

LETTER

Multiple natural enemies cause distance-dependent mortality at the seed-to-seedling transition

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Abstract

Specialised natural enemies maintain forest diversity by reducing tree survival in a density- or distance-dependent manner. Fungal pathogens, insects and mammals are the enemy types most commonly hypothesised to cause this phenomenon. Still, their relative importance remains largely unknown, as robust manipulative experiments have generally targeted a single enemy type and life history stage. Here, we use fungicide, insecticide and physical enclosure treatments to isolate the impacts of each enemy type on two life history stages (germination and early seedling survival) in three tropical tree species. Distance dependence was evident for five of six species-stage combinations, with each enemy type causing distance dependence for at least one species stage and their importance varying widely between species and stages. Rather than implicating one enemy type as the primary agent of this phenomenon, our field experiments suggest that multiple agents acting at different life stages collectively contribute to this diversity-promoting mechanism.

Keywords

Diversity maintenance, fungal pathogens, Janzen-Connell hypothesis, negative density dependence, seed dispersal, seed-to-seedling transition.

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INTRODUCTION

Density- or distance-dependent mortality plays a large role in maintaining diversity (Janzen 1970; Connell 1971; Wright 2002) and determining species abundance (Klironomos 2002; Comita *et al.* 2010; Mangan *et al.* 2010). Field studies in tropical and temperate forests show the widespread significance of this phenomenon (Harms *et al.* 2000; Hille Ris Lambers *et al.* 2002; Comita *et al.* 2010; Johnson *et al.* 2012). These findings have resulted in an increased research focus on determining the agents responsible (Packer & Clay 2000; Klironomos 2002; Bell *et al.* 2006; Bradley *et al.* 2008; Swamy & Terborgh 2010). Fungal pathogens, mammals and insects are often hypothesised to be the agents of distance- or density-dependent mortality (Janzen 1970; Connell 1971; Terborgh 2012). An understanding of the relative importance of different natural enemy types is limited, as research that has tested the impacts of certain enemy types individually (e.g. Packer & Clay 2000; Bell *et al.* 2006; Mangan *et al.* 2010; McCarthy-Neumann & Kobe 2010; Liu *et al.* 2012) is poorly equipped to assess which natural enemy types contribute most to this diversity-promoting mechanism.

The majority of studies that focused on the agents causing distance- and density-dependent mortality have targeted fungal pathogens, finding strong evidence for their involvement using a variety of approaches. Observational studies have found an increased prevalence of wilting or discoloration in seedlings near conspecific adults or at high conspecific density (e.g. Augspurger 1984; Alvarez-Loayza & Terborgh 2011). Other studies have shown survival of seedlings grown under greenhouse conditions to be greater in soil taken far from conspecifics than in soil taken near conspecifics (e.g. Packer & Clay 2000; Nijjer *et al.* 2007; Mangan *et al.* 2010; McCarthy-

Neumann & Kobe 2010; Liu *et al.* 2012). Field manipulations have used fungicide to show that fungal pathogens contribute to distance or density dependence (e.g. Hood *et al.* 2004; Bell *et al.* 2006; Kotanen 2007; Bagchi *et al.* 2010). When distance- or density-dependent mortality has been observed, all of the above studies have implicated fungal pathogens as a cause of the phenomenon, giving rise to the suggestion that fungal pathogens are a primary driver of distance- and density-dependent mortality (Bell *et al.* 2006; Bever *et al.* 2010). However, we argue that this conclusion may be premature, as uneven research effort across enemy types and methodological concerns associated with testing a single enemy type at a time limit the generality of existing studies.

Although fewer studies isolate effects of mammals or insects, both enemy types have been confirmed as agents of distance- or density-dependent mortality. Studies focused on mammals have reported distance- and density-dependent attack by mammals by observing signs of mammal predation (e.g. Wright & Duber 2001) and manipulating mammal access (e.g. Paine & Beck 2007; Hautier *et al.* 2010; Wotton & Kelly 2011) near and far from conspecifics or in locations of varying adult abundance. Other tests show no impact of mammals using similar observational (e.g. Visser *et al.* 2011) and manipulative approaches (e.g. Clark *et al.* 2012). Studies focused on insects include observational studies that show distance- and density-dependent attack by recording bore holes, mines and herbivory (e.g. Sullivan 2003; Norghauer *et al.* 2006a; Mangan *et al.* 2010; Visser *et al.* 2011). Manipulations have demonstrated distance-dependent impacts of insects at non-volant stages in one study (Sullivan 2003) and have shown that large insects were not a cause of distance dependence in another (Swamy & Terborgh 2010). At least two experimental manipulations have targeted all insects

using an insecticide, but either did not find survival to be distance-dependent (Hammond *et al.* 1999) or did not attribute density dependence observed in one species to insects (Gripenberg *et al.* 2014).

Simply comparing the number of single-enemy studies that show distance- or density-dependent impacts of each enemy type is not sufficient to assess the relative importance of natural enemy types. Because single-enemy studies do not measure impacts of unmanipulated – and potentially more important – enemy types, this approach could overestimate the importance of an enemy type if its impacts are widespread but weak and could underestimate impacts of enemy types that are rarely tested. Even in field studies where natural enemy exclusion appears to remove all distance or density dependence, it may be unsafe to assume that the excluded enemy is the sole agent. Studies that compare impacts of more than one enemy type within the same ecosystem are, in principle, much better equipped to determine the relative importance of enemy types. Simultaneous comparisons of multiple enemy types have been done using observational approaches (Yamazaki *et al.* 2009; Mangan *et al.* 2010; Alvarez-Loayza & Terborgh 2011). However, attributing the source of distance or density dependence using observations has been criticised due to the difficulty in detecting the actual cause of mortality without manipulations (Freckleton & Lewis 2006).

Experimental approaches that manipulate multiple enemy types provide the most robust approach for determining the relative importance of different natural enemies, as they make unambiguous comparisons between survival of plants exposed to different combinations of natural enemies. Indeed, several studies have used two or more size-based exclusion treatments. These studies effectively quantify the impact of larger natural enemies – usually a defined group of organisms such as mammals (Norghauer *et al.* 2006b; Wotton & Kelly 2011) and in at least one case larger insects (Swamy & Terborgh 2010) – vs. a group of smaller organisms including small insects and fungal pathogens. This approach gets to a part of the issue, but additional pesticide treatments are necessary to distinguish between fungal pathogens and insects (Freckleton & Lewis 2006). Approaches that isolate taxon-specific impacts are likely to provide more generalisable conclusions than are approaches that isolate impacts based on size alone, as individual species in taxonomically based groups are more likely to share similar life cycles and dispersal abilities, traits relevant to their role in distance dependence (Beckman *et al.* 2012). Gripenberg *et al.* (2014) may be the first study to independently target insects and fungal pathogens with pesticides, and yet in their study, density-dependent mortality was not observed in six of seven species-stage combinations, and the cause of density dependence was not attributed to either enemy type in the seventh. Existing studies have simply not separated impacts of fungal pathogens, insects and mammals to determine their relative importance.

Knowledge of differences in the source and strength of distance- and density-dependent mortality between life history stages is also limited, and this reduces our ability to model community dynamics and predict the consequences of seed disperser loss (Terborgh 2013). Mortality at seed and seedling stages is a key driver of plant demography (Wills *et al.*

1997; Harms *et al.* 2000), yet most studies of the source of distance and density dependence focus on only one of these life history stages, with most targeting seedlings alone (but see Wotton & Kelly 2011; Gripenberg *et al.* 2014). Existing single-enemy studies suggest that fungi may be more important and mammals less important at the seedling stage than at the seed stage (reviewed in Terborgh 2012). However, the difficulties involved with comparing disparate single-enemy studies also limit the ability to determine the relative importance of enemy types across life stages. From a demographic perspective, approaches that use data from a single life stage or enemy type (Mangan *et al.* 2010) to predict impacts on population or community dynamics may yield misleading results if the strength of distance dependence observed by one enemy type at one stage is not predictive of patterns observed across the full range of enemies and at other life stages.

In this study, we determined the relative importance of fungal pathogens, mammals and insects on mortality in the field at both seed and seedling stages for three forest species. We used seed additions, combined with fungicide, insecticide and physical enclosures to attribute sources of distance-dependent mortality at each stage.

METHODS

Study site and species

This study was conducted at three forest sites on the island of Saipan in the Mariana Island chain in the Western Pacific. The island experiences an average temperature of 27 °C with little annual variation, and receives approximately 2 m of precipitation per year with pronounced wet and dry seasons (Lander 2004). Experiments were conducted in three areas of limestone karst forest, the primary native forest type on the island, during the peak fruiting period in the early wet season. The three forest areas were separated by at least 500 m. We focused on three native, moderately common, fleshy-fruited forest tree species, *Aglaia mariannensis* Merr. (Meliaceae), *Morinda citrifolia* L. (Rubiaceae) and *Premna obtusifolia* R.Br. (Verbenaceae). The fruits of *A. mariannensis* contain one or two arillate seeds inside a fibrous pericarp, *M. citrifolia* species has fruits containing 100–200 seeds and *P. obtusifolia* produces single-seeded drupes.

Seed additions and exclusion experiments

We collected ripe fruits from at least 10 trees per species from several limestone forest sites, removed pulp from fruits and thoroughly mixed seeds prior to planting. Fruits were collected within 3 days of planting and seeds were removed from fruits within 24 h of planting. All seeds of a species were planted at all sites on the same day.

We planted seeds into plots either under adult conspecific canopies or under heterospecific canopies > 10 m from conspecifics. Placing ‘far’ plots further from conspecifics would have reduced the number of replicates because the focal species are relatively abundant across the forest. All plots were placed within 60 m × 60 m areas where tree locations have been mapped, therefore, we are confident that far plots have

no conspecifics within 10 m. Across the three forest sites, we established 36 plots each for *A. mariannensis* and *P. obtusifolia* and 30 plots for *M. citrifolia*, with half under the focal tree canopy and half far from conspecifics. To account for differences in light availability between plots, we used a spherical crown densiometer to record canopy density at each plot.

Within each plot, we established six circular 0.05 m² subplots separated from each other by at least 0.5 m, and randomly assigned treatments to each subplot. In five of the six subplots, we added seeds, and applied one of the following treatments: exclusion of fungal pathogens (fungicide), exclusion of insects (insecticide), vertebrate exclusion (physical exclusion), exclusion of all three mortality agents, or control (no exclusion) (treatments described in detail in *Natural enemy exclusion treatments* below). For *A. mariannensis*, *M. citrifolia* and *P. obtusifolia* we added 20, 40 and 50 seeds per subplot, respectively, to achieve seed densities scaling inversely with seed size. These densities are observed locally under adults; we matched these densities in far plots to isolate effects of distance, and used small subplots to minimise the area of high seed density far from conspecifics. In the sixth subplot, we did not add seeds but applied all treatments to measure maximum recruitment from background seed rain. We found a total of two seedlings across all species in these 'seed addition control' subplots and thus do not include data from these subplots in the analysis. In all plots, we marked any pre-existing seedlings to exclude these from our analysis; all species had fewer than five total pre-existing seedlings across all plots. During weekly checks, we recorded germination, marked all germinants and recorded survival of previously marked germinants. Germination of *A. mariannensis*, *M. citrifolia* and *P. obtusifolia* began after approximately 4, 6 and 1 weeks, respectively, and we monitored seedling survival for an additional 10 weeks for *A. mariannensis* and 5 weeks for *M. citrifolia* and *P. obtusifolia*.

Natural enemy exclusion treatments

For the exclusion of fungal pathogens, we used Ridomil Gold (Syngenta, Basel, Switzerland), a selective fungicide that has been used successfully in other experiments (Bell *et al.* 2006; Bagchi *et al.* 2010). We applied the fungicide weekly, in 50 mL of aqueous solution, at a concentration of 0.25 g m⁻².

For insect exclusion we used esfenvalerate (FenvaStar, LG International, Englewood Cliffs, NJ, USA). This pyrethroid insecticide, or its stereoisomer fenvalerate, has been used widely and existing evidence suggests that it does not impact plant survival (Carson & Root 2000; Mitchell 2003). We applied esfenvalerate bi-weekly, also in 50 mL of aqueous solution, at a concentration of 0.006 g m⁻². All other subplots were treated with an equal volume of water whenever treatments were applied.

To exclude rodents, we used closed-top cylindrical 1.3 × 1.3 cm mesh enclosures staked into the soil. As the wire mesh enclosures could influence the light environment, the subplots that did not have the rodent enclosure treatments were also covered with equally sized wire mesh enclosures, but with 13 × 13 cm openings cut into the side of the cylinder to allow rodent access.

Analysis

To analyse impacts of each natural enemy type on germination and seedling survival, we used generalised linear mixed effects models with a binomial error distribution in the R package *lme4* (Bates *et al.* 2013). In separate models for each species and each of the two stages, the response variable was the portion of seeds that germinated ('germination' stage) or the portion of germinants that survived to the end of the study period ('seedling survival' stage). The fixed effects included distance (under canopy or far) and each of the three enclosure treatments individually; canopy density was included as a covariate and site as a random effect. We derived estimates from a full model containing canopy density and interactions between distance and each enclosure treatment. The presence of an interaction between an enclosure treatment and distance would indicate that the agent causes distance-dependent mortality. The size of this interaction indicates how much more the treatment improves survival near conspecifics than it does far from conspecifics, or the distance-dependent effect of the agent that was excluded. A treatment effect without an interaction with distance would indicate that the agent is responsible for distance-independent mortality, or causing equally large impacts on mortality near and far from parent trees.

RESULTS

We observed distance-dependent mortality, with germination or survival greater far from conspecific canopies, in all but one life stage–tree species combination (Fig. 1, Table S1); no overall distance dependence was observed at the seedling stage of *Morinda citrifolia*. Insects caused distance-dependent mortality in germination of *Premna obtusifolia* and for seedlings of *Aglaia mariannensis* and *M. citrifolia* (Fig. 2). Rodents contributed to distance-dependent mortality of seeds for *P. obtusifolia* and *M. citrifolia*. Fungal pathogens caused distance-dependent mortality only for seeds of *M. citrifolia*. Fungi had the opposite impact on seedling survival of *M. citrifolia*, they appear to compensate for distance-dependent mortality caused by insects such that seedling survival of *M. citrifolia* in control plots was independent of distance. In two instances, the source of observed distance-dependent effects was not captured by our experimental treatments. For seeds of *A. mariannensis* and seedlings of *P. obtusifolia*, distance-dependent mortality was observed – 'total' distance dependence was non-zero (Fig. 2) – but no exclusion treatments increased survival near conspecifics more than they did in far plots. Survival was greater under more open canopies for *M. citrifolia* and *P. obtusifolia*, whereas canopy openness had little effect on survival for *A. mariannensis* (Table S1).

DISCUSSION

Our manipulative field experiments provide evidence that multiple types of natural enemies are important agents of distance-dependent mortality, which we observed in both germination and seedling survival stages of two tropical for-

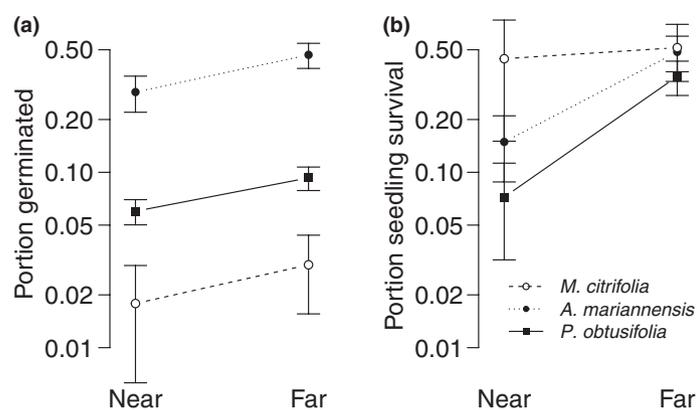


Figure 1 Distance-dependent survival of *Aglaia mariannensis*, *Morinda citrifolia* and *Premna obtusifolia* at germination (a) and early seedling (b) stages. Survival was greater far from adult conspecifics for each species and stage, except during the seedling stage of *M. citrifolia*. Survival data shown from untreated plots only. Error bars indicate ± 1 S.E.

est species and in the germination stage of the third species. Of six species-stage combinations, fungal pathogens were an agent of distance dependence in one species stage, insects in three species stages and mammals in two species stages. Although determining the relative importance of these enemy types in general will require the study of many more species and forests, these findings do not support the suggestion that fungal pathogens are the primary cause of this phenomenon (Bell *et al.* 2006; Bever *et al.* 2010). These results highlight the complexity of the mechanism underlying distance-dependent mortality and suggest a need for increased caution when integrating data on distance dependence across studies or life stages to model community dynamics.

This experimental approach demonstrated that multiple natural enemy types operate simultaneously to create distance dependence, and that natural enemy types can differ across life history stages. Insects were the most prevalent cause of distance-dependent mortality in this study; we suggest that their impacts may be underestimated in the literature because few manipulative experiments have targeted insects (but see Hammond *et al.* 1999; Gripenberg *et al.* 2014). Mammals caused more distance dependence at the seed stage than at the germination stage. This followed our predictions because the mammalian natural enemies in this system are rodents most commonly associated with seed predation, and supported previous suggestions based on observational, single-enemy, or single-stage studies (reviewed in Terborgh 2012). In both species stages in which mammals caused distance dependence, however, they played a supporting role – either fungal pathogens or insects were a stronger cause of distance-dependent mortality. Further research using similar methods to determine the relative importance of these enemy types may reinforce the suggested trend that mammals are weaker agents of distance and density dependence (Terborgh 2012). Finally, we were unable to attribute a source of distance dependence for two species stages in which we found distance-dependent mortality. These patterns could be interpreted as indirect evidence

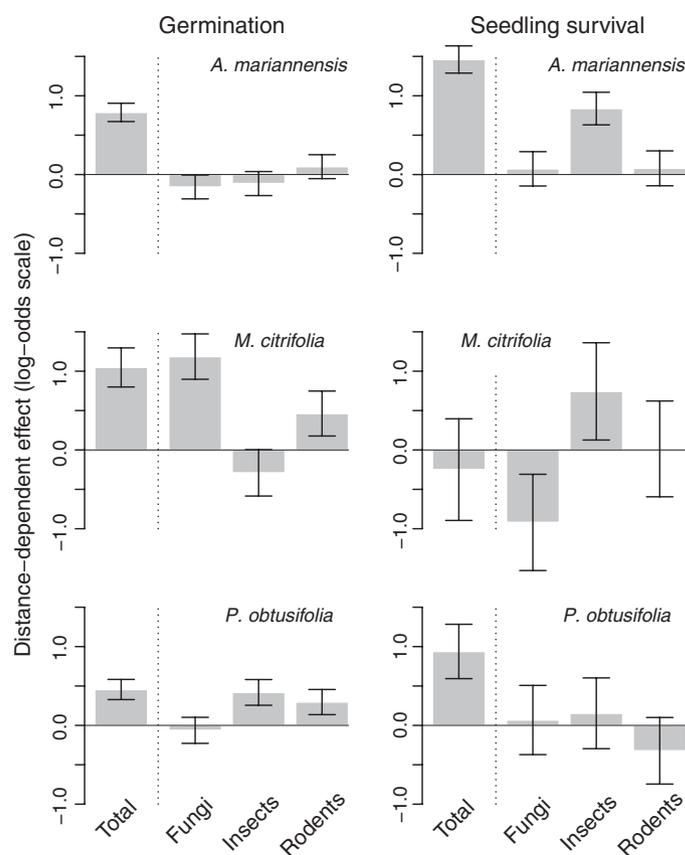


Figure 2 Source and strength of distance dependence for three tree species at germination and early seedling survival stages. Estimated effect sizes ('distance-dependent effect'; log-odds scale) indicate greater mortality near conspecific adults than far from adults when positive, the reverse when negative. Individual bars show the total observed distance-dependent impacts ('Total', left of dotted line), and the distance-dependent impacts attributable to fungal pathogens, insects, and rodents, assessed using fungicide, insecticide and physical enclosure treatments (right of dotted line). Error bars indicate ± 1 S.E.

for compensation among types of natural enemies (e.g. when fungal pathogens are excluded, the effects of insects may increase, masking some of the distance-dependent effects of fungal pathogens) or they could suggest that an even broader set of mortality agents is at work. For example, we did not manipulate competition, allelopathy, or certain less-often studied natural enemies such as bacteria and gastropods. These enemy types are seldom manipulated, although the impacts of both groups have been examined individually (Bradley *et al.* 2008; Pigot & Leather 2008).

Our results, showing large differences in the source and strength of distance dependence between species and between life history stages of the same species, have implications for the collection and use of distance- or density-dependent survival data. Specifically, our findings suggest that the strength of distance dependence at one life stage or caused by one enemy does not predict the strength of distance dependence at other life stages or caused by other enemies. Because of this, we caution against using distance-dependent survival data from experiments that target a single enemy type or a short

temporal window when predicting the consequences of seed disperser loss (Terborgh 2013) or linking distance dependence to population-scale dynamics (Mangan *et al.* 2010). Distance- or density-dependent survival data obtained by recording survival in the field (Harms *et al.* 2000; Comita *et al.* 2010) may be more appropriate for these purposes than are data obtained from manipulations that quantify single enemy types. These experiments indicate that, even when removal of one enemy type appears to completely remove the distance dependence observed in control plots, other enemy types can simultaneously contribute to distance dependence; this provides a further caution against attempting to determine the relative importance of natural enemy types by manipulating a single enemy type. The observed large differences in the strength of distance dependence between life stages reinforce the suggested need for more complex demographic models or long-term monitoring to link recruitment patterns to adult abundances (McConkey *et al.* 2012).

Testing the relative importance of natural enemy types provides information relevant for research that considers how spatial recruitment patterns and natural enemy specialisation impact diversity maintenance. Characteristics of the agents that cause distance dependence can greatly influence patterns of survival, and thus the probability that these dynamics maintain diversity (Adler & Muller-Landau 2005; Beckman *et al.* 2012). Natural enemies that specialise on certain plant species are thought to cause density dependence and insects and fungi exhibit high specialisation (Gallery *et al.* 2007; Beckman & Muller-Landau 2011; Sedio & Ostling 2013). Although some have suggested that mammals are usually generalists and less likely to produce distance dependence (Hammond & Brown 1998), mammals do have a role in generating distance and density dependence (Paine & Beck 2007; Hautier *et al.* 2010; Wotton & Kelly 2011), and did so for two species in this study.

Assessing the sources of mortality over the seed-to-seedling transition helps fill gaps in our understanding of the source and strength of factors limiting recruitment in tropical forests. In the first experiments testing impacts of multiple enemy types on pre-dispersal seed mortality for multiple tropical plants, Beckman & Muller-Landau (2011) note that the relative contribution of invertebrates, vertebrates and pathogens is poorly known at early life stages. Our results show that the source and severity of mortality at the seed-to-seedling transition is characterised by variability; the contribution of each enemy type varies widely by species, stage and between locations near and far from conspecifics.

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AUTHOR CONTRIBUTIONS

ECF, JTT and HSR designed field experiments. ECF collected field data, analysed data and wrote the first draft of the manuscript. All authors revised the manuscript.

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