

## Glues or poisons: which triggers vein cutting by monarch caterpillars?

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**Summary.** Late instar larvae of the monarch butterfly (*Danaus plexippus* L., Lepidoptera: Nymphalidae) deactivate the latex defense of milkweeds by chewing a furrow in the leaf midrib or petiole. The larva then feeds beyond the cut where latex outflow is minimal. If a larva does encounter latex exudate during feeding, it often returns to its initial cut to damage the midrib or petiole more extensively before resuming feeding. We used this response to latex as an assay for testing what cue triggers vein cutting. A sticky solution of polyethylene glycol and a mixture of cardenolides both were ineffective; drops applied to the mouthparts of feeding monarchs failed to elicit renewed vein cutting. Activity resided instead within a methanol extract of the supernatant obtained from centrifuged latex of the milkweed, *Asclepias curassavica* L. (Asclepiadaceae). Treatment with proteinase K did not eliminate activity documenting that the active compound is unlikely to be proteinaceous. Our results indicate that latex adhesiveness and low polarity cardenolides are relatively unimportant releasers of vein cutting. We propose that milkweeds contain noncardenolide stimulants of vein cutting; these compounds presumably serve a defensive role for milkweeds. Over 50 species of insects are known to employ vein-cutting before feeding on plants with canal-borne exudates; most of these species are dietary specialists like the monarch. Our behavioral assay provides a novel approach for identifying ecologically-significant compounds in the exudates of their diverse host plants.

**Key words.** Latex – laticifer – cardenolide – plant-insect interactions – monarch butterfly – *Danaus plexippus* – Lepidoptera – *Asclepias curassavica* – Asclepiadaceae

### Introduction

When damaged, many plants emit exudates such as latex, resin, or mucilage from elongate canals (Fahn 1979; Langenheim 2003). These secretory canals rank as one of the more ubiquitous plant defenses; over 20,000 species of plants produce latex alone (Lewinsohn 1991), some 8 % of all plant species. The exudates coat the mouthparts of foraging herbivores with noxious chemicals and seal wounds from pathogen attack (Farrell *et al.* 1991; Langenheim 2003).

Insects that specialize on plants with secretory canals often sever veins or cut trenches, thereby rupturing the canals and reducing exudation at their prospective feeding site (Dussourd & Denno 1991; Dussourd 1999; Evans *et al.* 2000). Presumably exudate constituents trigger vein cutting and trenching, although few studies have examined what plant cues elicit the behaviors or what exudate components are harmful for the insects.

In this paper, we use a novel bioassay to test if the adhesiveness of latex or poisonous cardenolides trigger vein cutting by monarch caterpillars, *Danaus plexippus* L. Late instar monarchs disarm latex canals in milkweeds by chewing a furrow in the leaf midrib or petiole (Brewer 1977; Dussourd & Eisner 1987; Zalucki & Brower 1992). The larva then consumes the distal portion of the leaf down to the furrow. Vein cutting serves to constrict and sever elongate latex cells (laticifers) that extend along the midrib and branch into peripheral leaf veins. The cells contain latex under pressure and ordinarily release copious fluid when damaged. But monarchs encounter little or no latex exudate during feeding because their vein cut isolates and drains branches of the laticifer cells that extend into the leaf (Dussourd & Denno 1991; Dussourd 1999).

If a drop of fresh latex from the milkweed *Asclepias syriaca* is placed in the path of a feeding monarch, the larva often stops feeding upon contacting the drop and returns to the leaf base to chew further on its earlier vein cut (Dussourd 1990). The larva reacts as though the latex drop exuded from the leaf, which would indicate that some laticifers remain intact and that further vein cutting is required to rupture all laticifers entering the leaf. The larval response to latex suggests that stimulants for vein cutting reside within this fluid.

Milkweed latex often contains a mixture of cardenolides that can occur at extraordinary concentrations (Shukla & Murti 1971; Nelson *et al.* 1981; Sady & Seiber 1991; Zalucki *et al.* 2001a). In *A. curassavica* latex, for example, the cardenolide concentration equals approximately 300 mg/g dry weight, ~50 times higher than levels in excised leaves with depressurized laticifers (Seiber *et al.* 1982). Cardenolides inhibit the Na<sup>+</sup>, K<sup>+</sup>-ATPase and thus are toxic to most herbivores (Malcolm 1991), including many caterpillars (Cohen 1983; Dussourd & Hoyle 2000).

Milkweed latex also coagulates upon exposure to air, becoming increasingly sticky before eventually hardening. Its adhesiveness is sufficient to glue small larvae to the

plant; indeed, some 30 % of the first instar monarchs on *A. humistrata* die embedded in latex (Zalucki & Brower 1992; Zalucki *et al.* 2001a). Late instar monarchs respond differently to fresh and dry latex. If a feeding monarch caterpillar encounters a drop of *A. syriaca* latex that has dried, the larva consumes the drop and underlying leaf tissue without pausing (Dussourd unpub. obs.). Only fresh latex triggers renewed vein cutting suggesting that the physical characteristics of latex, such as adhesiveness or viscosity, elicit the behavior. However, it is also possible that cardenolides and other allelochemicals in dried latex are less accessible to caterpillar taste receptors that detect dissolved chemicals. In this study, we tested whether the physical stickiness of latex or milkweed cardenolides elicit vein cutting by monarchs. We used resumption of vein cutting as a behavioral assay for testing which solution stimulates larvae to cut veins.

## Methods

Monarch larvae were reared to the final instar on potted *A. curassavica* grown in a greenhouse. The final instar larvae were deprived of food for two hours, then placed singly on potted *A. curassavica* plants and allowed to chew a furrow in a leaf. As soon as the larva began to feed, a series of 0.5-1  $\mu$ l drops of test solution (total 2  $\mu$ l/larva) were placed next to or directly on larval mouthparts using 5  $\mu$ l Wiretrol micropipettes. We recorded how many larvae stopped feeding to return to the base of the leaf to chew further on their vein cut. In all experiments, ten larvae were tested with each solution; each larva was tested with just one solution. Larvae were randomly assigned to treatment.

The following five experiments were completed using this vein-cutting assay.

### Experiment A

To determine if cardenolides elicit vein-cutting, monarch larvae were tested with a cardenolide extract from *Calotropis procera* obtained from Sigma Chemical Co., St. Louis. *C. procera* and *A. curassavica* have a similar profile of latex cardenolides that include uscharidin, uscharin, calactin, calotropagenin, calotoxin, and others (Seiber *et al.* 1982). Monarch larvae chew furrows in *C. procera* veins just like their vein cuts in *Asclepias* (Helmus unpub. obs.). The cardenolide mixture from *C. procera* was tested at 25 g/l to correspond to the cardenolide concentration reported for *A. curassavica* latex (Groeneveld *et al.* 1990). Three treatments were tested: fresh *A. curassavica* latex obtained from severed petioles, the cardenolide mixture dissolved in 20 % Tween 80, and a control solution of 20 % Tween 80. The surfactant Tween 80 was required to dissolve the cardenolides, many of which have poor solubility in water.

### Experiment B

To test if latex adhesiveness elicits vein-cutting, monarch larvae were tested with polyethylene glycol (MW 15,000-20,000, 0.5 g PEG/ml water), which forms a sticky solution that resembles drying latex. Larvae were tested with either PEG, fresh *A. curassavica* latex, or water as a control.

### Experiment C

To test further if latex adhesiveness is required for vein cutting, milkweed latex was centrifuged to separate the fluid portion from the large molecules that coagulate. The latex was collected by severing the tips of *A. curassavica* shoots and allowing a single drop per shoot to fall into a microcentrifuge tube held on ice.

A total of ~500  $\mu$ l latex was collected from five plants. The tube was spun at 24,000  $\times$  g for 15 minutes in a Beckman centrifuge (model #5415C) at 8 °C. The supernatant was removed and centrifuged for an additional 15 minutes. Combined pellet fractions were then resuspended in 300  $\mu$ l Tris buffer at pH 7 to match the pH of *A. curassavica* latex. We tested monarch larvae with four treatments: fresh *A. curassavica* latex, supernatant, pellet in Tris buffer, and Tris buffer control.

### Experiment D

In the preceding experiment, the supernatant triggered renewed vein cutting. To determine the polarity of the active compound(s), we dried the supernatant under vacuum, then extracted it three times per solvent first with methylene chloride, then methanol. The methylene chloride extracts were combined, dried and redissolved in 20 % Tween 80 equal in volume to the supernatant. The methanol rinses were treated similarly, but were redissolved in water. Lastly, the residual supernatant was extracted with water. Monarch larvae were tested with fresh *A. curassavica* latex, the methylene chloride, methanol and aqueous extracts, a 20 % Tween 80 control, and a water control. We anticipated that cardenolides, which are steroids, would occur primarily in the methylene chloride fraction. This experiment thus provides a second test of the importance of cardenolides as vein-cutting stimulants.

### Experiment E

To test if the vein-cutting stimulant is a protein, we treated the active methanol fraction with proteinase K, a nonspecific proteinase that digests most proteins (Kraus & Femfert 1976; Sambrook *et al.* 1989). Larvae were tested with three treatments: dried methanol fraction redissolved in 0.01M Tris, dried methanol fraction treated with proteinase K (Sigma, 1  $\mu$ g/ $\mu$ l) in 0.01M Tris (pH 7.8), proteinase K control in 0.01M Tris (pH 7.8). All three solutions were incubated for one hour at 37 °C before the bioassays to allow the proteinase to degrade proteins.

To test if the active methanol fraction contained cardenolides, we redissolved the extract in water, then spotted 5  $\mu$ l onto filter paper impregnated with 2,2',4,4'-tetranitrodiphenyl (TNDP). TNDP was synthesized using the procedure in Brower *et al.* (1972). When treated with 10 % aqueous NaOH, the TNDP reacts with cardenolides to produce a blue color. TNDP is commonly used as a diagnostic test for cardenolides, although some non-cardenolide ketones can also give positive responses (Sady & Seiber 1991). To estimate cardenolide concentrations, we prepared a concentration series of the cardenolide digitoxin and visually compared the intensity of spots produced by digitoxin and latex extracts.

## Results

All larvae tested with fresh *A. curassavica* latex resumed vein cutting, whereas none of the controls were active (Table 1). Interestingly, the cardenolide mixture and sticky PEG solution were both completely inactive (Table 1A, B) suggesting that neither cardenolides nor latex adhesives are vein-cutting stimulants. Activity resided within the transparent yellow supernatant fraction (Table 1C), which lacked the viscosity and adhesiveness of whole latex. Of the three solvent extracts tested, only the methanol fraction of the supernatant elicited vein cutting (Table 1D). Proteinase K did not alter its activity indicating that the active substance is unlikely to be a protein (Table 1E). When tested with TNDP, the methanol fraction showed a faint blue reaction similar in color to a control spot containing 50  $\mu$ g/ml digitoxin and much less distinct than the bright blue spot produced by the methylene chloride fraction. The slight response of the

**Table 1** Number of final instar monarch larvae on *A. curassavica* that resumed vein cutting after receiving 2  $\mu$ l of test solution. All solutions were tested with ten larvae; each larva was tested with only one solution. Asterisks indicate solutions that elicited vein cutting in significantly more larvae than the control ( $P < 0.05$  Fisher exact tests).

<b>A. Cardenolides</b>	
<i>Asclepias curassavica</i> latex	10*
Cardenolide extract from <i>Calotropis procera</i> in 20 % Tween 80	0
20 % Tween 80 control	0
<b>B. Adhesives</b>	
<i>A. curassavica</i> latex	10*
Polyethylene glycol (MW 15,000-20,000; 0.5 g/ml water)	0
Water control	0
<b>C. Centrifugation</b>	
<i>A. curassavica</i> latex	10*
Pellet from <i>A. curassavica</i> latex resuspended in Tris buffer	1
Supernatant from <i>A. curassavica</i> latex	10*
Tris buffer control	0
<b>D. Solvent fractionation</b>	
Supernatant from <i>A. curassavica</i> latex	10*
Methylene chloride extract redissolved in 20 % Tween 80	0
Methanol extract redissolved in water	8*
Aqueous extract	0
20 % Tween 80 control	0
Water control	0
<b>E. Protein digestion</b>	
Methanol extract of supernatant redissolved in water	7*
Methanol extract + proteinase K in Tris buffer	7*
Proteinase K + Tris buffer control	0

methanol fraction can be attributed to trace amounts of cardenolides or other substances that react with TNDP. The brighter reaction of the methylene chloride extract indicates that cardenolides occurred predominantly in this fraction, which did not elicit vein cutting (Table 1D).

## Discussion

Like many latices, milkweed latex becomes increasingly sticky as it dries. Our results suggest, however, that adhesiveness is not the cue that elicits vein cutting by final instar monarchs. The adhesive solution of PEG and the pellet fraction containing latex polymers were both completely inactive; none of the larvae revisited their vein cut, whereas all larvae tested with fresh latex resumed vein cutting.

Our results further indicate that low polarity cardenolides are ineffective stimulants of vein cutting. Monarch larvae sequester milkweed cardenolides, which are retained by the adults as a defense against predators (Reichstein *et al.* 1968; Brower 1969; Malcolm 1995). Several studies have documented that cardenolides incorporated into larval diets have little or no negative effects on monarchs (Seiber *et al.* 1980; Cohen 1983), apparently due to the monarch's altered Na<sup>+</sup>, K<sup>+</sup>-ATPase gene that confers insensitivity (Holzinger & Wink 1996). However, other papers report inverse correlations between monarch growth rate and cardenolide levels in milkweed hosts (Zalucki & Brower 1992; Zalucki *et al.* 2001a, b), and Malcolm (1995) suggests that very polar cardenolides are toxic to monarchs. We cannot exclude polar cardenolides as possible vein-cutting stimulants since these compounds may have been absent from the

Sigma cardenolide mixture we used and our active methanol fraction showed a faint reaction to TNDP. However, the complete lack of activity of both the cardenolide mixture and the cardenolide-rich methylene chloride fraction suggests that noncardenolide substances in *A. curassavica* trigger vein cutting. This view is supported by the observation that monarch larvae cut veins not only in cardenolide-rich *Asclepias*, but also in the milkweed vine *Cynanchum laeve* (Dussourd unpub. obs.). Cardenolides have not been reported from *Cynanchum* (Hoch 1961; Hegnauer 1964) and appear to be lacking throughout the *Cynanchinae* (S. Liede pers. comm.).

Milkweed latex contains diverse substances besides cardenolides. Various enzymes have been isolated (Shukla & Murti 1971; Giordani *et al.* 2000), including cysteine proteases, which occur in the latex of many plants and are toxic to some caterpillars (Arribere *et al.* 1998; Konno *et al.* 2004). However, it is unlikely that enzymes trigger vein cutting by monarchs because the active compound was soluble in methanol and proteinase K did not reduce its activity.

Noncardenolide chemicals that have been extracted from milkweed latex include resins, sterols, rubber and other terpenoids (Paul *et al.* 1943; Nielsen *et al.* 1977; Arribere *et al.* 1998; Giordani *et al.* 2000). Additional compounds have been isolated from sectioned or entire milkweed plants and might occur partly or entirely in the latex. These include pregnane glycosides, alkaloids, cyclitols, and flavonol glycosides found in various *Asclepias* species and other milkweeds (Abe & Yamauchi 2000; Abe *et al.* 2000; 2001; An *et al.* 2001; Haribal & Renwick 1998; Marion 1939; Warashina & Noro 2000). To our knowledge, no information is available on how these diverse chemicals affect monarch larvae. Their presence may explain not only why monarchs cut leaf veins, but also other

intriguing aspects of monarch biology such as why caterpillars often abandon milkweed plants that still have abundant foliage (Vickerman & de Boer 2002).

A chemical trigger for vein cutting has not been identified for any insect. However, three stimulants of trenching, a related behavior that also functions to deactivate defensive canals, have been identified for the cabbage looper, *Trichoplusia ni* (Dussourd 1997; 2003). Lactucin from lettuce latex, myristicin from parsley, and lobeline from cardinal flower all trigger trenching by naïve cabbage loopers feeding on *Plantago lanceolata*, a plant species that lacks secretory canals and ordinarily does not elicit trenching. The known toxicity of myristicin to diverse insects and the neuroactivity of lactucin, myristicin, and lobeline (refs. in Dussourd 2003) suggest that cabbage loopers cut trenches specifically to reduce their exposure to trenching stimulants. Identifying the vein-cutting stimulant(s) for monarchs thus has potential for uncovering a new milkweed defense, which would be a significant discovery given the historical importance of the monarch-milkweed system in chemical ecology (Malcolm 1995) and widespread public interest in this charismatic insect (e.g. Monarch Watch, <http://www.monarchwatch.org>). Monarchs develop exclusively on milkweeds (Vickerman & deBoer 2002), but other vein-cutting insects feed on diverse plants with secretory canals (Dussourd & Denno 1991; Dussourd 1993; Evans *et al.* 2000). The monarch assay could be adapted to these insects to guide the isolation of likely defensive compounds from their varied host plants as well.

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