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Failure-time Analyses of the Effectiveness of Larval Shield Defenses in Tortoise Beetles (Chrysomelidae: Cassidinae)[1]

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Abstract. Plant chemistry and predation are thought to be major factors responsible for the recurrent evolution of dietary specialization in herbivorous insects. However, their relative importance and the degree to which they interact to drive diet evolution remain unknown. The present study aims to test predictions of the 'nasty host plant hypothesis', which posits that an herbivore's diet becomes more restricted as its reliance upon novel host plant compounds that confer protection from predators increases. The tortoise beetle larval shield system affords a unique opportunity to examine how predation and host plant chemistry interact. Shields can be micro-manipulated, including removal, chemical modification and reattachment, without harm to the larvae. We subjected larvae of different diet breadths produced from basal and derived hosts to a predation bioassay and compared the relative effectiveness of their shields under different treatment conditions. Failure-time analyses, the most appropriate statistical approach for right-censored temporal data, revealed that specialist larvae were consistently less susceptible to predation than were generalists feeding on the same plant. Although generalists were as competent as specialists at handling non-polar host chemistry, specialists were better at manipulating more polar host-derived compounds, which are more likely to include novel chemistry. Host shifts may be constrained to only those plants that possess novel, polar compounds. The interaction between plant chemistry and beetle diet evolution may be one of escalation driven by predation, wherein specialists are increasingly more effective than generalists in the assimilation of host plant polar compounds into shield defenses.

Key words. Chrysomelidae, larval shield, *Azteca*, tortoise beetle, Hispinae, predation, chemical defense, failure-time analysis

1. INTRODUCTION

Approximately three-quarters of Earth's biodiversity is involved in a tri-trophic interaction among plants, herbivorous insects, and insect enemies (STRONG et al. 1984; SOUTHWOOD 1996). However, herbivory represents an ecological obstacle that few groups have been able to surmount and only nine of twenty-nine insect orders have succeeded in colonizing the plant resource spectrum. The vast majority of herbivorous insects are members of either the Lepidoptera or the Coleoptera. Chief among the many obstacles to plant-feeding are (1) bottom-up, plant related physiological factors, and, (2) top-down, ecological factors (FUTUYMA & KEESE 1992; DYER & FLOYD 1993). The major bottom-up obstacle is plant secondary chemistry, i.e., those compounds not involved in photosynthesis or respiration. Over 20 thousand such compounds have been discovered so far and most of them have known resistance or defensive functions. The major top-down obstacle is predation.

What is the relationship between the astonishing array of plant secondary compounds and insect enemies? The fact is that the vast majority of herbivorous insects are dietary specialists that can feed on only one or a few related plants (EHRLICH & RAVEN 1964; FUTUYMA & KEESE 1992; BERNAYS & CHAPMAN 1994). Why an insect would evolve a narrower, rather than a broader diet, presents an ecological conundrum: the likelihood of failing to locate a suitable host plant increases in the short life span of an individual insect with a narrow diet range. How plant chemistry and predation interact and their relative importances in the recurrent evolution of dietary specialization remains unknown.

Selection by both natural enemies and plant chemistry could in concert, result in a net narrowing of the herbivore's host range, providing that range limitation affords better protection. We wished to test the 'nasty host plant hypothesis' (hereafter ***nhph***), an hypothesis derived and modified from the parasitoid/insect host literature for application to the broader host plant/insect herbivory/predator reality of the Earth (from GUALD et al. 1992). At the micro-evolutionary time scale, ***nhph*** predicts that an insect will evolve a narrower diet, if, through the assimilation of the noxious compounds acquired from its host, it is rendered less vulnerable to its natural enemies. In macroevolutionary time, ***nhph*** also predicts that host shifts will always be to more, rather than less noxious plants. Specifically ***nhph*** predicts that, (1) when grown on the same host, the specialist should "handle", i.e. sequester, the host's chemistry better than the corresponding generalist does, both quantitatively and qualitatively; (2) specialists should fare better than generalists when subjected to natural predators in bioas-

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says; (3) derived specialists will fare better than basal specialists do in bioassays, and finally; (4) derived specialists will have novel compounds in their defenses that account for the difference in efficacy.

While evidence exists both for (SMILEY et al. 1985; STAMP & BOWERS 1992; DYER & FLOYD 1993; MONTLLOR & BERNAYS 1993; METCALF 1994; DYER 1995; VENCL & MORTON 1998) and against ***nhph*** (STAMP 1992; RANK et. al. 1996; DOBLER et al. 1997; KÖPF et al. 1998; BECERRA & VENABLE 1999), a test of its ability to explain how narrow diets evolve, or to predict the direction of host shifts, is still lacking. Such a test would require a combinatorial approach that includes chemical, and mechanistic analyses, field ecology, within a framework of phylogenetically informed experiments (FUTUYMA 2000). The present study presents preliminary results from an ongoing research program underway in Panamá testing the specific predictions of ***nhph***.

Our study system, the larval tortoise beetle shield, is well suited for the investigation of how plant chemistry and predation might interact to favor the evolution of narrow diet breadths. Tortoise beetle larvae are soft-bodied, leaf surface grazers, and as such, are very apparent and predictable targets for predators and parasitoids. Their shields are composite structures formed from the exuviae and accumulated fecula (Fig. 1A). Shields are attached to a mobile infrastructure, the furca, which emanates from the tip of the abdomen (Fig. 1B). Tortoise beetle larvae possess a bizarre telescoping anus that serves to precisely deposit fecal material on the furca-exuvia complex (Fig. 1C). Shields can be aimed and rapidly waved in the path of an attacking enemy (Fig. 1D). In addition to being physical barriers, shields have been shown to contain a plant-derived, chemical component that significantly enhances their effectiveness as an anti-predator defense (GÓMEZ 1997; GÓMEZ et al. 1999; MÜLLER & HILKER 1999; VENCL et al. 1999). Shields are suitable for examining the host plant chemistry/predation interaction because they can be easily removed, chemically modified, and then reattached without otherwise harming the larvae (OLMSTEAD & DENNO 1993; VENCL et al. 1999).

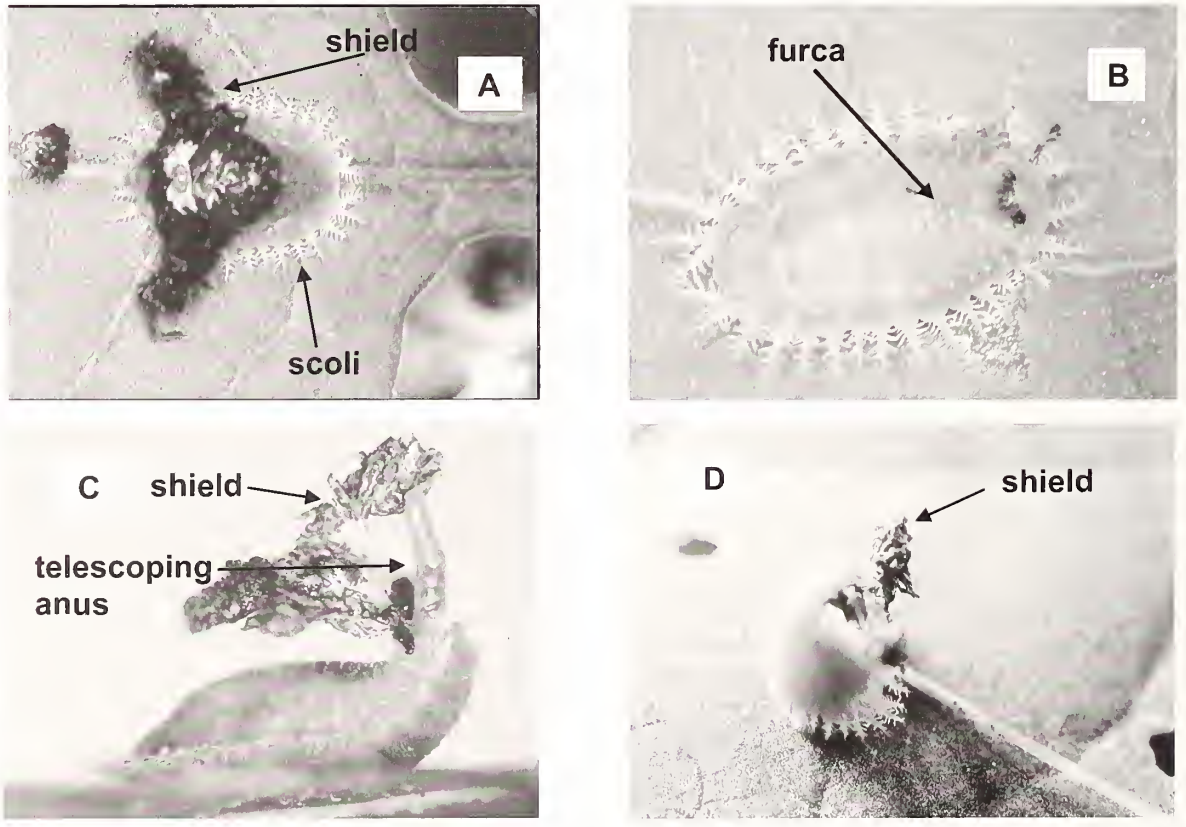

Fig. 1. Tortoise beetle larval shield system. (A) Dorsal aspect of shield (dark structure) and larval body with anterior oriented to the right. Scoli project laterally around the larval body and are probably sensory receptors. Three cast skins (exuviae) clearly visible within shield matrix; (B) De-shielded larvae with two-tined furca emanating from tip of abdomen and projecting above the larva; (C) Lateral view of larva with telescoping anus extended and applying fecula to shield; and (D) Shield tilted and waving in direction of perturbation (cat whisker) touching anterior end of larva.

Our objective was to test the predictions of ***nhph*** by examining the relative susceptibilities of generalist and of specialist tortoise beetle larvae to predation by a ubiquitous generalist ant predator, *Azteca,* in the Panamanian rain forest. We compared the effectiveness of shield defenses of three tortoise beetle species with contrasting diet ranges. To control for the potentially confounding effects of host plant chemistry on the behavior of predators, larvae of both diet-range types were reared on the specialist's host plant, members of the morning glory family, Convolvulaceae. We made two specialist-generalist dietary contrasts: (1) *Acromis sparsa*, a specialist on *Merremia umbellata,* against the generalist, *Chelymorpha alternans,* hereafter *Cm*, also reared on *M. umbellata* ; and (2) *Stolas plagiata,* a specialist on *Ipomoea phillomega,* once more against the generalist, *C. alternans,* hereafter *Cp,* also reared on *I. phillomega.* To address the importance of larval shield chemistry on ant behavior, shields of each species-host plant combination were subjected to micromanipulation and one of four leaching treatments prior to bioassay trials.

2. METHODS

Bioassays were conducted in Gamboa, Republic of Panamá, between 8:00 AM and 12:00 PM, during July, August, and September of 2003. *Azteca* ants (Hymenoptera: Formicidae: Dolichoderinae) are common, fierce, generalist predators in Neotropical lowland rainforests (CARROLL 1983; HÖLLDOBLER & WILSON 1990). We used *A. lacrymosa* as an assay agent. This species builds large carton nests attached to the boles of trees. They are extremely aggressive, strongly recruiting, and foraged primarily in the area in and around their home tree. One month prior to the onset of the bioassay experiments, we set potted individuals of *M. umbellata* and *I. phillomega* at the base of the home tree. Host vines were placed in contact with the tree trunks to enable the ants to use the host plants as foraging areas. We encouraged routine patrolling of *M. umbellata* and *I. phillomega* vines by the ants by regularly baiting host plant leaves with larva-sized tuna fragments.

Shield micromanipulation. Larval sibships of each species-host plant combination were equally divided at random among the following treatment groups: (1) water (H_2O); (2) methanol (MeOH); (3) both (H_2O followed by MeOH); and (4) unleached (intact) control. So that we could clearly observe the effects of diet range on shield chemistry in the absence of larval behavior, ant bioassay experiments were done using fourth-instar larvae freshly killed by freezing for 5 min. We immediately removed each larval shield by placing fine forceps between the tines of the furca and gently lifting the shield away from the body. Shields were then soaked for 25-30 min in a solvent bath agitated every five min (or two consecutive baths of 12-15 min each in the case of larvae assigned to the treatment with both H_2O and MeOH). After soaking, shields were dried on paper toweling under an incandescent light bulb and slow fan for 45 min. Each shield was re-attached to the larval furca using rapid-setting, fumeless, water-insoluble craft glue (DAP) that had been warmed to 28° C for five min to minimize setting time. Larvae with re-attached shields were allowed to stand and dry for at least 20 min before bioassays were begun. Controls consisted of the shield removal and reattachment manipulations, but no leaching.

To ensure high levels of ant activity on the host plants during bioassay trials, 45 min before the experiments began we baited each plant with pieces of tuna of about the same size as a fourth-instar larva. Each trial consisted of the presentation of an individual larva to foraging ants on either *M. umbellata* or *I. phillomega.* Initially, individuals from each treatment group were randomly assigned to different host plants with dice. Following the first round of testing the delegation of larvae to host plants was constrained by the previous round, such that no treatment group was tested on the same plant more than once nor tested consecutively. Only one trial was done on a given host plant before moving to the next tree, and a minimum of five min elapsed between each trial.

Using soft forceps, we placed each experimental larva near the center of a host plant leaf along the mid-vein. This formed the bioassay test arena. To avoid contamination, forceps were dedicated to a single treatment and were dipped in water and whipped to dryness between trials. A trial was started if there were at least two, but no more than five ants foraging on the leaf. Each trial lasted five minutes, or until the test larva was captured. A capture event was considered to be the movement of the test larva ≥ 1 cm toward the leaf petiole by the ants. Trials were recorded with a Panasonic digital video camera (PV-DV951) mounted on a tripod positioned such that the entire test leaf was included in the field of view. We started video recording at first contact of an ant with the experimental larva and measured the number of seconds elapsed between the start of the trial and a capture event (or the end of the trial period, whichever came first). Between 35 and 45 replicates of each solvent treatment were done for each of the four larval species-host plant combinations.

Statistical analyses. We examined the effects of larval diet range and shield chemistry on the time to capture by *Azteca* ants using failure-time statistics (PROC LIFETEST; SAS v. 9.0) (reviewed by FOX 2001). In contrast to classical methods such as ANOVA that compare either the total number of captures at the end of the experimental time interval or the mean time to capture among treatment groups, failure-time methods

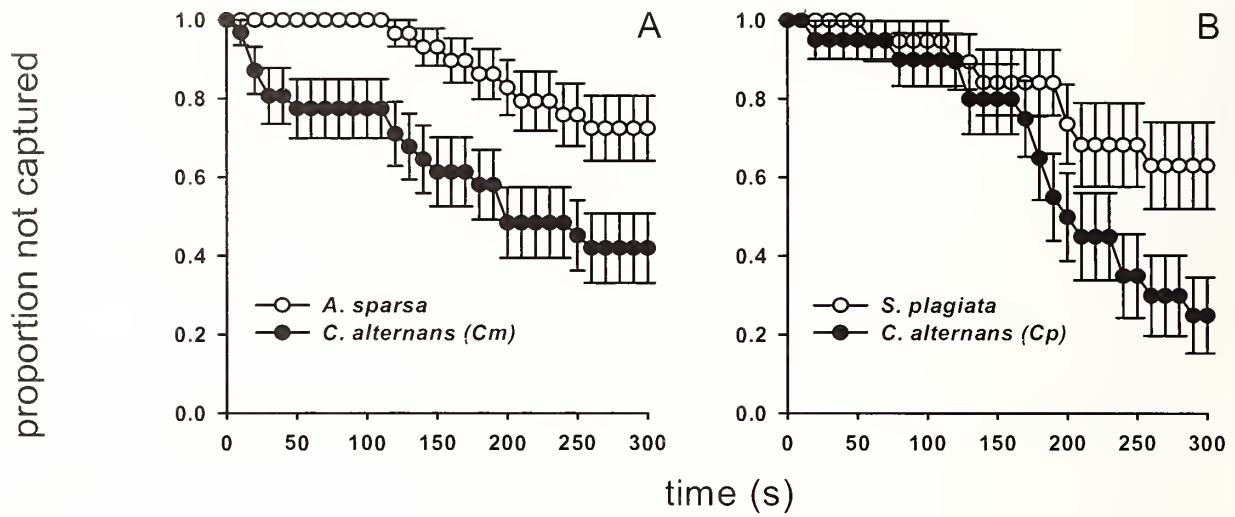

Fig. 2. Cumulative capture curves for specialist (open circles) and generalist (filled circles) tortoise beetle larvae with intact, unleached shields in Azteca ant bioassay. Larvae of both diet ranges were raised on either (A) *Merremia umbellata* (Contrast #1) or (B) *Ipomoea phillomega* (Contrast #2). Data points are the cumulative fraction of the cohort not yet captured (mean ±SE) for each 10 s interval in the bioassay. Both contrasts were statistically significant at the 0.05 level based on Bonferroni-corrected pairwise comparisons following a Wilcoxon signed-rank test for heterogeneity among all four groups ($P = 0.0040$).

compare the distributions of capture times over the entire experimental period. Times to the occurrence of a failure event (e.g., capture of a larva by ants) do not typically meet the distributional assumptions required by traditional parametric approaches. Such data often lack equal variances and normal distributions. In addition, many of the trials ended before a capture event was recorded (so-called right-censored data). As a consequence, the ultimate fate of the experimental larva beyond the 5 min census interval was unknown. Analysis of variance and related tests are unable to account for censored data, however, failure-time methods are not so limited. Cumulative capture functions were compared using a Wilcoxon signed rank test followed by pairwise multiple comparisons to determine specific differences between treatment groups (KALBFLEISCH & PRENTICE 1980). Significance levels were corrected using the sequential Bonferroni technique (Dunn-Sidák method; SOKAL & ROHLF 1995). This method is less conservative than the standard Bonferroni technique but ensures that an appropriate experiment-wise error rate is maintained.

Differences among groups in the proportion of larvae captured were assessed with likelihood-ratio chi-squared tests of independence (SOKAL & ROHLF 1995).

3. RESULTS

Failure-time analyses revealed pronounced differences between generalist and specialist larvae with both untreated and solvent-leached shields in susceptibility to predation by *Azteca* ants. The relative performance through time of generalists and specialists with untreated shields is shown in Figure 2. In both diet range contrasts, the specialists were consistently less susceptible to predation than were generalist larvae. Specialist larvae were also significantly less likely than generalists to have been captured by the end of a trial (Contrast #1: $G^2 = 5.78$, $P = 0.016$, Contrast #2: $G^2 = 5.92$, $P = 0.015$).

Tortoise beetle shield chemistry. The effects of solvent leaching of shields on larval susceptibility to predation were striking. In both contrasts, regardless of diet range, the decay in capture curves for shields leached by some or all solvents (H_2O, MeOH or both) was significantly steeper than in curves for intact, unleached shields (Figs 3 and 4; Table 1). For both specialists, shield leaching by H_2O had stronger effects on larval capture rates than leaching by MeOH. In contrast, there appears to be an interaction in the effects of different solvents on shields of generalist larvae (although our experimental design unfortunately does not allow for a statistical test of this possibility). Leaching by MeOH had a larger (negative) effect than leaching by H_2O on capture rates of *C. alternans* grown on *Merremia umbellata* (Contrast #1) compared to untreated controls. However, the opposite pattern exists for *C. alternans* grown on *Ipomoea phillomega* (Contrast #2) (Figs. 3 and 4; Table 1).

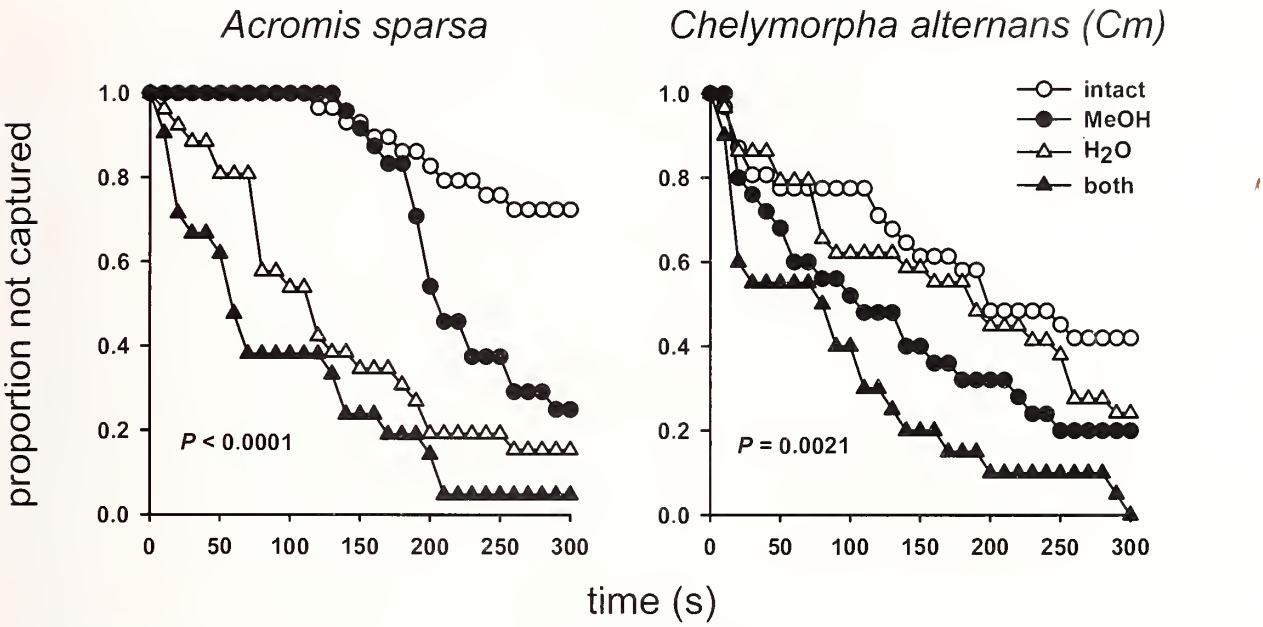

Fig. 3. Cumulative capture functions for specialist (*A. sparsa*) and generalist (*C. alternans, Cm*) tortoise beetle larvae raised on *Merremia umbellata* (Contrast #1) with intact and solvent-leached shields in the *Azteca* ant bioassay. *P*-values are from a Wilcoxon signed-rank test for heterogeneity among groups. Statistical results of Bonferroni-corrected pairwise comparisons are presented in Table 1. Error bars were eliminated for clarity.

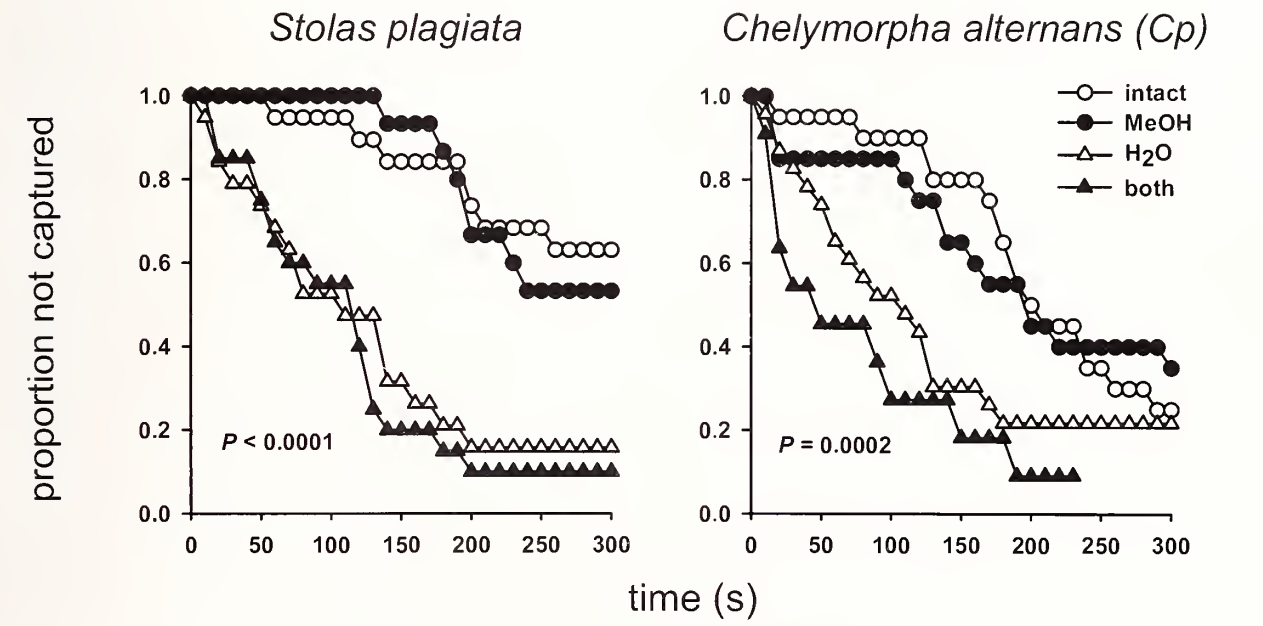

Fig. 4. Cumulative capture functions for specialist (*S. plagiata*) and generalist (*C. alternans, Cp*) tortoise beetle larvae raised on *Ipomoea phillomega* (Contrast #2) with intact and solvent-leached shields in the *Azteca* ant bioassay. *P*-values are from a Wilcoxon signed-rank test for heterogeneity among groups. Statistical results of Bonferroni-corrected pairwise comparisons are presented in Table 1. Error bars were eliminated for clarity.

Table 1. Pairwise multiple comparisons of cumulative capture curves for specialist (*A. sparsa* and *S. plagiata*) and generalist (*C. alternans*) tortoise beetle larvae with intact and solvent-leached shields in the *Aztèca* ant bioassay (Figs 3 and 4). Larvae of both diet ranges were raised on either *Merremia umbellata* (Contrast #1) or *Ipomoea phillomega* (Contrast #2). To keep the experiment-wise error rate at the 0.05 level, comparisons were done using a sequential Bonferroni approach (Dunn-Sidák method; Sokal and Rohlf 1995) following Wilcoxon signed-rank tests. Individual comparisons marked with an asterisk (*) were statistically significant.

	Contrast #1		Contrast #2	
Shield type	***A. sparsa***	***Cm***	***S. plagiata***	***Cp***
intact v. MeOH	*	*ns*	*ns*	*ns*
intact v. H_2O	*	*ns*	*	*
intact v. both	*	*	*	*
MeOH v. H_2O	*	*ns*	*	*
MeOH v. both	*	*ns*	*	*
H_2O v. both	*ns*	*	*ns*	*ns*

4. DISCUSSION

4.1. Are specialists better defended than generalists?

Our findings support the central tenet of ***nhph***, the 'nasty host plant hypothesis', which posits that when both are feeding on the same host plant, specialist tortoise beetle larvae will be better defended against predation by their shields than are generalist larvae in an ant bioassay. We found that specialists with unleached, intact shields clearly outperformed their generalist counterparts in both diet contrasts we tested. Furthermore in both contrasts, we observed that all solvent leaching treatments (H_2O, MeOH, and both) significantly degraded shield effectiveness, regardless of diet range. This latter finding supports the idea that shields have a critically important chemical component that consists of both polar and non-polar compounds.

4.2. Is the chemical defense derived from the host plant?

Although a definitive answer to the question of the provenance of shield compounds must await comparisons of plant and shield chemistries, evidence from this study supports the idea that shields are fortified with host-derived metabolites. If a shield system were based on autogenous synthesis, shield chemistry would be the same regardless of diet and thus one would not expect to observe differences in shield performance. When we compared the shield performances of the same beetle, *C. alternans*, reared on different host plants, we observed that effect of a particular leaching treatment depends on which plant the generalist fed upon, supporting the idea that at least part of larval shield chemistry is host-derived.

In all the other fecal shield-bearing species, including several tortoise beetles studied so far, shield chemistry is based on precursors obtained from the host plant (Gómez 1997; Morton & Vencl 1998; Vencl & Morton 1998; Müller & Hilker 1999). Fecula-laced shields may well represent a type of sequestered defense. If their chemical constituents were found to be host-derived, the shields of the tortoise beetles in this study would also strongly resemble sequestering types of defenses.

4.3. Do specialists sequester host chemistry better than generalists?

Our findings lend support to the ***nhph*** prediction that specialists are more competent than are their generalist counterparts at handling host plant chemistry. Our data show that the H_2O leaching treatment, which removed many of the more polar compounds from the larval shields, significantly increased the susceptibility of both specialist larvae to predation. In contrast, the MeOH leaching treatment had a greater impact on the generalist feeding on one host but not the other. Superior chemical sequestration might involve one or more of the following strategies: greater bio-concentration of a particular compound, differential sequestration of a variety of compounds, or the modification of the compounds.

Many specialist herbivores are known to have enhanced mechanisms for the sequestration and/or the transformation of plant metabolites into defensive compounds (Bowers 1988; Pasteels et al. 1983, 1988; Denno et al. 1990). Some specialist herbivores have been found to more efficiently excrete or egest host plant secondary compounds (Self et al. 1964; Berenbaum 1983; Ferguson et al. 1985; Metcalf 1994). We think that the tortoise beetle shield system is a type of sequestration

process that is relatively inexpensive (OLMSTEAD & DENNO 1993), possibly because tortoise beetle specialists are superior at harvesting and modifying host metabolites compared to their generalist counterparts feeding on the same hosts.

Since they cannot readily transit cell membranes, nonpolar compounds are arguably more difficult to manipulate because they first must be modified in order to contain them within the larval gut (DUFFY 1980). The process of gut compartmentalization requires that a compound be made more polar through mechanisms like hydroxylation or conjugation (DUFFY 1980; BOWERS 1988). Our data suggest that given identical dietary inputs, specialists are more competent at transforming less polar into more polar compounds, and thus disproportionately fortifying their fecula with more polar compounds, compared to their generalist counterparts. The identity and defensive characteristics of these more polar shield constituents must await future structural elucidation. Suffice it to say that many classes of polar substances are well known to have deterrent and toxic characteristics. Some of these more polar compound classes include pyrrolizidine alkaloids, phenolics, cardenolides, sapogenines, and flavonoids.

4.4. Adaptation or accident?

How herbivorous insects use their host plants for chemical defense, i.e., by processes of sequestration, may entail quite different physiological mechanisms from those used by tortoise beetles for processing host compounds into shield fecula. Does shield formation require special adaptations or are shields passive consequences of host consumption? Based on findings from shield-forming leaf beetles in other chrysomelid subfamilies, VENCL & MORTON (1998) have suggested that shields are not 'default' waste tanks, but instead are adaptations for defense. They argue that since shields contain a highly culled subset of ingested host derived precursors, some of which are nutrients and some of which are modified within the larval gut, fecal shield formation must have entailed the evolution of specialized enzymes that now serve the triple defensive functions of: (1) selective compound egestion through compartmentalization, (2) compound bio-concentration, and (3) compound bio-activation (VENCL et al. 1999). The unusual telescoping anus of tortoise beetles is *de facto* evidence of a specialized adaptation for the precise deposition of fecula on the shield framework (see Fig. 1B). There is good evidence from another tortoise beetle, *Plagiometriona clavata,* supporting the contention that shields with fecular retention represent specialized adaptations for predator defense (VENCL et al. 1999). For example, palmitic acid is one of the 'discarded' compounds that eventually ends up in *P. clavata's* shield. An erstwhile nutrient, it occurs in the fecula in relatively higher concentrations than it does in the host. Palmitic acid also elicits necrophoresis (undertaker behavior) in ants, whereby anything emitting it gets placed on the ant nest's dump (BLUM 1970). Palmitic acid therefore appears to be more beneficial as part of the larval defense system than as a dietary nutrient.

Our findings for tortoise beetles are the first instances where specialists have been shown to derive an advantage (enemy-free space) over their respective generalists when reared on the specialist's host plant. This conclusion is in overall agreement with previous studies on Lepidoptera (STAMP & BOWERS 1992; DYER & FLOYD 1993; CORNELIUS & BERNAYS 1995, but see STAMP 1992). It is important to note that the Lepidoptera and herbivorous Coleoptera represent well over half the insects attacking plants and most of these herbivores are dietary specialists (STRONG et al. 1984). More work is necessary to determine if the relationship between the effectiveness of plant-derived, anti-predator chemical defenses and specialization is a general one that has influenced the evolution of diet range in beetles and possibly in other phytophagous insects.

We are in the process of determining the origins and elucidating the chemical structures of the compounds responsible for the effects observed in this study. At a macroevolutionary level, ***nhph*** predicts that derived specialists will have novel, more polar compounds in their defenses that account for increased shield efficacy when compared to generalists or to basal specialists. If so, then the remarkably robust tortoise beetle radiation might have been fostered by the colonization of increasingly more chemically complex dicotyledonous plants. A process of defensive escalation may have enhanced the likelihood of beetle speciation. Whether host shifts have always been to plants containing more, rather than less potent chemistry must await further studies of more basal specialists and generalists in the tortoise beetle radiation. It is also essential to determine if selection on shield chemistry is diffuse, or whether each predator's selective impact is idiosyncratic, in which case, we would expect specificity in targeting of particular shield chemicals. We are currently undertaking studies to clarify these related issues.

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