR, cucurbitacins influence the behaviour of several important crop pests, including *Diabrotica balteata* LeConte, the banded cucumber beetle, *Diabrotica barberi* Smith and Lawrence, the northern corn rootworm, *Diabrotica undecimpunctata howardi* Barber, the southern corn rootworm, and *Diabrotica speciosa*, a crop pest in Central and South America.

Studies have shown that, when WCR eat crystalline cucurbitacins for 2 days, they excrete 85% of the material and permanently sequester the

Table 4.1 Effects of cucurbitacins on the feeding of *Cucurbitaceae*-associated leaf beetles (modified from Matsuda, 1988). Phagostimulation by cucurbitacin analogues is represented by a **+**. Apart from D.W.T. Tallamy (unpublished), data are from Chambliss and Jones (1966); Sinha and Krishna (1970); Metcalf *et al.* (1980); Ferguson *et al.* (1983); Nishida and Fukami (1990); Mehta and Sandhu (1992); Metcalf and Metcalf (1992); Lewis and Metcalf (1996); Eben *et al.* (1997); Tallamy *et al.* (1997a); and Abe *et al.*, 2000).

	Cucurbitacin analogues						
Species	A	B	C	D	E	I	L
Diabrotica undecimpunctata undecimpunctata	−	+	−	+	+	+	−
Diabrotica undecimpunctata howardi	+	+	+	+	+	+	+
Diabrotica vigifera vigifera	−	+	−	+	+	+	+
Diabrotica longicornis	−	+	−	+	+	+	−
Diabrotica cristata	−	+	−	+	+	+	−
Diabrotica balteata	+	+	−	+	+	+	−
Diabrotica speciosa	−	+	−	+	−	−	−
Cerotoma arcuata	−	+	−	+	−	−	−
Acalymma vittatum	+	+	−	+	+	+	−
Acalymma trivittatum	−	+	−	+	+	+	−
Aulacophora foveicollis	+	+	+	+	−	−	−
Aulacophora femoralis	−	+	−	−	+	+	−
Aulacophora nigripennis	−	+	−	−	−	−	−
Aulacophora lewisii	−	+	−	+	+	+	−
Trachyscelida sp.	−	−					

remainder in their fat bodies, cuticles, haemolymph, spermatophores and developing eggs (Ferguson and Metcalf, 1985; Andersen *et al.*, 1988; Tallamy *et al.*, 2000). There is good evidence that, regardless of the cucurbitacin configuration eaten, beetles transform it through glycosylation, hydrogenation, desaturation and acetylation into 23,24-dihydrocucurbitacin D (Andersen *et al.*, 1988; Nishida *et al.*, 1992).

There are decided defensive benefits to cucurbitacin sequestration. Beetles that have eaten cucurbitacins become highly distasteful and are readily rejected by predators such as mantids, mice and finches (Ferguson and Metcalf, 1985; Nishida and Fukami, 1990; D.W. Tallamy, unpublished data). Sequestered cucurbitacins may also discourage parasitoids such as tachinid flies in the genus *Celatoria*, although this has never been tested. Moreover, when cucurbitacins have been sequestered in eggs and larvae, both of which are denizens of pathogen-rich damp soil, survival after exposure to the entomopathogen *Metarhizium anisopliae* is significantly improved (Tallamy *et al.*, 1998). This may explain why females shunt 79% of the cucurbitacins that are not excreted into their eggs or the mucus coating of the eggs (Tallamy *et al.*, 2000).

Despite the benefits to female WCR from eating bitter cucurbit tissues, it is males rather than females who actively seek these compounds in nature. In a field trial quantifying the sex ratio of beetles that came to cucurbitacin-rich fruits of *Cucurbita andreana,* Tallamy *et al.* (2002) found that 99% of the 224 WCR found at the fruits over a 5-day period were males. This result concurs with the male-biased sex ratios frequently found in cucurbitacin traps (Shaw *et al.*, 1984; Fielding and Ruesink, 1985). Apparently females rely on males for their primary source of cucurbitacins (Tallamy *et al.*, 2000). Males sequester 89% of the cucurbitacins not excreted after ingestion in their spermatophores and pass them to females during copulation. Whether such behaviour imparts a mating advantage to WCR males has not been investigated.

Pharmacophagy, Luperines and Cucurbits

One of the most interesting relationships between insect herbivores and their host plants occurs when a herbivore seeks a phytochemical that is toxic to other animals. Such behaviour has focused attention on luperine chrysomelids for decades (Webster, 1895; Metcalf *et al.*, 1980; Nishida and Fukami, 1990; Nishida *et al.*, 1992; Tallamy *et al.*, 1999). As discussed above, cucurbitacins are phagostimulants for many luperine adults (Metcalf *et al.*, 1980; Nishida and Fukami, 1990; Tallamy *et al.*, 1997b) and larvae (DeHeer and Tallamy, 1991), despite their extreme bitterness and their ability to kill or repel most invertebrate and vertebrate herbivores (David and Vallance, 1955; Watt and Breyer-Brandwijk, 1962; Nielsen *et al.*, 1977; Tallamy *et al.*, 1997a). Of greatest interest are the species of luperines that do not (and apparently cannot) feed on cucurbit roots as larvae, but consume pure crystalline cucurbitacins as adults when given the chance.

The relationship between non-cucurbit specialists and cucurbitacins differs from that of most other insect herbivores that feed upon biotoxins. Many specialist herbivores use toxic phytochemicals as host recognition cues (Fraenkel, 1959; Feeny *et al*., 1970), while others encounter defensive compounds incidentally and unavoidably (Brower *et al*., 1972). These organisms, however, often exhibit elaborate behaviours to minimize exposure to their hosts' toxins (Dussourd, 1993).

Luperines such as WCR, in contrast, have been described as pharmacophagous insects (Nishida and Fukami, 1990) because they search for particular phytochemicals for purposes other than primary metabolism or host recognition (Boppré, 1990). Pharmacophagy was first described for certain Blattaria (Blattellidae, Blattidae), Orthoptera (Tettigoniidae, Gryllidae, Pyrgomorphidae), Coleoptera (Chrysomelidae, Cerambycidae), Lepidoptera (Danaidae, Ctenuchidae, Riodinidae, Pericopidae, Ithomiidae, Arctiidae, Noctuidae) and Diptera (Tephritidae, Chloropidae) which leave nutritionally suitable host plants to seek noxious pyrrolizidine alkaloids (Meinwald *et al*., 1969; Pliske, 1975; Schneider *et al*., 1982; Boppré *et al*., 1984; Krasnoff and Dussourd, 1989; Boppré, 1990). As with insects pharmacophagous toward pyrrolizidine alkaloids, there is no evidence that pharmacophagous luperines seek cucurbitacins to satisfy a nutritional requirement (although, once ingested, cucurbitacins may substitute for cholesterol under some conditions (Halaweish *et al*., 1999)). Nor can cucurbitacins serve as a cue for host plant recognition. By definition, there are no cucurbitacins in the hosts of pharmacophagous luperines!

The most frequently cited benefit of pharmacophagy is defence (Boppré, 1984). Whether obtained through pharmacophagy or specialization on cucurbits, cucurbitacins persist in the cuticle, fat bodies and haemolymph (Ferguson *et al*., 1985; Andersen *et al*., 1988) and provide protection against predators (Ferguson and Metcalf, 1985; Nishida and Fukami, 1990) and/or pathogens (Tallamy *et al*., 1998). Perhaps because of their defensive benefits, both cucurbitacins and pyrrolizidine alkaloids have also become an integral component of the reproductive behaviour of participating species (Dussourd *et al*., 1991; LaMunyon and Eisner, 1993; Tallamy *et al*., 2000). In both cases, the pharmacophagous agent is consumed directly by females and/or is sequestered by males and passed within spermatophores to females. Females, in turn, shunt the majority of these materials to developing eggs.

Origins of Cucurbitacin Pharmacophagy

Ancestral host hypothesis

If defence and mating advantages are benefits imparted to all pharmacophagous insects, the selective maintenance of such behaviour is no mystery. The origins of insect pharmacophagy, however, are controversial and poorly understood. Although larval host plants of pharmacophagous taxa, by definition, do not produce the chemical that is subsequently sought by adults (Boppré, 1984), larvae of a few ithomiid and danaid relatives of species pharmacophagous to pyrrolizidine alkaloids do, in fact, develop on plants containing pyrrolizidine alkaloids (Edgar *et al.*, 1974). Thus, students of pyrrolizidine alkaloid pharmacophagy have cautiously concluded that, at some point in the evolutionary history of the diverse group of insects exhibiting this behaviour, immatures must have developed on plants that contained the pharmacophagous compound (Pliske, 1975; Boppré, 1978; Edgar, 1982; Trigo and Molta, 1990). This concept was modified by Dussourd (1986) to suggest that variation in the host's production of the compound selected for receptors that enables adults with inadequate supplies of alkaloids to supplement their needs through pharmacophagous forays to non-host sources.

The evolution of cucurbitacin pharmacophagy in the Luperini has been viewed similarly (Metcalf, 1979, 1994; Metcalf *et al.*, 1980), because: (i) cucurbitacins are phagostimulants for some species of both Old World Aulacophorina and New World Diabroticina (Table 4.1); (ii) at least two genera of Luperini (*Acalymma* and *Aulacophora*) are larval host specialists on cucurbits (Wilcox, 1972a; Monroe and Smith, 1980); and (iii) all pharmacophagous species tested to date are most stimulated by cucurbitacin B, the most ubiquitous of the 46-plus known cucurbitacin configurations (Hill *et al.*, 1991). Luperine responses to cucurbitacins are thought to be derived from plesiomorphic traits that arose from an ancestral host relationship with the *Cucurbitaceae*. Under this hypothesis, the phagostimulatory response to cucurbitacins in species that currently develop only on non-cucurbitaceous host plants is a relic of a long-lost need for host recognition and is a trait that is currently maintained through secondary selection from protection benefits associated with cucurbitacin consumption (Ferguson and Metcalf, 1985; Tallamy *et al.*, 1998). The majority of luperine species, then, are assumed to have undergone host shifts away from cucurbits to plants in other families. It is important to note here that pharmacophagous visits to cucurbits by adult luperines that have developed on other plants have vastly inflated luperine host records for *Cucurbitaceae* (e.g. Metcalf, 1994; Jolivet and Hawkeswood, 1995). Unfortunately, our knowledge of true larval hosts in the Luperini is extremely limited because the larvae of all species develop underground on roots and are seldom collected or correctly identified with their respective adults.

Loose receptor hypothesis

There is intuitive appeal to assuming an ancient relationship between luperine ancestors and early cucurbits, but it is not the most parsimonious explanation for why so many extant luperine lineages do not use cucurbits as larval hosts. Recent evidence from other pharmacophagous insects suggests that an ancestral association with a particular compound may not be necessary to promote the evolution of pharmacophagy (Tallamy *et al.*, 1999). Studies of cantharidin, a noxious, volatile monoterpene produced *de novo* as a nuptial gift exclusively by male meloid and oedomerid beetles, provide a number of examples whereby exposure to a novel and potentially deleterious compound results in phagostimulation rather than deterrence. Cantharidin attracts and stimulates feeding in several diverse taxa, including pyrochroid, endomychid, anthicid, staphylinid and chrysomelid beetles, ceratopogonid, sciarid and anthomyiid flies, braconid wasps and mirid and tingid bugs, which may have had no evolutionary exposure to the compound (Young, 1984; Frenzel *et al.*, 1992; Frenzel and Dettner, 1994; Mafra-Neto and Jolivet, 1994; Eisner *et al.*, 1996). It is possible, however, that canthariphagy arose because of the coincidental acceptance of cantharidin as a novel agonist by peripheral receptors with less than perfect specificity rather than from adaptive responses to a historically familiar compound.

The neurophysiological basis of peripheral perception is extraordinarily complex in insect gustatory systems (Frazier, 1986; Simmonds *et al.*, 1990; Städler, 1991; Schoonhoven *et al.*, 1992; Mullin *et al.*, 1994). In the simplest terms, feeding behaviour is stimulated if the chemoreception of phagostimulants exceeds the chemoreception of feeding deterrents (Dethier, 1980). In caterpillars and possibly all insects, taste sensilla contain cells specialized for the production of either deterrent and stimulatory inputs, or, more probably, neurons capable of producing both deterrent and stimulatory inputs (Frazier, 1986). Receptor sites on these cells can be highly specific (tight) or less specific (loose). Strychnine, for example, is a compound novel to phytophagous insects, but it readily depolarizes activation channels, leading to deterrent input in most insects. The binding requirements at these sites are sufficiently loose for a variety of molecular structures to meet the polarity and configuration specifications for binding there. The loose characteristics of receptor sites with deterrent capabilities may be adaptive because they protect the central nervous system from exposure to damaging novel compounds (Frazier, 1992).

Critical to the loose receptor hypothesis is the fact that relatively loose binding properties of receptor sites can also enable novel and sometimes deleterious compounds to trigger feeding behaviour. There are several mechanisms by which this can happen (Frazier, 1986, 1992). Some molecules bind at receptor sites leading to deterrent inputs, but, rather than depolarizing the activation channels, they simply block them. Without inhibitory inputs, even small amounts of phagostimulants, including amino acids present in the insects' saliva, are sufficient to activate the stimulatory inputs at the sensillum and elicit feeding. Activation leading to deterrent inputs can also be inhibited when particular molecules block the stimulus removal system. Finally, loose stimulatory receptor sites themselves can encourage phagostimulation by novel compounds with the appropriate configuration and polarity at binding sites.

This is apparently the mechanism by which the peptide aspartame mimics the carbohydrate sucrose at vertebrate receptors, a mimicry upon which much of the sweetener industry is based. Another example of a loose receptor exists within WCR as well. WCR larvae are highly attracted to carbon dioxide (Strnad *et al.*, 1986; Hibbard and Bjostad, 1988) and dichloromethane (Jewett and Bjostad, 1996). Apparently, the size and polarity of the chlorine atoms in dichloromethane are similar to carbonic acid, the water soluble form of carbon dioxide (Jewett and Bjostad 1996).

How might the loose receptor hypothesis explain cucurbitacin pharmacophagy in luperines? Although data are few, a review of the adult feeding habits of luperines suggests that there is a perfect correlation between adult affinity for pollen and cucurbitacin phagostimulation: pollen is a substantial component of the adult diet in species stimulated to eat by cucurbitacins (*Diabrotica*, *Acalymma*, *Aulacophora*), while species in which the adult diet is largely confined to foliage are repelled by cucurbitacins (*Cerotoma trifurcata*, *Trachyscelida* spp., all Luperina) (Metcalf *et al.*, 1980; Sinha and Krishna, 1970; D.W. Tallamy, unpublished data). One scenario, then, is that the phagostimulatory response to cucurbitacins arose through adult feeding behaviours rather than through larval host dependencies.

The link between pollen feeding and cucurbitacin phagostimulation may be based on similarities in structures of common pollen constituents and cucurbitacins (Tallamy *et al.*, 1999). Amino acid neuroreceptors exhibiting g-aminobutyric acid (GABA)/glycine pharmacology are located on the maxillary galeae of *Diabrotica* and have been implicated in the perception of both antifeedants and phagostimulants (Mullin *et al.*, 1992; Chyb *et al.*, 1995; Hollister and Mullin, 1998; Kim and Mullin, 1998). Cucurbitacins do not occur in pollen (Andersen and Metcalf, 1987), but pollen and meristematic tissues favoured by adult pharmacophagous luperines are enriched with similar mid-polar (mildly lipophilic) compounds such as brassinosteroids, ω-3-linoleic acid-containing lipids and hydroxycinnamic acid-polyamine amides, along with polar low-molecular-weight neutral amino acids, including GABA (Barber, 1971; Stanley and Linskens, 1974; Erhardt and Baker, 1990; Marquardt and Adam, 1991; Feldlaufer *et al.*, 1993; Mullin *et al.*, 1993; Lin and Mullin, 1999). Many of these compounds elicit phagostimulatory responses from *Diabrotica* amino acid receptors (Mullin *et al.*, 1994; Hollister and Mullin, 1998; Lin and Mullin, 1999), either alone or while interacting at multiple sites. In particular, some pollen sterols potentiate the amino acid agonists in pollen (Chyb *et al.*, 1995; Hollister and Mullin, 1998; Kim and Mullin, 1998). Current data suggest that it is these same taste neurons that are depolarized by cucurbitacins (Mullin *et al.*, 1994).

Cucurbitacins are structurally similar to many sterols, sharing both their hydroxylation and their stereochemistry (Dinan *et al.*, 1997a,b). The unusual 4,4-dimethyl-5-ene structure of cucurbitacin B, for example, orients the 3-one group below the ring plane (Mullin *et al.*, 1994) and thus closely resembles GABA-acting 3a-ol pregnane steroids (Purdy *et al.*, 1990). Like pregnane steroids, cucurbitacins potentiate amino acids such as alanine, serine, praline and GABA (compounds present in pollen and saliva alike) and trigger a feeding response in WCR even at very low doses (Mullin *et al*., 1994). The structural similarity between cucurbitacins and many sterols also makes these molecules into powerful antagonists at insect ecdysteroid receptors (Dinan *et al*., 1997a,b). It is possible that selection on luperine amino acid receptors for the loose perception of pollen constituents simultaneously rendered these receptors susceptible to cucurbitacin depolarization. Since ancestral chrysomeloid beetles were associated with polliniferous food sources even before the division of the cerambycid and chrysomelid lineages in the early Cretaceous (Samuelson, 1994), it is likely that in certain lineages pollen feeding and the evolution of taste neurons associated with pollen feeding preceded luperine interactions with cucurbits and their cucurbitacins. Thus, the response of Luperini to cucurbitacins may not reflect an ancestral larval association with cucurbitacin-producing plants as suggested for luperines in particular (Metcalf, 1979) and implied by pharmacophagous theory in general (Edgar, 1982). Rather, it may be the product of a physiological coincidence mediated by the molecular configuration of cucurbitacins that promotes binding at a steroidal site on amino acid receptors.

Under this scenario, beetles that evolved to exploit cucurbit pollen because of its abundance and nutritional richness (Reddi and Aluri, 1993) were, regardless of their larval host, serendipitously placed in contact with cucurbitacins, which are components of cucurbit anthers and flower petals, but not pollen (Andersen and Metcalf, 1987). In the early stages of the interaction, beetles that were stimulated to feed on cucurbitacins would have suffered reduced fitness. Cucurbitacin avoidance, however, could not have evolved without a significant tightening of the pollen-adapted amino acid receptors, compromizing the detection of an evolutionarily entrenched adult food source. Repeated exposure to cucurbitacins would have favoured physiological tolerance, while advantages gained from their defensive properties would have encouraged beetle associations with cucurbitacin sources.

Phylogenetic Resolution

Whether or not cucurbitacin pharmacophagy in the luperine arose through ancestral host specialization on cucurbits or a stochastic similarity between the molecular structure of cucurbitacins and pollen sterols may never be known definitively, but mapping larval host range, cucurbitacin phagostimulation and pollen use on a reconstructed luperine phylogeny will infer much about the evolution of this behaviour. We have learned a great deal about phylogenetic relationships among the Luperini in recent years (Gillespie *et al*., 2003, 2004), but our knowledge of the distribution of cucurbitacin phagostimulation, pollen use and larval host range remains scanty. Nevertheless, Gillespie *et al*. (2003, 2004) provide a phylogenetic analysis of host range and cucurbitacin phagostimulation that is sufficiently robust to test the primary predictions of the ancestral host hypothesis (Fig. 4.2). Using partial DNA sequences from two gene regions, phylogenetic trees have been reconstructed for over 100 luperine taxa under several optimality criteria (equally weighted parsimony, differentially weighted parsimony and maximum likelihood), all of which unambiguously suggest that the rootworms are a paraphyletic assemblage of beetles. Interestingly, the Old World cucurbit specialists (*Aulacophora* and possibly related genera) and the New World cucurbit specialists (*Acalymma*) do not share a common ancestor at the tribal level within the subfamily Galerucinae. The luperine subtribe Diabroticina, which contains the cucurbit specialists *Acalymma* spp. and several other genera that are pharmacophagous towards cucurbitacins, shares affinities with two other galerucine tribes, the Metacyclini and the Galerucini. The subtribe Aulacophorina, which contains the cucurbit specialists in the genus *Aulacophora*, is a derived lineage within the third and largest rootworm subtribe, the Luperina. The remaining tribes of the Galerucinae, the Oidini and the Hylaspini, as well as the rogue diabroticine genus *Trachyscelida*, fluctuate in their placement in these rootworm lineages (Gillespie *et al.*, 2004); however, the authors point out that the undersampling of these lineages has clearly affected the stability of their phylogenetic position throughout the analyses. Recent analyses with thorough samplings of the Oidini and Hylaspini are adding more stability to the entire galerucine tree, and the mode of rootworm paraphyly discussed above is still strongly supported (Gillespie *et al.*, 2004).

If cucurbitacin pharmacophagy developed through an ancestral host association with Cucurbitaceae, then genera specializing on cucurbits (*Acalymma* and *Aulacaphora*) are predicted to be basal to other lineages within the Luperini. Moreover, if the behaviour is very old and arose before the breakup of Gondwana, as implied by the worldwide distribution of species specializing on cucurbits (*Acalymma* and *Aulacaphora*), and derived pharmacophagous species are expected to belong to the same or related lineages; that is, pharmacophagy should be a monophyletic trait. This evolutionary scenario is examined on a reconstructed phylogeny of the Galerucinae (Fig. 4.2A). When a single origin for a luperine–cucurbit association in an ancestor to this hypothetical *Aulacophora*/*Acalymma* lineage is assumed, it must be placed at the root to the Galerucinae *sensu stricto* (minus the flea beetles). Assuming that cucurbit specialization preceded cucurbitacin pharmacophagy in the hypothetical galerucine ancestor, this explanation for the evolution of pharmacophagy suggests that the majority of galerucine lineages, totalling over 4000 described species (Wilcox, 1972a,b), have shifted to other host plants outside the *Cucurbitaceae*, with only two genera retaining cucurbit specialization. This is clearly not the most parsimonious explanation for the evolution of cucurbitacin–luperine associations. To adopt this hypothesis, one must explain why the majority of species in such a successful beetle radiation have aborted a host plant affiliation that provides

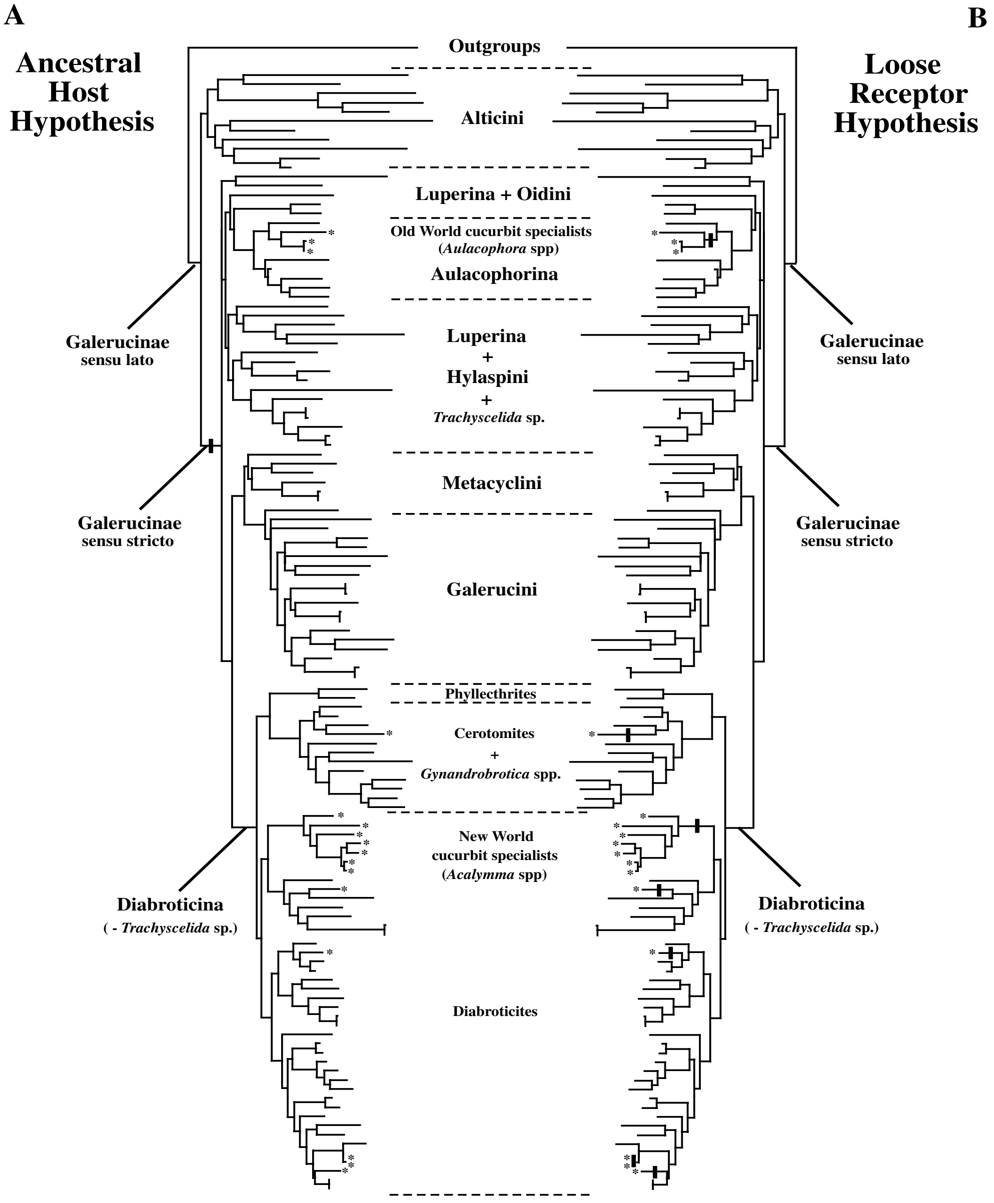

Fig. 4.2. Mirror image phylogram showing the loss and gain of cucurbitacin feeding under two evolutionary scenarios (redrawn from Gillespie *et al*., 2004). Bold horizontal bars denote the gain of cucurbitacin feeding, and asterisks represent lineages known to utilize cucurbitacins. (A) The phylogram on the left (ancestral host hypothesis) illustrates cucurbitacin feeding in aulacophorine and diabroticine lineages as a plesiomorphic remnant of an ancestral host association between the *Cucurbitaceae* and Luperini, Metacyclini, Oidini, Hylaspini and Galerucini. (B) The phylogram on the right illustrates cucurbitacin feeding in some aulacophorine and diabroticine lineages as a product of convergent evolution in these geographically isolated lineages.

highly selective advantages in the form of chemical defence and/or beneficial mating strategies. Moreover, it is difficult to suppport this hypothesis if an ancestral association between Cucurbitaceae and an immediate aulacophorine/diabroticine ancestor is perceived as the origin for pharmacophagy.

In contrast, the loose receptor hypothesis carries no assumptions about the position of cucurbit specialists in the phylogeny of the Luperini. It does, however, assume that the evolution of pollen specialization preceded or is directly correlated with cucurbitacin pharmacophagy. Under this evolutionary scenario, pharmacophagy by convergent evolution unrelated to larval host use explains the independent origin of cucurbitacin utilization in Old and New World rootworm lineages (Fig. 4.2B). Given the strongly supported paraphyletic relationships between the Aulacophorina and Diabroticina, the loose receptor hypothesis is currently the more parsimonious model explaining the evolution of Cucurbitaceae/Luperini associations.

It is important to note that cucurbitacin feeding may have arisen independently in basal lineages of both the Aulacophorina and the Diabroticina and then proceeded to cucurbitacin pharmacophagy via host switching as the ancestral host hypothesis predicts. Whether or not the ancestral host hypothesis supports the evolution of cucurbitacin pharmacophagy in Old World cucurbit feeders will remain an open question until more taxa are sampled and more is known about the host plants used by the basal lineages within the Aulacophorina. Within the Diabroticina, however, a well-supported ancestor is beginning to emerge (Fig. 4.2). A monophyletic group of bean-feeding species, comprized of taxa sampled from the Phyllecthrites, Cerotomites and the genus *Gynandrobrotica* (Diabroticites), is basally separated from the remaining Diabroticina (- *Trachyscelida* sp.). It is possible that the association between *Acalymma* spp. (Diabroticina) and cucurbits occurred in an ancestor to the Diabroticites, with pharmacophagy arising in other genera (such as *Diabrotica*, *Paratriarius* and *Isotes*) as a means of retaining the benefits imparted by cucurbitacin sequestration that are not provided by other host plants. This scenario, however, does not explain the affinity *Cerotoma arcuata*, a legume feeder, has with cucurbitacins (Nishida and Fukami, 1990). Because this species is grouped within the basal bean-feeding lineage (Cerotomites), we cannot account for its cucurbitacin affinity by an ancester that once specialized on cucurbits. Thus, it is easier to explain cucurbitacin associations as independently arising through convergence caused by the similarities of cucurbitacins and pollen compounds that favour pharmacophagy. While the loose receptor hypothesis is consistent with Gillespie's phylogenetic reconstructions of the Luperini, more data from cucurbitacin sensitivity bioassays are needed to differentiate between cucurbit pollen feeders and species that incorporate cucurbitacins into their life cycles from non-pollen cucurbit tissues (Gillespie *et al.*, 2004).

WCR Adult Attraction to Volatile Semiochemicals from Cucurbits

LeConte (1868) based the species, *D. virgifera*, on two specimens found on 'wild gourd', so the association of WCR with members of the *Cucurbitaceae* dates back to the earliest published record for the species. *Diabrotica* spp., in general, have been associated with blossoms of varying *Cucurbita* spp. (Fronk and Slater, 1956; Howe and Rhodes, 1976; Bach, 1977; Fisher *et al.*, 1984). Andersen and Metcalf (1987) examined selected *Cucurbita* spp. and cultivars for differences in floral volatile release, blossom cucurbitacin content and pollen content of male blossoms, and correlated these data to preference by *Diabrotica* beetles. *D. undecimpunctata howardi* were primarily found on *Cucurbita maxima*, which were the only blossoms to contain detectable levels of cucurbitacins and also released the greatest quantity of volatiles. WCR most preferred *C. maxima*, but were also found in significant quantities on one of the cultivars of *Cucurbita pepo*. Pollen content did not vary significantly between the cultivars and was actually highest on *Cucurbita moschata*, the least attractive species to WCR beetles, indicating that pollen was not responsible for differences in beetle preference.

Andersen (1987) identified 22 of the 31 major components of *C. maxima* floral aroma. Metcalf and Lampman (1991, and references therein) evaluated these compounds by themselves and in blends for their attraction to diabroticite beetles in varying field settings. They also tested synthetic analogues of these compounds by varying functional group type, position, etc. Metcalf and Lampman (1991) speculated that ancestral diabroticite rootworms coevolved with primitive *Cucurbitaceae* species and that antennal sensory receptors evolved from those tuned to such compounds as cinnamaldehyde into receptors more specifically attuned to compounds that are currently being produced from cucurbit plants.

Metcalf and Metcalf (1992, and references therein) discussed a series of experiments which resulted in the development of a three-component blend of 1,2,4-trimethoxybenzene, indole and cinnamaldehyde (the TIC mixture), which was attractive to WCR in the field. The blend had a synergistic effect in that the combination showed more than a twofold increase in rootworm response than would have been expected from an additive effect of the individual compounds. Metcalf and Metcalf (1992) referred to this blend as a highly simplified *Cucurbita* blossom volatile aroma.

Although a behavioural bioassay is the only reliable means of evaluating an entire complement of compounds in a semiochemical blend, electroantennogram (EAG) recording is usually helpful in detecting the most important components in the blend. This technique is a simple method for electrophysiological detection of the responses of insect antennae to volatile semiochemicals (Roelofs, 1984). Hibbard *et al.* (1997b) used an EAG-driven isolation and identification schemes to identify (*E*,*E*)-3,5-octadien-2-one, (*E*,*Z*)-2,6-nonadienal, (*E*)-2-nonenal, 2-phenethanol, benzyl alcohol, and 6,10-dimethyl-5,9-undecadien-2-one from extracts of buffalo gourd (*C. foetidissima* Humboldt, Bonpland and Kunth) root powder, the behaviourally active component of semiochemical baits for adult corn rootworm control programmes. Cossé and Baker (1999) isolated several of these same compounds from buffalo gourd root powder and demonstrated that (*E*,*E*)-3,5-octadien-2-one was attractive to *D. barberi* adults.

Volatile Semiochemicals from Maize

Prystupa *et al*. (1988) demonstrated that WCR are attracted to maize silk. Abou-Fakhr *et al*., (1996) demonstrated that the senescing portions (the brown portion that protrudes from the tip of the ear) of maize silks elicited strong EAG responses from WCR adults, while the green portions (under the husk or very young, green silk beyond the husk) did not elicit a significant EAG response. Hibbard *et al*. (1997a) went on to isolate and identify the primary EAG-active components from brown maize silk as tridecan-2-one, (*E*,*E*)-3,5-octadien-2-one, (*E*,*Z*)-2,6-nonadienal, and (*E*)-2-nonenal. Hammack (1996) demonstrated that (*E*)-6,10-dimethyl-5,9-undecadien-2-one (geranylacetone) was highly attractive to *D. barberi* and also attractive to WCR. In a reevaluation of the most EAG-active fractions from maize silk, Hibbard *et al*. (1997b) found 6,10-dimethyl-5,9-undecadien-2-one as one of the smaller peaks present. The phenyl propanoids 2-phenethanol and benzyl alchohol were also present, but were not found in the fractions with the most EAG activity.

Volatile Semiochemicals from Both Maize and *Cucurbita*: a Role Connecting the *Poaceae,* the *Cucurbitaceae,* and the *virgifera* Group?

Both maize silk extracts and buffalo gourd root extracts are composed predominantly of free fatty acids and hydrocarbons; yet in both extracts neither the fatty acid portion of the extract nor the hydrocarbon fraction was EAG-active (Hibbard *et al*., 1997a,b). When EAG-driven isolations and identifications were used with WCR, the primary compounds with EAG activity in both extracts included (*E*,*E*)-3,5-octadien-2-one and 6,10-dimethyl-5,9-undecadien-2-one (Hibbard *et al*., 1997a,b), both of which have been shown to be attractants for *D. barberi* (Hammack, 1996; Cossé and Baker, 1999). The phenyl propanoids 2-phenethanol and benzyl alchohol were also present from both plant species, which again are attractive to *D. barberi* (Metcalf and Metcalf, 1992). The same general chemistry appears to play a role in both the *Poaceae* and the *Cucurbitaceae* in terms of biological activity for WCR and *D. barberi* adults. As noted above, despite specialization on the *Poaceae*, adult WCR and *D. barberi* feed compulsively on bitter cucurbitacins when presented the opportunity, and they are attracted to volatiles from *Cucurbita* blossoms (Metcalf and Metcalf, 1992, and references therein). According to

Metcalf and Metcalf (1992) the *Cucurbitaceae* were the ancestral host of all Diabroticites, but the *virgifera* group, which includes the WCR, currently specializes on the *Poaceae*. If the ancestral host hypothesis is correct, volatile compounds which are produced from both the *Cucurbitaceae* and the *Poaceae* may have been partially responsible for the host switch from the *Cucurbitaceae* to the *Poaceae* for the *virgifera* group of *Diabrotica*. The results of Gillespie *et al*. (2003), though, call into question the ancestral host hypothesis as an explanation for the affinity of some Diabroticites to the *Cucurbitaceae* and give additional credence to the loose receptor hypothesis (Tallamy *et al*., 1999) described above. Ancestral members of the *virgifera* group that specialized on the *Poaceae* may have initially found *Cucurbitaceae* plants through volatile attractants which are released by both groups. These compounds may still play a role today, although compounds not yet identified from maize appear to play a more important role.

Parakairomones

Given the drastically different responses of different *Diabrotica* species to small changes in chemical structure, Metcalf and Metcalf (1992, and references therein) evaluated the attractiveness of a series of compounds modified slightly from *Cucurbita* blossom volatiles. Adding a methoxy group to natural compounds dramatically increased its effectiveness in attracting adult beetles. Although 4-methoxycinnamaldehyde has not yet been documented to be produced by *Cucurbita* blossoms, it is 2750-fold more attractive than cinnamaldehyde, which was isolated and identified from *Cucurbita* blossoms as attractive. Similarly, 4-methoxyphenethanol was 6200-fold more attractive to *D. barberi* than phenethanol (Metcalf and Lampman, 1991). Metcalf and Metcalf (1992) used this information to speculate on the make-up of the receptors responsible for detecting these chemicals. It is these more attractive methoxy analogues of natural compounds which are generally used as lures today.

Use of Volatiles and Cucurbitacin-based Baits in Rootworm Management

Semiochemicals have been used in a number of ways to assist in corn rootworm management. Shaw *et al*. (1984) developed a ‘vial trap’ made from 60 ml amber-coloured plastic snap caps in which holes were drilled to allow rootworm beetles to enter. Levine and Gray (1994) then used this design to develop thresholds for predicting economic damage in areas with the rotation-resistant biotype (see also Spencer *et al*., Chapter 6, this volume). Acetate transparency film was sprayed with a mixture of water and carbaryl and sprinkled with squash powder before placing it in the vial. Cucurbitacins from the squash served as a feeding stimulant and arrestant, but did not attract beetles. Beetles typically fed on the carbaryl and died. Rondon and Gray (2003) went beyond the maize-soybean rotation and looked at beetle numbers in oat (*Avena sativa* L.) stubble and alfalfa (*Medicago sativa* L.) with both the vial trap and the Pherocon® AM yellow sticky trap. Unfortunately, the vial trap is not currently commercially available.

Whitworth *et al*. (2002) evaluated a series of traps with and without volatile attractants. They found that traps baited with volatile attractant lures captured more beetles than unbaited traps. Lure constituents affected the species of beetle attracted to the trap. Traps baited with 4-methoxycinnamaldehyde attracted more WCR, traps baited with eugenol were more attractive to *D. barberi* and traps baited with *trans*-cinnamaldehyde were most attractive to southern corn rootworm larvae. They also found that a new trap developed by Trécé (Salinas, California) was as effective as or more effective than traditional monitoring techniques and, because it lacks sticky material, may be more accepted by consultants and growers, who are often averse to using sticky traps. The trap uses volatile attractants and a 'stun pill' containing buffalo gourd root powder and 3.9% carbaryl. Although not reported in Whitworth *et al*. (2002), Trécé's website (http://www.trece.com/) reports a threshold of 200 beetles/trap/week to reduce egg laying below economic levels the subsequent year. This number is reduced by half when areawide management (see Gerber *et al*., Chapter 11, this volume) is in place. The trap is commercially available (Trécé, Salinas, California).

Pruess *et al*. (1974) demonstrated that adult control prior to egg laying in continuous maize could be used to prevent economic damage from larval feeding the following season. Adult control in conjunction with field scouting has been used or recommended by a number of professional crop consultants in Nebraska and several other states (Meinke, 1995). Metcalf *et al*. (1987) documented that a dry bait containing cucurbitacins impregnated with a reduced rate of insecticide resulted in substantial reduction in adult corn rootworm populations. Since that time, a large amount of effort has gone into optimizing the formulations. Current versions of the bait contain cucurbitacins, a toxicant (which one depends on the product) and a non-toxic edible carrier. The bait uses a 95–98% less toxic active ingredient than traditional beetle management insecticides. Commercial products have been made by several companies (Slam and Adios, Microflow Co., Memphis, Tennessee; Invite, Florida Foods Products, Inc., Eustis, Florida; and CideTrak, Trécé, Inc., Salinas, California). Use of a semiochemical bait for controlling adult corn rootworm beetles has been applied on an areawide basis with some success (Chandler, 2003; Gerber *et al*., Chaper 11, this volume), but whether this programme will be taken over by growers after it is over remains to be seen. Metcalf *et al*. (1987) suggested that the effectiveness of cucurbitacin-based baits could be increased should bait efficiency increase, but, according to Lance and Sutter (1991), the addition of volatile attractants did not increase the efficiency of adult rootworm control and commercial companies have not added volatile attractants to their formulation. Hammack (2003) suggested that attractants could be used to concentrate beetles in an area of the field that is subsequently controlled with cucurbitacin-based baits, but this approach has not been attempted.

While a complete understanding of WCR–cucurbit relations has not been achieved, considerable data have been generated. The sum of these data indicate that the most parsimonious explanation for the relationship is the loose receptor hypothesis first proposed by Tallamy *et al.* (1999). Regardless of the true explanation for the relationship, it provides unique opportunities for the management of WCR. Utilizing cucurbit-based attractants and feeding stimulants in WCR management may prove even more fruitful down the road than they have been to date.

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