Functional interactions among tortoise beetle larval defenses reveal trait suites and escalation

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ORIGINAL PAPER

Functional interactions among tortoise beetle larval defenses reveal trait suites and escalation

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Abstract Whereas the "escape-and-radiate" plant-herbivore scenario predicts that reciprocating cycles of defense-counter defense foster the evolution of traits with increasing efficacy that accumulate during clade diversification, coevolutionary models of herbivore responses to their enemies remain unexplored. Quantitative information is scarce about how defensive traits perform, interact and become functionally integrated. Moreover, there are few studies that have combined performance and phylogenetic information to detect patterns of trait assembly and trends in defense efficacy. Using field demonstrations of effectiveness and phylogenetic reconstructions, we evaluated patterns of trait precedence and suite assembly by comparing the larval defenses of two beetles, *Acromis sparsa* and *Chelymorpha alternans*, which both feed on the leaf surfaces of the same plant, have shields containing host-derived deterrent chemicals and form aggregations. Additionally, female *A. sparsa* guard their larvae. Using an ecologically relevant bioassay, we quantified the extent to which: (1) gregariousness, size, maternal care and shields affected survival; (2) defenses interacted, and; (3) derived traits and suites outperformed ancestral ones. Regression models ranked traits revealing synergistic interactions. Shields interacted with gregariousness to form the strongest suite. Maternal care contributed to overall higher survival in *A. sparsa*, an advantage lost after female removal. Phylogenetic reconstruction revealed a sequence of trait accumulation and suite formation. The combined performance-phylogenetic approach revealed: (1) multi-trait interactions amplified effectiveness; (2) a sequence of novel trait origins was followed by suite assembly, and; (3) an incremental trend in defense efficacy congruent with escalation. Multi-trait interactions fostered suite assembly that likely conferred the advantage of enhanced survival in the precarious leaf surface adaptive zone.

Keywords Failure-time analysis · Shield · Gregariousness · Maternal care · Bayesian · Character reconstruction · Cassidinae · Splits analysis

Introduction

In contrast to their adult stage, externally leaf-feeding larval insects are flightless, slow moving and soft-bodied. Because they are so vulnerable, herbivorous larvae often suffer mortality rates approaching 90% (Stamp and Casey 1993 and references therein; Cornell et al. 1998; Hunter 2000). Due to the strong impact of numerous enemies,

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exposed leaf-feeding (i.e., ectophagous) larval herbivores have evolved remarkably diverse arsenals of morphological, chemical and behavioral defenses (Gross 1993; Zalucki et al. 2002). Larval defenses, namely, those resistance traits that increase survival in the presence of enemies, are likely to be complex for three reasons. Firstly, a defensive trait should seldom be expected to function in isolation because varied or persistent enemies can select among several traits resulting in both negative (trade-off) and positive performance correlations (Abrams 2000; reviewed by Relyea 2003; Mikolajewski et al. 2006). Secondly, if by deploying traits together, an herbivore gains greater protection (i.e., from synergisms) than from the sum of their separate effects, then selection should favor trait integration into multi-trait suites (e.g., Ghalambor et al. 2003). Such trait suites can thus acquire their own evolutionary trajectories (Agrawal et al. 2009a). Thirdly, synergistic interactions may result in the functional melding of many formerly developmentally uncoupled traits, analogous to the processes of modular organization, and plasticity that are thought to facilitate both trait integration and change in morphology (West-Eberhard 2003). Hence, trait interactions may not be adequately described by classic bi-variate correlations (Agrawal and Fishbein 2006). Trade-offs, if they occur, would be expected at the level of the multi-trait phenotypes that form overall defensive strategies within lineages (Stephens and Wiens 2008; Armbruster et al. 2009). However, neither the prevalence of such multi-trait interactions, directional trends in trait effectiveness, nor the replacement of older, multi-trait strategies by newer ones with higher performance, are well explored within a phylogenetic context (Härlin and Härlin 2003; Agrawal 2007; Futuyma and Agrawal 2009).

In perhaps the only well-investigated multi-trait larval defense found in several lepidopteran lineages, caterpillars have recurrently evolved gregarious behavior and unprofitability (unpalatability), in conjunction with warning signals that form an aposematic suite (Sillén-Tullberg 1988). However, interactions among traits that might foster suite formation have scarcely been quantified. In a seminal study measuring trait correlations, Gentry and Dyer (2002) demonstrated that gregarious caterpillars that regurgitated unpalatable fluids, thrashed, and bit attacking wasps, fared better than solitary caterpillars using these same tactics. In addition to the broader possibility of multi-trait interactions increasing defense potency, the expected order of trait evolution (viz. trait precedence) is an unresolved issue that also bears on the evolution of trait interactions and multi-trait suite formation. For example, theoretical predictions are conflicting about whether a caterpillar defense should precede, or follow the evolution of either a warning signal or gregariousness. Phylogenetic analyses using independent contrasts suggest that caterpillar defenses generally precede the evolution of aposematism and gregariousness (Tullberg and Hunter 1996; reviewed by Ruxton and Sherratt 2006). However, neither trait precedence, multi-trait interactions nor suite assembly have not been rigorously examined in other major herbivore lineages with externally feeding (i.e., ectophagous) immature stages, such as those of the Coleoptera (e.g., Chrysomelidae), epilachnine Coccinelidae, Heteroptera (Membracidae), or Orthoptera (Acrididae).

Here, we present an analysis of larval defenses in the tortoise beetle lineage (Chrysomelidae: Cassidinae). Our aim is to evaluate the roles of ecological performance and phylogenetic history in steering the functional assembly of multi-trait suites within this lineage. We quantify the relative potency and interactions among gregariousness, size, unpalatability and maternal care in the larval defense repertoires of two closely related beetle species, *Acromis sparsa* and *Chelymorpha alternans*. Because they both feed on the same host plant, *Merremia umbellata* (Convolvulaceae), their larvae confront the same enemies as they graze openly on leaves (e.g., Windsor et al. 1992; Cuignet et al. 2008). The larvae of both species have an unpalatable defense in the form of a physical and chemical barrier, or shield, which is mobile and fortified with host-derived chemicals (Vencl et al. 2005, 2009). Only *A. sparsa* employs a maternal care defense tactic, wherein females guard their offspring from egg until adult emergence (Windsor 1987).

We measured trait effectiveness against a major larval predator, the ant *Azteca lacrymosa*, in field bioassays. In conjunction with these performance bioassays, we used a phylogenetic approach to infer the order of trait evolution and to detect patterns of trait assembly that might have been associated with historical shifts in trophic mode from concealed, internal feeding (endophagy) to exposed feeding on leaf surfaces (ectophagy), and between host use (monocot to eudicot). The specific objectives of this study were to test whether or not: (1) group size, larval size, maternal care, or unpalatability (shields) enhanced survival; (2) traits interacted to form functional suites; (3) *A. sparsa's* overall defense was superior to *C. alternans'* and, finally; (4) a sequence of trait origin and assembly reflected an incremental directional trend (escalation) in defense effectiveness. The tortoise beetle species we studied provide a test of these conjectures, because the life history of both beetle are syntopic as well as sympatric.

To address these hypotheses, three sources of evidence are required. These are: (1) functional information about trait efficacy and multiple trait interactions; (2) comparative performance data from related species with and without derived traits, preferable in similar ecological circumstances, and; (3) a resolved phylogeny. We sought evidence of transitions from less potent to more potent defenses, or from simple traits to more complex, multi-trait suites that would constitute the discernable imprint of escalation within the tortoise beetle clade. In the limit, we wanted to know if our experimental findings, in combination with defensive trait reconstructions, were consistent with the appealing, but largely unsubstantiated hypothesis that strong selection by enemies has promoted the evolution of increasingly more effective armamentaria in tortoise beetles, and perhaps also of the robust tortoise beetle radiation.

We will present performance evidence in support of hypotheses (1), (2), and (3) for the study's focal species, as well as phylogenetic evidence in support of hypothesis (4), for a set of genera, including the study's species, representing the main lineages within the tortoise beetle radiation. By extension, all four hypotheses are taken to support the idea that more potent defensive traits and suites subtend the diversification of 3,000+ of the most derived tortoise beetles.

Methods

Study organisms

The larvae of *C. alternans* Boheman feed on the leaves of at least eight morning glory (Convolvulaceae) species, but prefer *M. umbellata* (L.) Hallier, which is the only host known for *A. sparsa* Boheman (Windsor et al. 1992). Sympatric throughout Mesoamerica, both species feed openly on leaf surfaces (i.e., ectophagous) and likely confront a similar suite of enemies there (larvae of both species form tightly knit aggregations or "rosettes" (viz. cycloalexy)), and also possess "shields" that can be oriented to thwart attacking enemies (Fig. 1a). Shields are made of accumulated cast skins and feces, which are attached to a highly movable fork-like caudal process that emanates from the penultimate abdominal segment (Fig. 1a). Applied precisely by a telescoping anus to the caudal process, feces contain host-derived chemical derivatives that significantly increase shield effectiveness, and make what otherwise would be a simple physical barrier, into a potent chemical defense (Vencl et al. 1999, 2005; Gómez et al. 1999). However, the maternal care strategy is only employed by *A. sparsa*, wherein females guard their offspring from egg to adult emergence using plowing and blocking maneuvers (Fig. 1b).

We used the aggressive and strongly recruiting generalist predatory ant, *A. lacrymosa* Forel (Hymenoptera: Formicidae: Dolichoderinae), in bioassays to measure defense effectiveness. As major predators of ectophagous larvae in lowland rainforests, *Azteca* ants (Carroll 1983; Hölldobler and Wilson 1990) are sympatric with the study's beetle species over much of their range (Forel 1900; Windsor et al. 1992).

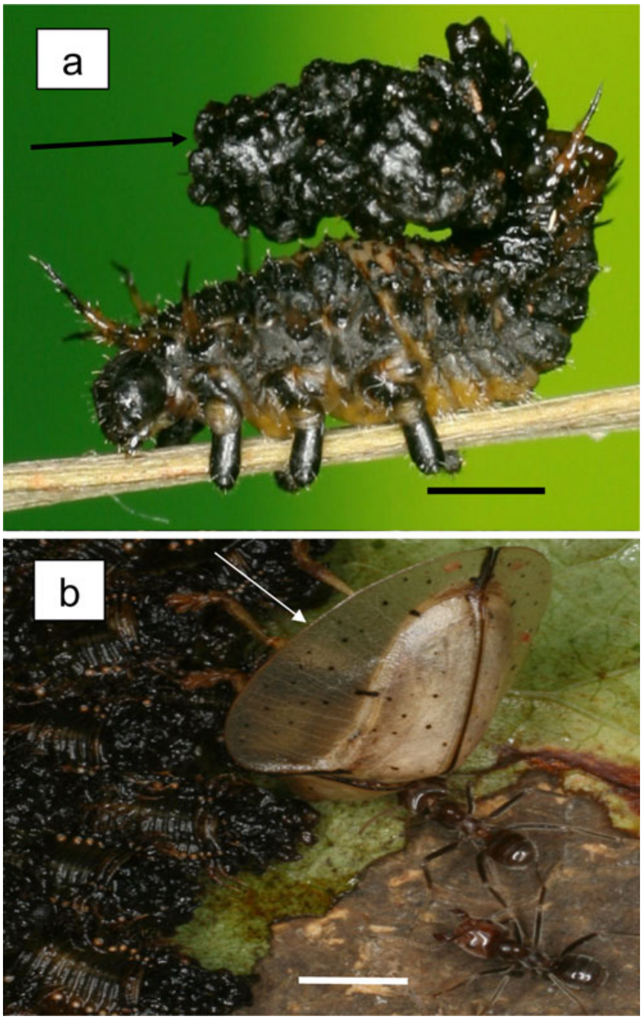

Fig. 1 **a** Second instar *Chelymorpha alternans* larva showing its shield (*arrow*), which is formed by the accumulation of fecula (glistening material) and cast larval skins attached to a movable caudal appendage emanating from the penultimate tergum (bar= 2 mm). **b** Female *Acromis sparsa* using a plowing maneuver to defend her 3rd instar brood from *Azteca lacrymosa* ant attackers. Note the slightly transparent, highly expanded elytra (*arrow*) (bar=4 mm)

Collection and husbandry

From April to September 2006, we collected 64 and 71 egg masses and broods of *C. alternans* and of *A. sparsa* (including females and their broods), respectively, from their mutual host, *M. umbellata*, a common, viny colonist of forest edges, gaps, and roadsides in the vicinity of Gamboa, Republic of Panamá (9°07′ N, 79°41′ W: 55 m). To augment field material, we collected additional adults to establish three long-term breeding colonies of both species at the Smithsonian Tropical Research Institute station in Gamboa. To exclude predators and parasitoids, the colonies were maintained in a 2×2.5 m conical insect tent (1×1 mm mesh), at ambient conditions, supplied with potted *M. umbellata* vines. Each colony produced between ten and 15 egg masses per week, which averaged 30.79±4.6 (n=41) and 30.75±5.6 (n=40) larvae of *C. alternans* and *A. sparsa*, respectively. Egg masses were collected daily and each transferred to a 473-ml plastic food cup containing plastic mesh for aeration and moistened filter paper. After hatching, each cup was supplied daily with a fresh, intact host leaf until the brood was used in a bioassay.

Bioassay protocol

To determine the relative importance of shields, group size, larval size and maternal care defenses, and to detect interactions among them, we used three experimental manipulations of larvae living in groups: (1) shield present or absent; (2) small group (≤15 larvae) or large group (>15 larvae), and in the case of *A. sparsa*; (3) mother present or absent. Larval instar size (small larva <3rd instar or large larva ≥3rd instar) represented an un-manipulated covariate. The group category was based on the mean egg mass of 31 for both species. The larval size category was derived from the midpoint of the five instar developmental period. For statistical analyses, each larva was categorized dichotomously by presence or absence of each of the treatment variables above. For bioassays of *A. sparsa*, there were (total groups:large groups:larvae ≥3rd instar): 65:36:584 with shields and maternal care; 86:14:258 with shields and without maternal care; 39:18:222 with maternal care and no shields, and; 63:16:274 without shields or maternal care. For *C. alternans*, there were (total groups:large groups/ larvae ≥3rd instar): 142:41:605 with shields, and; 95:38:239 without shields. In total, we monitored the survival of 2,728 *A. sparsa* and 2,613 *C. alternans* larvae.

Since shields, group size, and maternal care traits are physically independent, we could manipulate them by procedures that did not otherwise alter the larvae (Olmstead and Denno 1993). Larvae were capable of evading capture by walking or using their shields (if not removed) to deflect attackers. Group size treatments consisted of the removal of larvae to produce groups of various sizes. Female removal treatments and group size reductions were made prior to presentation. Due to the time involved, shield removal treatments were prepared the night before. For no-shield treatments, we removed shields by placing fine forceps between the tines of the caudal process and gently lifting the shield away from the body. Controls for shield removals and group size reductions consisted of touching each larva with forceps to simulate the removal manipulations.

Bioassays were conducted near Gamboa from July to September 2006, between 8:00 a.m. and 12:00 p.m. Serving as a bioassay test arena, we used a raised platform, measuring 45×60 cm, attached to the bole of a tree with an *A. lacrymosa* nest. Vines and fallen branches with active ant trails connected to the platform formed clearly defined avenues across the platform's surface, which varied from five to eight daily.

A bioassay trial consisted of the presentation of a single larval group, which had been subjected to a treatment or treatment combination, as defined above. Using soft forceps, we placed a host leaf with the attached treatment 1 cm from an active foraging trail on the platform. A trial began after the first ant antennated the treatment. Each trial lasted 5 min, a time period long enough to record ant responses to defenses but short enough not to generate mass attacks which can last more than 20 min. A larva was deemed captured when the ants carried it ≥1 cm sideways or backwards and we recorded this time as the interval from the first ant contact, which began the trial. Individual trials were separated from 1 another by 3 to 5 min and conducted along different trails on the platform. Delays between trials and variable locations served to minimize recruitment interactions between trials during the course of each morning's experiments.

Behavior and larval size measurements

Larval escape behavior was recorded. Since the effectiveness of many larval defenses is thought to be size-dependent (see Reavey 1993), we weighed (weight/dry) a sample of 2nd and of 4th instar larvae and their shields to the nearest 10^{-4} g (Sartorius TE646) and then estimated scaling relationships between instar, larval size, and shield weight.

Statistical analyses

We analyzed the capture times of individuals within groups using a failure-time approach, the Cox proportional hazards model. Times to the occurrence of events (e.g., capture times of larvae by ants) rarely meet the distributional assumptions of traditional parametric approaches, chiefly because experimental periods often end before an event is recorded (i.e., a right-censored event). Failure-time methods, like the Cox model, compare the distributions of capture times throughout the entire bioassay period, including censored data (Fox 2001; Allison 1995).

The Cox proportional hazards analysis fits a multiplicative model that is designed to detect interactions among covariates that act on the hazard function to change the risk of capture (PHREG procedure; SAS 2004). The model assumes that the degree of risk varies among treatments but the time of hazard (exposure during the 5 min bioassay period) is the same for all individuals. That is, the risk of capture for an individual larva at any given time is a function of its particular set of defense covariates. Defense covariates do not make the predators arrive sooner or later, since we start the bioassay upon their arrival, but they do influence vulnerability once the predators discover the larva. If for example, a defense covariate has a significantly negative coefficient, it decreases the risk of capture, thereby increasing the larva's survival time in the bioassay. For each covariate with a significant coefficient, the analysis also generates a hazard ratio, which is the ratio of hazard functions between individuals with and without the covariate. For example, the smaller the defense variable's ratio, the lower an individual's risk and the longer it is likely to survive (controlling for all other covariates).

After estimating survival functions for defense variables of individuals within larval groups, we then compared the intact defenses of the two species, and in the case of *A. sparsa*, with and without maternal care. Differences between species' survival functions were statistically compared using the Wald χ^2 implemented by the TPHREG procedure (SAS 2004).Finally, in order to rank defensive traits statistically and to detect interactions among them, we compared proportional hazard models with different trait combinations using the branch-and-bound option implemented by the PHREG procedure (SAS 2004). Large differences in Wald χ^2 values identified significant trait interactions.

Phylogenetic analysis: taxon trees, trait trees, and trait evolution

We obtained phylogenetic hypotheses of species relationships based on Bayesian and maximum parsimony (MP) analyses of a combined molecular and morphological matrix. Study taxa included species representing 20 genera in the subfamily Cassidinae and two species in the outgroups Donaciinae and Galerucinae (Table 1). The data consisted of 12S mtDNA sequences and morphological traits (culled from Hsiao and Windsor 1999 and from Chaboo 2007, respectively; Online Resource Table 1).

Table 1 Presence or absence of seven focal defense-related traits used in character reconstructions for 21 beetle genera

Cassidinae Genus[a] or outgroup	Trophic mode[b]	Shield	Fecal component	Larval behavior	Gregarious larvae cycloalexy[c]	Maternal care	Host family
Plateumaris[d]	Endophagous (under water)	Absent	None	Solitary	Absent	Absent	Monocot
Ophraella[e]	Ectophagous	Absent	None	Solitary	Absent	Absent	Eudicot
Alurnus	Endophagous	Absent	None	Solitary	Absent	Absent	Monocot
Prosopodonta	Endophagous	Absent	None	Solitary	Absent	Absent	Monocot
Cephaloleia	Endophagous	Absent	None	Solitary	Absent	Absent	Monocot
Imatidium	Endophagous	Absent	None	Solitary	Absent	Absent	Monocot
Microrhopala	Endophagous	Absent	None	Solitary	Absent	Absent	Eudicot
Anisostena	Endophagous	Absent	None	Solitary	Absent	Absent	Eudicot
Spilophora	Ectophagous	Present	None	Solitary	Absent	Absent	Monocot
Oediopalpa	Ectophagous	Present	None	Solitary	Absent	Absent	Monocot
Calyptocephala	Ectophagous	Present	None	Solitary	Absent	Absent	Monocot
Spaethiella	Ectophagous	Present	Present	Solitary	Absent	Absent	Monocot
Laccoptera	Ectophagous	Present	Present	Solitary	Absent	Absent	Eudicot
Metrionella	Ectophagous	Present	Present	Solitary	Absent	Absent	Eudicot
Charidotella	Ectophagous	Present	Present	Solitary	Absent	Absent	Eudicot
Physonota	Ectophagous	Present	Present	Gregarious	Absent	Absent	Eudicot
Echoma	Ectophagous	Present	None	Gregarious	Present	Present	Eudicot
Chelymorpha	Ectophagous	Present	Present	Gregarious	Present	Absent	Eudicot
Stolas	Ectophagous	Present	Present	Gregarious	Present	Absent	Eudicot
Eugenysa	Ectophagous	Present	Present	Gregarious	Present	Present	Eudicot
Acromis	Ectophagous	Present	Present	Gregarious	Present	Present	Eudicot
Omaspides	Ectophagous	Present	Present	Gregarious	Present	Present	Eudicot

Traits derived from: personal observation; Windsor et al. 1992; Hsiao and Windsor 1999; Chaboo 2007

[a] From less to more derived genera

[b] Endophagous is feeding concealed inside the host plant, and ectophagous is feeding exposed on leaf surfaces

[c] Tight, rosette-like larval formation

[d] Outgroup in the Donaciinae

[e] Outgroup in the Galarucinae

Twenty 12S sequences were downloaded from GenBank, aligned using CLUSTALW and manually adjusted using the color editor in MacClade (Maddison and Maddison 2008). For the Bayesian analysis of the 12S data, the general time-reversible nucleotide substitution model was applied, with invariant sites estimates and assuming a discrete gamma distribution (GTR+I+G). The molecular model was chosen based on Likelihood Ratio Tests using the program ModelTest 3.7 (Posada and Crandall 1998). For Bayesian analysis of morphological data, a likelihood model was assumed (Mk: Lewis 2001). In this model, the rates of gains and losses are equally likely, and rates of change among characters differ following a gamma distribution. Bayesian analyses were performed using two chains, each run for 1 M generations. Trees were sampled every 100 generations. Stationarity (as determined by the convergence of likelihood values) was reached after 20,000 generations, and consensus trees were obtained after discarding the first 200 trees as "burnin". For maximum parsimony (MP) analysis gaps were treated as missing characters. All traits were unordered and 364 parsimony informative characters were included. Starting trees were obtained by 1,000 random additions and no branch-swapping; these were used as the starting points for MP analyses as implemented in PAUP* 4.0b10 (Swofford 2001). During MP searches, TBR branch-swapping, no steepest descent option and the MulTrees option were in effect. Bootstrap analyses (1,000 runs) were done using MP with similar search options, but for the use of simple addition of taxa. The phylogenetic tree was viewed using TreeView (Page 1996). The evolution of seven focal defense-related traits was inferred using ancestral state reconstruction and trait-tree reconstruction (Table 1; personal observation; Windsor et al. 1992; Hsiao and Windsor 1999; Chaboo 2007). Ancestral states were reconstructed across trees (a set of 972 highest likelihood trees from the Bayesian runs) using ML as implemented in Mesquite (Maddison and Maddison 2008). An ancestral node was assigned to the state with the higher likelihood value if the difference between the likelihoods of alternative states was greater than two, and no state was assigned if the difference was lower (Pagel 1999; Maddison and Maddison 2008). The components of an evolving entity are expected to be related as a phylogenetic tree (Williams 1992). In other words, if a set of traits forming a loosely integrated complex, or "defensive suite," evolved together, they are expected to be related in a tree-like configuration, that is, as a "trait tree." Conversely, if different traits are related as a tree, then they may be inferred to make up an evolving, integrated complex entity or "defensive suite." Such complex entities are inferred to exist due to integrating forces (developmental, ecological) that may act during the lifetime of the organism, but are the result of evolutionary forces (e.g., Geeta 2003). In order to detect whether the defense-related traits in this study might have evolved as an integrated suite, we determined whether they are related as a "trait tree" using two phylogenetic analyses, MP, and neighbor-joining (NJ). We also assessed the strength of the tree structure in the data using the Splits analysis. We estimated trait trees using a matrix consisting of a subset of seven defense-related traits (Table 1). We used PAUP* to implement NJ and MP methods, the robustness of whose results was assessed using 1,000 bootstrap searches (Swofford 2001). Conflict in the data was detected using Splits analysis (Bandelt and Dress 1992; SplitsTree: Huson 2002). Splits analysis results in graphs in which parallel edges weaken evidence of tree structure (e.g., pointing to weak developmental integration; see Geeta 2003). In our analysis, tree structure was taken as supporting the hypothesis of integrated evolution of the defense-related traits, while parallel edges were accepted as a rejection of that hypothesis.

Results

All defense variables significantly increased survival during the ant bioassay (Tables 2 and 3). Moreover, interactions among variables were synergistic (Tables 4 and 5).

Shield effects

For both species, shielded larvae fared significantly better than their unshielded counterparts. Solitary *A. sparsa* and *C. alternans* larvae with shields survived longer than did their de-shielded counterparts in both species (χ^2=6.69; P=0.0097; n=78 and χ^2=9.93; P=0.0016; n=119, respectively). Larvae of *A. sparsa* and *C. alternans* with shields experienced a 76% and 46% reduction in the predation risk, respectively, compared with aggregated larvae without shields (Tables 2 and 3). Shields were ranked as the second most important defense component in both species (Tables 4 and 5). Solitary, shielded larvae of both species survived equally well (χ^2=1.86; P=0.1722; n=100). However, solitary *C. alternans* tried to escape 23% of the time (31/133 bioassays), while *A. sparsa* rarely attempted to escape (1/209).

Group effects

With all the other defense covariates held constant in the hazard analysis, *A. sparsa* larvae living in large groups experienced an 90% reduction in individual predation risk, the highest afforded by any other single defense covariate (Table 2). Group size ranked as the single most important defensive component (Table 4). The predation risk for a *C. alternans* larva residing in a large group was reduced by

Table 2 The risk of predation in the *Azteca* ant bioassays for an individual *Acromis sparsa* larva estimated by the Cox proportional hazards model as a function of group size, shield, instar size, and maternal care

Parameter[a]	Parameter[b] estimate	Standard error	Wald[c] χ^2	P	Hazard ratio[d]
Group size	−2.28152[e]	0.18440	153.0785	<0.0001	0.102
Shield	−1.44148	0.16755	74.0166	<0.0001	0.237
Maternal care	−1.12058	0.11627	47.4337	<0.0001	0.326
Larval size	−0.76652	0.17407	19.3908	<0.0001	0.465

[a] Large group size (>15 larvae); shield (intact fecal shield present); large larval size (≥3rd instar); maternal care (guarding mother present)

[b] A negative parameter estimate indicates a reduction of risk

[c] The Wald χ^2 statistic determines if the parameter estimate is significantly increasing or decreasing risk based on a χ^2 distribution with one degree of freedom

[d] The hazard ratio measures the relative risk of predation for an individual possessing a particular parameter compared with an individual lacking it, all other factors being equal. If the hazard ratio is less than one, for example 0.237 in the case of an individual larva with a shield, the risk of predation, controlling for all other covariates, is only 24% of the risk for an individual lacking a shield

[e] A negative slope indicates reduced risk during the bioassay

69% compared with a larva residing in a small group (Table 3). For a *C. alternans* larva, gregariousness also ranked as the most important defense (Table 5).

Maternal care effects

With all other factors held constant, larvae with maternal care were 67% less vulnerable to predation as unguarded larvae (Table 2). Overall, maternal care by itself was ranked as a weaker defense, well below group membership and shields (Table 4).

Larval size effects

Large larval size was an important contributing factor prolonging survival in the bioassay for both species. In *A. sparsa*, 4th instar larvae were nearly sixfold larger than 2nd instar larvae (2nd: X±SE=0.0112±0.001 mg, n=30; 4th: X±SE=0.0641±0.0009 mg; n=31). With all factors held constant, a large *A. sparsa* larva was 54% less likely to be captured than a smaller larva (Table 2), despite larval size being ranked as the least important factor (Table 4). Likewise, fourth instar *C. alternans* larvae are nearly five times larger than 2nd instars (2nd: X±SE=0.0152±0.0015 mg, n=30; 4th: X±SE=0.0713±0.0032 mg, n=20). Large larval size played a minor, but significant role in increasing survival rates in this species, accounting for a 34% reduction in predation risk but was ranked as the least important factor (Tables 3 and 5).

Trait interactions

To discern interactions among *A. sparsa* defenses, we compared models with the "best" combinations of traits among the multi-defense models. Of the two-defense models, the combination of the large group size and shields produced a goodness of fit (χ^2=262.8) higher than their combined values (χ^2=244.4), indicating a strong multiplicative (synergistic) interaction between them (Table 4). Although the inclusion of all defenses in the saturated model produced the highest score, the next most robust model incorporated large groups, shields and maternal care, revealing a functional coupling among these traits (χ^2=290.5; Table 4).

As noted above, large group size was by far the most important factor prolonging larval survival in *C. alternans* (Table 5). Although shields were extremely important for *C. alternans* survival, they afforded larvae the

Table 3 The risk of predation in the *Azteca* ant bioassays for an individual *Chelymorpha alternans* larva estimated by the Cox proportional hazards model as a function of group size, shield, and instar size

Parameter[a]	Parameter estimate[b]	Standard error	Wald[c] χ^2	P	Hazard[d] ratio
Group size	−1.16518[e]	0.19848	34.4619	<0.0001	0.312
Shield	−0.62061	0.20733	8.9599	0.0028	0.538
Larval size	−0.42097	0.21262	3.9200	0.0477	0.656

See footnotes (captions) in Table 2

Table 4 Ranking of defense models with group size, instar size, shield, and maternal care variables based on failure-time analysis of *Acromis sparsa* larval vulnerability in the *Azteca* ant bioassays

Number of variables	χ^2 rank[a]	Variables included in model[b]			
1	191.1510	Group size			
1	53.3693	Shield			
1	41.8973	Maternal care			
1	26.1561	Larval size			
2	262.7885	Group size	Shield		
2	230.8184	Group size	Maternal care		
2	209.5855	Group size	Larval size		
2	84.7504	Shield	Maternal care		
3	290.5412	Group size	Shield	Maternal care	
3	280.6677	Group size	Shield	Larval size	
3	250.9882	Group size	Maternal care	Larval size	
3	112.5131	Shield	Maternal care	Larval size	
4	309.9066	Group size	Shield	Maternal care	Larval size

[a] The branch-and-bound procedure finds the four best models containing individual variables, up to the single model containing all of the defense variables. The criterion used to include variables is based on the global Wald χ^2 statistic. By comparing two models with the same number of explanatory variables, the algorithm ranks them according to which model, of all possible models with a given number of covariates, has the higher global score χ^2 statistic

[b] See variables defined in Table 2

highest level of resistance when expressed in combination with large group size. The large group/shield trait suite produced by far the most robust two-factor defense model, and the interaction between these traits appears to improve survival more than the sum of their separate contributions (χ^2=39>χ^2=31; Table 5). Despite its low rank, large instar size, when expressed with groups and shields, also formed a strongly interacting suite in *C. alternans* (χ^2=41.9; Table 5).

Comparison between species with intact defenses

When we measured the overall survival of larvae living in groups with intact maternal guarding and shields defenses, *A. sparsa* survived significantly longer than did its gregarious, shielded counterparts (χ^2=8.063; P<0.0045; n=65 and 102 groups, respectively). However, removal of guarding females from shielded groups rendered *A. sparsa* larvae as vulnerable to predation as gregarious *C. alternans* (χ^2=0.1852; P=0.667; n=41 and 70, respectively).

Phylogenetic hypotheses and character evolution

Bayesian analysis of the combined data yielded a phylogenetic hypothesis of species relationships that was consistent with previous analyses of the molecular and morphological data (Fig. 2; Hsiao and Windsor 1999; Chaboo 2007). MP results (not shown) were generally consistent with the Bayesian tree, but not as well supported. However, the well-supported position of *Spaethiella* in the Bayesian tree was not supported in the MP tree. Well-supported portions of the Bayesian tree included the clades of interest (Fig. 2; clades A and B). The ML reconstructions of ancestral states across different topologies showed the same sequence in trait evolution (Fig. 3; Electronic supplementary Table 2). Leaf surface feeding and shields may have evolved at least

Table 5 Ranking of defense models with large group size, instar size, and shield variables based on failure-time analysis of *Chelymorpha alternans* larval vulnerability in the *Azteca* ant bioassays

Number of variables	χ^2 rank[a]	Variables included in model[b]		
1	24.0171	Group size		
1	6.5697	Shield		
1	4.8778	Larval size		
2	38.7206	Group size	Shield	
2	34.0404	Group size	Larval size	
2	8.1265	Larval size	Shield	
3	41.9642	Group size	Shield	Larval size

[a] See footnote caption in Table 4

[b] See variables defined in Table 2

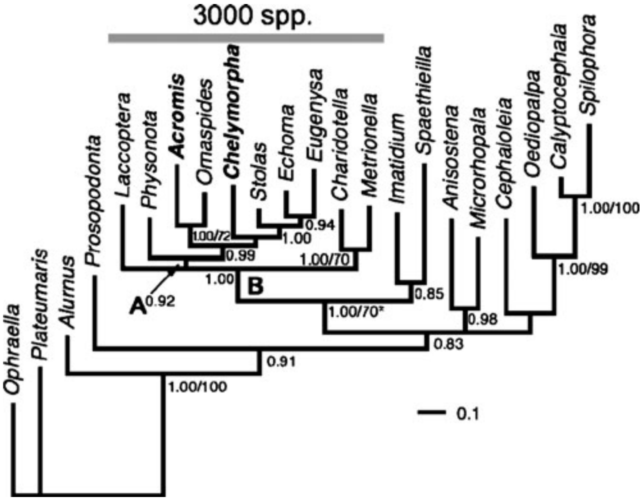

Fig. 2 Phylogeny showing the evolution of larval defenses in tortoise beetles. The phylogram shows the clades of interest (*A*, *B*, and *C*) based on Bayesian 50% consensus. *Numbers at nodes* are the posterior probability (PP) of the clade>0.75%. Percent bootstrap values in maximum parsimony (MP) analysis follow slashes after PPs. *Number above the horizontal bar* indicates the estimated number of species in the relevant clades. *Asterisk* MP support without *Spaethiella*. The studies' genera in *bold*

three times (Fig. 3; nodes 13, 19, 38). In one of these instances, a fecal component was later added to the shield (node 19), which may well have included host-derived compounds. It appears that dietary specialization on the more chemically complex eudicots likely preceded the fecal innovation (see Electronic supplementary Table 2: host family). The fecal shield was followed by the evolution of gregariousness (node 25), and finally by maternal care (Fig. 3; nodes 30 and 33). The fecal shield + gregariousness suite arose in the ancestor of clade A (Fig. 3; node 25; see Electronic supplementary Table 2: shield). Maternal care evolved twice in clade A (Fig. 3; nodes 30, 33).

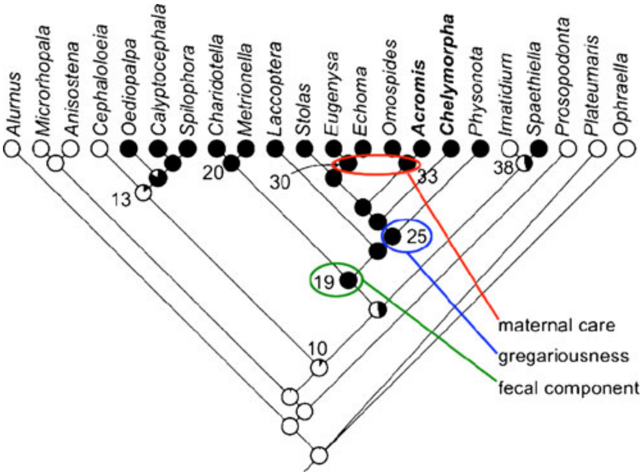

Fig. 3 Maximum-likelihood reconstructions of defense-related traits on a tree obtained in Bayesian analysis. *Circles* at nodes show the proportional likelihood of having a shield (in *black*). Terminal nodes in *black* have shields. The ancestral tortoise beetle shifted to feeding on the leaf surface at node 10. *Ellipses* around nodes indicate on the cladogram where chemically enhanced shields (i.e., with fecal component; *green*), gregariousness (*blue*) and maternal care (*red*) traits evolved. The studies' genera in *bold*

Trait analysis reveals tree-like relationships among the defense-related traits. The NJ reconstruction analysis of defensive traits suggests a tree-like structure in trait evolution (Fig. 4a). That is, trait states are nested. At the base are endophagous (i.e., feeding concealed inside the host), solitary, shieldless larvae with variable monocot and eudicot feeding habits (e.g., *Plateumaris, Alurnus, Anisostena, Cephaloleia, Imatidium, Microrhopala, Prosopodonta*). Next, a variable set of mixed defense-trait associations appears with ectophagy (i.e., feeding exposed on leaf surfaces) and shields (stage I: seen in *Calyptocephala, Oediopalpa, Spilophora*). Then, ectophagy and shields with a fecal component (viz. chemical) become linked (stage II: in *Charidotella, Laccoptera, Metrionella, Spaethiella*). By stage III, ectophagy, fecal shields, and gregariousness (cycloalexy, a tight circular larval group formation) become linked as a well-supported (74% bootstrap) suite (*Stolas, Chelymorpha*). The assembly of cycloalexy with maternal care (stage IV: e.g., *Acromis, Eugenysa*, and *Omaspides*) completes the sequence of changes (Fig. 4a). Taxa in clade A show stages III–IV of the evolution of most complex suite of characters (see Fig. 2).

The Splits analysis also supported a tree-like, sequential structure in trait state evolution indicating trait integration in defense evolution (Fig. 4b). Due to the loss of the fecal component in *Echoma's* shield, stage II is characterized by parallel edges, which indicates a lack of trait integration. Connected in a tree-like manner in stages III and IV, traits have become well integrated into suites (Fig. 4b).

Discussion

Interactions among defensive traits

Our experiments demonstrated that: (1) traits contributed differentially to survival, and (2) particular trait combinations enhanced survival above that afforded by their individual effects. Shields by themselves were an important factor increasing survival in both *A. sparsa* and *C. alternans*. In combination with gregariousness, shields formed a formidable defense suite, with a level of resistance higher than any other trait combination among the unsaturated models. Despite its low ranking, maternal care contributed to five of the eight multi-component unsaturated defense models. The findings that: (1) *A. sparsa* larvae with a full complement of defense traits are more resistant to attack than are *C. alternans*, and; (2) female removal

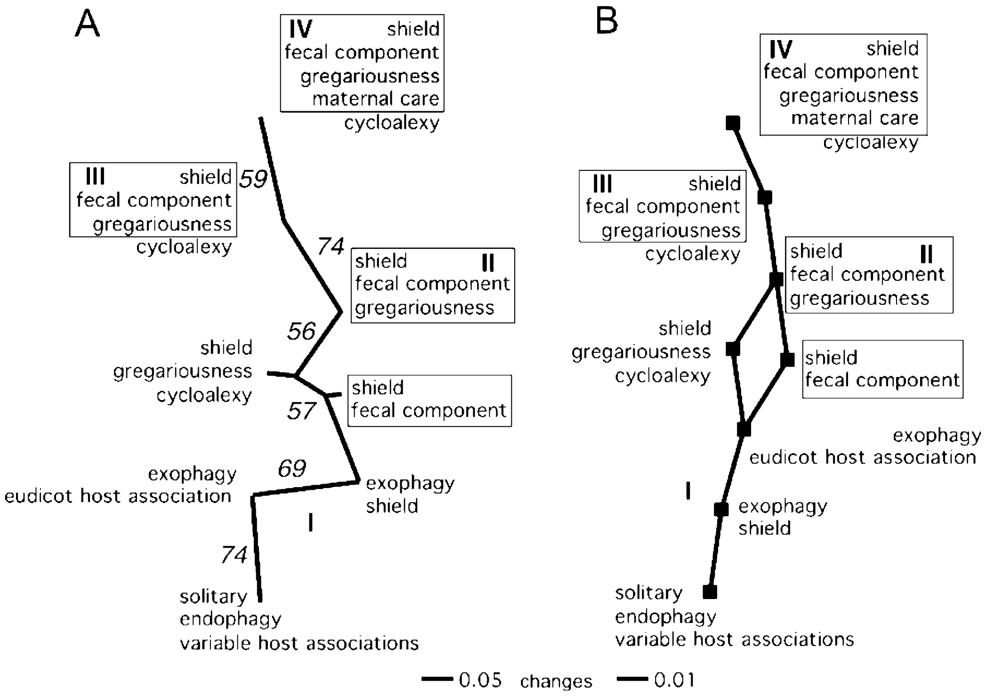

Fig. 4 Trait trees derived from **a** neighbor-joining and **b** SplitsTree reconstruction analyses. Numbers along branches of the NJ tree represent bootstrap values of >50%. Boxed-in traits signify integrated suites revealed in the failure-time analyses of the *Azteca* ant bioassays. Stage I consists of ectoophagy and host specialization associated with a variable set of traits. Stage II consists of ectoophagy and host specialization invariably associated with fecal shields. Cycloalexy is added at stage III. Finally, maternal care evolves by stage IV. SplitsTree analysis (*B*) supports the NJ results in that stages I and III–IV are related in a tree-like fashion (groupings shown as *filled squares*), while the rest of the trait states show reticulation (*parallel edges in the central part of the graph*). Taxa for each stage listed in text

eliminated this advantage, further support the conclusion that the superiority of the *Acromis* defense stems from the interactions of the maternal care tactic with other traits, in particular the group trait. The power of the maternal care/shield combination was relatively low, perhaps indicating a physical conflict between the female's ability to easily traverse larvae and large shield size (see Tallamy 2005).

For many larval herbivores, size matters. Invertebrate predators may be limited to prey small enough to be subdued with minimum effort (Reavey 1993). For example, ants can capture Eastern tent caterpillars (*Malacosoma americanum*) only within the first 3 weeks of development (Tilman 1978). Likewise, the effectiveness of shields appears to scale positively with size. In both beetle species, the significantly negative slopes for large size in the hazard analysis indicate that as larvae grow, their chance of survival increases coincidently with the four to fivefold increase in body weight between the 2nd and 4th instars. These findings agree with foraging theory and empirical studies, which show that prey selection is governed by an inverse relationship between prey size and handling effort (Stephens and Krebs 1987; see Olmstead and Denno 1993). Although it contributed significantly to overall resistance in both species, larval size interacted with shields much more strongly in *C. alternans*. We initially assumed that *A. sparsa's* smaller shields would be less effective than those of *C. alternans*. The smaller shields of *A. sparsa* (and also of *Echoma*) may represent a physical trade-off with the maternal defense because they are less of a hurdle to females as they maneuver over larvae (e.g., Cocroft 2002; Tallamy 2005). The lack of interaction between shields and size in *A. sparsa* may be due to the advantage of maternal care or the result of a stronger shield defense (see below).

Evolutionary ecology of tortoise beetle larval defenses

As a first step in documenting an escalation scenario, we sought support for a trend in defense efficacy using performance comparisons. The comparisons demonstrated that individuals with derived traits or complex suites survived longer than individuals with singlet ancestral traits. There was an incremental trend in trait performance in *A. sparsa* larval defenses from: solitary < solitary + shield < solitary + fecal shield < group < fecal shield + group < fecal shield + group + maternal care. There was a similar escalating trend in *C. alternans* (without maternal care). As a second step, we used phylogenetic analyses to show that this ecological trend is paralleled by an evolutionary trend going from singlet traits to more effective multi-trait suites. Phylogenetic optimization (ML) of traits revealed the following sequence in the evolution of novel defense adaptations (starting from solitary larvae): shield → fecal shield → group → maternal care. The observation that *A. sparsa's* fecal shield + group + maternal care suite out performed *C. alternans'* fecal shield + group suite, which lacked the maternal care trait, demonstrated a incremental pattern in performance due to the addition of a novel, derived trait. An evolutionary basis to this pattern was inferred from trait analyses (NJ and Splits), which revealed that these defensive traits evolved in an integrated manner.

Based on previous chemical ecological data presented above and the experimental and phylogenetic data presented here, we propose the following sequence in the origin and assembly of tortoise beetle larval defenses: (1) coincident with the shift to the leaf surface by the ancestral tortoise beetle (Fig. 3; node 10), dorsal appurtenances, lacking a fecal component, functioned initially as simple physical barriers in solitary larvae, and appear to have evolved at least three times; (2) a fecal component, laced with host-derived metabolites, was then added, perhaps twice, to the shield armature to form a novel, more effective physical-chemical barrier defense (first at node 19 and then at node 38); (3) subsequent dietary specialization on more chemically complex eudicots augmented the fecal shield innovation in the lineage leading to clade A (Fig. 3 node 19; see Wink 2003); (4) gregariousness then evolved once (node 25) and became functionally integrated with the fecal shield suite in clade A, which includes *Acromis* and *Chelymorpha*, and; (5) maternal care evolved at least twice in clade A, which includes *Acromis*, from a gregarious, shielded ancestor in response to persistent threats from several enemies that might have managed to circumvent the fecal shield-group defense suite (Figs. 2 and 3; nodes 30, 33).

From ancestral to derived members of a clade, the historical imprint of an incremental trend, i.e., escalation, would be discernible as a pattern of increased: (1) trait number; (2) trait effectiveness, or; (3) defense potency, wherein multi-trait interactions have promoted trait “assembly” (functional integration) and suite formation (Vermeij 1987; Agrawal 2007; Armbruster et al. 2009). The results of our combined phylogenetic-performance study demonstrated all three patterns in larval defense evolution. Firstly, a number of novel defensive traits accumulated in certain lineages within the tortoise beetle phylogeny. Secondly, traits evolved in a sequence of increasing effectiveness. Thirdly, these traits show synergistic interactions and are evolutionarily assembled into more potent multi-trait suites. Finally, the incremental directional trend in efficacy appears to be correlated with increased diversification. For example, the fecal shield/gregarious defense suite characterizes the nested clade A, which accounts for almost half of the entire Cassidinae (*sensu lato*) beetle radiation of 6,000 species.

A sequence of trait origin, accumulation and suite assembly, reflecting a pattern of escalation, is an inherent corollary of Ehrlich and Raven's (1964) “escape-and-radiate” hypothesis. In this view, novel defensive traits, acting as barriers, accumulate through repeated cycles of defense-counter defense coevolution. A lineage bearing a novel defense is released from the depredations of its erstwhile enemies and should diversify. This scenario further entails escalation, wherein the efficacy of a defense increases from basal to derived taxa (Vermeij 1987). Analogous to Ehrlich and Raven's (1964) host plant-herbivore model, Singer and Stireman propose a model in which variation in enemy-free space afforded by host plants, chiefly by means of refugia or sequesterable metabolites, has driven cyclical defense/counter defense coevolution between insect herbivores and their enemies. According to this view, novel sequesterable metabolites for instance, represent avenues of release from enemy pressure that could foster herbivore diversification. This hypothesis might be tested by comparisons between diversification rates of herbivore lineages with different trophic modes (ectophagy vs. endophagy) and which use different defensive strategies. The applicability of the escape-and-radiate model to interactions between herbivorous insects and their natural enemies and the impact of novel defensive adaptations, such as anti-predator behaviors and the sequestration of plant secondary compounds, on herbivore diversification rates are essentially unexplored (Singer and Stireman 2005; Vamosi 2005). Our comparison of diversities within the tortoise beetle lineage, although preliminary, appears to lend support to a “tri-trophic niche” perspective. This hypothesis, however, would need to be verified in evolutionarily replicated instances.

Although both the Ehrlich–Raven and Singer–Stireman models assume a central role for novel trait evolution in fostering diversification, Vermeij's (1987) escalation concept further predicts that overall defense, rather than the evolution of more potent novel traits *per se*, can incrementally increase during clade diversification. The fossil record reveals several reciprocal prey–predator diversifications (Vermeij 1987). Support is accumulating from theoretical modeling (e.g., Abrams 2000; Bowers et al. 2003) and empirical studies (Mitter et al. 1988; Nosil and Crespi 2006; Vamosi 2003; Stephens and Wiens 2008) for the idea that enemies not only drive phenotypic divergence in prey defenses, but also the diversification of their prey (reviewed by Vamosi 2005; e.g., Mallet and Joron 1999; Farrell and Sequira 2004; Nosil and Crespi 2006). Several recent phylogenetically informed empirical studies suggest that novel trait evolution in plant defenses show convergent patterns of suite formation and directional trends correlated with diversification (Farrell et al. 1991; Armbruster et al. 2009; Agrawal et al. 2009b; Becerra et al. 2009). Despite Futuyma's (1987) caveat that it may be difficult to determine whether escalation begets diversification or vice versa, we interpret our results to be consistent with the emerging view that defenses do not function in isolation, but instead become assembled into functionally integrated multi-trait suites, which likely have conferred the advantage of enhanced larval survival, and possibly also of diversification in the dangerous leaf surface adaptive zone.

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