2012

The relationship between variable host grouping and functional responses among parasitoids of *Antispila nysaefoliella* (Lepidoptera: Heliozelidae)

Candace Low
Cornell University, cl754@cornell.edu

Sonja J. Scheffer
USDA-ARS, sonja.scheffer@ars.usda.gov

Matthew L. Lewis
USDA-ARS, matthew.lewis@ars.usda.gov

Michael W. Gates
USDA-ARS, michael.gates@ars.usda.gov

MOLECULAR ECOLOGY

Molecular Ecology (2012) **21**, 5892–5904 doi: 10.1111/mec.12061

The relationship between variable host grouping and functional responses among parasitoids of *Antispila nysaefoliella* (Lepidoptera: Heliozelidae)

CANDACE LOW,* SONJA J. SCHEFFER,† MATTHEW L. LEWIS† and MICHAEL W. GATES†‡

*Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, NY 14853, USA, †Systematic Entomology Laboratory, USDA-ARS, Beltsville, MD 20705, USA, ‡Systematic Entomology Laboratory, USDA-ARS, National Museum of Natural History, Smithsonian Institution, Washington, DC 20013-7012, USA

Abstract

Our study investigated the importance of variability in the parasitoid community as a source of selection on host group size using a field population of the tupelo leafminer, *Antispila nysaefoliella* Clemens, which specializes on tupelo, *Nyssa sylvatica* Marsh. Larvae were collected from leaves with variable numbers of larvae and screened for parasitism using polymerase chain reaction of mitochondrial cytochrome oxidase I using markers designed specifically for amplifying parasitoid DNA while excluding host DNA. This method of selective PCR was effective for detecting the presence and identifying species of immature stages of three hymenopteran superfamilies: Chalcidoidea, Ichneumonoidea and Platygastroidea, which represented 83.4%, 16.0% and 0.6% of the total detectable parasitism, respectively. Our resulting sequences were then calibrated with sequences from identified adult parasitoids that had been either reared or field-captured. A cluster analysis revealed 10 distinct clades that showed differences in attack patterns with respect to host traits and season. Total parasitism followed an inverse density-dependent or density-independent pattern with respect to host density (number per leaf). However, when parasitoid taxa were considered separately, one clade, which could be a cryptic species of *Pnigalio maculipes* Crawford (Chalcidoidea: Eulophidae), was found to increase its per leaf attack rate with host density. Our results suggest that parasitoid community composition and differences among species in their attack strategies can play a large role in determining the adaptive advantage of host grouping.

Keywords: community ecology, dilution effect, DNA barcoding, field survey, functional response, life history strategy, mtDNA COI

Received 27 December 2011; revision received 20 August 2012; accepted 23 August 2012

Introduction

For many decades, empirical and theoretical studies have investigated the cost–benefit continuum of grouping with an effort to understand the selective pressures that shape grouping behaviour, group size or clutch size (Hamilton 1963, 1971; Clark & Mangel 1986; Coster-Longman *et al.* 2002; Krause & Ruxton 2002; Jackson *et al.* 2005; Tosh *et al.* 2006; Low 2008a; Ioannou *et al.* 2009; Morrell *et al.* 2011). One well-documented benefit of grouping is safety from predation or natural enemy attack (Clark & Mangel 1986; Coster-Longman *et al.* 2002; Krause & Ruxton 2002). However, being part of a larger group can also increase the risk of detection by predators, intensify resource competition, make parental care more costly and reduce resource quality through synergistic effects (Caraco & Wolf 1975; Clark & Mangel 1986; Turner & Pitcher 1986; Hughes *et al.* 2002; Low 2008a). The evolution of grouping and its impacts on

Correspondence: Candace Low, Fax: + 607-255-8088;
E-mail: cl754@cornell.edu

ecological and social interactions continues to be actively researched because of its key role in the organization of biological systems and its nonlinear dynamical effects (Buss 1981; Ives 1989; Avilés 1999; Krause & Ruxton 2002; Fordyce & Shapiro 2003; Lett *et al.* 2004; Solomon & Crist 2008).

Every organism exists either singly or with others, and each numerical context is associated with social and ecological conditions that carry potential fitness consequences for the individual. Therefore, achieving the optimal size of an egg clutch, flock or herd, for example, is a basic but dynamic ecological and evolutionary problem that all organisms must solve (Charnov 1976; Hassell & May 1986; Charnov & Skinner 1988; Kagata & Ohgushi 2004). However, selection gradients can be highly variable across time, space and with respect to the life history stage of the study organism, and optima are not likely to be static. Therefore, to fully understand the significance of adaptive grouping as a defence strategy, the functional response of the selective agent(s) to prey (or host) group size and its variability across time and prey (or host) ontogeny must be considered explicitly. Specifically, knowledge of the functional form by which natural enemies respond to prey traits, in general, is essential for making accurate predictions of ecological and evolutionary dynamics (Abrams 1982; Abrams & Matsuda 1997; Benrey & Denno 1997; Schmitz *et al.* 1997; Schreiber *et al.* 2000; Reader & Hochuli 2003; Krivan & Eisner 2006; Abrams *et al.* 2007).

In this study, we conducted an ecological survey using molecular genetics to determine the patterns of parasitism associated with host group size in a natural population of the tupelo leafminer, *Antispila nysaefoliella* Clemens (Lepidoptera: Heliozelidae), which is a specialist of tupelo, *Nyssa sylvatica* Marsh (Cornales: Nyssaceae). We tested the prediction that if group size is a response to parasitism, then variation in group size should correspond to the attack preferences and behaviours of the parasitoid community collectively. However, if there is high variability in the assemblage of parasitoid species, then would host grouping patterns correspond to the attack behaviours of the parasitoid community at large or to particular species of parasitoids? We expected that host group size would be optimized either for the average attack behaviour of all parasitoid species or only for the most frequent parasitoid taxa, depending on whether grouping is a generalized defence or not.

The current study applies a molecular genetics approach that has been little used in ecological studies– to quantify the potential effectiveness of host grouping as a defence strategy against a parasitoid community. Because of the minute sizes of the insects (host and parasitoid) and extreme variation of host traits, a novel approach was necessary to accurately assess a large sample of hosts gathered from the field. Compared with traditional methods used to detect and identify parasitoids within hosts, such as dissection and rearing, respectively, our study applied selective amplification of parasitoid DNA to quantify the timing, presence and species of parasitoids from within a background of host DNA (Greenstone 2006; Gariepy *et al.* 2007, 2008; Kaartinen *et al.* 2010; Santos *et al.* 2011; Smith *et al.* 2011; Gariepy & Messing 2012). By using taxon-specific primers, we were able to assign parasitism (confirm presence and identify species) and minimize sources of error that are typical of traditional methods due to: the difficulties of seeing and finding minute eggs, larvae, or pupae within a host, and rearing immature stages to adults that would then have to be identified using morphological keys or DNA sequencing (Hebert *et al.* 2003). Our approach simplified the data gathering process, and increased both accuracy and throughput.

Materials and methods

Natural history

The study site is located within a privately owned mixed deciduous forest in Clarke County, Virginia (39° 00.85′N, 78°03.88′W). In the spring, soon after the leaves of *Nyssa sylvatica* have flushed, adults emerge and persist in high abundance for ~4 weeks. Mating occurs near dusk and oviposition occurs throughout the day. Adults can be found easily by sight on the undersides of leaves or moving between leaves and branches. Multiple females have been observed to search and oviposit on the same leaves, either in succession or simultaneously. Therefore, larvae that occur on the same leaves are not necessarily all from the same female. Observational and unpublished experimental data indicate that eggs deposited in the spring lay dormant on leaves for several months before hatching into larvae. This suggests that timing of egg hatch can determine the realized larval group size, which may be under selection by forces that occur after the adult female is long gone. The current study provides the first empirical evidence on the potential importance of the interaction between larval group size and emergence timing on survival from natural enemies.

Typically around the first or second week of August each year, larvae emerge nearly synchronously. Then, 7–15 days later, a second cohort may emerge on the same leaves (Fig. S1). Low (2010) demonstrated that the presence of the earlier cohort delays the emergence of the subsequent cohort, which suggests a mechanism by which larvae may be able to regulate their group size

intrinsically. Group sizes have ranged from one to 48 larvae per leaf and from 0.02 to 0.8 larvae per cm^2 leaf area, with 4.8 ± 4.9 (mean ± 1 SD) larvae per leaf averaged across leaves with larvae only. Of the total leaves sampled (N = 3674; 2001, 2002, 2004), 59% contained at least one *Antispila nysaefoliella* larva. Actively feeding larvae can be found on leaves until complete leaf fall, which occurs during late October. By this time, however, all or most larvae will have completed all necessary feeding and exited from the leaves for pupation in the leaf litter. Completion of larval feeding is marked by the creation of a double-sided shield from the leaf epidermal layers within which the larva prepares for pupation. Once the shield is formed, the larva detaches from the leaf by perforating the shield's perimeter (from within), and still bound inside the shield, descends to the leaf litter. This leaves a distinctive oval-shaped hole in the leaf, which marks the end of the leaf-mining stage (Fig. S1).

In general, leaf-mining larvae burrow into the leaf mesophyll immediately after egg hatch and tunnel through the leaf tissue by feeding (or 'mining'). Body size and mine size are correlated because of the association between growth and feeding. Because *A. nysaefoliella* feeds in both upper and lower layers of the leaf mesophyll, the mine becomes a highly visible semi-transparent blotch-shaped 'window' composed only of the leaf cuticle layers (Hering 1951; Johnson & Lyon 1991). The mines of *A. nysaefoliella* always begin at a leaf vein, expand radially, and become more oblong-shaped during later instars (Fig. S1). Given that mines are more apparent than the larvae themselves, they are useful for finding larvae, retaining a historical record of their development and survival (head capsules and molt skins), and serving as proxies of larval size and developmental stage.

Leaf-mining insects are typically prone to extremely high levels of hymenopteran parasitism, with some species routinely experiencing parasitism rates exceeding 50% (Hawkins 1994). Such high rates of parasitism appear not to be the case in *A. nysaefoliella*. In previous years (2001, 2002, 2004), rearing and dissection of *A. nysaefoliella* mines and larvae from this site found confirmed parasitism rates ranging from 4.9 to 12.5% (N = 5374 *A. nysaefoliella* larvae; Low 2008 and unpublished data; Fig. S2). Mortality from predation and all other (unknown) sources have ranged from 0.9–3.9% and 7.0–21.5%, respectively. Because of the inherent difficulty of diagnosing sources of mortality, any larvae that were dead without obvious indication of parasitism or predation (e.g., mines ripped open with larva missing) were scored as 'other/unknown'. It is very likely that a proportion of these could be attributed to parasitism, and other factors, such as host plant defences, pathogens, and inadequate microclimate conditions for development. Hymenopteran parasitoids previously reared from *A. nysaefoliella* include: *Pseudognamptodon* sp. (Braconidae); *Chrysocharis assis* Walker, *Closterocerus cinctipennis* Ashmead, *Closterocerus trifasciatus* Westwood, *Pediobius* nr. *adelphae* Peck, *Pnigalio maculipes* Cr, *Pnigalio minio* Westwood, probably *Aprostocetus* sp. (all Eulophidae); and *Leptacis* sp. (Platygastridae).

Sampling

Host mine size served as an index for host ontogeny and parasitoid preferences, and seasonal timing provided information on the order of entry and temporal prevalence of parasitoid taxa. Therefore, we designed our sampling regime to capture the natural variation in parasitoid community over host ontogeny and season, and then subsampled within this collection to represent a balanced assay of host group sizes. Basic field protocol is described as follows. Small branches with leaves were sampled from 4 trees (~10 m apart) on 10 dates in 2006: 3, 8, 13, 21, 23, 27, 30 August, and 4, 9, 16 September (ordinal dates 215, 220, 225, 233, 235, 239, 242, 247, 252, and 259). From each tree, *c.* 4–6 branches in the lower to middle strata of the canopy were clipped from the ground using a hand held pruning pole, which extended ~5 m from the ground. Next, whorls of leaves were removed from the branches and put into large plastic zip bags labelled with the tree number and containing a paper towel to absorb moisture. Later, these were stored in a standard refrigerator (1.5 °C) during the sample preparation and processing, which took *c.* 2–3 days per sample date. Mines of each leaf were dissected and inspected for larvae using a dissecting microscope. The field collection yielded over 4000 larval samples. From this, we subsampled to create a balanced representation of: each tree, each sample date, and full range of larval densities. (See Fig. S3 for sample distributions.) Mine sizes (cm^2) were measured from scanned digital images using Sigma Scan 5.0. The statuses of unused specimens were recorded before being stored in 100% ethanol and at −80 °C.

When parasitoid pupae were discovered, they were reared in microcentrifuge tubes at room temperature. Prior to mounting and identification of successfully reared adults, their DNA was extracted and sequenced for a portion of COI using the methods described below. We also included COI sequences that we obtained from morphologically identified adult parasitoids from 2006 through 2008 in order to supplement the species diversity in our molecular reference dataset.

Molecular screening and cluster analysis

Our objectives were to: (i) detect the presence of parasitoids at any developmental stage through taxon-specific

PCR amplification of a 498 bp region of COI; and (ii) identify molecularly detected parasitoids by sequencing the COI amplifications and comparing the resulting sequences to those we obtained from morphologically identified adult specimens.

To design parasitoid-specific COI primers, we used a variety of published insect COI primers (Simon *et al.* 1994) to amplify and sequence COI from *A. nysaefoliella* as well as from morphologically identified adult parasitoids that we reared from *A. nysaefoliella* (see methods below). We aligned host and parasitoid COI sequences in Sequencher v. 4 (Gene Codes Corp., Ann Arbor, MI); alignment was straightforward and no indels were observed. Using this alignment, we designed new parasitoid-specific COI primers [forward: HYMCO2222 (5′-ATA TTT TAA TTY TNC CNG GAT TYG G-3′); reverse: HYMCO2769 (5′-CWG ART AWC GWC GRG GTA TWC CIC-3′)] to amplify a 498 bp portion of COI from all parasitoid taxa while precluding amplification of *A. nysaefoliella* host DNA. No multiple peaks were found within sequence data.

Genomic material was extracted from each *A. nysaefoliella* larva or parasitoid larva/pupa using the insect protocol B of the QIAGEN DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA). For PCR amplifications, we used Takara Ex Taq Polymerase™ (Takara Mirus Bio, Madison, WI) in reaction volumes of 25 μL. Cycling conditions were 95 °C for 2 min, 92 °C for 15 s, 58 °C for 20 s, 72 °C for 1 min, repeat last three steps 12 times, 92 °C for 10 s, 45 °C for 10 s, 72 °C for 1 min, then repeat the last three steps 34 times, 72 °C for 7 min, then hold at 4 °C. In every set of PCR reactions, we included both positive (adult parasitoid genomic DNA from this study that we had previously sequenced) and negative controls (blank). PCR product was purified with Exo-SapIt (USB, Cleveland, OH). Sequencing reactions were carried out using the amplification primers (above) and Big Dye Terminators v3.1 kits, and the sequence data were collected by fractionation using an ABI 3130XL automated DNA sequencer (both from Applied Bioysytems, Foster City, CA, USA).

Sequences from each parasitoid amplification were aligned, proofread, and exported as consensus sequences using Sequencher v.4 (Gene Codes Corp.); this program was also used to align the consensus sequences from each individual into a final alignment file. Because COI is a protein-coding gene, alignment was straightforward, and no indels were observed. Clustering of the sequences by overall similarity was performed using neighbour-joining (NJ) in PAUP* 4.0 Beta (Swofford 2005). *Leptacis* (Platygastridae) COI sequences were chosen to represent the outgroup taxa in the analysis. Using the patterns of sequence clustering generated from the NJ tree, we assigned probable species identity to parasitoid sequences obtained from nonadult parasitoid amplifications. Molecular voucher specimens are stored at the National Museum of Natural History (Washington, DC, USA) and sequences have been deposited in GenBank (Table S2; Accession numbers JN800360–JN800398; www.ncbi.nlm.nih.gov).

Ecological analyses

With parasitoid species assigned to clades of the NJ tree, we analysed the patterns of host use and temporal distribution with standard parametric tests. First, we determined the average rates of attack by each species out of the total number of host larvae sampled (n = 1524). Second, we tested the main effect of clade on mine size using analysis of variance of *ln*-transformed data, followed by post hoc comparison tests (Bonferroni method). Third, we used linear regression analysis to test the effect of group size on per capita attack rates for each parasitoid species. Lastly, we used data on the presence or absence of parasitism for each host larva (n = 1524) to determine the significance of mine size as a predictor variable for the risk of parasitism using logistic regression. This was analysed separately for each temporal cohort because of postulated differences in environmental and life history tradeoffs between cohorts.

Results

Parasitoid clades and prevalence

In total, we screened 1524 *A. nysaefoliella* larvae from 250 leaves having mine densities ranging from 1 to 22 per leaf. Amplification using parasitoid-specific COI primers resulted in the detection of 144 cases of parasitism (9.5%). Sequences from these amplifications were compared to those collected from reared and morphologically identified adult parasitoids using NJ clustering analysis. Parasitoid sequences could be attributed to one of 10 clusters representing various species of eulophids, braconids, and playgastrids (Fig. 1). In a few cases, one or two sequences were different enough from related adult sequences that an extra species or two may have been detected, but these are generally singletons and so few in number that they were lumped with their related sequences. In the case of *Pnigalio maculipes*, morphologically identified adult specimens came out in two highly distinct sister clades, both of which were well-represented in the larval screening sequences (Fig. 1). Because there is no formal taxonomic differentiation between these clades, we will call these *P. maculipes* 1 and *P. maculipes* 2 for the purposes of this study. *Pnigalio maculipes* 1 was the most common parasitoid, accounting for 47.2% of the total cases of parasitism, fol-

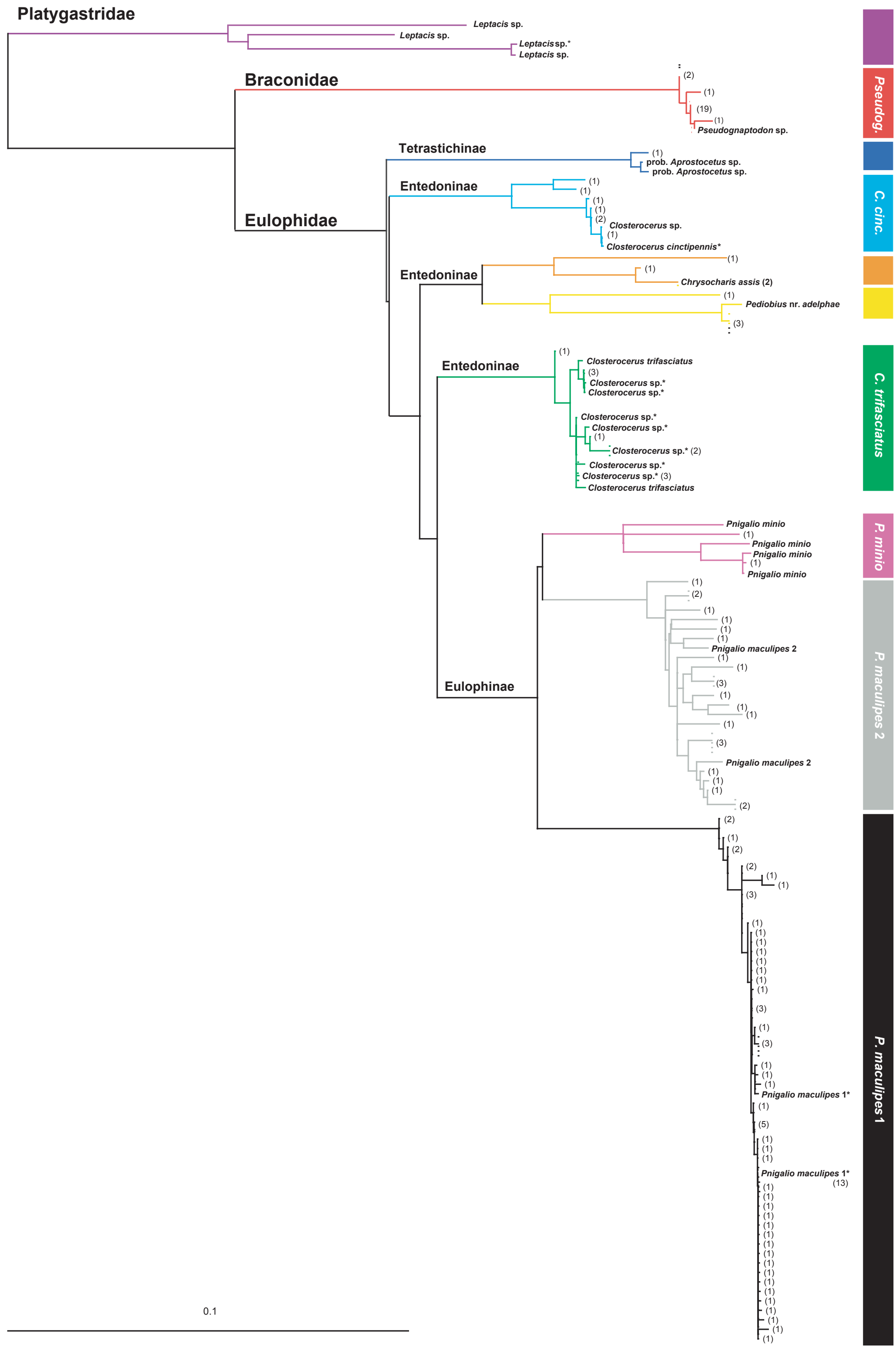


Fig. 1 Neighbour-joining tree using 163 mtDNA COI sequences (450 bp) of Hymenoptera. Tips with species names indicate an identified adult specimen. Otherwise, the number of samples belonging to a particular haplotype is indicated in parentheses. Coloured bars correspond to clade assignments and are consistent with colour legends in subsequent figures. An asterisk next to species name indicates that the specimen was reared from *Antispila nysaefoliella*, rather than field captured. All specimens were identified by M. Gates, except for *Leptacis* sp., which were identified by M. Buffington (NMNH).

lowed by: *Pseudognamptodon* sp. (16.0%), *Pnigalio maculipes* 2 (13.9%), *Closterocerus trifasciatus* (10.4%), *Closterocerus cinctipennis* (4.2%), *Pediobius* nr. *adelphae* (2.8%), probably *Aprostocetus* sp. (2.1%), *Pnigalio minio* (1.4%), *Chrysocharis assis* (1.4%), *Leptacis* sp. (0.7%).

Parasitoids were detected on all sample dates and across the full range of mine sizes (or larval ontogeny) (Figs. 2 and 3). However, there were qualitative temporal differences in parasitoid species composition and a tendency for assortment with respect to host mine size (Fig. 2). Species of *Pnigalio* sp. overlapped broadly in time but tended to be less overlapping with respect to host size, although this was not statistically significant (Fig. 2). *Pseudognamptodon* sp. was the most common late-season parasitoid and attacked significantly larger mine sizes than the second most common late-season parasitoid, *C. cinctipennis* (Fig. 2). Univariate analysis of variance showed that parasitoid clade had a significant effect on the average mine size attacked using *ln*-transformed data ($F = 11.56$; d.f. = 8, 133; $P < 0.001$). Significance values of all post hoc comparisons are presented in Table S1 and notated in Fig. 2. In brief, *Pseudognamptodon* sp. attacked mines that were significantly larger than most of the common species: *Chrysocharis* sp., *C. cinctipennis*, *P. maculipes* 1 and *P. maculipes* 2. In addition, *Closterocerus cinctipennis* differed from probably *Aprostocetus* sp., *Pediobius* nr. *adelphae*, *C. trifasciatus*, *P. maculipes* 1 and P. *maculipes* 2. Both *P. maculipes* clades differed from *Pseudognamptodon* sp., *Pediobius* nr. *adelphae* and *C. cinctipennis*, but not from each other. *Closterocerus trifasciatus* differed only from *C. cinctipennis* and *Pnigalio minio* did not differ significantly from any clade.

Risk from the host's perspective

As in previous years, there were two temporal cohorts of *A. nysaefoliella*, which occurred during days 210–225 (cohort 1) and days 230–260 (cohort 2), approximately.

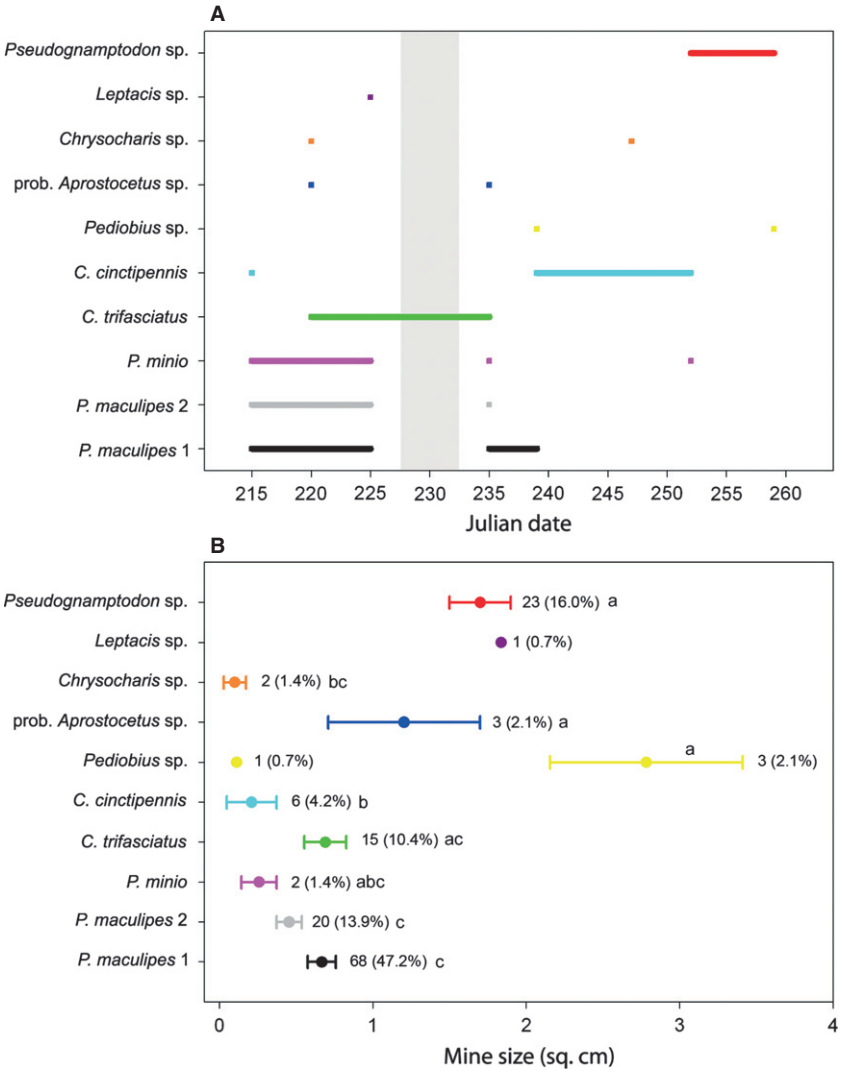


Fig. 2 Distribution of parasitoid clades across time and the average mine sizes parasitized. (A) Horizontal bars indicate the detection of a clade in consecutive sampling dates, whereas a single dot represents its presence during an isolated sampling date. Shaded region marks the transition period between cohorts. (B) The average mine size (±1 SE) attacked by each parasitoid. The sample size is indicated by the number next to each bar. Significant differences between clade notated by absence of shared letters. Values are reported in Table S1.

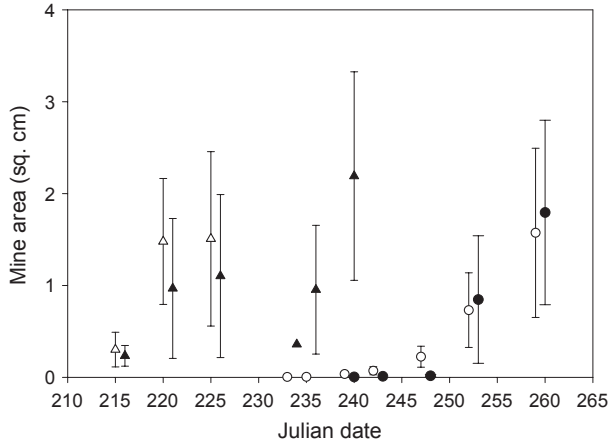


Fig. 3 Mine sizes of parasitized larvae (solid symbols) and unparasitized larvae (open symbols) for each sample date (mean ± 1 SD). Data for each date are offset for visual clarity. Triangles symbolize larvae from the first cohort, and circles symbolize the second cohort. (For more details on the temporal delay in *Antispila nysaefoliella*, see Low 2010.)

These cohorts were separated by a distinct break (at days 225–233, Figs 3 and S4) in larval activity where most of the earlier cohort had reached the pupation stage and exited the leaves. After several days, however, a widespread hatching of new larvae marked the beginning of the second cohort.

Of total parasitism, 78.5% (113) occurred with larvae of cohort 1. This includes parasitism of samples from days 210 to 225, as well as, most of those detected in samples from days 230 to 240. These latter parasitism events were included in cohort 1 because the advanced development of the parasitoid larvae or pupae indicated that they had attacked larvae of the first cohort (Fig. 3). By day 230, all surviving host larvae had exited the leaves for pupation. Hence, there are no measurements of nonparasitized larvae of the first cohort during days 230–240. The second larval cohort emerged on day 233 (Fig. 3). The leaves that were sampled often contained the mining damage from cohort 1 but no active larvae from cohort 1.

The parasitism rate of the first sample date of cohort 1 (day 215: 24.2%, $n = 227$) was markedly higher than even the first 14 days combined for cohort 2 (days 233–247: 0.57%, $n = 695$ larvae). Using mine size as a proxy, larvae from each cohort would be similar in body size and development on days 215 and 247, respectively (Fig. 3). Overall, the rate of parasitism was much lower for cohort 2 (2.9%, $n = 1060$) in comparison to cohort 1 (24.3%, $n = 464$).

Parasitism risk varied over time and with parasitoid species. Logistic regression analyses of parasitism risk suggest that the likelihood of parasitism is higher for small larvae in cohort 1, but higher for large larvae in cohort 2 (Fig. 4). This difference in risk may be due to the difference in the dominant parasitoid species and their respective host preferences. During cohort 1, *Pnigalio maculipes* 1 was the most common and attacked small larvae (early instars) early in the season, whereas during cohort 2, *Pseudognamptodon* sp. was the most common and attacked large larvae (late instars) at the end of the season (Fig. 2).

Variation in response to host group size

Regression analysis of the combined species data indicated that the number of parasitized larvae (y) increased with host group size (x) (model: $y = 0.94 + 0.12x$; $R^2 = 0.066$, d.f. = 79, $P = 0.02$; Fig. 5A); however, this pattern was driven solely by three outlier values (i.e. three leaves) of *P. maculipes* 1, which were two standard deviations above the mean values of both the total number and the proportion of larvae parasitized. These were leaves with 10, 12, and 13 larvae, of which 10 (100%), 11 (92%), and 12 (92%) were parasitized, respectively. When these particular values were removed from the analysis, there was no longer a significant positive association between the number of larvae parasitized and larval group size ($R^2 = 0.027$, d.f. = 76, $P = 0.15$), which then allowed the per capita risk of parasitism (y) to decline with group size (x) (model: $y = 0.81–0.27*\log(x)$; $R^2 = 0.46$, d.f. = 79, $P < 0.001$; Fig. 5B). When *P. maculipes* 1 was analysed separately, this survival benefit (i.e. dilution effect of group size) was no longer apparent ($R^2 = 0.09$, d.f. = 18, $P = 0.21$). Data are plotted as both the total number and proportion of larvae that were

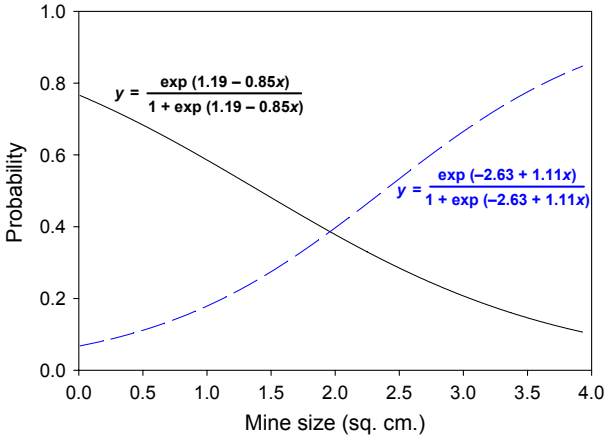


Fig. 4 The likelihood of parasitization with respect to mine size determined by logistic regression. Curves represent temporal cohorts. Solid line: cohort 1, $R^2 = 0.09$, d.f. = 1, $P < 0.001$; Dashed line: cohort 2, $R^2 = 0.12$, d.f. = 1, $P < 0.001$.

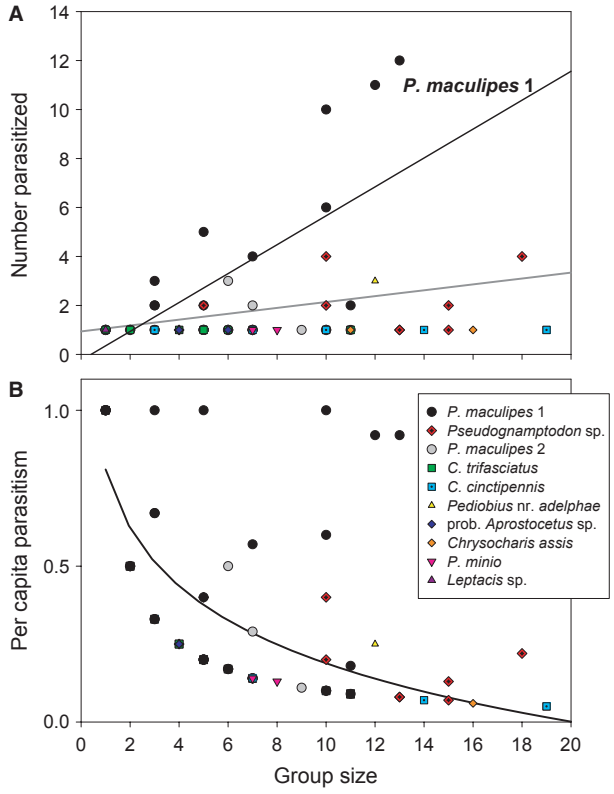


Fig. 5 The distribution of parasitism across larval group sizes. In (A), linear regression lines of number of hosts parasitized by group size for *Pnigalio maculipes* 1 alone (black line, $R^2 = 0.066$, d.f. = 79, $P = 0.002$) and all species combined (gray line, $R^2 = 0.027$, d.f. = 76, $P = 0.15$). In (B), the proportion of larvae parasitized (or per capita risk) by all parasitoid taxa estimated using a regression fit to a logistic model ($R^2 = 0.46$, d.f. = 79, $P < 0.001$). The lower panel (B) illustrates the decreasing per capita risk despite the increase in the number parasitized with host group size (A). The legend is sorted in order of prevalence of each parasitoid species.

parasitized on a leaf to illustrate both the risk to a group (Fig. 5A) and to an individual (Fig. 5B), respectively.

Of the 1524 larvae screened, we had initially identified through visual inspection during sample preparation that 93.9% (1431) were alive and showed no signs of parasitism or infection. Therefore, total mortality was estimated to be 6.1% using traditional methods of visual inspection. Our molecular survey was able to estimate of 9.5% mortality due to parasitism. Taking the all the mines together, including those that were empty (i.e. larva was missing), ~15.3% (476 out of 3104) were considered as no longer alive or missing, and assigned as an 'unknown' source of mortality.

Discussion

The objective of this study was to determine the patterns of parasitism in association with the traits of a host, *Antispila nysaefoliella*, with particular attention to host group size and the functional responses of parasitoid species. In order to assess parasitism, we used a molecular approach to detect and identify parasitoids within or associated with *A. nysaefoliella* larvae and pupae. The parasitism rate of 9.5% that we observed for *A. nysaefoliella* using this approach is fairly low compared to that reported for many other leafminers (Hawkins 1994). However, estimates of parasitism obtained during previous years (2001, 2002, 2004) from rearing *A. nysaefoliella* larvae and pupae have ranged from 5% to 13%, suggesting that a low rate of parasitism may be typical of this species, or at least this population (and across both cohorts). We speculate that the comparatively low rates of parasitism observed in *A. nysaefoliella* may be due to several factors. First, the late seasonal emergence of this species coincides with decreasing temperatures and generally reduced biotic activity. Therefore, parasitoids may simply be less abundant when larvae of *A. nysaefoliella* are available. The observation that parasitism is much lower in second cohort supports this. Second, this species has unique and dramatic seismic behaviours that are likely to function as defensive responses to parasitoids (Low 2008b, 2012). This behaviour would provide *A. nysaefoliella* with a distinct survival benefit that has not been reported in other leaf-mining species, especially to later instar larvae (Low 2008b). Overall, despite the low rate of parasitism in this population, there was nevertheless considerable variation in the attack behaviours, prevalence, and timing among parasitoid species. This suggests variability in the selective background for *A. nysaefoliella*. In the following sections, we discuss our results and the general implications of natural variation in parasitoid attack patterns.

Patterns of selection by parasitoids

Our molecular survey of parasitism in *A. nysaefoliella* detected 144 hymenopteran parasitoids, which were assigned to 10 separate species based on clustering of their DNA sequences with those from morphologically identified adult specimens. The different parasitoid species varied in timing of entry, host size selection, and attack rate in relation to host group size. The more common taxa, *Pnigalio maculipes* (1, 2), *Closterocerus* spp., and *Pseudognampton* sp., had relatively narrow temporal ranges in host attack. In contrast, the less common taxa, *Leptacis* sp., *Chrysocharis assis*, probably *Aprostocetus* sp., *Pediobius* nr. *adelphae*, and *Pnigalio minio*, did not appear to be restricted to any particular time window. However, the low rate of discovery of these taxa precludes us from drawing any firm conclusions about their distributions.

Given the synchrony of host ontogeny, the range of host sizes that would be available to temporally restricted parasitoid species would be somewhat limited. Therefore, it might be expected that co-occurring parasitoid species would also largely overlap in the host sizes or stages that they attack. Although we did not find the differences to be statistically significantly, the size ranges between species that co-occur in time tend to be more narrow than broadly overlapping, especially given the very small size range of *Pnigalio* sp. and comparing between haplotypes and species (Fig. 2). Furthermore, the observed overlap of host size between *P. maculipes* (both clades) and *C. trifasciatus* appears to be potentially offset by their temporal asynchrony, where *Closterocerus trifasciatus* becomes the most prevalent at days 233–235 when it may no longer be direct competition with *P. maculipes* (Figs 2 and S5). Although these data suggest that phenology and host size might be important in niche partitioning among these parasitoid species, competitive interactions among parasitoid larvae within host larvae (e.g. one parasitoid consuming another) might also lead to the appearance of temporal niche partitioning. However, the typical low rates of parasitism suggest that multi-parasitism necessitated by a competitive environment is unlikely since hosts do not seem to be a limiting resource. More sampling and experimentation are needed for a better understanding of the parasitoid ecology of this system.

Pnigalio maculipes

Pnigalio maculipes was the most common and prevalent species to attack *A. nysaefoliella*, causing 61% of the total detectable parasitism (Figs 2 and S5). It also appeared as one of the earliest species and was the only species to aggregate attacks at the leaf level (Figs 2 and 5). One explanation for the observed high rate of attack per leaf (i.e. outlier data) is that the high density of *P. maculipes* increases competition for hosts, and therefore, would favour an increased time investment of adult parasitoids at any single leaf. Optimal foraging theory would predict this to be true if the probability of finding another suitable patch is reduced (Charnov 1976). On the other hand, the overall rate of parasitism of *A. nysaefoliella* was quite low (9.5%), especially when compared to rates in other systems, which suggests that commonly range from 20% to 80% (Stiling *et al.* 1982; Connor & Cargain 1994; Godfray 1994; Hawkins 1994). The parasitism rates for both day 215 and for the entire cohort 1 was 24%, which also strongly supports that resources might not have been limiting even during this early part of the season. In addition, *A. nysaefoliella* may not be its only host resource (Krombein *et al.* 1979). Moreover, given that *P. maculipes* was one of the first parasitoids to enter the system, it would have primacy in access to newly emerged larvae. On the other hand, the early entry might be associated with a perceived, rather than actual, level of resource limitation, and the condition of going from an environment of few hosts (or none) may generate a response that is based on state-dependent or relative conditions—rather than actual or absolute conditions (Murdoch & Stewart-Oaten 1989; Rosenheim & Rosen 1991, 1992; Visser & Rosenheim 1998; Schreiber & Vejdani 2006).

Lastly, an alternate, but not exclusive, hypothesis is that *P. maculipes* 1 may be aggregating attacks because this is a better strategy given that females oviposit in the mine on or near the larva, rather than directly into the body (or onto the cuticle) of the host. This allows larvae to hatch from eggs, and then, search for the hosts themselves which could effectively reduce the expected handling costs for a female. Perhaps, a strategy of depositing multiple eggs per patch could be less costly if larvae can find and attach themselves to a host on their own. This could be adaptive especially in a resource-limited or time-limited environment and if a female was *not* egg-limited (Casas *et al.* 2000; Rosenheim *et al.* 2000). That is, she could be quite efficient in distributing eggs and have a high rate of success (in parasitizing a host) if larvae can invest in part of the effort. Females of endoparasitic species that oviposit directly into the host integument or body cavity have been reported to spend considerable amounts of time searching and handling each host (Casas 1989; Connor & Cargain 1994; Godfray 1994).

Disruptive selection of host life history traits

The two cohorts observed for this population of *Antispila nysaefoliella* may represent a life history strategy that has evolved in response to temporal variation in parasitism risk, and variation in the functional response of parasitoids to host density (or group size). Timing of emergence for *A. nysaefoliella* and for other sessile or sedentary species, not only impacts the potential risks that are experienced, but also influences defence effectiveness by directly determining the size of a group or cohort. Low (2010) showed experimentally that the removal of the earlier cohort of larvae triggers the emergence of otherwise dormant eggs that had been deposited on leaves 3–4 months earlier. This suggests that there is a mechanism in place that allows eggs to hatch in response to particular cues, and that a temporal delay in egg hatch could be adaptive and advantageous under particular ecological conditions.

For example, if absolute levels of parasitism are higher during the early cohort, then it may be a safer strategy to be a member of the later cohort and feed more slowly

and achieve a larger final size (Low *et al.* 2009). However, a potential disadvantage of being part of the second cohort is the rapid reduction in resource quality and quantity from leaf damage, leaf senescence, and herbivory. On the other hand, direct resource competition is not a problem when parasitism rates are high for the early cohort; and leaf senescence is less problematic, in general, for leafminers that are highly specialized in choosing particular leaf tissues and manipulating the leaf photosynthesis and longevity (Kaiser *et al.* 2010). Finally, a possible confounding factor is the effect of seasonal temperatures on slowing metabolic processes and feeding activity of late-feeding larvae, and subsequent increases in exposure (Benrey & Denno 1997; Fordyce & Shapiro 2003). Of course, this might also affect the foraging activity of parasitoids, and possibly even more so, compared to leafminers which are protected within a safe and more stable microhabitat (Connor & Taverner 1997). Therefore, overall safety could actually increase despite an increase in exposure time due to slower growth rates.

Safety in numbers

Our results provide evidence that grouping may be effective as a general strategy to reduce the per capita rate of parasitism, rather than a specialized strategy against any single parasitoid species. This is also consistent with previous findings (Low 2008a), which indicate that although host grouping increases (patch) detectability, its saturating function with respect to group size allows for a numerical dilution of risk. On the other hand, our results suggest that this benefit relies on the particular response form (functional response) adopted by a particular species and the prevalence of that species. We found that the most common parasitoid, *P. maculipes* 1 increased the number of hosts attacked (per leaf) with host group size, enough to remove the benefit of grouping. Therefore, if all parasitoid species attacked in this same pattern, grouping would no longer provide a survival benefit. Moreover, grouping would then become a liability rather than a source of greater safety for the individual if large groups are detected more frequently (Low 2008a).

In ecological studies, the consequences of group size are often studied as density-dependent or spatially density-dependent effects in predator-prey, host-parasitoid, or metapopulation dynamics (Walde & Murdoch 1988; Krivan & Eisner 2006; Saccheri & Hanski 2006). Nevertheless, whether the fitness consequences of group size are studied implicitly or explicitly, the form of the functional response is what identifies the shape or gradient that characterizes the selective pressure (i.e. the predator, parasitoid, or consumer). Most studies to date tend to use a particular and single functional response form to describe the behaviour of the predator or parasitoid populations (Casas *et al.* 1993; Bolker *et al.* 2003). However, our results demonstrate that the functional response can be diverse and dynamic with respect to the assemblage of species, which verifies the importance of explicit consideration of variation in functional response forms within communities of interacting species (Kato 1996; Cressman *et al.* 2004; Vamosi 2005; Saccheri & Hanski 2006).

Conclusion

Host-parasitoid systems have been a centrepiece for many studies in evolutionary biology and in both basic and applied ecology. Drivers of host-parasitoid dynamics have been attributed to, for example, nonrandom search by parasitoids (May 1978; Chesson & Murdoch 1986), spatial aggregation of hosts (May 1978; Walde & Murdoch 1988; Murdoch & Stewart-Oaten 1989), invulnerable host stage classes (Murdoch *et al.* 1997), variable durations of host instars and stage structure (Nisbet & Gurney 1983; Godfray & Hassell 1987, 1989) and life cycle synchrony (Gordon *et al.* 1991). In support of theoretical studies, our results provide natural history evidence showing that multiple mechanisms are in play in natural populations and can be dynamic with respect to host trait variation and time. However, our data also suggest that natural parasitoid assemblages are very diverse and host populations may respond differently to variable risk environments with defence repertoires that have evolved to deal with variability. Because the implications from host-parasitoid models are far reaching, an accurate view of the selective forces acting on the host population and the adaptive response of the host are essential for achieving both generality and application in ecological and evolutionary dynamics studies.

Acknowledgements

We are extremely grateful to John Endler, Roger Nisbet and Todd Oakley for their support and initial discussions on this project; Blandy Experimental Farm, Al Uy and Tom Starmer for logistical help and laboratory space; and Stonebridge Farm for access to their property. The manuscript was improved greatly by suggestions from Rosemary Gillespie and three anonymous reviewers. This project was supported by U.S. National Science Foundation Grants, DBI-0904395 and DEB-0608392, both awarded to C.L. USDA is an equal opportunity provider and employer.

References

Abrams PA (1982) Functional responses of optimal foragers. *American Naturalist*, **120**, 382–390.

Abrams PA, Matsuda H (1997) Prey adaptation as a cause of predator-prey cycles. *Evolution*, **51**, 1742–1750.

Abrams PA, Cressman R, Krivan V (2007) The role of behavioral dynamics in determining the patch distributions of interacting species. *American Naturalist*, **169**, 505–518.

Avilés L (1999) Cooperation and non-linear dynamics: an ecological perspective on the evolution of sociality. *Evolutionary Ecology Research*, **1**, 459–477.

Benrey B, Denno RF (1997) The slow-growth-high-mortality hypothesis: a test using the cabbage butterfly. *Ecology*, **78**, 987–999.

Bolker B, Holyoak M, Krivan V, Rowe L, Schmitz O (2003) Connecting theoretical and empirical studies of trait-mediated interactions. *Ecology*, **84**, 1101–1114.

Buss L (1981) Group living, competition, and the evolution of cooperation in a sessile invertebrate. *Science*, **213**, 1012–1014.

Caraco T, Wolf LL (1975) Ecological determinants of group sizes of foraging lions. *American Naturalist*, **109**, 343–352.

Casas J (1989) Foraging behavior of a leafminer parasitoid in the field. *Ecological Entomology*, **14**, 257–265.

Casas J, Gurney WSC, Nisbet R, Roux O (1993) A probabilistic model for the functional response of a parasitoid at the behavioural time-scale. *Journal of Animal Ecology*, **62**, 194–204.

Casas J, Nisbet RM, Swarbrick S, Murdoch WW (2000) Eggload dynamics and oviposition rate in a wild population of a parasitic wasp. *Journal of Animal Ecology*, **69**, 185–193.

Charnov EL (1976) Optimal foraging, the marginal value theorem. *Theoretical Population Biology*, **9**, 129–136.

Charnov EL, Skinner SW (1988) Clutch size in parasitoids: the egg production rate as a constraint. *Evolutionary Ecology*, **2**, 167–174.

Chesson PL, Murdoch WW (1986) Aggregation of risk: relationships among host-parasitoid models. *American Naturalist*, **127**, 696–715.

Clark CW, Mangel M (1986) The evolutionary advantages of group foraging. *Theoretical Population Biology*, **30**, 45–75.

Connor EF, Cargain MJ (1994) Density-related foraging behaviour in *Closterocerus tricinctus*, a parasitoid of the leaf-mining moth, *Cameraria hamadryadella*. *Ecological Entomology*, **19**, 327–334.

Connor EF, Taverner MP (1997) The evolution and adaptive significance of the leaf-mining habit. *Oikos*, **79**, 6–25.

Coster-Longman C, Landi M, Turillazzi S (2002) The role of passive defense (selfish herd and dilution effect) in the gregarious nesting of *Liostenogaster* wasps (Vespidae, Hymenoptera, Stenogastrinae). *Journal of Insect Behavior*, **15**, 331–350.

Cressman R, Krivan V, Garay J (2004) Ideal free distributions, evolutionary games, and population dynamics in multiple-species environments. *American Naturalist*, **164**, 473–489.

Fordyce JA, Shapiro AM (2003) Another perspective on the slow-growth/high-mortality hypothesis: chilling effects on swallowtail larvae. *Ecology*, **84**, 263–268.

Gariepy TD, Messing RH (2012) Development and use of molecular diagnostic tools to determine trophic links and interspecific interactions in aphid-parasitoid communities in Hawaii. *Biological Control*, **60**, 26–38.

Gariepy TD, Kuhlmann U, Gillott C, Erlandson M (2007) Parasitoids, predators and PCR: the use of diagnostic molecular markers in biological control of Arthropods. *Journal of Applied Entomology*, **131**, 225–240.

Gariepy T, Kuhlmann U, Gillott C, Erlandson M (2008) A large-scale comparison of conventional and molecular methods for the evaluation of host-parasitoid associations in non-target risk-assessment studies. *Journal of Applied Ecology*, **45**, 708–715.

Godfray HCJ (1994) Parasitoids: Behavioral and Evolutionary Ecology. Princeton University Press, Princeton, New Jersey.

Godfray HCJ, Hassell MP (1987) Natural enemies can cause discrete generations in tropical insects. *Nature*, **327**, 144–147.

Godfray HCJ, Hassell MP (1989) Discrete and continuous insect populations in tropical environments. *Journal of Animal Ecology*, **58**, 153–174.

Gordon DM, Nisbet RM, DeRoos AM, Gurney WSC, Stewart RK (1991) Discrete generations in host-parasitoid models with contrasting life cycles. *Journal of Animal Ecology*, **60**, 295–308.

Greenstone MH (2006) Molecular methods for assessing insect parasitism. *Bulletin of Entomological Research*, **96**, 1–13.

Hamilton WD (1963) The evolution of altruistic behavior. *American Naturalist*, **97**, 354–356.

Hamilton WD (1971) Geometry for the selfish herd. *Journal of Theoretical Biology*, **31**, 295–311.

Hassell MP, May RM (1986) Generalist and specialist natural enemies in insect predator prey interactions. *Journal of Animal Ecology*, **55**, 923–940.

Hawkins BA (1994) Pattern and Process in Host-Parasitoid Interactions. Cambridge University Press, Cambridge.

Hebert PDN, Cywinska A, Ball SL, DeWaard JR (2003) Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **270**, 313–321.

Hering EM (1951) Biology of Leaf Miners. S-Gravenhage, Berlin.

Hughes WO, Eilenberg HJ, Boomsma JJ (2002) Trade-offs in group living: transmission and disease resistance in leaf-cutting ants. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **269**, 1811–1819.

Ioannou CC, Morrell LJ, Ruxton GD, Krause J (2009) The effect of prey density on predators: conspicuousness and attack success are sensitive to spatial scale. *American Naturalist*, **173**, 499–506.

Ives AR (1989) The optimal clutch size of insects when many females oviposit per patch. *American Naturalist*, **133**, 671–687.

Jackson AL, Brown S, Sherratt TN, Ruxton GD (2005) The effects of group size, shape and composition on ease of detection of cryptic prey. *Behaviour*, **142**, 811–826.

Johnson WT, Lyon HH (1991) Insects That Feed on Trees and Shrubs. Cornell University Press, Ithaca, NY.

Kaartinen R, Stone GN, Hearn J, Lohse K, Roslin T (2010) Revealing secret liaisons: DNA barcoding changes our understanding of food webs. *Ecological Entomology*, **35**, 623–638.

Kagata H, Ohgushi T (2004) Conflict between optimal clutch size for mothers and offspring in the leaf miner, *Leucoptera sinuella*. *Ecological Entomology*, **29**, 429–436.

Kaiser W, Huguet E, Casas J, Commin C, Giron D (2010) Plant green-island phenotype induced by leaf-miners is mediated by bacterial symbionts. *Proceedings of the Royal Society B-Biological Sciences*, **277**, 2311–2319.

Kato M (1996) Effects of parasitoid community structure upon the population dynamics of the honeysuckle leafminer, *Chromatomyia suikazurae* (Diptera: Agromyzidae). *Researches on Population Ecology (Kyoto)*, **38**, 27–40.

Krause J, Ruxton GD (2002) Living in Groups. Oxford University Press, New York.

Krivan V, Eisner J (2006) The effect of the Holling type II functional response on apparent competition. *Theoretical Population Biology*, **70**, 421–430.

Krombein KV, Hurd Jr PD, Smith DR (1979) Catalog of Hymnoptera in America North of Mexico, Smithsonian Institution Press, Washington, District of Columbia.
Lett C, Auger P, Gaillard JM (2004) Continuous cycling of grouped vs. solitary strategy frequencies in a predator-prey model. *Theoretical Population Biology*, **65**, 263–270.
Low C (2008a) Grouping increases visual detection risk by specialist parasitoids. *Behavioral Ecology*, **19**, 532–538.
Low C (2008b) Seismic behaviors of a leafminer, *Antispila nysaefoliella* (Lepidoptera: Heliozelidae). *Florida Entomologist*, **91**, 604–609.
Low C (2010) The presence of active larvae delays the emergence of conspecifics in the tupelo leafminer, *Antispila nysaefoliella*. *Evolutionary Ecology Research*, **12**, 545–553.
Low C (2012) An experimental test of the seismic behaviors of *Antispila nysaefoliella* (Lepidoptera: Heliozelidae). *Florida Entomologist*, **95**, 16–20.
Low C, Wood SN, Nisbet RM (2009) The effects of group size, leaf size & density on larval performance. *Journal of Animal Ecology*, **78**, 152–160.
May RM (1978) Host-parsitoid systems in patchy environments: a phenomenological model. *Journal of Animal Ecology*, **47**, 833–843.
Morrell LJ, Ruxton GD, James R (2011) The temporal selfish herd: predation risk while aggregations form. *Proceedings of the Royal Society B-Biological Sciences*, **278**, 605–612.
Murdoch WM, Stewart-Oaten A (1989) Aggregation by parasitoids and predators: effects on equilibrium and stability. *American Naturalist*, **134**, 288–310.
Murdoch WW, Briggs CJ, Nisbet RM (1997) Dynamical effects of host size- and parasitoid state-dependent attacks by parasitoids. *Journal of Animal Ecology*, **66**, 542–556.
Nisbet RM, Gurney WSC (1983) The systematic formulation of population models for insects with dynamically varying instar duration. *Theoretical Population Biology*, **23**, 114–135.
Reader T, Hochuli DF (2003) Understanding gregariousness in a larval Lepidopteran: the roles of host plant, predation, and microclimate. *Ecological Entomology*, **28**, 729–737.
Rosenheim JA, Rosen D (1991) Foraging and oviposition decisions in the parasitoid *Aphytis lingnanensis*: distinguishing the influences of egg load and experience. *Journal of Animal Ecology*, **60**, 873–893.
Rosenheim JA, Rosen D (1992) Influence of egg load and host size on host-feeding behavior of the parasitoid *Aphytis lingnanensis*. *Ecological Entomology*, **17**, 263–272.
Rosenheim JA, Heimpel GE, Mangel M (2000) Egg maturation, egg resorption and the costliness of transient egg limitation in insects. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **267**, 1565–1573.
Saccheri I, Hanski I (2006) Natural selection and population dynamics. *Trends in Ecology & Evolution*, **21**, 341–347.
Santos AMC, Besnard G, Quicke DLJ (2011) Applying DNA barcoding for the study of geographical variation in host-parasitoid interactions. *Molecular Ecology Resources*, **11**, 46–59.
Schmitz OJ, Beckerman AP, Litman S (1997) Functional responses of adaptive consumers and community stability with emphasis on the dynamics of plant-herbivore systems. *Evolutionary Ecology*, **11**, 773–784.
Schreiber SJ, Vejdani M (2006) Handling time promotes the coevolution of aggregation in preclator-prey systems. *Proceedings of the Royal Society B-Biological Sciences*, **273**, 185–191.
Schreiber SJ, Fox LR, Getz WM (2000) Coevolution of contrary choices in host-parasitoid systems. *American Naturalist*, **155**, 637–648.
Simon C, Frati F, Beckenbach A, Crespi B, Liu H, Flook P (1994) Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America*, **87**, 651–701.
Smith MA, Eveleigh ES, McCann KS, Merilo MT, McCarthy PC, and Van Rooyen KI. (2011) Barcoding a quantified food web: crypsis, concepts, ecology and hypotheses. *PLoS One*, **6**, 1–12.
Solomon NG, Crist TO (2008) Estimates of reproductive success for group-living prairie voles, Microtus ochrogaster, in high-density populations. *Animal Behaviour*, **76**, 881–892.
Stiling PD, Brodbeck BV, Strong DR (1982) Foliar nitrogen and larval parasitism as determinants of leafminer distribution patterns on *Spartina alterniflora*. *Ecological Entomology*, **7**, 447–452.
Swofford DL (2005) PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.
Tosh CR, Jackson AL, Ruxton GD (2006) The confusion effect in predatory neural networks. *American Naturalist*, **167**, E52–E65.
Turner FT, Pitcher TJ (1986) Attack abatement: a model for group protection by combined avoidance and dilution. *American Naturalist*, **128**, 228–240.
Vamosi SM (2005) On the role of enemies in divergence and diversification of prey: a review and synthesis. *Canadian Journal of Zoology-Revue Canadienne De Zoologie*, **83**, 894–910.
Visser ME, Rosenheim JA (1998) The influence of competition between foragers on clutch size decisions in insect parasitoids. *Biological Control*, **11**, 169–174.
Walde SJ, Murdoch WW (1988) Spatial density dependence in parasitoids. *Annual Review of Entomology*, **33**, 441–466.

C.L. studies the evolutionary ecology of insect defences to gain insight into the mechanisms that stabilize plant-insect-enemy systems. She has focused her research on the defense strategies of the tupelo leafminer since 2000, and has uncovered many of this system's natural history attributes. S.J.S. conducts research on the evolution and ecology of plant-insect interactions with particular emphasis on leaf-mining and galling insects. M.L.L. is a molecular biologist who contributed extensive laboratory training, protocols, and troubleshooting for the molecular component of this study. M.G. studies taxonomy and systematics of Chalcidoidea, particularly Eurytomidae and Eulophidae, from the Neotropics. He is interested in plant-insect interactions, especially gall induction by arthropods and diversity in general.

Data accessibility

Final DNA sequence assembly and the ecological data are available in the Dryad Digital Repository: doi: 10.5061/dryad.68v7r. Summary of parasitoid and host DNA sequences listed in Table S2: Genbank acces-

sion numbers: JN800360-JN800398. These include only the sequences of identified parasitoid adults or distinct haplotypes of hosts. Voucher specimens were identified by M. Gates (Chalcidoidea), M. Buffington (Platygastroidea), R. Kula (Braconidae, *Pseudognamptodon* sp.), and D. Davis (initial specimens of host, *A. nysaefoliella*) and are archived at the National Museum of Natural History, Washington, DC, USA.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Significance values of differences in mine sizes attacked between clades tested using Bonferroni method of multiple comparisons.

Table S2 List of sequences from parasitoid adults either reared from *A. nysaefoliella* or caught directly from the field, and from the host, *A. nysaefoliella*.

Fig. S1 Leaves of *Nyssa sylvatica*. Panel (A) shows 14 larger mines ('a') and 12 smaller mines ('b'). The set of smaller mines represent a second larval cohort. Within the open space of each mine, you can see a single larva. The darker portion at the 'base' of each mine is the accumulation of frass, which traces from a leaf vein marking the mine's origin. In (B), two leaves are shown; each with a single completed mine with a distinctive punch hole (at 's') left behind after the larva created a shield and exited the leaf for pupation in the litter.

Fig. S2 These are estimates of per capita mortality risk due to: (A) parasitism, (B) predation, and (C) unknown causes. Leaves were sampled in 2001, 2002, and 2004. In 2001 and 2004, leaves were also sampled nondestructively in which marked leaves were left intact on trees and larvae were monitored over their lifetime. The nondestructive samples are noted as '2001.1' and '2004.1'. In total, 5374 larvae were sampled.

Fig. S3 Distribution of samples by tree and sample date (A) and larval group size (B), expressed as the proportions of the total sample of 215 leaves.

Fig. S4 Distribution of mine sizes of parasitized larvae (solid symbols) and unparasitized larvae (open symbols) for each sample date. Data for each date are offset for visual clarity. Triangles symbolize larvae from the first cohort, and circles symbolize the second cohort. (The means are reported in the main text in Fig. 3.For more details on the temporal delay in *A. nysaefoliella*, see Low 2010.)

Fig. S5 A total of 1524 larvae from 250 leaves were screened for parasitism. This resulted in 144 identified parasitisms, or 9.5%. The bars represent the percentage of larvae that were parasitized by each clade per sample date. The legend symbols are ranked in order of most to least prevalent among parasitoid clades. The numbers above each bar are the number of larvae/number of leaves. Sampling occurred from 3 Aug (day 215) to 16 Sep 2006 (day 259).

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

Table S1. Significance values of differences in mine sizes attacked between clades tested using Bonferroni method of multiple comparisons. The data were ln-transformed. *Leptacis* sp. and one haplotype of *Pediobius* sp. were excluded because these contained only one data point each.

Clade	*Pseud*	*Chrys*	*Quad*	*Pedio*	*C. cinc*	*C. trif*	*P. min*	*P. mac* 2	*P. mac* 1
Pseudognamptodon sp.	-	**0.002**	1.000	1.000	**<0.001**	0.112	0.478	**<0.001**	**<0.001**
Chrysocharis sp.	**0.002**	-	0.099	**0.003**	1.000	0.205	1.000	0.817	0.567
Quadrastichius sp.	1.000	0.099	-	1.000	**<0.001**	1.000	1.000	1.000	1.000
Pediobius nr. *adelphae*	1.000	**0.003**	1.000	-	**<0.001**	0.426	0.282	**0.050**	**0.038**
C. cinctipennis	**<0.001**	1.000	**<0.001**	**<0.001**	-	**<0.001**	1.000	**<0.001**	**<0.001**
C. trifasciatus	0.112	0.205	1.000	0.426	**<0.001**	-	1.000	1.000	1.000
P. minio	0.478	1.000	1.000	0.282	1.000	1.000	-	1.000	1.000
P. maculipes 2	**<0.001**	0.817	1.000	**0.050**	**<0.001**	1.000	1.000	-	1.000
P. maculipes 1	**<0.001**	0.567	1.000	**0.038**	**<0.001**	1.000	1.000	1.000	-

Table S2. List of sequences from parasitoid adults either reared from *A. nysaefoliella* or caught directly from the field, and from the host, *A. nysaefoliella*. Identifications were made by M. Gates (Chalcidoidea), M. Buffington (Platygastroidea), R. Kula (Braconidae, *Pseudognamptodon* sp.), and D. Davis (initial specimens of host, *A. nysaefoliella*). "Accession" is the number assigned to the nucleotide sequence by GenBank (submission "BankIt1478712", http://www.ncbi.nlm.nih.gov/genbank/). "Voucher" is the number assigned by the authors for DNA sample and the physical specimen, if available, that is archived at the National Museum of Natural History. Specimens labeled with "sp." could not be resolved further because of equivocal or missing morphological features. All specimens were collected from a stand of *Nyssa sylvatica* located in Clarke Co., Virginia, from 2005-2008. Duplicate sequences were not submitted, and hence, only 29 of the 163 total hymenopteran sequences are listed here.

Accession	Voucher	Order	Superfamily	Family	Subfamily	Species
JN800360	ANX0003	Lepidoptera	Incurvarioidea	Heliozelidae		*Antispila nysaefoliella* Clemens
JN800361	ANX0020	Lepidoptera	Incurvarioidea	Heliozelidae		*Antispila nysaefoliella* Clemens
JN800362	ANX0188	Lepidoptera	Incurvarioidea	Heliozelidae		*Antispila nysaefoliella* Clemens
JN800363	ANX0288	Lepidoptera	Incurvarioidea	Heliozelidae		*Antispila nysaefoliella* Clemens
JN800364	ANX0011	Lepidoptera	Incurvarioidea	Heliozelidae		*Antispila nysaefoliella* Clemens
JN800365	ANX0469	Lepidoptera	Incurvarioidea	Heliozelidae		*Antispila nysaefoliella* Clemens
JN800366	ANX0012	Lepidoptera	Incurvarioidea	Heliozelidae		*Antispila nysaefoliella* Clemens
JN800367	ANX0209	Lepidoptera	Incurvarioidea	Heliozelidae		*Antispila nysaefoliella* Clemens
JN800368	ANX0165	Lepidoptera	Incurvarioidea	Heliozelidae		*Antispila nysaefoliella* Clemens
JN800369	ANX0004	Lepidoptera	Incurvarioidea	Heliozelidae		*Antispila nysaefoliella* Clemens
JN800370	ANX0347	Hymenoptera	Platygastroidea	Platygastridae	Platygastrinae	*Leptacis* sp. Förster
JN800371	ANX0346	Hymenoptera	Platygastroidea	Platygastridae	Platygastrinae	*Leptacis* sp. Förster
JN800372	ANX0274	Hymenoptera	Platygastroidea	Platygastridae	Platygastrinae	*Leptacis* sp. Förster
JN800373	ANX0350	Hymenoptera	Platygastroidea	Platygastridae	Platygastrinae	*Leptacis* sp. Förster
JN800374	ANX1769	Hymenoptera	Ichneumonoidea	Braconidae	Gnamptodontinae	*Pseudognamptodon* sp. Fisher
JN800375	ANX0101	Hymenoptera	Chalcidoidea	Eulophidae	Tetrastichinae	*Quadrastichius* sp. Girault
JN800376	ANX0343	Hymenoptera	Chalcidoidea	Eulophidae	Tetrastichinae	*Quadrastichius* sp. Girault
JN800377	ANX1781	Hymenoptera	Chalcidoidea	Eulophidae	Entedoninae	*Closterocerus cinctipennis* Ashmead
JN800378	ANX1789	Hymenoptera	Chalcidoidea	Eulophidae	Entedoninae	*Closterocerus cinctipennis* Ashmead
JN800379	ANX1768	Hymenoptera	Chalcidoidea	Eulophidae	Entedoninae	*Chrysocharis assis* Walker
JN800380	ANX1772	Hymenoptera	Chalcidoidea	Eulophidae	Entedoninae	*Chrysocharis assis* Walker
JN800381	ANX1767	Hymenoptera	Chalcidoidea	Eulophidae	Entedoninae	*Pediobius* nr. *adelphae* Peck
JN800382	ANX1778	Hymenoptera	Chalcidoidea	Eulophidae	Entedoninae	*Closterocerus trifasciatus* Westwood

Accession	Voucher	Order	Superfamily	Family	Subfamily	Species
JN800383	ANX1775	Hymenoptera	Chalcidoidea	Eulophidae	Entedoninae	*Closterocerus trifasciatus* Westwood
JN800384	ANX1785	Hymenoptera	Chalcidoidea	Eulophidae	Eulophinae	*Pnigalio minio* Walker
JN800385	ANX1776	Hymenoptera	Chalcidoidea	Eulophidae	Eulophinae	*Pnigalio minio* Walker
JN800386	ANX1790	Hymenoptera	Chalcidoidea	Eulophidae	Eulophinae	*Pnigalio minio* Walker
JN800387	ANX1800	Hymenoptera	Chalcidoidea	Eulophidae	Eulophinae	*Pnigalio minio* Walker
JN800388	ANX1780	Hymenoptera	Chalcidoidea	Eulophidae	Eulophinae	*Pnigalio maculipes* Crawford
JN800389	ANX1783	Hymenoptera	Chalcidoidea	Eulophidae	Eulophinae	*Pnigalio maculipes* Crawford
JN800390	ANX0103	Hymenoptera	Chalcidoidea	Eulophidae	Eulophinae	*Pnigalio maculipes* Crawford
JN800391	ANX0344	Hymenoptera	Chalcidoidea	Eulophidae	Eulophinae	*Pnigalio maculipes* Crawford
JN800392	ANX0352	Hymenoptera	Chalcidoidea	Eulophidae	Entedoninae	*Closterocerus* sp. Westwood
JN800393	ANX0351	Hymenoptera	Chalcidoidea	Eulophidae	Entedoninae	*Closterocerus* sp. Westwood
JN800394	ANX0349	Hymenoptera	Chalcidoidea	Eulophidae	Entedoninae	*Closterocerus* sp. Westwood
JN800395	ANX0353	Hymenoptera	Chalcidoidea	Eulophidae	Entedoninae	*Closterocerus* sp. Westwood
JN800396	ANX0348	Hymenoptera	Chalcidoidea	Eulophidae	Entedoninae	*Closterocerus* sp. Westwood
JN800397	ANX0342	Hymenoptera	Chalcidoidea	Eulophidae	Entedoninae	*Closterocerus* sp. Westwood
JN800398	ANX0100	Hymenoptera	Chalcidoidea	Eulophidae	Entedoninae	*Closterocerus* sp. Westwood

Supplementary figures

Fig. S1 Leaves of *Nyssa sylvatica.* Panel (A) shows 14 larger mines ("a") and 12 smaller mines ("b"). The set of smaller mines represent a second larval cohort. Within the open space of each mine, you can see a single larva. The darker portion at the "base" of each mine is the accumulation of frass, which traces from a leaf vein marking the mine's origin. In (B), two leaves are shown; each with a single completed mine with a distinctive punch hole (at "s") left behind after the larva created a shield and exited the leaf for pupation in the litter.

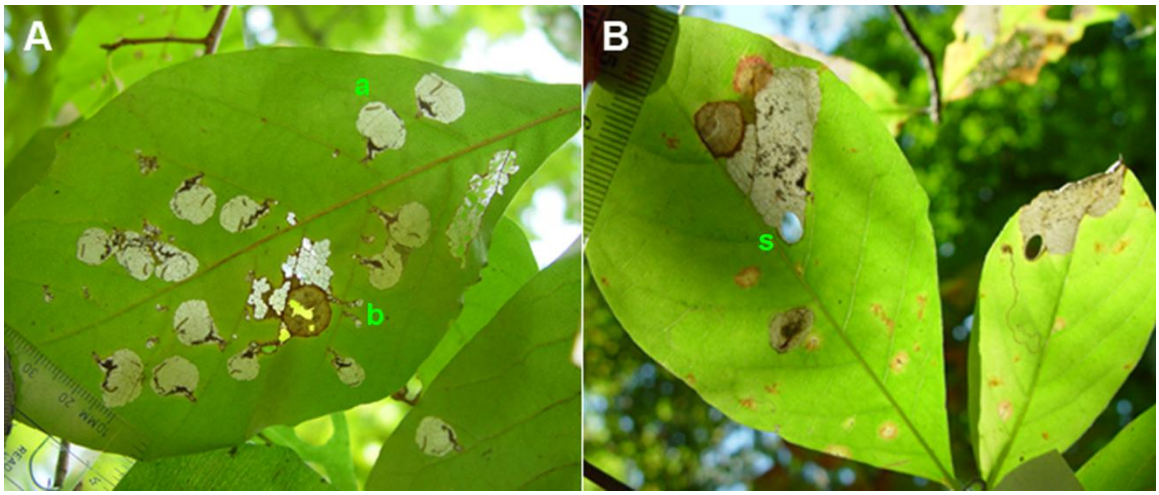
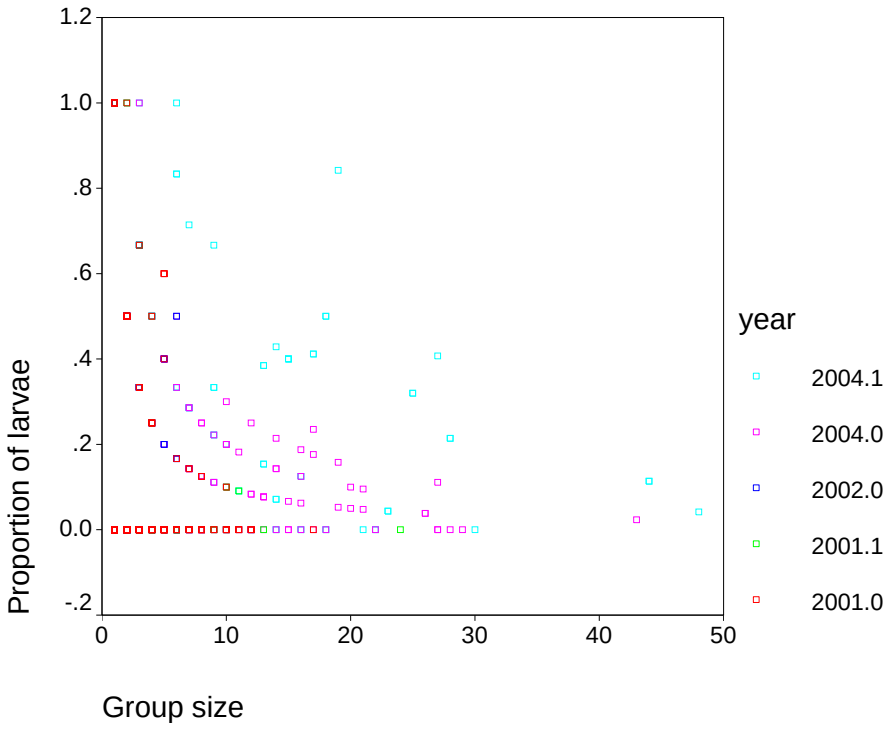
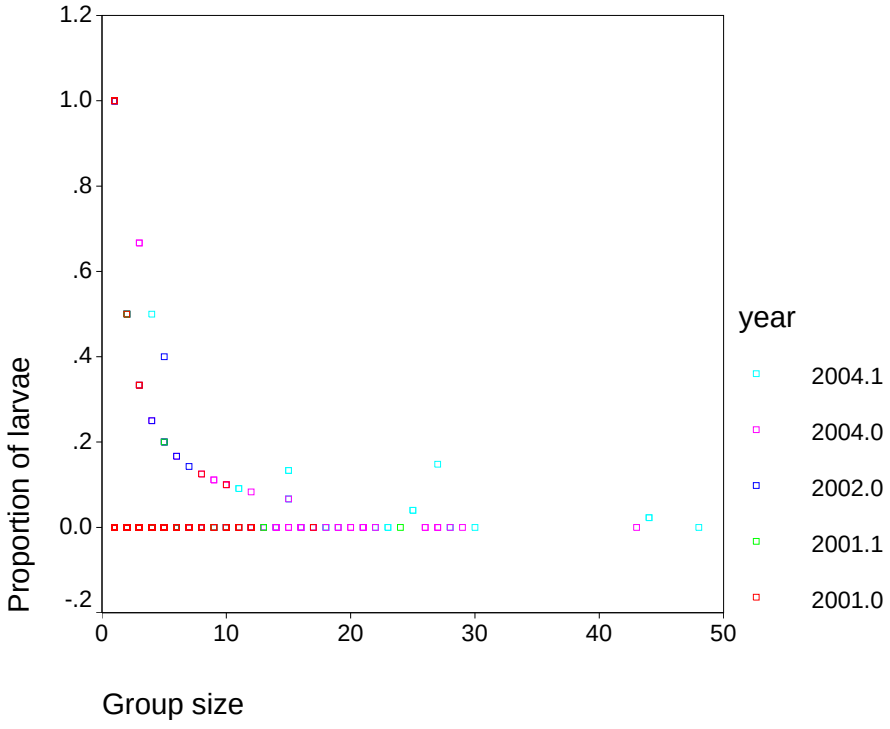


Fig. S2 These are estimates of per capita mortality risk due to: (A) parasitism, (B) predation, and (C) unknown causes. Leaves were sampled in 2001, 2002, and 2004. In 2001 and 2004, leaves were also sampled nondestructively in which marked leaves were left intact on trees and larvae were monitored over their lifetime. The nondestructive samples are noted as "2001.1" and "2004.1". In total, 5,374 larvae were sampled.

A. Per capita parasitism



B. Per capita predation



C. Per capita mortality from unknown causes

Although nondestructive sampling allowed for individual larvae to be tracked throughout their lifetime, the inability to inspect hosts for parasitism increased the number of assignments to "unknown", which is apparent in sample 2004.1.

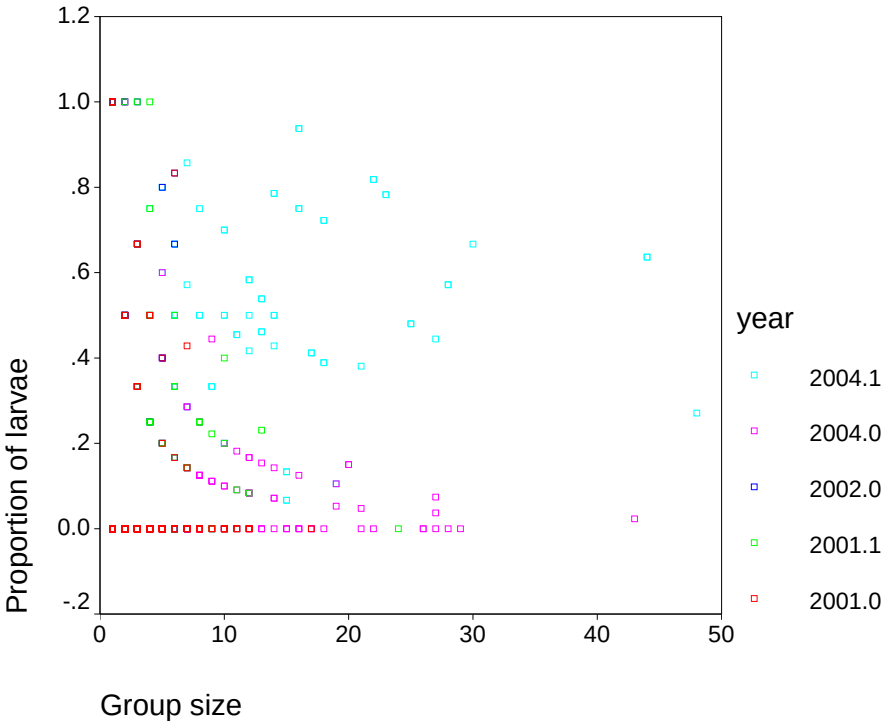


Fig. S3 Distribution of samples by tree and sample date (A) and larval group size (B), expressed as the proportions of the total sample of 215 leaves.

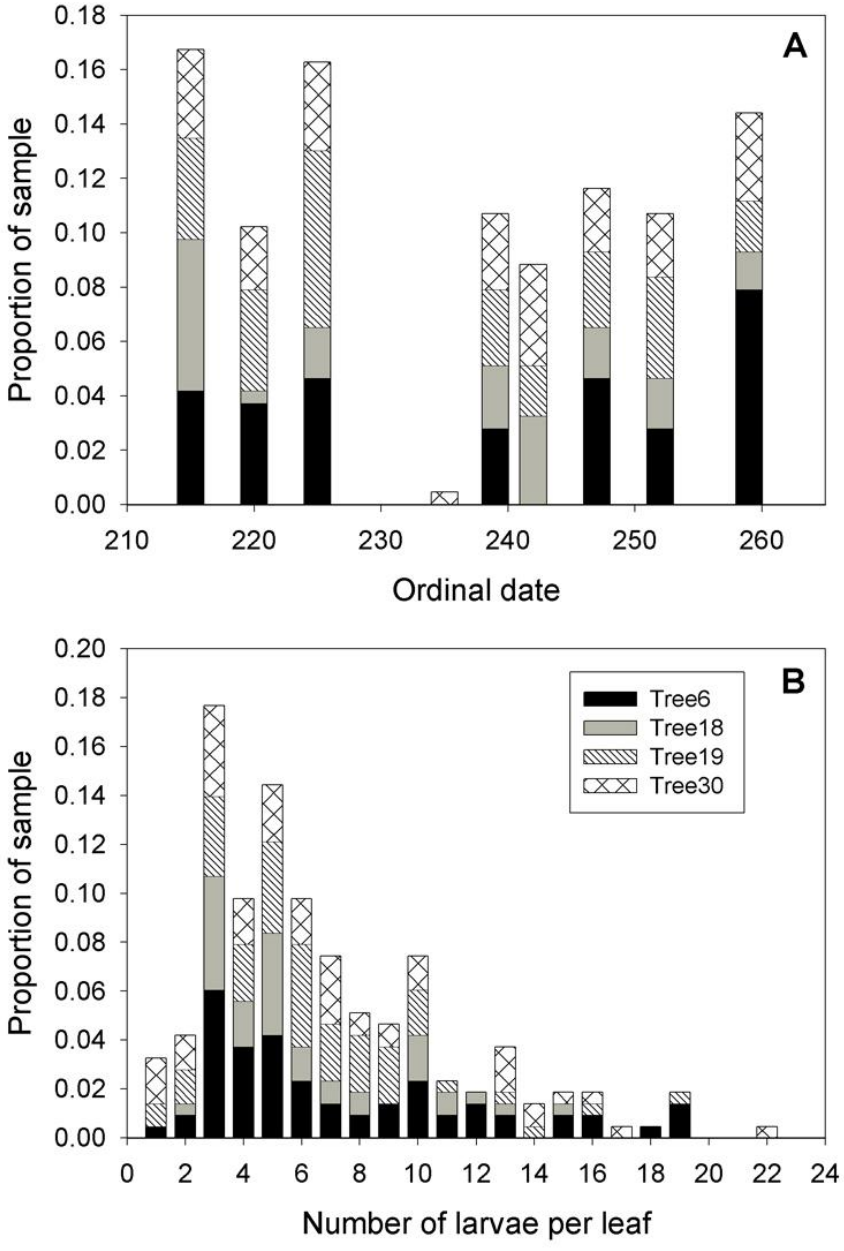


Fig. S4 Distribution of mine sizes of parasitized larvae (solid symbols) and unparasitized larvae (open symbols) for each sample date. Data for each date are offset for visual clarity. Triangles symbolize larvae from the first cohort, and circles symbolize the second cohort. (The means are reported in the main text in fig. 3. For more details on the temporal delay in *A. nysaefoliella*, see Low 2010.)

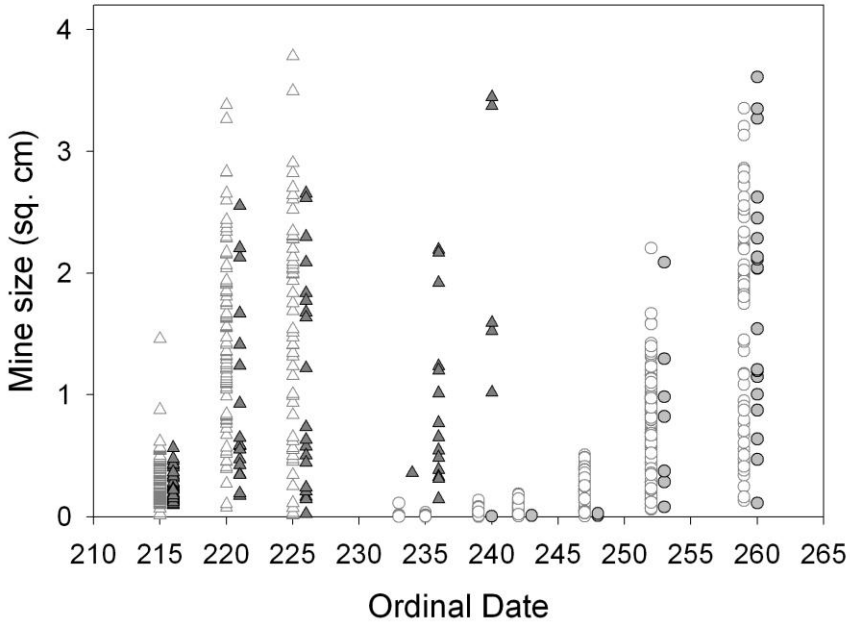


Fig. S5 A total of 1,524 larvae from 250 leaves were screened for parasitism. This resulted in 144 identified parasitisms, or 9.5%. The bars represent the percentage of larvae that were parasitized by each clade per sample date. The legend symbols are ranked in order of most to least prevalent among parasitoid clades. The numbers above each bar are the number of larvae/number of leaves. Sampling occurred from 3 Aug (day 215) - 16 Sep 2006 (day 259).