

The role of herbivorous insects and pathogens in the regeneration dynamics of *Guazuma ulmifolia* in Panama

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Academic editor: C. V. Castilho | Received 27 September 2018 | Accepted 19 February 2019 | Published 6 March 2019

<http://zoobank.org/2CAF6A71-CFEA-412C-8F0A-CE11D76ADB65>

Citation: Solé R, Gripenberg S, Lewis OT, Markesteijn L, Barrios H, Ratz T, Ctvrticka R, Butterill PT, Segar ST, Metz MA, Dahl C, Rivera M, Viquez K, Ferguson W, Guevara M, Basset Y (2019) The role of herbivorous insects and pathogens in the regeneration dynamics of *Guazuma ulmifolia* in Panama. Nature Conservation 32: 81–101. <https://doi.org/10.3897/natureconservation.32.30108>

Abstract

A significant proportion of the mortality of rainforest trees occurs during early life stages (seeds and seedlings), but mortality agents are often elusive. Our study investigated the role of herbivorous insects and pathogens in the early regeneration dynamics of *Guazuma ulmifolia* (Malvaceae), an important tree species in agroforestry in Central America. We reared pre-dispersal insect seed predators from *G. ulmifolia* seeds in Panama. We also carried out an experiment, controlling insects and pathogens using insecticide and/or fungicide treatments, as well as seed density, and compared survivorship of *G. ulmifolia* seeds and seedlings among treatments and relative to untreated control plots. We observed (1) high pre-dispersal

attack (92%) of the fruits of *G. ulmifolia*, mostly by anobiine and bruchine beetles; (2) negligible post-dispersal attack of isolated seeds by insects and pathogens; (3) slow growth and high mortality (> 95%) of seedlings after 14 weeks; (4) low insect damage on seedlings; and (5) a strong positive correlation between seedling mortality and rainfall. We conclude that for *G. ulmifolia* at our study site the pre-dispersal seed stage is by far the most sensitive stage to insects and that their influence on seedling mortality appears to be slight as compared to that of inclement weather. Thus, the regeneration of this important tree species may depend on effective primary dispersal of seeds by vertebrates (before most of the seed crop is lost to insects), conditioned by suitable conditions in which the seedlings can grow.

Keywords

Anobiidae, fungal pathogen, negative density-dependence, rainforest, seed predation

Introduction

In tropical rainforests a significant proportion of tree mortality occurs during the early life stages of the plant (seeds and seedlings), and patterns of mortality at these early stages of life may be critical in influencing the diversity of older individuals (Massad 2013; Green et al. 2014). There is growing evidence that interactions between plants and host-specific insect herbivores and pathogens could be a central mechanism for explaining plant diversity (Leigh et al. 2004). A mechanism proposed to facilitate the coexistence of tree species in tropical forests is negative density-dependence or the so-called Janzen-Connell effect (Janzen 1970; Connell 1971; Bell et al. 2006; Comita et al. 2010; Bagchi et al. 2014). Seeds are most likely to disperse to sites near their parent trees, but seeds and seedlings are also more likely to survive away from parents where they are less likely to be attacked by host-specific insects and pathogens, which may disperse contagiously from parent trees. Negative density-dependence is the result of the proliferation of species-specific herbivores and pathogens among hosts in areas of high conspecific plant densities, and the negative correlation between enemy propagation and the distance from parent trees to their nearby offspring (Janzen 1970; Connell 1971; Bell et al. 2006; Comita et al. 2010; Bagchi et al. 2014).

Insects, more than herbivorous mammals, are responsible for most herbivory on seedlings in tropical rainforests (Cruz and Dirzo 1987). In addition, many insects, but very few mammals, preferentially attack seeds or plants near conspecific adults (Hammond and Brown 1998). While there have been many studies assessing insect herbivory (foliar damage) and resulting seedling mortality (review in Massad 2013), community studies targeting the insects feeding on rainforest seedlings are relatively rare (Basset 1999; Alvarez-Loayza and Terborgh 2011). Ecologists often infer the impact of insect herbivores on plants by measuring the apparent, or rate of, leaf damage (Coley 1983; review in Lowman 1984), because of the relatively low density of herbivores in tropical rainforests (Novotny and Basset 2000). However, such studies are known to underestimate the magnitude and impact of herbivory significantly because herbivores may eat whole leaves, or disproportionately damage buds and young or expanding leaves (Lowman 1984). This issue may be critical during the seed-to-seedling transition (germinating seed or very young seedling). Although damage by sap-sucking insects on

seeds or seedlings may be difficult to quantify, it may nevertheless lower plant fitness significantly via direct action or the spreading of diseases (Nault and Ammar 1989).

Seedling establishment and survival in tropical rainforests may also be influenced by pathogens, especially fungal pathogens (Augspurger 1984; Mangan et al. 2010; Bagchi et al. 2014). For example, Augspurger (1984) reported that on Barro Colorado Island in Panama, dependent on the tree species, 2–74% of seedlings may be dead from disease two months after germination. Disease incidence is often host-density dependent and may have the potential to regulate host spatial distribution (Augspurger 1984). Further, interactions between herbivory and fungal pathogens may in some cases be significant and affected by anthropogenic disturbance.

One way to assess the effect of insect herbivores or fungal pathogens on rainforest seeds or seedlings is to exclude them with (a) insecticide and/or fungicide or (b) mesh enclosures in treatment plots and compare survivorship of seeds or seedlings between treatment plots and control plots (Swamy and Terborgh 2010; Bagchi et al. 2014). Bagchi et al. (2014) used method (a) to study the role of insects and fungal pathogens in causing mortality to seeds and young seedlings in a rainforest in Belize. They found that spraying insecticides on seeds and germinating seedlings increased overall seedling survival dramatically (by a factor of 2.7). The benefits of insecticide application varied among plant species, so that excluding insects changed the structure of the seedling community considerably. These initial results suggest that insects and pathogens play an important role in driving plant dynamics and diversity.

Several studies have used insecticide and fungicide applications to assess the role of insects and pathogens in maintaining plant diversity and causing conspecific negative density-dependent plant survival as predicted by the Janzen–Connell hypothesis (e.g. Bell et al. 2006; Bagchi et al. 2014; Gripenberg et al. 2014; Fricke et al. 2014). However, to our knowledge, none of the insecticide studies have followed the fate of the seeds and seedlings in close enough detail to allow any conclusions to be drawn regarding which stage in the early life of plants was most likely to suffer from enemy mediated mortality, and how insecticide treatment would impact insect herbivores specific to these stages. Moreover, since most studies assessing the role of enemies on plant recruitment have applied insecticides and fungicides separately, little is known about the potential interactive effects of insect and pathogen attacks (e.g. Bagchi et al. 2014; Gripenberg et al. 2014).

The main objectives of this study were to investigate the impacts of insect herbivores and fungal pathogens on the early regeneration of a tree species of great importance in agroforestry in Central America and Mexico, *Guazuma ulmifolia* Lam. (Batis et al. 1999). *Guazuma ulmifolia* is one of the most common trees in agricultural areas in Central America, where it grows in fields, primary and secondary forests (Orwa et al. 2009). Elucidating the major causes of mortality of this species at an early stage can help in drafting recommendations to foster the regeneration of this species and forest reestablishment during the process of rehabilitation of degraded soils (Griscom et al. 2009).

Our protocol involved assessing both the damage on seeds caused by insect predators and the potential joint effects of insect herbivores and fungal pathogens on seed germina-

tion and the survival of young seedlings. We then evaluated (1) which stage (seed, seed-to-seedling transition, seedling) is particularly vulnerable to insect herbivores and pathogens; (2) whether the negative impact of insect herbivores at the seedling stage is higher than that of fungal pathogens; and (3) whether seed or seedling density affects the action of insect herbivores and fungal pathogens, as predicted by the Janzen-Connell hypothesis (Alvarez-Loayza and Terborgh 2011). Finally, with these data in hand, we briefly discuss their implication for the regeneration of *G. ulmifolia* in agroforestry landscapes of Central America.

Material and methods

Study sites

Insect seed predators were reared from *Guazuma ulmifolia* fruits collected on Barro Colorado Island (BCI) and at a nearby site, El Charco (ELC). As the use of insecticide is not allowed on BCI, our experiment assessing the role of insects and pathogens at the seed-to-seedling transition stage was conducted at ELC. Barro Colorado island is a biological reserve (9.15N, 79.85W, elevation 120–160 m) of 1,500 ha covered by lowland rainforest and created by the opening of the Panama Canal in 1914. A permanent ForestGEO vegetation plot (Anderson-Teixeira et al. 2014) of 50 ha is located in the centre of the island. The annual average rainfall is 2551 mm and annual average daily maximum air temperature is 26.3 °C. Details about the island and the rainforest plot are reported in Anderson-Teixeira et al. (2014). ELC (9°502.58'N, 79°39048.24'W, elevation 100 m) is ca 20 km from BCI and is part of the 19,545 ha Soberania National Park. There is a one ha ForestGEO plot at ELC and salient differences between the vegetation on BCI and that of ELC and other ForestGEO plots in the area are discussed in Pyke et al. (2001). ELC is a secondary rainforest while BCI includes both mature secondary forest and old-growth forest. Annual average rainfall is also lower (2050 mm) at ELC than at BCI (Pyke et al. 2001).

Study plant

Guazuma ulmifolia (Lam. 1789, Malvaceae, “Guácimo” in Panama) was selected for this study because of the relatively high availability of seeds at the study sites. It is also an important tree species in agroforestry, being used for firewood, medical treatment, the production of living fences and rehabilitation of degraded soils by fostering forest regeneration (Batis et al. 1999; Griscom et al. 2009). Other advantages of selecting this species in the context of our study included long-lived seeds (+18 months, Sautu et al. 2006) and rather quick germination (10–143 days after sowing, Roman et al. 2012; average 52.5 days: Sautu et al. 2006).

Of the seven species in the *Guazuma* genus, *G. ulmifolia* is the only one to occur in Panama (Robyns and Cautrecasas 1964). The tree is 4–25 m tall, with a broad and

irregular crown and grey bark. It grows at low or medium elevation, in lowland dry or wet forests, from Mexico to Paraguay, most commonly in areas where annual rainfall is 700–2500 mm (average 1,956 mm), and in a variety of soils (Francis 1991; CONAFOR 2018). In Panama, it is commonly observed on the Pacific side, where it flowers and produces fruits from January to July. The species is partially deciduous during the dry season (Roman et al. 2012). *Guazuma ulmifolia* is a pioneer species and usually constitutes only a small percentage of basal area in secondary forests (Francis 1991). On BCI, the 50 ha plot of old growth forest includes 64 individuals of this species (Forest GEO 2018). At ELC *G. ulmifolia* is not present in the small one ha plot (and was also not recorded within or near our experimental blocks) but is relatively frequent nearby (i.e., within 500 m).

Individual *Guazuma* trees can produce up to 5,000–10,000 fruits per crop. The capsule of the fruit is elliptical or hemispherical, measuring between 18–25 mm long, and 14–22 mm wide. When mature, it is black (Suppl. material 1: Plate S1), contains a hydrophilic mucilage, and includes 60–68 small seeds averaging 2 mm length and 0.0042 g dry weight (Robyns and Cautrecasas 1964; Francis 1991; Roman et al. 2012). Fruits usually mature from September to April and may be observed for long periods on trees (Batis et al. 1999). Fruits are eaten by peccaries, tapirs, deer, agoutis and squirrels, as well as domestic livestock; bats are efficient seed dispersers (Janzen 1983; Roman et al. 2012). In Mexico and Costa Rica 12–42% of seeds have been reported to have been destroyed by several species of bruchine beetles (Johnson and Kingsolver 1971; Janzen 1975, 1983).

Insect rearing from seeds

For rearing insects we collected 320 fruits freshly fallen (without apparent decomposition) from *G. ulmifolia* on BCI. They were obtained between 2011–2013 from 22 trees (average 14.5 fruits collected per tree). To assess pre-dispersal attack (i.e., while seeds are still on trees) of *G. ulmifolia* fruits, in 2016 we collected 100 fruits directly from the crown of 5 trees near the 1 ha plot in ELC (20 fruits collected per tree). In both cases fruits were stored in individual plastic pots, lined with tissue paper and covered with 1 mm mesh netting for ventilation and to avoid subsequent colonization/contamination of fruits. Rearing pots were checked twice weekly, and any emerging insects were collected, preserved, mounted and then identified with the assistance of taxonomists (see Acknowledgements) and/or with molecular techniques (Ratnasingham and Hebert 2013). Fruits were stored for 3 months and then dissected to check for the presence of developing larvae. We obtained DNA Cytochrome c oxidase subunit I (COI, ‘DNA barcode’) sequences from legs of representative insect specimens and we used Barcode Index Numbers (BINs) derived from insect sequences to delineate species (Ratnasingham and Hebert 2013). Molecular data were uploaded in the Barcode of Life project “BCISP” (<http://www.barcodinglife.org/>). Insect vouchers were deposited at the Fairchild Museum and Smithsonian Tropical Research Institute in Panama.

Monitoring of seeds and seedlings

Ten experimental blocks (B01 to B10) were set up near the permanent plot at ELC (coordinates in Suppl. material 1: Table S1; the distance between blocks varied from 16 to 69 m). Each block (5.5 m × 2.5 m) consisted of 8 quadrats of 1 m², in which 4 treatments were applied to two seed densities (10 and 25 seeds per m², see below). Control quadrats (coded C10 and C25) were sprayed with water, insecticide quadrats (I10 and I25) were sprayed with insecticide, fungicide quadrats (F10 and F25) were sprayed with fungicide, and combined treatment quadrats (I+F10 and I+F25) were sprayed with insecticide and fungicide. Quadrats (one replicate of each treatment combination per block) were located at random within each block (Suppl. material 1: Table S1). Litterfall traps on BCI (Wright et al. 2004) were used to estimate natural *G. ulmifolia* seed densities. In 2013, average seed density of *G. ulmifolia* at ELC was 1 seed m⁻² y⁻¹ (n=50 traps, L. Markesteijn and O. Lewis unpubl. data). On BCI, for the period 1987–2011, seed density for this species was 6.1 seeds m⁻² y⁻¹ (n=200 traps, Wright et al. 2004; S.J. Wright unpubl. data). The density of seeds buried in the soil was much lower, < 0.6 seeds m⁻² (Dalling et al. 1997). Hence for our experiment we considered a high natural seed density of 10 seeds m⁻² y⁻¹ and a very high seed density of 25 seeds m⁻² y⁻¹.

We used the insecticide ENGEO 247 SC (Syngenta SA, Basel, Switzerland) with active ingredients thiamethoxam (a neonicotinoid) 141 g L⁻¹ and lambda-cyhalothrin (a pyrethroid) 106 g L⁻¹. According to the manufacturer, thiamethoxam has root-, leaf- and stem-systemic activity, while Lambda-cyhalothrin has a strong contact action. We used 0.0025 ml m⁻² in 50 ml sprayed on each quadrat every week, as recommended by the manufacturer and used in previous studies (e.g. Bagchi et al. 2014). ENGEO is a broad-spectrum insecticide, commonly used against herbivorous insects in crops. At the volume used here, ENGEO has very few ecotoxic and health risks. Similarly, we used the fungicide AMISTAR TOP 325 SC (Syngenta SA, Basel, Switzerland), with active ingredients difenoconazole 125 g L⁻¹ and azoxystrobin 250 g L⁻¹. AMISTAR was sprayed at levels of 0.1 ml m⁻² in 1000 ml on each quadrat every week, as used by Bagchi et al. (2014) and at a slightly higher level than recommended by the manufacturer (0.05 ml m⁻²). Difenoconazole is a broad-spectrum fungicide, while azoxystrobin is a systemic fungicide commonly used in agriculture.

In total, 1,400 fresh seeds of *G. ulmifolia*, collected from different trees near ELC, were sown into the experimental blocks in May 2016 at the onset of the rainy season. Prior to sowing, capsules were opened and seeds were checked for insect damage. Only apparently intact seeds were sown. To mimic near natural conditions, seeds were not treated to increase germination rates (Sautu et al. 2006). Blocks were first cleared of litter and seeds, and seeds of *G. ulmifolia* were then sown and the litter carefully replaced. Within each quadrat seeds were sown individually in 25 areas of 20 × 20 cm delineated by a sowing frame. In quadrats with a seed density of 10 seeds m⁻² y⁻¹, seeds were positioned in 10 randomly selected 20 × 20 cm areas, whereas at a seed density of 25 seeds m⁻² y⁻¹, each seed occupied one of the 25 areas. Each seed was coded and marked with a plastic coffee stirrer for the purpose of monitoring. After sowing, each quadrat was

sprayed weekly with a hand mister with 50 ml of water (control) or with 50 ml of the corresponding treatment for a total duration of 14 weeks (to August 2016). After this date surviving seedlings were measured and monitored at a lower frequency until May 2017, one year after the start of the experiment. Every week, the status of each seed or seedling was scored individually as Seed (no germination), Seedling (germination occurred, seedling growing, no insect damage), Damaged (seedling growing with damage) and Dead (seedling germinated but subsequently died). We initially planned to score seedlings in the Damaged category further using 10 different subcategories (including chewing, mining, galling, skeletonizing, etc.), but only three of these categories proved to be useful for *G. ulmifolia* seedlings: (a) leaf chewing (on side of leaves; possible agents Lepidoptera and Orthoptera); (b) shrivelled and/or necrotic areas (possible agent: fungi); and (c) unknown reason (i.e., damage could not be attributed with certainty). Seedlings of species other than *G. ulmifolia* were removed from quadrats throughout the monitoring period. At the end of the experiment, we measured the height of surviving seedlings.

Statistical analyses

We first ensured that there was no or little spatial pseudoreplication in our data by running simple Mantel tests (1,000 randomizations) between the dependent variables (see below) and the coordinates of the blocks. There was no obvious spatial autocorrelation for any of the response variables: germinated seeds: Mantel's $t = 0.092$, $p = 0.303$; dead seedlings: $t = 0.117$, $p = 0.248$; damaged seedlings: $t = -0.079$, $p = 0.654$. We used general linear models (GLM) to test the effect of treatment (Control, Fungicide, Insecticide and Insecticide + Fungicide), seedling density (10 or 25 seeds) and the interaction between these two fixed effects on the three dependent variables, at the end of the 14 monitoring weeks: number and percentage of germinated seeds, seedling mortality (number and percentage of dead seedlings) and number and percentage of seedlings damaged. All statistical analyses were conducted using R v 3.3.3 (R Development Core Team 2011) in the RStudio (Racine 2012) integrated development environment. The `glm` function was used with dependent variables expressed as percentages to compare the two seed densities. The significance of each fixed effect in the GLMs was assessed using the `Anova` function of the package 'car' in R (Fox and Weisberg 2017) and is provided in the Results section together with the likelihood-ratio chi-square ($LR\chi^2$) and the corresponding degrees of freedom. We empirically modelled seedling survival over time with a rational model ($y = (a + b * x) / (1 + c * x + d * x^2)$; where y is the number of seedlings growing and x is the time in weeks), which in almost all cases tested had the highest coefficient of determination (R^2) and the lowest Akaike information criterion corrected (AICC) of all models tested with CurveExpert Professional (Hyams 2011). We tested significant differences between fitted models using Kolmogorov-Smirnov two samples tests. We used available rainfall data from BCI (http://biogeodb.stri.si.edu/physical_monitoring/research/barrocolorado) to check for possible correlation with the mortality of seedlings at ELC. Means \pm s.e. are reported in text and figures.

Results

Insects reared from fruits

The insects reared from the fruits of *G. ulmifolia* are summarized in Table 1, notes on their ecology are presented in Appendix S1, and some species are illustrated in Plate S1. They included 15 species, mostly Coleoptera. Six beetle species (four Bruchinae, *Amblycerus* spp. and two Anobiinae, *Lasioderma* spp.) can be considered as seed predators, are likely to be host-specific to *G. ulmifolia* and represented 69% of individuals reared. Further, one lepidopteran seed predator was also reared and considered to be generalist (Appendix S1). The percentage of Anobiinae to the total number of insects reared was higher at ELC (90%) than at BCI (38%). Faunal differences between ELC and BCI may partly be due to pre- and post-dispersal fruit attack as the percentage of fungal-feeders, scavengers or sapromycetophagous insects reared was higher at BCI (26.3%) than at ELC (5.4%). At ELC, out of the 100 fruits obtained from trees and reared (i.e., pre-dispersal stage), 92% of fruits were attacked by Anobiinae.

Seed germination

After 14 weeks of monitoring, 36.1% of the 1,400 sown seeds had germinated (Suppl. material 1: Table S2). Peak germination occurred during the onset of the rainy season, in the third week of monitoring (24.1%). The average percentage of germinated seeds per block ranged from 30.8% (quadrats I25) to 42.0% (quadrats F10) but varied little between treatments and seed densities (Fig. 1; Suppl. material 1: Table S2). This was confirmed by the GLM, which indicated no significant effect of combined insecticide and fungicide treatment on germination ($LR\chi^2 = 0.17$, $df = 3$, $p = 0.98$), seed density ($LR\chi^2 = 1.57$, $df = 1$, $p = 0.20$), or their interaction ($LR\chi^2 = 1.30$, $df = 3$, $p = 0.72$).

Survival, growth and mortality of seedlings

Overall, seedling mortality was very high, and in total 95.7% of germinated seeds were dead by Week 14 (Suppl. material 1: Table S3). Average seedling mortality (percentage of dead seedlings to total germinated seeds) per block varied little and ranged from 88.6% in quadrats F25 to 97.8% in quadrats I10 (Fig. 2). GLM models confirmed that (a) treatments had no effect on the survival of seedlings ($LR\chi^2 = 5.24$, $df = 3$, $p = 0.15$); (b) seed densities had no effect on seedling survival $LR\chi^2 = 0.0048$, $df = 1$, $p = 0.94$); and (c) the interaction between the two variables had no impact on seedling survival ($LR\chi^2 = 1.30$, $df = 3$, $p = 0.72$). Seedling survival over time could be fitted to rational models (Fig. 3; range of R^2 0.899 to 0.968). At densities of 10 seeds per m^2 , seedling survival was similar in control and I10 quadrats. However, it was slightly better in F10 quadrats than in control quadrats and this difference was significant

Table 1. Summary of insects reared (total number of individuals per species) from fruits of *G. ulmifolia* at BCI (320 fruits) and ELC (100 fruits). Barcode Index Number (BIN) indicated when available and is linked to available insect pictures. Host specificity was inferred from a larger study on insect seed predators on BCI (Gripenberg et al. 2018).

Taxa	BIN	BCI	ELC	Notes
Coleoptera				
Chrysomelidae – Bruchinae				
<i>Amblycerus cistelinus</i> (Gyllenhal, 1833)	BOLD:ACG0463	72	0	Seed predator, host specific on <i>G. ulmifolia</i>
<i>Amblycerus</i> sp. bru39SG	BOLD:ACJ3956	39	3	Seed predator, host specific on <i>G. ulmifolia</i>
<i>Amblycerus</i> sp. bru30SG	BOLD:ABW8381	15	0	Seed predator, host specific on <i>G. ulmifolia</i>
<i>Mimosstes</i> sp. 1YB	–	0	1	Seed predator
Ptinidae – Anobiinae				
<i>Lasioderma</i> sp. ano2SG	BOLD:ACF0770	242	291	Seed predator, host specific on <i>G. ulmifolia</i>
<i>Lasioderma</i> sp. ano4SG	–	1	0	Seed predator
Bostrichidae				
<i>Lyctus</i> sp.	–	0	2	Wood borer
Silvanidae				
<i>Abasverus advena</i> (Waltl, 1832)	–	1	0	Fungal-feeder, cosmopolitan pest of stored products
<i>Cathartus</i> sp. 1SG	–	115	0	Probably fungal-feeder
Unidentified ELC sp. 1	–	0	1	Probably fungal-feeder
Unidentified ELC sp. 2	–	0	1	Probably fungal-feeder
Cucujidae				
Unidentified – cuj1SG	–	63	0	Ecology unknown
Nitidulidae				
<i>Stelidota</i> sp. 5SG	–	2	0	Sapromycetophagous?, generalist, reared from several hosts
Unidentified larva	BOLD:ACL7065	29	0	Sapromycetophagous?, may be larvae of <i>Stelidota</i> sp. 5SG
<i>Stelidota</i> sp. 6SG	–	0	1	Sapromycetophagous?, generalist, reared from several hosts
Curculionidae – Scolytinae				
<i>Xyleborus</i> sp. 1YB	–	0	1	Wood borer
Corylophidae				
Unidentified Corylophidae	–	0	11	Fungal-feeder
Lepidoptera				
Autostichidae				
<i>Deoclona xanthoselene</i> (Walsingham, 1911)	BOLD:ACF0463	51	0	Seed predator, generalist, reared from several hosts
Blastobasidae				
Blas lep37SG	BOLD:ABV2151	2	1	Scavenger, generalist, reared from several hosts
Blas lep31SG	BOLD:ABV2158	1	2	Scavenger, generalist, reared from several hosts
Hymenoptera				
Braconidae				
Unidentified Braconidae	–	0	1	Parasitoid
Pteromalidae				
Unidentified Pteromalidae	–	1	7	Parasitoid
TOTAL		634	323	

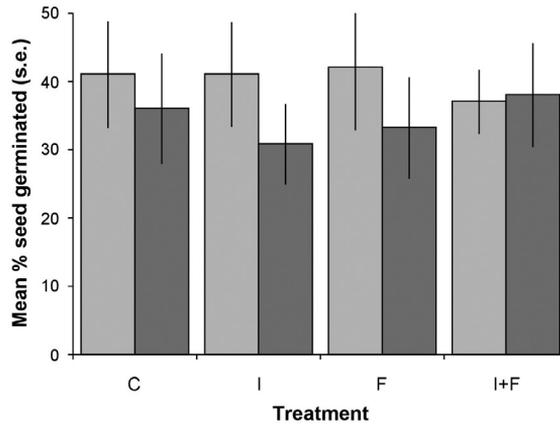


Figure 1. Average percentage of germinated seeds per block, detailed by treatment (C = control, I = insecticide, F = fungicide, I+F = insecticide and fungicide) and seed densities (light bars: 10 seeds m⁻² y⁻¹; dark bars: 25 seeds m⁻² y⁻¹).

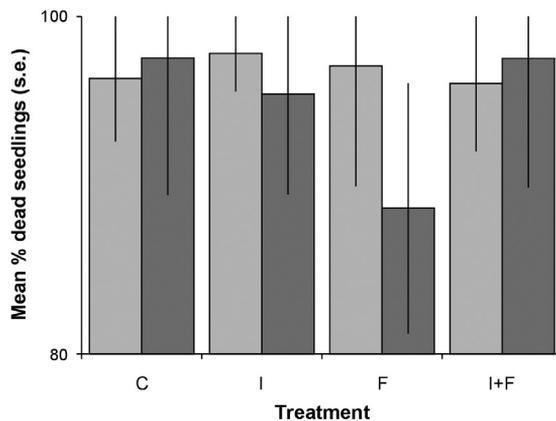


Figure 2. Average percentage of dead seedlings per block, detailed by treatment (C, I, F, I+F, coded as in Fig. 1) and seed densities (light bars: 10 seeds m⁻² y⁻¹; dark bars: 25 seeds m⁻² y⁻¹).

(Kolmogorov-Smirnov two samples test between the two fitted models, max. diff. = 0.267, $p = 0.002$). Patterns of survival of seedlings were similar when sown at densities of 10 seeds per m² or 25 seeds per m². At densities of 25 seeds per m², seedling survival was higher in F25 quadrats than in control quadrats (max. diff. = 0.327, $p < 0.0001$), and also slightly higher in I+F25 quadrats than in control quadrats (max. diff. = 0.228, $p = 0.011$). However, these differences were small (Fig. 3).

The highest seedling mortality was in weeks 4 and 5 (weeks of the 27 May and 3 June 2016), with a total of 77 and 86 dead seedlings, respectively. During week 4, only one week after the highest rate of seed germination (Fig. 3), 25.7% of seedlings growing the preceding week died. Weekly seedling mortality was not correlated with the

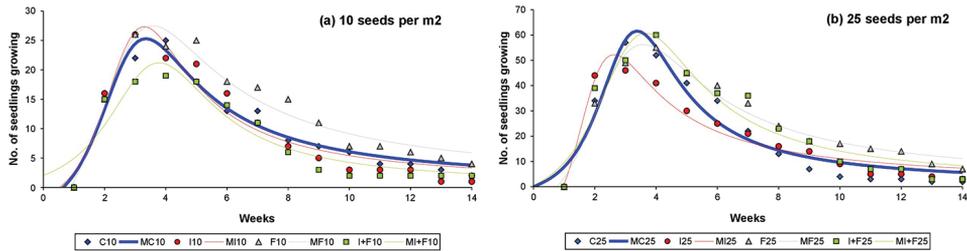


Figure 3. Number of seedlings growing plotted against time for each treatment (coded as in Fig. 1 and plotted with different colors) and seed densities of **a** 10 seeds per m² and **b** 25 seeds per m². Observations indicated by symbols, fitted rational models indicated by continuous lines (control identified by thick blue line).

amount of rainfall every monitoring week ($r = -0.08$, $p > 0.80$; excluding weeks 1 and 2 during which few seeds germinated and no seedlings died). However, the cumulative mortality of seedlings during weeks 3 to 14 was strongly and positively correlated with the cumulative amount of rainfall during the same period ($r = 0.93$, $p < 0.0001$).

After 14 weeks of monitoring, only 24 seedlings were alive, including 9 damaged seedlings, spread only over 4 blocks. The rational models estimated that after one year of monitoring, a total of 9 seedlings for the whole experiment would still be alive. This was not the case: the last two seedlings died on 5 May 2017, one year after the beginning of the experiment, in block 07, quadrat I+F25. The growth and biomass of surviving seedlings in week 14 were also rather slow and low. They measured on average 30.0 ± 1.91 mm and the two last seedlings to die were 125 and 140 mm tall. Since the sample size of surviving seedlings in week 14 was low, we performed a simple ANOVA that indicated that neither treatment ($F_{3,1} = 0.896$, $p = 0.48$) nor seed density ($F_{3,1} = 0.713$, $p = 0.42$) had any effect on seedling height.

Damage to seedlings

Overall, 100 out of 506 seedlings that germinated were damaged (19.8%, Suppl. material 1: Table S4). In 80% of cases, damage was identified as originating from leaf-chewing insects, 19% of cases involved fungal necrosis, whereas the final 1% could not be attributed with certainty. The mean percentage of damaged seedlings relative to total germinated seeds per block varied from 15.5% (F10) to 25.2% (C10), with no clear positive effect of insecticide (Fig. 4). GLM models indicated that neither the combined insecticide and fungicide treatment ($LR\chi^2 = 0.60$, $df = 3$, $p = 0.89$), nor seed density treatments (density $LR\chi^2 = 0.27$, $df = 1$, $p = 0.60$), nor their interaction ($LR\chi^2 = 0.26$, $df = 3$, $p = 0.98$) had a significant effect on the number of damaged seedlings. The relatively low percentage of seedlings damaged, as compared to the mortality of apparently undamaged seedlings (80.2%), suggests that insects were not responsible for most seedling mortality. This was confirmed by the rarity with which insects were observed damaging seedlings during day-time fieldwork.

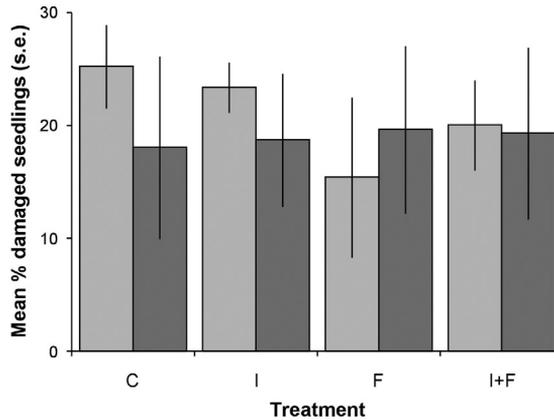


Figure 4. Average percentage of damaged seedlings per block, detailed by treatment (C, I, F, I+F, coded as in Fig. 1) and seed densities (light bars: 10 seeds m⁻² y⁻¹; dark bars: 25 seeds m⁻² y⁻¹).

Discussion

Which stage is particularly sensitive to insect herbivores?

Regarding this question, we observed that: (a) pre-dispersal attack of the fruits of *Guzuma ulmifolia* is very high (up to 92% of fruits attacked) and the result of the action of several species of insect seed predators, mainly anobiine and bruchine beetles (*Lasioderma* spp. and *Amblycerus* spp.). (b) In Panama, and contrary to what was observed in Costa Rica (Janzen 1975), anobiine beetles were responsible for most of the fruit damage, not bruchine beetles. (c) Post-dispersal attack of isolated *G. ulmifolia* seeds appears to be rare, as rates of seed germination (overall 36%) were not affected significantly by either insecticide or fungicide treatments, nor by seed density. (d) Once seeds germinated, seedling mortality was high (> 95% after 14 weeks of monitoring) but was not reduced by our insecticide or fungicide treatments and was not affected by seed density.

Because of very high pre-dispersal attack of *G. ulmifolia* fruits at ELC, the number of viable seeds that can germinate on the ground is probably considerably reduced. Janzen (1975) indicates that in Costa Rica in almost all *G. ulmifolia* fruits attacked by *Amblycerus cistelinus*, all seeds in the fruit are destroyed by the time the larva matures. The same author reports that anobiine beetles drill through large number of seeds but that they rarely kill > 50% of the seeds in a fruit and infest usually < 5% of fruits in the field. Although we have no precise rate of seed attack per se, it presumably remains high because of the high infestation rate of fruits and should be a strong limiting factor in the early regeneration of *G. ulmifolia* at ELC. The distance to other reproductive conspecific trees, as well as the dispersal distance of insect seed predators, could also explain different rates of fruit attack per tree (Janzen 1975), but this was not quantified in this study. Fruits stay on trees for a long time; they are dormant and immature

for 5–6 months and then mature over 3 months (Janzen 1982). *Amblycerus* spp. attack fruits when maturing (Janzen 1975), but long periods of fruit exposure on trees may be favorable to other seed predators, such as anobiine beetles.

In contrast, we believe that post-dispersal attack of *G. ulmifolia* seeds by insects is infrequent. *Amblycerus* spp. do not oviposit on fallen fruits even if there are many on the ground (Janzen 1975). Our insecticide treatment did not affect the rate of germination of isolated seeds, which anyway are small and hence unlikely to be attacked by insects (Moles et al. 2003). Although *G. ulmifolia* seeds lack polyphenols as defensive compounds, they invest in protective tissue, as almost 70% of the seed dry weight is made up by a hard seed coat (Gripenberg et al. 2018; S. Gripenberg, pers. obs.). This could further impede the attack on isolated seeds. Furthermore, the lack of improved seed germination or seedling survival in our insecticide and fungicide treatments (see below) suggests that the pre-dispersal seed stage is by far the stage most sensitive to insect enemies.

Is the negative impact of insect herbivores at the seedling stage higher than that of fungal pathogens? Agents of seedling mortality

In relation to this question we further observed that: (a) seedling survival over time was weakly and positively influenced by the fungicide treatment. (b) There was a strong positive correlation between weekly cumulative seedling mortality and cumulative rainfall. (c) About 20% of seeds that germinated were damaged, mostly by leaf-chewing insects, but neither the treatments nor seed density affected the number of damaged seedlings. (d) Insect herbivores were not observed feeding on seedlings during day-time. (e) Seedling growth was slow (3cm tall after 14 weeks), and all 506 seedlings that germinated out of 1,400 seeds sown were dead after one year.

Leaves of *G. ulmifolia* on mature trees are readily attacked by a variety of insect herbivores (Janzen 1983), but reports of damage on conspecific seedlings are, to the best of our knowledge, not available. Insects feeding on *G. ulmifolia* seedlings may be active during night-time, as suggested by the low but nevertheless standing leaf damage suffered by seedlings (16% of germinated seeds). It is also unlikely that seedling mortality could be explained by the near presence of conspecific mature trees and contagion of insect herbivores, as *G. ulmifolia* is not very common at ELC. These arguments, along with the lack of insecticide treatments on the survival of seedlings or their damage, suggest that insect herbivores (leaf-chewing and sap-sucking insects) infrequently attacked *G. ulmifolia* seedlings at ELC, and are not primarily responsible for seedling mortality. This could be explained by the low foliar biomass represented by these seedlings (Basset 1999).

Apart from a weak positive effect of fungicide treatment on seedling demography (survival in time; as reported in Bagchi et al. 2014), our data do not provide evidence for fungal pathogens being important in the early regeneration of *G. ulmifolia*. When seedlings were damaged, and the source of damage could be unequivocally scored,

more seedlings were apparently damaged by insects rather than by fungi. However, it may be very difficult to score visually seedlings damaged by fungi. Furthermore, our fungicide treatment may have been more effective against endophytic pathogens than soil pathogens, which may attack both seeds and seedlings (Mangan et al. 2010). The lack of fungicide effects may also be explained differently. Strobel et al. (2007) reported on a strain of *Muscodor albus* isolated in leaves of *G. ulmifolia* in Ecuador and able to colonize conspecific seedlings. This endophytic fungus produces a series of volatile organic compounds with antibiotic activity that collectively act to kill other fungi or bacteria, while there are no external symptoms on its host. It may be argued that this fungus lives in its host in a symbiotic condition, providing protection from pathogens while surviving and growing on plant nutrients (Strobel et al. 2007). If *G. ulmifolia* produces its own fungicides, then the lack of strong effect of our fungicide treatment is not surprising.

July 2016 should have been a period of active growth for *G. ulmifolia* seedlings that germinated in May 2016. July 2016 was the second rainiest July (monthly sum 486.8mm) on BCI since records began in 1925, only surpassed by July 1956 (496.6mm; http://biogeodb.stri.si.edu/physical_monitoring/research/barrocolorado). In these conditions, high rainfall may have caused soil anoxia and some rot in seeds and seedlings, as suggested by the strong correlation between cumulative rainfall and seedling mortality during the experiment. Further, *G. ulmifolia* seedlings have a relatively low tolerance to flooding (Lopez and Kursar 2003), and heavy rain may also damage stems and foliage or uproot the seedlings. Thus, we strongly suspect that the main agent of seedling mortality during the experiment was unfavorable rainfall conditions, but we stress that the patterns recorded may not apply under all conditions.

Does seed or seedling density affect the action of enemies? Relations with the Janzen-Connell hypothesis

For the densities considered in this study, post-dispersal seed and seedling density did not greatly affect the negative action of insect herbivores and fungal pathogens. The lack of difference in seedling survival between control quadrats sown at two different seed densities also suggests that intra-specific competition of seedlings is not responsible for their high mortality (Alvarez-Loayza and Terborgh 2011), at least for the densities considered in this study, which were high as compared to natural seed densities for this species in Panama.

Since post-dispersal attack of *G. ulmifolia* seeds and seedlings by insects at ELC was infrequent, this suggests that Janzen-Connell effects due to insect herbivores specifically, if they exist, may be rather weak for this tree species. Comita et al. (2010) examined the strength of negative density-dependence for 180 plant species on nearby BCI, investigating seedling survival as function of conspecific seedling abundance (Suppl. material 1: Table S2 in Comita et al. 2010), they found that the strength of conspecific neighbor effects were rather weak for *G. ulmifolia*, which ranked 158th

out of 180 species (i.e. in the lower quartile of species). Nonetheless their data indicate negative density-dependence for *G. ulmifolia*, raising the question of which biotic agent may be responsible.

Since *Amblycerus* spp. only oviposit in fruits on trees (Janzen 1975), they are probably not involved directly in this negative density-dependence. Perhaps a more plausible explanation is that rapid ingestion of *G. ulmifolia* fruits by dispersal agents (mammals, birds and bats; Janzen 1982) results in small clusters of seeds, germinating close together corresponding to a single defecation (Howe 1989; Lawson et al. 2012). Seeds and seedlings would be unlikely to be attacked by insects, as suggested by our experiment, and these clusters are potentially very isolated from other conspecifics (Lawson et al. 2012).

Possible biases in our study

First, as indicated by further monitoring of blocks after 14 weeks, seeds were unlikely to germinate after the end of our experiment. This suggests that the length of the study and the germination rate of seeds were appropriate for our experiment (and see Grip-enberg et al. 2014). Yet, the overall growth of seedlings was slow: after nearly 4 months of monitoring, most seedlings were dead and survivors were < 5cm tall, whereas in greenhouse conditions after the same time period, seedlings are usually 30–40 cm tall (Francis 1991). Seedling biomass, not measured in the present study, was presumably low with, on average, only 3–5 leaves amounting to 4 cm² for each survivor at the end of the experiment. Further, seedlings survived only a short time at ELC: 27.5% of germinated seedlings survived only one week. In these conditions, selecting a different tree species for our experiment, with rapid growth and high foliar biomass, would have perhaps helped to quantify better seedling mortality due to insect herbivores.

Second, the overall germination rate of *G. ulmifolia* seeds (36%) was higher at ELC than that reported in the literature for Panama (natural conditions, 20%: Sautu et al. 2006). Many seeds germinated quickly, within three weeks of being sown. This suggests that light limitation at ELC was not a major factor compromising seedling performance of *G. ulmifolia* at the early stages of the life cycle of this species, which was the focus of our study. Indeed, this pioneer species can grow in a variety of habitats (Francis 1991), but we acknowledge that (1) our experimental conditions might have been different from open habitats where *G. ulmifolia* also grows and (2) that the longer-term survival of the seedlings and saplings of this species might be compromised by limited light. Whether light-demanding pioneers are ultimately able to grow to maturity in a given location will depend on changes to the light environment *subsequent* to establishment. In a longer-term perspective, light gaps will form in the forest subsequent to seedling establishment, providing initially shaded seedlings with the conditions necessary to grow towards maturity. Thus, it is relevant to look at the early stages of seedling growth under relatively shaded conditions because the outcome of this phase in plant demography sets the template for future establishment.

Third, an unknown proportion of seeds was also probably lost in our experiment before germination. We observed damage due to vertebrates crossing the blocks (i.e., excavated soil, seed labels chewed), most likely caused by agoutis, *Dasyprocta punctata*, and coatis, *Nasua narica*. We also suspect that ants, particularly *Atta* spp., removed some of the seeds used in the experiment. In Colombia, several ant species, such as *Atta cephalotes*, are known to remove preferentially in open pastures small tree seeds including *G. ulmifolia* (Escobar-Ramírez et al. 2012). On BCI, a high proportion of *G. ulmifolia* seeds are removed secondarily, presumably by ants (Ruzi et al. 2017). However, our insecticide treatment at ELC should have impacted negatively such seed removal by ants. Heavy rainfall may have also washed some of the small seeds out of the experimental blocks. The very small size of the seeds made it impossible to search for individual seeds at the end of the experiment and assess their fate. Selecting tree species with large seeds would thus be useful for future experiments. We believe that pre-germination loss to vertebrates is probably infrequent because of the low biomass represented by isolated seeds, but we cannot quantify loss of seeds due to removal by ants or by rainfall.

Our factorial design insured that all treatment groups were exposed to the same experimental conditions. All experimental blocks were thus likely to be affected in the same way by additional environmental factors (such as light conditions, seed removal by vertebrates and rainfall) which were not controlled in our study.

Conclusion

Our study generated new information on the role of natural enemies in the survival of *Guazuma ulmifolia* at the early stages of the life cycle. We conclude that the pre-dispersal seed stage is by far the stage most vulnerable to insect enemies. Also that the main agent of seedling mortality during the experiment was probably inclement rainfall, and that Janzen-Connell effects that may be specifically due to insect herbivores are probably rather weak for this tree species. The regeneration of *G. ulmifolia* in the forest of ELC appears to be difficult to account for but not to be the outcome of the action of insect herbivores after seeds are dispersed. This illustrates that each tree species may respond differently to pathogens and herbivores (Gripenberg et al. 2014), and that further work should aim at tracking the possible existence of suites of species that share a common response, perhaps partly dictated by conserved morphological and chemical traits (Paine et al. 2012).

What are the implications of our findings for the regeneration of this important tree species in agroforestry? Without addressing what may be suitable conditions for seedling growth, the regeneration of this species may further depend on effective primary dispersal of seeds by vertebrates before most of the seed crop is lost to insects. In this situation, local conservation of dispersal agents may be crucial to enhance escape of seed predation by insects. For example, artificial roosters for frugivorous bats, which disperse seeds of *G. ulmifolia* (Roman et al. 2012), may greatly help the regeneration of this species and the reestablishment of vegetation in disturbed areas (Silveira et al. 2011).

Acknowledgements

We thank the Smithsonian Tropical Research Institute in Panama for logistical support. D. Catalina Fernandez, Indira Simon Chaves and Marjorie Cedeño collected most of the insect material on BCI, which was identified by H.B., John Heppner, Josef Jelinek, M.M., Geoffrey Morse, M.R. and Henry Stockwell. Stephany Arizala helped with fieldwork. This work was supported by the Czech Science Foundation [16-20825S to Y.B.]. Field work on BCI was largely funded by a postdoctoral grant from the Academy of Finland to S.G. Grants from the Smithsonian Institution Barcoding Opportunity FY013 and FY014 (to Y.B.) and in-kind help from the Canadian Centre for DNA Barcoding allowed sequencing of insect specimens. Y.B. and H.B. are members of the Sistema Nacional de Investigación, SENACYT, Panama. S.G. holds a Royal Society University Research Fellowship. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the USDA; USDA is an equal opportunity provider and employer.

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Supplementary material I

Supplementary tables S1-S4, Appendix S1, Plate S1

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Link: <https://doi.org/10.3897/natureconservation.32.30108.suppl1>