

Testing for enemy-mediated density-dependence in the mortality of seedlings: field experiments with five Neotropical tree species

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The coexistence of plant species in species-rich tropical forests can be promoted by specialised enemies acting in a negatively density-dependent manner. While survival of tropical tree seedlings is often negatively density-dependent, the causes have rarely been identified. We tested whether insects and plant pathogens cause density-dependent seedling recruitment and survival in five forest tree species in Belize, Central America. We manipulated densities of seeds or newly germinated seedlings in small (1 m² or 0.25 m²) plots close to fruiting conspecific trees. Using a factorial design, we excluded enemies from subsets of the plots with fungicides and insecticides. Seed germination (for two species) and early seedling survival (for all species) were monitored at approximately weekly intervals for up to eight weeks, during the period when plants are likely to be most susceptible to natural enemies. In *Terminalia amazonia*, seed germination was negatively density-dependent and the proportion of seeds germinating increased when insects were excluded. However, the magnitude of the insecticide effect was independent of density. The only significant density effect for survival of young seedlings was in *Acacia polyphylla*; counter to expectation, seedling survival was higher at high densities. In a few cases pesticide application had a significant effect on seedling survival, but in only one case (*Terminalia amazonia*) was a significant pesticide × density interaction detected. Our results caution against generalising from studies conducted on a single species at a single time and place and illustrate the challenges of experimentally testing for enemy-mediated negative density-dependence. Experimental outcomes are likely to depend on the spatial scale at which the principal enemies disperse and respond to plant density, and the timescales over which they act. Gathering information on these variables will improve our understanding of the natural histories of tropical forest species and help inform the design of future experiments.

According to the Janzen–Connell hypothesis, the coexistence of plant species in tropical forests – and the consequent high levels of alpha diversity (Valencia et al. 1994) – is promoted by specialised plant enemies (Janzen 1970, Connell 1971). By killing a larger proportion of their host in areas where it is common, host-specific enemies can create vacant sites for other plant species to colonise – thus promoting local diversity. Several decades after its formulation, the Janzen–Connell hypothesis remains a leading explanation for the high levels of plant diversity in tropical forests (Wright 2002, Leigh et al. 2004, Carson et al. 2008, Terborgh 2012).

While enemy mediated density-dependent effects can occur at any stage of a plant's life cycle (Schupp and Jordano 2011), seeds and young seedlings may be particularly susceptible (Clark and Clark 1985, Gilbert 2002). A substantial proportion of mortality occurs in these early stages of the plant life cycle (Harms et al. 2000, Hille Ris Lambers et al.

2002), and can leave a strong imprint on the distribution and diversity in larger size classes (Wills et al. 1997, Hubbell et al. 1999). Numerous studies on the fate of seeds and seedlings of tropical tree species have demonstrated widespread negative density-dependence (Harms et al. 2000, Comita et al. 2010, Metz et al. 2010, Paine et al. 2012). However, the mechanisms driving density-dependence have received less attention. In six tropical tree species, Mangan et al. (2010) showed that the growth and survival of seedlings grown in soil collected near conspecific trees was substantially lower than in soil from near heterospecifics, suggesting that host-specific soil biota reduce recruitment close to members of the same species. While Mangan et al. (2010) found little evidence of a role for aboveground enemies, these may be an important source of mortality for other species. Given their high degree of diet specialisation, certain groups of herbivorous insects have been identified as particularly likely

contributors to Janzen–Connell effects (Wilson and Janzen 1972, Janzen 1980, Hammond and Brown 1998, Novotny et al. 2010).

While many studies have assessed seed and seedling survival under different densities (sometimes taking a manipulative approach; Hyatt et al. 2003, Carson et al. 2008), relatively few studies have attempted to exclude enemies to establish the agents causing density-dependence (but see Bell et al. 2006, Norghauer et al. 2006, Swamy and Terborgh 2010). A potentially powerful approach is to use pesticides targeted at specific groups of enemies in combination with manipulative density treatments (Freckleton and Lewis 2006). By applying targeted pesticides to seedlings growing under different conspecific densities and assessing subsequent differences in seedling survival between pesticide treated seedlings and untreated control seedlings grown under similar densities, it is possible to assess the extent to which enemies contribute to density-dependent seedling mortality. At our field site in Belize, experiments of this kind have revealed that strong density-dependent seedling mortality in one tree species, *Pleradenophora longicuspis* (Euphorbiaceae) is driven by fungal pathogens causing rapid mortality within the first four weeks after seed germination (Bell et al. 2006, Bagchi et al. 2010). However, it remains unclear how widespread such effects are for other species, and whether other categories of plant enemy may also be important in driving density-dependence at this critically susceptible seedling stage.

In this paper, we describe field experiments testing for enemy mediated density-dependent mortality in five tree species, extending our earlier experiments to manipulate both pathogenic fungi and – for four of the species – insect herbivores. The aim of our study is to test whether the survival of seeds and newly germinated seedlings varies with conspecific density and whether any observed density-dependent effects are weakened in the absence of enemies. If insects and/or pathogens cause density-dependent seedling mortality, we predict 1) that seedling mortality should be higher in high density plots than in low density plots in the control treatment; 2) that seedling mortality should be lower in pesticide-treated plots than in control plots; and 3) that the decrease in seedling mortality following pesticide application should be greater in the high density treatment than in the low-density treatment (i.e. a significant pesticide treatment \times density interaction).

Methods

Study site and species

Field experiments were established in the vicinity of Las Cuevas research station (16°43'53"N, 88°59'11"W) in the Chiquibul Forest, Cayo District, Belize. The site is within one of the largest tracts of remaining forest in Central America and its vegetation has been classified as deciduous seasonal forest and deciduous/semi-evergreen seasonal forest (Wright et al. 1959). Annual rainfall is approximately 1500 mm (Johnson and Chaffey 1973) with a wet season typically from June to January and a dry season from

February to May. Timber has been extracted from the forest since the 1920s through selective logging operations (Bridgewater et al. 2006). The site lies within an area frequently affected by hurricanes; much of the current vegetation around Las Cuevas is regrowth following disturbance caused by hurricane Hattie in 1961.

Our experiments targeted five tree species, all of which are locally common: *Acacia polyphylla* (Fabaceae), *Cedrela odorata* (Meliaceae), *Cordia alliodora* (Boraginaceae), *Cryosophila stauracantha* (Arecaceae) and *Terminalia amazonia* (Combretaceae). These species represent a range of plant families, seed size and shade tolerance, and were drawn from a larger pool of potential taxa in this community without prior information on density-dependence in seedling survival and enemy-mediated mortality. *Cordia alliodora*, *T. amazonia* and *C. odorata* all have wind-dispersed seeds that tend to disperse over relatively large areas around the parent tree. The seed and seedling shadows surrounding reproducing individuals of *A. polyphylla* tend to be narrower. Many of the seeds of the palm *C. stauracantha* fall within a few meters of the parent tree, creating dense aggregations of seeds and seedlings. With the exception of *C. stauracantha*, which has an extended fruiting period, seeds of all study species are dispersed towards the end of the dry season, with germination occurring early in the wet season.

Experimental protocol

In 2007, 2008 and 2009 we conducted a set of experiments to test for enemy-mediated negative density-dependence in seed germination and/or in the survival of newly recruited seedlings for our study species. The basic experimental design was similar for all species, and followed the approach used by Bell et al. (2006). For each of the species 6–12 blocks, each comprising six 1-m² plots (0.25 m² for *C. alliodora*), were established close to fruiting tree individuals. Within each block, the plots were distributed haphazardly < 1 m apart, avoiding trees and larger saplings. Small volumes of pesticides applied at close range to the targeted seeds and seedlings prevented cross-contamination between plots. Densities of seeds or seedlings were manipulated to two levels: high and low (see species-specific details in Table 1 and Supplementary material Appendix A1). We assessed ambient seedling densities through preliminary surveys; for each species, one of the experimental densities represented a typical seedling density range (except for *T. amazonia*, where our density manipulation followed a slightly different scheme; Supplementary material Appendix A1). Within each block, half of the plots were randomly assigned to the 'low density' treatment and the other half to the 'high density' treatment. In each density category, pest pressure was suppressed in a subset of the plots using fungicides or an insecticide, while the remaining plots served as controls exposed to ambient levels of pest activity. This resulted in a design with one plot per density \times treatment combination in each block.

To reduce the activity of fungal pathogens (true fungi as well as oomycetes), 'fungicide plots' were treated weekly with a combination of two systemic pesticides, Ridomil Gold (active ingredient metalaxyl) and Amistar (active ingredient azoxystrobin), which were applied according to

Table 1. Summary of experimental designs for the different study species. For comparison, we also include information for the experiment on *Pleradenophora longicuspis* at the same field site conducted by Bell et al. (2006).

Species	Trees	Blocks per tree	Plot size	Pesticides used	Type of density manipulation	Initial difference in density between low and high density plots (L:H) ^a	Length of census intervals (days)	Duration of experiment (days)
<i>A. polyphylla</i>	6	1	1 m ²	Ridomil + Amistar; Engeo	seedlings uprooted	1:3.3 (15:49)	7–8	43
<i>C. odorata</i>	3	2	1 m ²	Ridomil + Amistar; Engeo	seedlings uprooted	1:8.4 (16:134)	6–9	34
<i>C. alliodora</i>	4	3	0.25 m ²	Ridomil; Amistar	seedlings uprooted	1:8.1 (25:202)	6–7	34
<i>C. stauracantha</i>	6	1	1 m ²	Ridomil + Amistar; Engeo	seeds added	1:41 (49:2000)	7–21	96
<i>P. longicuspis</i>	1	6	0.25 m ²	Ridomil	seedlings uprooted	1:6.1 (25:153)	7	35
<i>T. amazonia</i>	1	6	1 m ²	Ridomil + Amistar; Engeo	seeds added (high density) or removed (low density)	1:7	5–8	34

^aRelative difference in seed or seedling density between low and high density plots at the start of the experiments. Absolute densities are shown in brackets. For the high density treatment, an across-plot average is given. For *T. amazonia*, absolute seed densities were not assessed.

manufacturer's guidelines (Ridomil: 0.25 g m⁻² dissolved in 50 ml of water; Amistar: 0.005 g m⁻² dissolved in 50 ml of water). Both pesticides protect against true fungi as well as oomycetes, have low toxicity to non-target organisms and have little inhibitory direct effects on arbuscular mycorrhizal root colonization in temperate grasslands (Maron et al. 2011), crops (Diedhiou et al. 2004), and in *C. alliodora* and *C. odorata* seedlings at our study site (Miles 2008). (The combined application of Ridomil and Amistar will hereafter be referred to as the 'fungicide' treatment.) For all species (except *C. alliodora*, which did not include an insecticide treatment) we treated 'insecticide plots' with Engeo (active ingredient thiamethoxam) applied weekly according to manufacturer's guidelines (0.0025 ml m⁻² dissolved in 50 ml of water) and 50 ml of water. To account for potential effects of added moisture on seed germination and seedling survival, control plots were sprayed with 100 ml of water. The experimental treatments for *C. alliodora* focused only on pathogens and were designed to separate the potential effects of oomycetes and true fungi. To this end, Ridomil, which primarily targets oomycetes (through the active ingredient metalaxyl), and Amistar, a broad-spectrum fungicide that excludes true fungi as well as oomycetes, were applied as separate treatments. Due to practical constraints (notably, difficulties in locating seedling carpets for some species and differences in the extent of the seedling carpets among species), the number and spatial distribution of blocks, the method for density manipulation, and the temporal extent of the monitoring of seed and/or seedling fate differed slightly between the study species (summarised in Table 1, details in Supplementary material Appendix A1). Since the aim of this study was to determine the prevalence of enemy-mediated negative density-dependence in this community, not to compare specific responses among plant species, the experimental design was allowed to differ among species. Importantly, the variations in experimental methodology and design do not preclude testing the hypothesis of enemy-mediated negative density-dependence in seed and seedling survival of individual species.

Statistical analyses

Seedling survival

The mortality of newly germinated seedlings under different density and pest regimes was analysed for all five species using an analysis equivalent to a Cox-proportional hazard model (Egli and Schmid 2001). Since plots were censused at multiple points in time (to reduce the risk of newly recruited seedlings being undetected), we used seedling mortality between subsequent censuses as our unit of observation. New seedlings that emerged between two censuses were not included when deriving the response variable for the census interval in question. Hence, our response was the proportion of individuals detected at census t that were dead at census $t + 1$. For each study species we modelled the proportion of dead individuals as a function of pesticide and density treatment and their interaction. Census period was included as a categorical factor to allow the overall mortality rate to change over time without making specific assumptions about the temporal patterns of mortality. To account for differences in the length of census intervals, we included the logarithm of census length as an offset term. The correlation among repeated measures of seedling mortality was accounted for by including normally-distributed random effects for blocks and plots. Since the response was a proportion (fraction of seedlings dying between two censuses), and since initial analyses suggested that the data were overdispersed, we assumed a beta-binomial distribution of errors and the models were fitted with a complementary log-log link function. All models were fitted using the `gamlss` library in R 2.15.1 (Rigby and Stasinopoulos 2005).

The support for our three predictions regarding insect and/or pathogen-caused density-dependent mortality was assessed by comparing the parameter estimates for each factor against that of low density plots sprayed with water (control \times low density). Prediction 1, that seedling mortality should be higher in high density plots than in low density plots in the control treatment, would be supported by a significant (positive) coefficient value for 'high density'.

Significant negative parameter estimates for ‘fungicide treatment’ and ‘insecticide treatment’ provide support for prediction 2, that seedling mortality should be lower in pesticide-treated plots than in control plots. Finally, significant, negative coefficient values for the interaction terms ‘fungicide treatment × high density’ and ‘insecticide treatment × high density’ indicate whether the effects of pesticide treatment are density-dependent. If enemies cause negative density-dependence (prediction 3), the coefficient values of the interactions must differ significantly from that of the intercept. Differences in parameter estimates between control × low density and other treatment combinations were assessed based on hierarchical Wald tests and their associated p-values. To facilitate comparison with results reported by Bell et al. (2006), we report weekly survival rather than mortality in the figures and throughout the text.

Seed germination

For *T. amazonia* and *C. stauracantha* (for which density manipulations were implemented at the seed stage; Table 1, Supplementary material Appendix A1), we also tested whether the number of germinated seeds differed among the density and pesticide regimes. The number of seedling recruits in the experimental plots was modelled as a function of pesticide treatment and density using a similar approach as above for each species. Variation among blocks was modelled by including an intercept term for each block as a normally-distributed random effect. In the case of *C. stauracantha*, the response was the number of seeds (out of 49 tagged seeds in each 1-m² plot; Methods) germinating during the experiment. The models were fitted using the glmer function in R (lme4 package; Bates et al. 2012), assuming Poisson-distributed residuals as there was no evidence of residual over-dispersion. For *T. amazonia*, the protocol for density manipulation involved moving approximately 75% of seeds from the low density to the high density treatment (Supplementary material Appendix A1), so the expected ratio of seedling abundance in high versus low density plots was $(1 + 0.75)/0.25 = 7$, if recruitment success is

unrelated to local seed densities and/or natural enemies. To test whether the number of seeds germinating in the different density treatments deviated from the expected 1:7 relationship we included the expected number of seedlings (under no density-dependence) as an offset term in the models. Initial models of *T. amazonia* seed germination assuming a Poisson distribution for the errors showed evidence of residual overdispersion. Therefore, the model was fitted assuming a negative binomial distribution of residuals using the gamlss library.

Results

Seedling survival

Weekly seedling survival for untreated seedlings in the low density treatment ranged from 63% for *Cordia alliodora* to 97% for *Cryosophila stauracantha*. The density treatment had little effect on seedling survival (Fig. 1, Table 2a) and the only statistically significant effects were found in *Terminalia amazonia* and *Acacia polyphylla*. In *Acacia polyphylla*, contrary to our predictions, survival was higher in high density plots. In the case of *T. amazonia*, survival was lower in the high density treatment although the significant interaction between density and pesticide treatment in this species (Table 2a) suggests that this effect was only observed in the fungicide treated plots. Likewise, pesticide treatment did not generally have a dramatic effect on seedling survival (Fig. 1, Table 2a). In *A. polyphylla*, both insecticide and fungicide application caused a statistically significant increase in seedling survival, however the magnitude of the effect of pesticides was rather small: weekly seedling survival rates were 7 and 4% higher in the fungicide and insecticide treatments, respectively, compared to the control treatment. In *C. stauracantha*, fungicide did not significantly affect survival, and while insecticide application significantly increased survival it did so only by 1%. In *C. alliodora*, application of both fungicides (Ridomil and Amistar) increased seedling

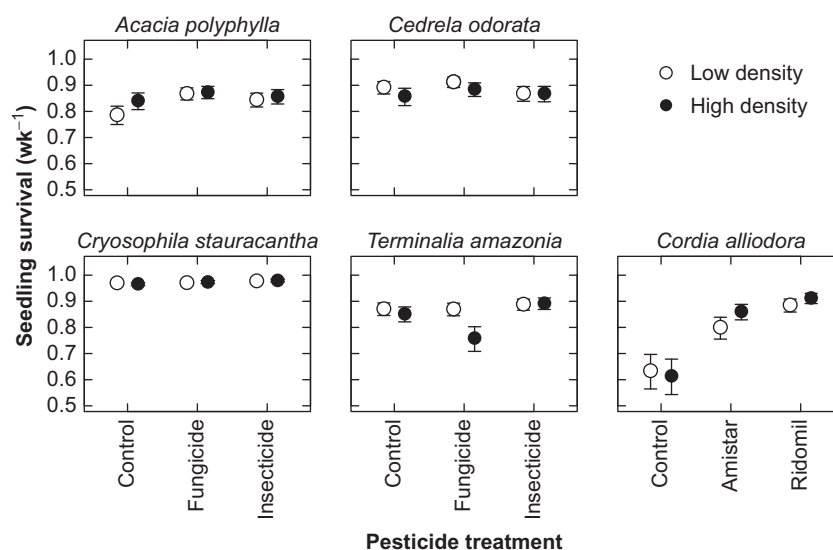


Figure 1. Model-adjusted weekly seedling survival rates in each treatment combination for each studied species.

Table 2. Results from species-specific generalised linear mixed-effects models of (a) seedling mortality and (b) germination (for exact model structure, see methods). The Wald-statistic was used to test for significance of the individual coefficients in the model. The 'Control × low density' treatment combination was set as the baseline (intercept) against which other parameters were compared. A negative value of the Wald-statistic indicates decreased mortality (increased survival) in the corresponding treatment. The standard deviations of the random effects for block and subplot are also provided.

(a)

Effect	<i>A. polyphylla</i>		<i>C. odorata</i>		<i>C. alliodora</i>		<i>C. stauracantha</i>		<i>T. amazonia</i>	
	Wald	p	Wald	p	Wald	p	Wald	p	Wald	p
Intercept	-27.62	<0.001	-25.45	<0.001	-13.62	0.001	-27.39	<0.001	-29.3	<0.001
Fungicide	-3.60	<0.001	-1.78	0.076	-	-	-0.91	0.363	-3.21	0.002
Insecticide	-2.18	0.030	-0.02	0.986	-	-	-2.49	0.013	-2.34	0.021
Amistar	-	-	-	-	-7.34	0.001	-	-	-	-
Ridomil	-	-	-	-	-10.64	0.001	-	-	-	-
Density_High	-2.08	0.039	1.64	0.103	1.51	0.128	-0.08	0.935	2.86	0.005
Fungicide × Density_High	1.40	0.163	-0.01	0.991	-	-	-0.70	0.487	2.30	0.002
Insecticide × Density_High	1.25	0.212	-1.05	0.295	-	-	-0.64	0.524	-0.82	0.411
Amistar × Density_High	-	-	-	-	-1.84	0.067	-	-	-	-
Ridomil × Density_High	-	-	-	-	-1.27	0.206	-	-	-	-
	Sigma		Sigma		Sigma		Sigma		Sigma	
Block	1.35		2.08		2.60		3.65		1.53	
Subplot	1.34		2.06		2.57		3.65		1.52	

(b)

Effect	<i>C. stauracantha</i>		<i>T. amazonia</i>	
	Wald	p	Wald	p
Intercept	48.13	<0.001	25.53	<0.001
Fungicide	0.41	0.683	0.01	0.995
Insecticide	1.39	0.164	6.10	<0.001
Density_High	0.71	0.478	-7.67	<0.001
Fungicide × Density_High	-0.04	0.969	-0.24	0.813
Insecticide × Density_High	-0.06	0.955	0.16	0.872
	Sigma		Sigma	
Block	<0.001		1.05	

survival, making Ridomil treated seedlings 42% more likely and Amistar treated seedlings 32% more likely to survive than seedlings in the control plot. There was a negative, although non-significant, trend in the interaction between fungicide and density, with Amistar treatment decreasing mortality of *C. alliodora* seedlings in the high density treatment slightly more than in the low density treatment (Table 2). In *T. amazonia*, there was a significant fungicide × density interaction (Fig. 1). Under fungicide treatment, seedling survival decreased in high density plots while seedling survival in low density plots remained similar to that in control plots.

Seed germination

In *C. stauracantha*, the experimental treatments had no significant effect on seed germination (Fig. 2, Table 2b). In *T. amazonia*, the relative number of seedling recruits was higher in low density plots than in high density plots, implying that the probability of seeds germinating is higher under low seed densities. In this species, the relative number of seedling recruits was also higher when insects were excluded. Since the effect of insecticide application was similar under both density regimes (Fig. 2, Table 2b), there was no evidence for insects causing the observed negative density-dependence in seed germination success.

Discussion

Despite experimental manipulations resulting in up to 40-fold differences in seedling densities between high and low density plots (Table 1), the survival of young seedlings typically differed little between density treatments for the five species in our study (for a qualitative summary of the results,

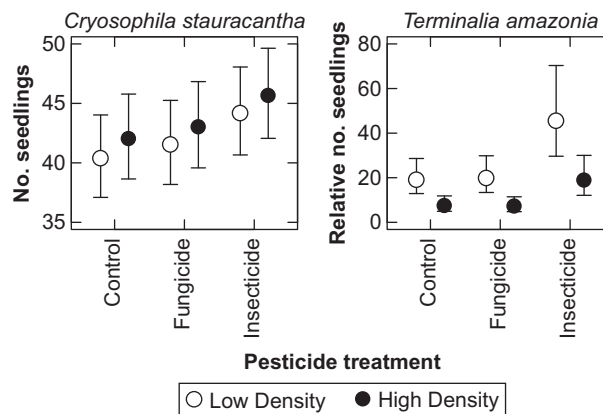


Figure 2. Model-adjusted absolute or (in the case of *Terminalia amazonia*) relative number of seedlings in different density and pesticide treatment combinations.

Table 3. Qualitative summary of results on (a) seedling survival and (b) germination. The information in the brackets indicates the direction of the effect on seedling survival or seed germination.

(a)

Species	Density effects in control treatment	Effect of insecticide on seedling survival	Effect of fungicide on seedling survival	Pesticide × Density interaction
<i>Acacia polyphylla</i>	yes (+)	yes (+)	yes (+)	no
<i>Cedrela odorata</i>	no	no	no	no
<i>Cordia alliodora</i>	no	not tested	yes (+); both fungicides	no
<i>Cryosophila stauracantha</i>	no	yes (+)	no	no
<i>Terminalia amazonica</i>	no	no	no	yes (high density, fungicide-treated seedlings survive better than untreated control seedlings)

(b)

Species	Density effects in control treatment	Effect of insecticide on germination	Effect of fungicide on germination	Pesticide × Density interactions
<i>Cryosophila stauracantha</i>	no	no	no	no
<i>Terminalia amazonica</i>	yes (–)	yes (+)	no	no

see Table 3). Similarly, the effects of enemy exclusion on seedling survival were either absent or small, and in none of the studied species did we find support for enemy-mediated negative density dependence in the survival of young seedlings. Enemy mediated negative density-dependence is thought to be important in promoting the coexistence of tropical plant species (Janzen 1970, Connell 1971), and we have repeatedly documented this mechanism at a small spatial scale for another tree species at our study site (Bell et al. 2006, Bagchi et al. 2010). Below we discuss specific results and methodological implications of our study.

Species-specific responses to density and pesticide treatments

Acacia polyphylla was the only species for which we detected a significant effect of seedling density on survival and, contrary to our predictions, untreated seedlings survived better when growing at high densities. In some cases, apparent positive density-dependence in seedling survival could result from higher seedling numbers in favourable microhabitats, for example if these habitats promote high seed germination success (Grubb 1977, Bagchi et al. 2009). However, this is not likely to be the case in our experiment since density treatments were randomly allocated to plots. Another potential explanation for positive density-dependence is enemy satiation (Crawley 1992). While enemies do seem to play a role in seedling survival in this species, with positive effects on seedling survival observed for both fungicide and insecticide treatments, these were independent of density suggesting that the strength of insect and fungal pathogen interactions was not strong enough to cause negative density-dependent recruitment patterns for *A. polyphylla*. It is also possible that positive density-dependence was caused by satiation of enemies not affected by our experimental treatments, such as non-insect herbivores. Alternatively, positive density-dependence may arise from a mechanism unrelated to plant enemies, for example through positive density-dependence in the action of plant mutualists such as mycorrhizae. These possibilities cannot be resolved using the current data. The only other case where density had a

significant effect (seed germination in *Terminalia amazonica*) was consistent with our prediction: a substantially larger proportion of seeds germinated in the low density plots.

Pathogen exclusion significantly increased seedling survival in two species (*Cordia alliodora* and *A. polyphylla*), with weekly survival increased by 40% and 7% respectively. These results suggest that pathogens play a role in early seedling mortality in these species. In *C. alliodora*, two types of fungicides that target different taxa were applied as separate treatments. In both treatments, we recorded a significant difference in seedling survival between fungicide treated plots and water-sprayed control plots. According to manufacturer guidelines, Ridomil is primarily effective against oomycete *Phytophthora* spp. and a small subset (three genera: *Alternaria*, *Peronospora* and *Septoria*) of the many fungal and oomycete genera targeted by Amistar. These genera all contain significant plant pathogens as well as species that are parasites of other fungi (e.g. *Cladosporium*), endophytes of asymptomatic leaf tissue (e.g. *Colletotrichum*), saprotrophs, moulds, and other life histories. Therefore it is important to consider that fungicides not only protect plants against pathogens, but may also change fungal species abundances and interactions both within plants and in the soil community. Given these complex interactions, conclusions about the relative importance of fungi or oomycetes as pathogens in this system must be made with great caution. In line with our predictions of density-dependence, there was a trend for Amistar application to have a stronger impact on seedling survival in the high density plots than in the low-density plots. A similar trend was not found in the Ridomil treatment. Using similar experimental manipulations, including Ridomil application to seedlings in the field, Liu et al. (2012), found *Fusarium oxysporum* to be an important fungal pathogen of their focal species but not in a negatively-density dependent manner. Together, these results highlight the importance of diseases in forest systems while demonstrating that the nature of these interactions may not always be density-dependent.

Insecticide application had an effect on seedling survival in *A. polyphylla* and *Cryosophila stauracantha* and on seed

germination success in *T. amazonia*. In *A. polyphylla* and *Cryosophila stauracantha*, weekly seedling survival was 4% (*A. polyphylla*) and 1% (*C. stauracantha*) higher in insecticide treated plots than in control plots, and in *T. amazonia*, the relative number of seedling recruits was 150% higher in insecticide plots than in control plots, suggesting that insects do indeed play a role in the early survival of these species.

In all the above-mentioned cases, pesticide effects were independent of density and so our key prediction of enemy-mediated negative density-dependence was not supported by our data. The only significant pesticide \times density effect that we recorded was unexpected: seedling survival in the high-density treatment of *T. amazonia* was significantly lower following fungicide application. There are several potential reasons for this. In principle, seedlings could die as a result of phytotoxic effects of the fungicides or due to unintentional side effects of fungicides on associated organisms such as beneficial soil organisms and foliar endophytes. Phytotoxicity is unlikely to have occurred from the weekly foliar applications used in our field experiments, which should also have had limited impact on non-target and soil organisms. Furthermore, since the exclusion of beneficial organisms should also affect fungicide-treated seedlings in the low-density plots, some other explanation seems more likely. One possibility is a shift in the strength of other mortality factors following enemy exclusion. If the exclusion of pathogens results in higher seedling survival, intraspecific competition for resources could become strong enough to increase mortality in the high density plot. Since there is little evidence for strong inter- and intraspecific competition among seedlings (Paine et al. 2008, Svenning et al. 2008), the likely mechanism driving the significant negative fungicide \times density interaction in *T. amazonia* remains unknown.

Methodological insights on the potential importance of scale

Little is known about the spatial and temporal scales at which different groups of enemies respond to the densities of their hosts (Lewis and Gripenberg 2008), and it may be that density manipulations carried out at the scale of 1-m² plots have little effect on the behaviour of certain enemies. Two studies (Harms et al. 2000, Bagchi et al. unpubl.) detecting negative density-dependence at the seed-to-seedling transition stage (a period which comprises both seed germination and survival at the early seedling stages) in a large number of species used plots of similar size to ours. However, these studies did not manipulate conspecific densities and utilised natural variation in density over relatively large spatial scales (1 to 50 ha) instead. Because local (1 m²) seedling densities may covary with densities at larger spatial scales, correlations between small-scale seedling density and mortality in the studies by Harms et al. (2000) and Bagchi et al. (unpubl.) may therefore reflect enemy responses to adult, seed, or seedling host densities in the wider landscape rather than to fine-scale seed or seedling densities. Furthermore, if enemies attacking seeds or seedlings aggregate on conspecific adults (as suggested by Janzen 1970), or enemy abundance is more strongly determined by the roots, litter, or canopy of the adult tree than by ephemeral increases in seed or seedling densities, small-scale

manipulations of seed or seedling densities are unlikely to affect mortality greatly.

Our experiments also differ from the studies by Harms et al. (2000) and Bagchi et al. (unpubl.) in terms of their temporal scale. The experiments reported here were relatively short-term (experimental treatments maintained for up to eight weeks, compared to durations of months or years) and tracked germinating seeds and seedlings that were only weeks to months old. We concentrated on the earliest stage in plant establishment because of evidence from earlier experiments at our field site that suggested that pathogen induced mortality in one species, *Pleradenophora longicuspis*, predominantly occurred in the first weeks after germination (Bell et al. 2006, Bagchi et al. 2010). However, longer sampling allows more time for enemy effects to accumulate, potentially making it more likely that enemy-mediated effects on seedling mortality will be detected. Furthermore, longer experiments are more likely to overlap with transient conditions suitable for pathogen or herbivore outbreaks that could have important effects on seedling survival. For example, germinating seeds of ten *Cecropia* species showed no survival benefit from eight weeks of fungicide application, even though fungal pathogens caused significant mortality during four months of seed bank incubations (Gallery et al. 2010). While vertebrate seed predators and herbivores can drive rapid negative density-dependence (Paine and Beck 2007, Bagchi et al. 2011), our short-term results suggest that insect and pathogen-mediated mortality of very young seedlings is low for the majority of our studied species, but it could prove to be important over longer time scales.

While the spatial and temporal scales of our density manipulations and survival monitoring may have been too restricted to find any enemy-mediated density effects on seedling survival in our five studied species, the studies by Bell et al. (2006) and Bagchi et al. (2010) in even smaller experimental plots (0.25 m²) demonstrated marked effects of density on pathogen induced mortality in *Pleradenophora longicuspis* seedlings on similarly short time-scales. This serves to illustrate the system-specific aspects of experimental outcomes. In the case of *P. longicuspis*, the newly germinated seedlings are attacked by fungal pathogens that are very effective in killing hosts growing in dense aggregations. These pathogens are primarily spread through physical contact between neighbouring seedlings and through rain splash (Swinfield et al. 2012). In such cases, thinning of seedling carpets may have dramatic impacts on the success of the spread of the pathogen, even at small spatial scales. On the other hand, if seedling survival is affected by pathogens already present in the soil, environmental factors triggering outbreaks would have stronger effects than density-manipulations of contemporary seedling densities.

A more efficient though challenging route for manipulative field experiments of the type described here might therefore be a dual approach, where the first step involves identifying the likely identities of the enemies affecting the plant species in question (Alvarez-Loayza and Terborgh 2011), the ontogenetic stages that the enemies attack, and the temporal window across which substantial enemy-mediated mortality occurs (e.g. through pesticide applications in a density-independent context). Only when this

information is available would factorial experiments testing for a pesticide \times density interaction be carried out. The lack of information on the relevant spatial scales and magnitudes across which different types of enemies respond to variation in host densities remains, but it seems likely that more mobile enemies (e.g. insects, vertebrates) require larger scale experiments than most soil-borne pathogens. In cases where enemies attack multiple ontogenetic stages of the plant (for example herbivores feeding on seedlings and adult foliage), it may also be necessary to allow experimental plots to vary in their range of conspecific densities in the neighbourhood. Ideally, experiments could be nested within mapped vegetation plots (to allow assessment of the spatial scale across which enemies respond). Where this is not possible, establishing plots both near and far from adult trees – as done by Swamy and Terborgh (2010) for seedling enemies, and Gallery et al. (2007) for fungal seed pathogens – is an alternative.

Summary and conclusions

Adopting a protocol almost identical to the one used by Bell et al. (2006) to detect strong pathogen-caused negative density-dependence in the tree *P. longicuspis*, we failed to detect any enemy-mediated negative density-dependence in the survival of young seedlings of five other, haphazardly selected tree species in the same community. This suggests that small-scale insect and/or pathogen-mediated negative density-dependent mortality of newly germinated seedlings is a far from ubiquitous phenomenon and cautions against generalising the results of a species-specific study. Our study illustrates some of the challenges of conducting manipulative experiments testing for enemy-mediated negative density-dependence in seedling survival. Decisions must be made about the spatial scale, type (seed addition, seedling thinning) and magnitude of the density manipulation, which enemies are targeted and what methods are used to exclude them, and the time period over which the experimental treatments are maintained. Each of these decisions can potentially have an impact on the experimental outcome. Nevertheless, continued manipulative field-based experiments are required to reveal the processes behind negative density-dependence patterns such as those reported by Harms et al. (2000), Comita et al. (2010), and Paine et al. (2012).

Acknowledgements – SG and RB are joint first authors. We thank the late Nicodemus Bol, Matthew Bol, Jimmy Boucher, Ed Miles and Claire Addis for help with fieldwork and Timothy Paine for helpful comments on a previous version of the manuscript. The study was supported by NERC grant NE/D010721/1 to Owen Lewis and Robert Freckleton. Sofia Gripenberg was supported by the Academy of Finland (grant no. 126296 and 138299).

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Supplementary material (available online as Appendix oik-00835 at <www.oikosoffice.lu.se/appendix>). Appendix A1.