Geographical and interspecific variation and the nutrient-enrichment hypothesis as an adaptive advantage of myrmecochory

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In myrmecochory, the relocation of diaspores to ant nests may lead to the enhancement of plant fitness because ant nests and their middens are often richer in essential nutrients than surrounding areas. This idea is the basis of the nutrient-enrichment hypothesis (NEH), which suggests that nutrient enrichment may be a major selective influence in the evolution of myrmecochory. However, there is little evidence regarding whether the greater plant performance and fitness enhancement in ant nests is due to nutrient enrichment or other benefits of directed dispersal. Here, we present the results of a large-scale seed-sowing experiment that tests the NEH in the ant-dispersed perennial herb *Helleborus foetidus*, exploring geographical and inter-ant taxa variation. Experiments were conducted in three well-separated regions of the Iberian Peninsula, targeting the nests of major and minor local ant dispersers (nine ant species in total) and the soil beneath maternal plants as seed destinations. Seedling emergence, survival and early establishment rates, as well as variation in soil characteristics, were obtained for each seed destination at each region. Our results do not fully support the NEH in our study system. Instead, we found that the advantage of ant nest soil for establishment in *H. foetidus* was conditional. Differences in soil fertility and concomitant differences in seedling establishment between ant nests and beneath the canopy of maternal plants were observed in some regions and for some ant species, but not in others. Thus, the conditional outcomes arise from inconsistencies among regions, between stages of seedling regeneration and among ant species in the advantages of being dispersed to nests. Because variation in the guilds of ant dispersers of myrmecochore plants across their ranges is common, this study illustrates the need to consider geographic and inter-ant taxa variation for a complete evaluation of the NEH.

Ants can influence plant growth, survival and reproductive output via their effects on soil properties (Beattie and Hughes 2002). Numerous studies have shown notable differences in the chemical, physical and/or microbial environments of ant nests relative to the surrounding soil as a result of ant activity (reviewed by Folgarait 1998, MacMahon et al. 2000). Thus, ant nests contribute significantly to the formation and maintenance of the spatial heterogeneity of soil nutrients (McGinley et al. 1994, Wagner et al. 2004, Wagner and Jones 2006) and play a major role in regulating vegetation diversity and composition in diverse ecosystems (Culver and Beattie 1983, Dean et al. 1997, Farji-Brener and Ghermandi 2004, Whitford et al. 2008).

In myrmecochory (i.e. seed dispersal by ants), relocation of diaspores to ant nests may enhance plant fitness because ant nests and their middens are often richer in essential nutrients than surrounding areas (Beattie 1985, Hanzawa et al. 1988). This idea is the basis of the nutrient-enrichment hypothesis (NEH), which has been perceived as a major selective advantage in the evolution of the myrmecochory in certain geographical areas (Beattie 1985, Wenny 2001). In particular, the NEH seems to be relevant in temperate forests in the Northern Hemisphere (Culver and Beattie 1978, Beattie and Culver 1983, Hanzanwa et al. 1988, Giladi 2006).

However, studies conducted in regions in the Southern Hemisphere that contain an important fraction of myrmecochore flora (i.e. the South African Fynbos and Australian sclerophyllous vegetation) have provided contradictory results as to whether the NEH is an adaptive advantage derived from myrmecochory (Rice and Westoby 1986, Bond and Stock 1989, Giladi 2006).

Some limitations have constrained the full corroboration of the NEH in the context of myrmecochory. First, it is often difficult to discern whether the increase in certain components of plant fitness at ant nests is a direct result of nutrient enrichment or a consequence of other benefits of directed dispersal, such as lower levels of post-dispersal predation, greater protection against herbivores, decreased plant competition or optimal light conditions for plant establishment (Giladi 2006). Second, plant performance at ant nests has usually been contrasted with performance at random seed destinations away from ant nests (Culver and Beattie 1983, Horvitz and Schemske 1986, Oostermeijer 1989, but see Boyd 2001). Given that the destination of the seeds dispersed by ants is non-random and highly directional (i.e. seeds will be dispersed mainly to ant nests; Wenny 2001), a more realistic approach would be to contrast plant performance at ant nests against plant performance beneath the maternal plant canopy

(Boyd 2001). Third, although seminal papers on the NEH have indicated that variation in the ant species analyzed is one of the main sources of variation in the results of NEH validation studies (Culver and Beattie 1980, Beattie and Culver 1983), few works, if any, have adopted a multi-ant species approach for testing the NEH. This is a critical issue because 1) the magnitude and sign of the differences in chemical and physical soil properties between ant nests and surrounding areas depend on the ant species involved (Beattie and Culver 1983, Dauber and Wolters 2000, Whitford et al. 2008), and 2) the evolutionary effects of multiple interacting ant species on plant traits cannot be evaluated accurately without knowing whether there is any variation in dispersal service among different ant partners (Bronstein et al. 2006, Giladi 2006).

Studies aiming to corroborate the NEH have been conducted within local frameworks. However, there are important reasons to extend the exploration of the NEH geographically. In ant–seed dispersal mutualisms, the guild of ant dispersers frequently varies in space, particularly geographically (Garrido et al. 2002, Manzaneda et al. 2007); as a consequence, the outcome of the interaction may vary across geographic regions (Manzaneda et al. 2005a, Rey and Manzaneda 2007). In addition, ants' ability to alter the soil depends on the original nature of the soil background (Frouz et al. 2003), which is expected to vary geographically.

Here, we present the results of a multi-site seed-sowing experiment aimed at testing the NEH as an adaptive advantage of myrmecochory for the ant-dispersed perennial herb *Helleborus foetidus* (Ranunculaceae). This species is widely distributed throughout western Europe and tolerates huge variation in climate (its range encompasses both humid Eurosiberian and dry/subhumid Mediterranean biogeographical domains), soil types (subsequently nutrients) and ant disperser guild (Manzaneda et al. 2007). This species will allow us to explore whether the validation of the NEH depends on any of these variables. We focused on seedling establishment as a measure of early maternal plant fitness. Any validation of the NEH should at least corroborate the following predictions: 1) the probability of seedling establishment is higher in ant nests than beneath the maternal plant canopy (i.e. undispersed seeds) due to enhanced germination and/or survival rates; 2) soils in ant nests and beneath the maternal plant canopy differ in some or all of the chemical and/or physical soil parameters related to soil fertility; and 3) some differences in soil fertility are predictably associated with a higher probability of seedling establishment (e.g. higher fertility in nests corresponds to higher seedling survival). We acknowledge that validation of these predictions would not provide full support of the NEH, but they certainly constitute the minimum set of conditions necessary to corroborate the hypothesis. Likewise, the non-refutation of some of these premises in some regions and/or for some ant species may denote that the NEH is conditional (sensu Bronstein 1994).

Methods

Study system and sites

Helleborus foetidus is a perennial herb that is distributed throughout central and southern western Europe. It typically grows in the mountains at middle to high elevations and occupies patchy scrublands and the understory of deciduous and mixed forests. Its flowers have 1–6 carpels, each containing 10–16 elaiosome-bearing seeds. The elaiosome is fleshy and accounts for 3–15% of the fresh mass of the diaspore (total diaspore mass ranges between 5 and 23 mg). Fruit maturation and seed shedding occur in June–July. Ants rapidly remove diaspores and carry them to their nest. The intact seeds are then discarded within the nest or on waste-middens. Rodents (mainly wood mice) are important seed predators and may consume seeds directly from the plant (Fedriani et al. 2004) or later at ant nests (Manzaneda et al. 2005a). Nests of the main ant dispersers, however, have been shown to be safe microhabitats for diaspores (Manzaneda et al. 2005a). The ant species associated with *H. foetidus* in the Iberian Peninsula have been characterized elsewhere (Garrido et al. 2002, Manzaneda et al. 2007). These species are diverse in species composition, size, and dispersal behavior and vary spatially at fine and large scales (Manzaneda et al. 2007). Despite the diversity of the ant diaspore collectors, most dispersal is conducted by just one or two species in many localities, suggesting a specialized system (Manzaneda and Rey 2009). Most seeds germinate during the second spring (February–May) after they entered the seed bank (Garrido et al. 2009). Many seedlings die after the first summer due to water stress (Manzaneda et al. 2005b), but those that survive this first dry period have a higher probability of recruitment and becoming established (Garrido et al. 2005). Because the majority of the diaspores remain undispersed to ant nests (individual seed removal varied between 0 and 30% in our study sites, Manzaneda and Rey 2008), seedling recruitment also occurs in the vicinity of the parent plants (Garrido et al. 2005, 2007). Posterior parent-offspring competition seems to be common because juvenile mortality remains stable over time (Rey unpubl.). Seed dispersal to ant nests may likely reduce such intra-specific competition. Water and irradiance stresses are important determinants of the spatial variation in seedling survival (Garrido 2003, Garrido et al. 2007); survival is favored by the alleviation of such stresses by the canopy of the surrounding vegetation (Ramírez et al. 2006). However, recruitment in this species is limited not by microsite but by seed dispersal and to some extent by fecundity (Rey et al. 2006). Nutrients are also thought to be relevant to the recruitment dynamics of this species (Garrido 2003), but the role of nutrient availability on seed germination and seedling survival and its variation among potential seed destinations remains unknown.

The present study was conducted from September 2002 to October 2004 in three geographically separate regions on the Iberian Peninsula. One study region was located in the Eurosiberian domain, and the other two were located in the Mediterranean domain (Table 1). The study population in the southern region of Cazorla was within an area of 4 km^2 and located in a Mediterranean mixed forest of oaks *Quercus rotundifolia* and pines *Pinus nigra* ssp. *salzmanii*. The population in Peña Negra was located within an area of 2.5 km^2 in a deciduous oak forest (*Quercus pyrenaica*). Finally, the study population at the northern region of Caurel was located in an area of 3 km^2 in a patchy pine forest *Pinus sylvestris*. The summer accumulated rainfall values during the study period were 182.4, 89.8 and 41.1 mm for Caurel, Peña Negra and

Table 1. Locality names, geographical coordinates and elevations of study sites. The ecological characteristics of the ant species considered at each geographical region are also shown. The major ant dispersers in each population are in boldface.

Region	Locality names	Geographical coordinates	Elevation (m a.s.l.)	Species	Number of nests*	Dispersal behavior†	Nest midden⌠	Nest life span¥
Cazorla	Roblehondo	37°56′N, 3°52′W	1270	*Aphaenogaster iberica*	4	legitimate disperser	discrete	labile
				Camponotus cruentatus	5	legitimate disperser	discrete	stable
				Camponotus vagus	4	legitimate disperser	discrete	stable
				Cataglyphis velox	5	legitimate disperser	discrete	stable
				Pheidole pallidula	5	facultative disperser	discrete	labile
Peña Negra	Barranco del Toril	40°26′N, 5°18′W	1430	*Formica sanguinea*	3	legitimate disperser	discrete	stable
				Lasius emarginatus	4	facultative disperser	discrete	stable
Sierra del Caurel	Las Cruces	42°36′N, 7°6′W	1500	**Formica lugubris**	4	legitimate disperser	discrete	stable
				Lasius fuliginosus	4	facultative disperser	diffuse	labile

*Number of nests (replicates) located and used in the experiment at each *H. foetidus* population.
†Legitimate dispersers are ant species that behave predominantly as true seed removers. Facultative dispersers are ant visitors that behave indistinctly as in situ elaiosome consumers or occasional seed removers.
⌠Discrete nests present patent and persistent refuse piles and waste middens around nest entrances, while in diffuse nests, both refuse piles and/or waste middens are not present or are not evident around the entrances of the main nests.
¥Labile nests are typical of short-lived ant colonies, while stable nests correspond to long-lived colonies.

Cazorla, respectively, while means temperatures were 16.6, 18.1 and 21.8°C, respectively. These populations also represent very different types of background soils. In Cazorla, the soils originated from a variant of calcite or dolomite limestone bedrock (Herrera 2002). In Peña Negra, soils are acidic and sandy and originated from the weathering of granites, quartzite and slates (Pereira 1998), while in Caurel, *H. foetidus* mainly grows in limestone outcrops among schist and slates. Therefore, these three populations cover much of the natural range of *H. foetidus* on the Iberian Peninsula, representing the diverse ecological conditions in which this species occurs (see Manzaneda et al. 2005a, 2007 for more details about these locations).

Experimental design and procedure

To test the NEH, we performed a seed-sowing experiment with two treatments for a seed destination effect: seeds dispersed to ant nests 'nest' and seeds remaining beneath maternal plants 'undispersed'. The rationale behind this design is based on two arguments: 1) seed dispersal by ants is considered to be a non-random and highly directional process (Introduction) and 2) at the end of seed-shedding, seeds are basically found in one of two locations: undispersed beneath the plant or dispersed in ant nests (this is further confirmed by the observation that ant-seed dropping is negligible in our system; authors' unpubl.). In each population, we sowed the seeds at active ant nests. Each experimental unit consisted of 15×15 cm seed sowings with 25–30 seeds without the elaiosome sown at a depth of ~2–3 cm. Most ant dispersers considered here have superficial or subsuperficial waste middens, which roughly correspond to the seed burial depth used in our experiment. Each seed sowing location was protected with a $20 \times 20 \times 8$ cm wire-mesh cage to prevent rodent predation. In the 'nest' treatment, seeds were sown into the waste-midden or, when no defined midden was evident, close to the nest entrance (Table 1). In the 'undispersed' treatment, seeds were sown beneath the reproductive plant closest to the ant nest (i.e. 'nest' and 'undispersed' treatments were paired). The distance between the ant nest and each plant in the 'undispersed' treatment

ranged between 0.75 and 10 m, which is within the dispersal range of *H. foetidus* (Manzaneda unpubl.). To minimize the possible interference of alien seeds in the experiment, we manually removed all other seeds that we could find in each microsite (both beneath the plant and in ant nests). In each population, the seeds that were used were collected previously (in June–July 2002) from the same population. Seeds were stored in dry conditions at room temperature until sowing in September 2002.

To explore the suitability of nests of different species of ants for seedling establishment, we included both ant species that behave predominantly as true seed removers (legitimate dispersers, 'LD') and other ant visitors that behave indistinctly as in situ elaiosome consumers or occasional seed removers (facultative dispersers, 'FD'). Because ant species differ in visitation rates, we included both the ant species that showed the highest seed removal rates (major dispersers) and the ant species that visited the plants only occasionally. Therefore, ant species were categorized on the basis of on two distinct dispersal behaviors and two different plant visitation rates (Table 1). Detailed information about the dispersal behavior of the ants and their visitation rates may be found elsewhere (Manzaneda et al. 2007, Manzaneda and Rey 2008, 2009). In total, 38 nests of nine different ant species were included in the experiment (3–5 nests per ant species, Table 1). A total of 2075 seeds were sown in 76 locations.

No seedlings emerged during the first year, i.e. in 2002–2003 (consistent with the long latency period of *H. foetidus* seeds, Garrido et al. 2009). Seedling emergence began in February 2004. Sowings were checked every month during the emergence period (February–May) and bimonthly thereafter until October 2004 (i.e. the end of the first dry period for emerged seedlings). In each survey, we counted and marked newly emerged seedlings using colored bamboo sticks. The survival of the emerged seedlings was monitored until the end of the experiment.

Characterization of the seed sowing locations

Because canopy cover frequently influences seed germination and seedling establishment (Kitajima and Fenner 2000),

sowing locations were characterized in relation to canopy cover. In July 2004, we took a hemispheric photograph of the canopy cover above each sowing point at each sowing location. Pictures were oriented to the north and were taken at a height of 60 cm using a Nikon FC-E8 fisheye lens attached to a digital camera Nikon Coolpix 995. For each picture, we calculated the percent canopy cover openness using the image processing and analysis program WINPHOT v.5.0 (ter Steege 1996).

At the end of the experiment, we collected soil samples from our 76 seed sowing locations by extracting the superficial layer soil (top 15–20 cm) at each location. The soil samples taken from ant nests also included, when present, the organic refuse pile of the ant nest's middens, which is the natural final destination of the dispersed seeds. The textural (percent sand, silt, gravel and clay content) and chemical (pH, organic matter, organic carbon, percent carbonates, total Kjeldahl nitrogen, organic nitrogen, available potassium and Olsen phosphorus) characteristics of the samples were determined using standard soil analytical procedures. Soil analyses were conducted at the Inst. de Recursos Naturales y Agrobiología (C.S.I.C., Seville, Spain).

Data analysis

We fitted a generalized linear mixed model using the GLIMMIX procedure in SAS 9.1.2 with binomial errors and logit link function to determine whether seed destination affected seedling emergence (ratio of emerged seedlings to total number of seeds sown), seedling survival (ratio of surviving seedlings to number of emerged seedlings) or seedling establishment (ratio of surviving seedlings to number of seeds sowed). Analyses were conducted separately for each of these dependent variables. We considered seed destination, geographical region, and their interaction as fixed factors as well as each pair of nest and undispersed seed sowing locations as a random block effect nested within each region. When a significant interaction between main effects was detected, we conducted a 'test of simple main effects', which allows the effects of a given factor to be explored at the level of the other factors using the SLICE option in the LSMEANS statement of the MIXED procedure in SAS.

To analyze differences in emergence, survival and establishment between ant species and seed taking categories (major or occasional, i.e. FD or LD functional groups), we generated generalized linear models considering binomial error and the logit link function (GENMOD procedure in SAS) with species as main factor. It was impractical to incorporate seed sowing points in these analyses (as random blocks) based on the lack of convergence of the models due to frequent nil emergence, survival and recruitment in sowing points for most species. Thus, we opted to conduct event/trial tests, assuming each seed at the same sowing point was independent. Although this assumption may be somewhat unrealistic, the low emergence rates make density-dependent effects on survival and recruitment unlikely. These analyses were conducted only in the Cazorla region because the other regions had only two ant disperser species and fewer emerged seedlings (Results). Contrast tests were used to compare functional groups.

To compare ant nests with the micro-site beneath the plant and after controlling for regional soil variation, we performed a variance partitioning analysis. Because soil variables are typically correlated, a principal component analysis (PCA) was further used to identify independent factors (after varimax normalized rotation) that accounted for a combination of soil variables. Factor scores were subsequently used to explore the variability in soil properties between destinations and regions. In Cazorla (where the number of ant nests allowed the use of multivariate analysis), we also used univariate variance analyses and PCA to analyze differences in nest soil parameters between ant species and obtain factors describing species-specific ant nest soil characteristics. Then, factor scores were compared between ant species nests by ANOVA and subsequently compared between major and occasional dispersers and between LD and FD.

Results

Variation in seedling survival, emergence and establishment

Seed destination and probability of emergence, survival and establishment

Seedlings emerged from approximately 18% of the sown seeds (363 seedlings). The probability of emergence varied significantly between regions (Table 2). The average proportion of seedlings that emerged was higher in Cazorla (0.2 ± 0.46) and Peña Negra (0.2 ± 0.37) than in Caurel (0.04 ± 0.12). The effect of seed destination on seedling emergence depended on the region (Table 2, significant Region × Seed destination interaction). Tests of simple main effects showed that emergence was significantly affected by seed destination ($F = 5.26$, $p = 0.0219$) only in Cazorla.

Table 2. Results of the generalized linear mixed model testing the effect of geographical region and seed destination on the probability of emergence, survival and early establishment of *H. foetidus* seedlings. Significant effects (p > 0.05) are in boldface.

	Emergence			Survival			Establishment		
Fixed effects	DF	F	p	DF	F	p	DF	F	p
Region	2,70	6.66	**0.0023**	2,47	3.27	**0.046**	2,71	0.69	0.505
Seed destination	1,1974	1.28	0.2586	1,310	1.01	0.315	1,1973	0.24	0.626
Region × Seed destination	2,1974	4.57	**0.0105**	2,310	4.25	**0.015**	2,1973	0.77	0.461
Random effects		Z	p		Z	p		Z	p
Block (Region)		4.41	**< 0.0001**		3.38	**0.0004**		4.39	**< 0.0001**

In this region, seeds dispersed to ant nests had more than a two-fold higher likelihood of emerging than did seeds that remained undispersed (Fig. 1a). In contrast, in the other two regions, percent emergence was greater in seedlings from undispersed seeds than in seeds at ant nests (Fig. 1a), but only at Caurel was this trend close to significant (F = 1.36, p = 0.243; F = 2.93, p = 0.087, test values for Peña Negra and Caurel, respectively). The probability of emergence also varied among seed sowing locations within regions (Table 2).

After the first summer, 18% of the 363 emerged seedlings had survived. Yet, the probability of survival differed significantly among regions (Table 2). The proportion of surviving seedlings was high in Caurel (0.58 ± 0.12), intermediate in Cazorla (0.21 ± 0.33) and low in Peña Negra (0.043 ± 0.05). Again, the effect of seed destination on seedling survival was dependent on the region because the Region $\times$ Seed destination term was significant (Table 2). Tests of simple main effects showed that seed destination had an effect on seedling survival in Caurel only (F = 2.21, p = 0.031). In this region, seeds emerging in ant nests had a 2.5-fold greater probability of surviving than seeds emerging beneath the plant (Fig.1b). In the other two regions, seed destination did not affect seedling survival (p > 0.05, Fig. 1b). Again, seedling survivorship varied at small scales (Table 2).
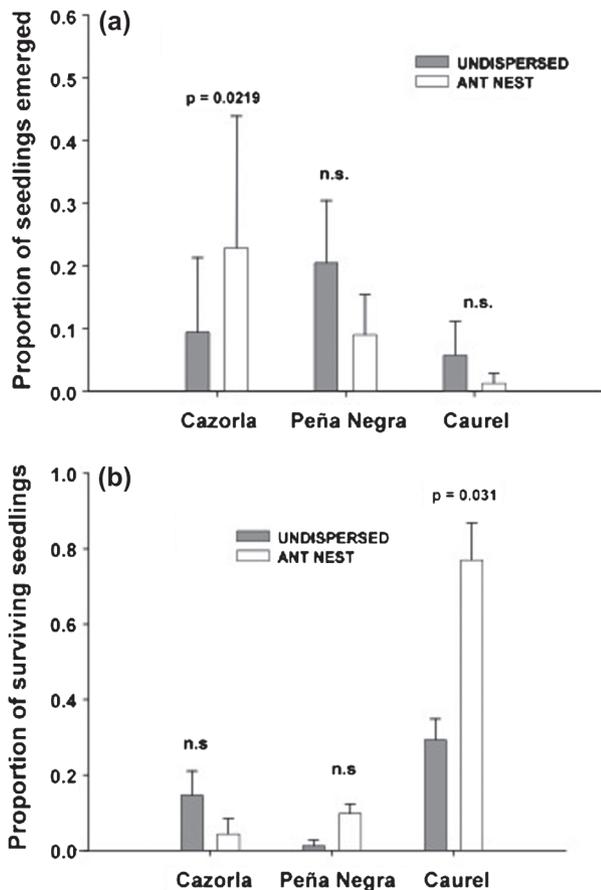

Figure 1. Proportion of (a) emerged seedlings and (b) surviving seedlings per sowing point in each region and seed destination. LS means $\pm$ 1 SE are represented. In each plot, significance p-values reflect tests of simple main effects (interaction slices) for the Region $\times$ Seed destination interaction effect.

Early seedling establishment was very low. Only 3.1% of sown seeds were able to establish seedlings after 10 months post-emergence. This trend was consistent among regions and independent of seed destination, as suggested by the lack of significance of the Region $\times$ Seed destination interaction term (Table 2). The probability of seedling establishment again varied significantly among sowing locations in the same region.

Ant species-specific and functional variation in emergence, survival and establishment

The probability of seedling emergence did not differ significantly among ant species (Wald $\chi^2 =$ 8.69, DF = 4,570, p = 0.07) in Cazorla. However, seedling survival and establishment in nests varied significantly among ant species (Wald $\chi^2 =$ 19.43, DF = 4,159, p = 0.0006; Wald $\chi^2 =$ 10.61, DF = 4,570, p = 0.0314, for survival and establishment, respectively). The highest percent seedling survival was observed at *Componotus vagus* nests, where survival was approximately 50% (Fig. 2A). The lowest survival was observed in *Aphaenogaster iberica* and *Componotus cruentatus* nests, while survival rates at *Cataglyphi velox* and *Pheidole pallidula* nests were intermediate (Fig. 2A). The probability of seedling establishment 10 months after emergence was significantly higher at the nests of *C. vagus*, *C. velox* and *P. pallidula* than those of *A. iberica* and *C. cruentatus* (Fig. 2B).

Seedling emergence, survival and establishment did not vary significantly between major and occasional ant dispersers ($\chi^2 =$ 0.40, DF = 1,18, p = 0.532, $\chi^2 =$ 0.84, DF = 1,18, p = 0.357; $\chi^2 =$ 0.065, DF = 1,18, p = 0.8, for emergence, survival and establishment, respectively) or between LD and FD dispersers ($\chi^2 =$ 0.118, DF = 1,18, p = 0.734; $\chi^2 =$ 0.58, DF = 1, 18, p = 0.44; $\chi^2 =$ 0.75, DF = 1,18, p = 0.39, for emergence, survival and establishment, respectively). Emergence, survival and establishment data for all functional groups are provided in Supplementary material Appendix 1.

Variation in soil characteristics

Variation between regions and seed destinations

Regional differences were found for 6 of 13 soil parameters, and destination differences were found for 5 of 13 parameters (Table 3). Parameters related to soil texture generally varied much more among regions than between seed destinations. In fact, the percentage of variance in soil texture properties that could be explained by variations between seed destinations was always < 12% (Table 3). Accordingly, we did not find significant differences between seed destinations in any of the soil variables related to soil texture (Table 3). In contrast, with the exception of the percentage of carbonates, P, and K, soil chemistry parameters varied more between seed destinations than among regions (Table 3). In particular, organic soil components differed significantly by destination (organic carbon, organic matter, N and C/N ratio), while pH differed both by destination and region (Table 3). Supplementary material Appendix 2 shows the means and standard deviations of soil properties at each region and seed destination.

The PCA of the correlation matrix of soil parameters (across the 76 sowing sites) revealed the existence of two
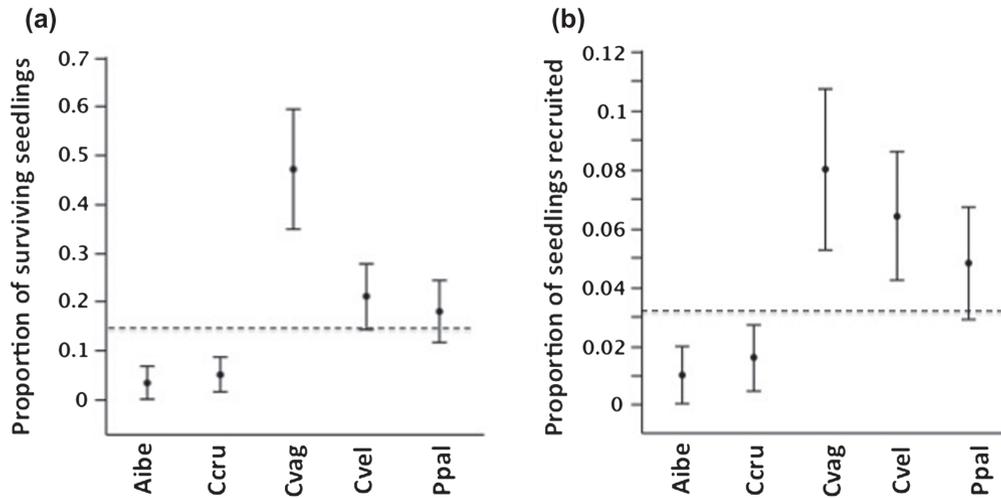
Figure 2. Proportions of (a) surviving seedlings and (b) recruited seedlings 10 months after emergence in the nests of five ant species from the southern region of Cazorla. LS means ± 1 SE are represented. In both plots, different letters indicate significant differences at p < 0.05. (Aibe = *A. iberica*, Ccru = *C. cruentatus*, Cvag = *C. vagus*, Cvel = *C. velox*, Ppal = *P. pallidula*). The horizontal line depicts the mean survival (a) and recruitment (b) beneath maternal plants in Cazorla region.

main independent factors that accounted for 67.43% of the variation in soil properties (Supplementary material Appendix 3). The first factor (PC1) represents a gradient of soil fertility (negative values describe infertile soils) and included the significant parameters of percent organic carbon, organic matter, N, organic N, the C/N ratio and, marginally, P (Supplementary material Appendix 3). The second factor (PC2) represents a gradient of soil texture properties (ranging from gravelly soils on the negative side of the gradient to less porous soils with a higher content of clay on the positive side) because it was negatively correlated with the percentage of gravel and positively correlated with the percentage of sand and silt (Supplementary material Appendix 3).

PC1 varied significantly between seed destinations, and the sign and magnitude of the variation was dependent on the geographical region (ANOVA on PC1: Region, F = 2.45, DF = 2,72, p = 0.1; Seed destination, F = 2.95, DF = 1,72, p = 0.093; Region × Seed destination, F = 4.26, DF = 2,72, p = 0.0018). Thus, soil fertility was significantly higher in ant nests than beneath maternal plants only in Caurel (posthoc test p = 0.0047; Fig. 3). On the contrary, fertility was significantly higher in soils beneath the plant than in ant nest soils in Cazorla (posthoc test p = 0.032; Fig. 3). No significant differences in soil fertility between seed destinations were found in Peña Negra (Fig. 3). PC2 varied significantly only across regions (ANOVA on PC2: Region, F = 42.09, DF = 2,72; p < 0.0001; Seed destination,

Table 3. Summary statistics and variance partitioning analysis (among regions and seed destinations) for soil variables obtained from 76 sowing points. The statistical significance for the variation of each variable among regions and seed destinations is given. Significant differences (p > 0.05) are in boldface. Variance partitioning was performed with a hierarchical design (ant nest was nested within region). The model was fitted with restricted maximum likelihood to take into account the unbalanced nature of this design.

| Soil parameter	Range	CV (%)	Explained variance (%)		Difference among regions		Difference between destinations	
			Among regions	Between destinations	F	p	F	p
Soil texture								
Clay (%)	12.10–48.70	36.75	62.12	7.88	14.28	**0.019**	1.92	0.141
Sand (%)	2.40–22.0	48.03	20.18	0.14	3.814	0.111	0.96	0.418
Gravel (%)	4.50–65.80	63.85	81.58	11.82	63.28	**< 0.0001**	0.19	0.902
Silt (%)	14.70–62.50	30.55	81.50	10.20	58.41	**< 0.0001**	0.09	0.963
Soil chemistry								
CaCO₃ (%)	< 0.50–46.0	149.01	82.28	0.12	56.46	**< 0.0001**	0.02	0.996
Organic carbon (%)	0.88–14.40	51.56	1.16	43.90	0.568	0.615	5.28	**0.004**
pH	5.10–8.30	13.77	38.62	22.80	20.68	**0.014**	3.38	**0.026**
P (mg kg⁻¹)	< 1.0–85.70	115.71	17.55	7.31	3.47	0.148	1.59	0.204
Organic matter (%)	1.53–24.80	51.56	25.83	43.45	1.17	0.434	5.24	**0.0043**
N (%)	0.07–1.224	59.32	30.71	18.55	2.64	0.206	2.17	0.109
Organic N (%)	0.085–1.225	50.34	1.15	43.93	0.57	0.614	5.28	**0.0041**
K (mg kg⁻¹)	41.0–663.0	62.16	81.17	1.25	34.97	**0.0012**	0.39	0.758
C/N	10.40–11.70	2.36	1.02	44.7	0.164	0.855	5.38	**0.0037**
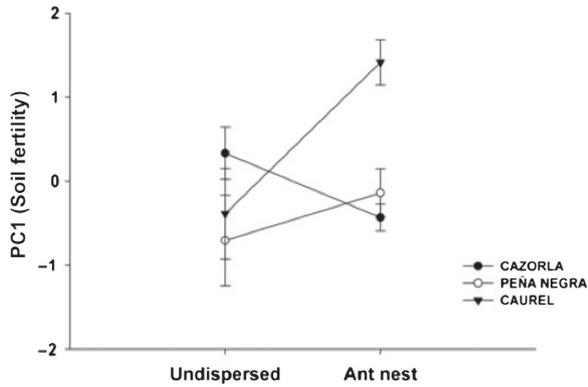
Figure 3. Variation in the soil fertility factor PC1 (LS mean ± 1 SE) between seed destinations in three regions from the Iberian Peninsula. Higher PC1 values indicate higher soil fertility.

$F = 0.123$, $DF = 1,72$, $p = 0.727$; Region × Seed destination, $F = 0.72$, $DF = 2,72$, $p = 0.72$).

Interspecific variation in soil characteristics in ant nests

PCA conducted on the correlation matrix of soil parameters across 23 ant nests in the Cazorla region revealed two independent gradients of variation in soil properties (Supplementary material Appendix 4), resembling the results previously found in the regional analysis (that is, PC1 was soil fertility and PC2 was soil texture; Supplementary material Appendix 4). In this case, PC1 varied significantly among ant species (ANOVA: $F = 4.39$, $DF = 4,18$, $p = 0.011$), denoting species-specific differences in soil fertility in nests in this region, while PC2 did not vary among ant species (ANOVA: $F = 0.49$, $DF = 4,18$, $p = 0.747$). *Campontus vagus* nests were significantly more fertile than nests from other ant species, which fell close together on PC1 (Fig. 4). Specifically, *C. vagus* ant nests were significantly richer than the rest of the nests in organic carbon, organic matter and organic nitrogen contents (Supplementary material Appendix 5). There was no significant difference in soil fertility between the nests of major and occasional ant dispersers ($\chi^2 = 2.37$, $p = 0.14$) or LD and FD dispersers ($\chi^2 = 0.0018$, $p = 0.97$).

Canopy cover ambient in seed destination

The percentage of canopy cover did not vary among geographical regions ($F = 1.19$, $DF = 2,70$, $p = 0.314$) or between seed destinations ($F = 0.75$, $DF = 1,70$, $p = 0.391$). However, canopy cover varied significantly between ant species ($F = 13.34$, $DF = 6.32$, $p = 0.037$). *Lasius emarginatus* and *L. fuliginosus* ant nests were significantly located in shadier areas than nests of the rest of the ant species (Supplementary material Appendix 6).

Discussion

Our results partially support the hypothesis that the NEH is an adaptive advantage derived from myrmecochory in our study system. In particular, this study has shown that differences in soil fertility-related properties and concomitant seedling survival between ant nests and the soil beneath the
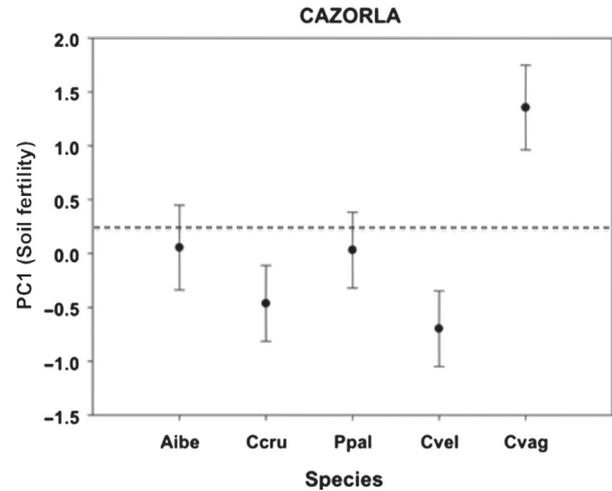

Figure 4. Variation in soil fertility PC1 (LS mean ± 1 SE) between the nests of five ant dispersers in Cazorla. Higher PC1 values indicate higher soil fertility. (Aibe = *A. iberica*, Ccru = *C. cruentatus*, Cvag = *C. vagus*, Cvel = *C. velox*, Ppal = *P. pallidula*). The horizontal line depicts the mean soil fertility (PC1) beneath maternal plants in Cazorla region.

canopy of maternal plants can be observed in some regions and for some ant species, but not others. Thus, we propose that the advantage of ant nests for seedling establishment is conditional and/or context dependent. This conditionality arises from differences among regions, between stages of seedling regeneration, and among ant species in the seedling establishment outcome between ant nests and beneath maternal plants. Our results concur with previous studies that have aimed to test the NEH in other myrmecochory systems, which also did not provide full support for this hypothesis (Horvitz and Schemske 1986, Rice and Westoby 1986, Bond and Stock 1989, Giladi 2006). Our results are also in agreement with previous studies conducted in the same study system, which detected context dependence in other advantages derived from myrmecochory (Manzaneda et al. 2005a). Moreover, our findings support the view that myrmecochory may pre-adapt plants to be successful in various environments (Bronstein et al. 2006, Lengyel et al. 2009, 2010).

Variation in the ant nest environment

Differences in the chemical and physical environments between ant nests and the surrounding soil have led to the suggestion of the NEH as an adaptive benefit derived from seed dispersal by ants (Hanzawa et al. 1988). In our study, however, we detected notable variations in the sign and/or magnitude of such differences between dispersal environments among regions and ant species. All of the parameters related to soil texture varied much more between regions than between seed destinations (Table 3). This result may be due to the large regional differences in the type of background soil and the apparently low ability of ants to modify the physical parameters of the original soil. Ants' limited ability to alter soil texture has been documented previously (Passos and Olivera 2002, but see Folgarait 1998, Dostál et al. 2005), which seems to be the case in this study. In contrast, chemical soil parameters generally varied more between seed

destinations than regions (Table 3). However, both the sign and the magnitude of the variation in soil fertility between seed destinations were dependent on the geographical region. Thus, the prediction that ant nests are significantly more fertile than the soils beneath the plant was only corroborated in the Caurel region and not in the two other regions. In Peña Negra, we found no significant differences in soil fertility between seed destinations, whereas in Cazorla, ant nests were significantly more infertile than soils beneath plants. This geographical inconsistency is likely caused primarily by the variability in ant species present and their ability to modify the soil chemistry and accumulate nutrients in nests (Fig. 4, Supplementary material Appendix 1, 6). Ants' ability to alter soil chemistry is highly species-specific (Culver and Beattie 1983, McGinley et al. 1994, Dauber et al. 2001) and dependent upon species-specific attributes, such as nest architecture, nest lability, age, size and depth of the colony, diet and relationship with the soil microbial biota (Wang et al. 1995, Whitford 1996, Dauber and Wolters 2000, Wagner et al. 2004, Dostál et al. 2005). Thus, large and long-lived colonies of ant species with high biological activity and with patent and persistent refuse piles (which prevent erosion and nutrient leaching at the soil surface) will have a superior ability to alter the soil chemistry and accumulate nutrients (Whitford 1996). In our study system, only *Formica lugubris* in the region of Caurel (where the NEH prediction was indeed corroborated) and of *Camponotus vagus* in Cazorla (the ant species with the most significantly fertile nests in this region; Fig. 4, Supplementary material Appendix 5) are representative of this type of nest. The nests of the other ant species presented labile or discrete middens, with relatively small or absent waste middens.

Unlike other studies that aimed to test the NEH, we compared the soil properties of ant nests with the maternal seed environment, rather than with the soils surrounding the nests, which in our opinion, is the most sensible strategy for testing the NEH. Our finding that ant nests frequently lack significant nutrient enrichment (or even exhibit nutrient impoverishment) relative to the soils beneath maternal plants may be related to the fact that mineralization and other soil chemical processes are often enhanced by the presence of plant cover (Burke et al. 1999).

Finally, canopy cover did not differ between seed destinations, and this result was constant in all three geographical regions. However, the canopy cover above ant nests varied significantly between ant species. The origin of these differences was beyond the scope of this study but may be mostly explained by ant species-specific affinities for habitat within each region.

Seedling establishment at ant nests

Seedling emergence
Diverse studies have shown enhanced seed germination and emergence rates in ant nests relative to their surroundings in different myrmecochory systems (Culver and Beattie 1980, Oostermeijer 1989, Gibson 1993, Horvitz and Schemske 1994, Farji-Brener and Ghermandi 2004, but see Rice and Westoby 1986, Boyd 2001). In our study, the effect of ant nests on the emergence of *H. foetidus* seedlings

was geographically inconsistent. Ant nests had neutral or negative effects on seedling emergence in two out of the three study regions. Only in Cazorla was emergence in ant nests significantly higher than emergence beneath maternal plants. In addition, this positive effect on emergence was constant across all ant disperser species and functional species in this region. Such geographical inconsistency is not surprising because differences (or lack of) in typical determinants of seedling emergence (soil texture, fertility and openness) between seed destinations also varied among regions (above). Our findings agree with the few works on myrmecochory that incorporated the spatial variation in studies of germination and of ant nest effects on seedling emergence, which also found considerable variation within and among populations (Gibson 1993, Horvitz and Schemske 1994). Interestingly, in Cazorla, emergence was congruent with differences in soil fertility. Thus, the probability of emergence increased with impoverishments in fertility and organic matter (correlates of PC1), which occurred significantly at ant nests. Elevated concentrations of nitrogen and, especially, of organic matter in the soil may inhibit germination and emergence due to 1) the presence of allelochemicals in the organic layer; 2) the often thick layer of organic stratum, which may limit the amount of light that seeds receive; and/or 3) the low water retention capacity of the superficial organic layer (Herr et al. 1999, Hilhorst and Karseen 2000). It is likely that the latter is an important determinant of the inverse association between emergence and fertility shown here because previous studies of the germination ecology of *H. foetidus* proved that soil water availability is a limiting factor for its seed germination in most habitats (Garrido 2003). Regardless, our results coincide with previous studies conducted in Mediterranean climate areas, which also demonstrated higher seedling emergence at infertile ant nests compared with their surroundings or the maternal footprint (Rice and Westoby 1986, Bond and Stock 1989).

Seedling survival and early recruitment
A positive influence of ant nests on plant recruitment through improvements in growth and/or survival rates has frequently been reported (Hanzawa et al. 1988, Gibson 1993, McGinley et al. 1994, Farji-Brener and Ghermandi 2008), but whether such improvements are ascribable to increments in the concentration of particular soil nutrients has rarely been proven (Giladi 2006, but see McGinley et al. 1994, Farji-Brener and Ghermandi 2008). Our results show geographical and taxonomic inconsistencies in the effects of ant nests on seedling survival. Against the predictions of the NEH, fertility neither differed between seed destination nor was linked to seedling survival or establishment in the Mediterranean regions of Cazorla and Peña Negra. However, in the humid Eurosiberian region of Caurel, ant nests positively affected seedling survival (2.5-fold advantage), although this positive influence did not extend to establishment (because of the extremely low seedling emergence in this region). Interestingly, in Caurel, ant nests were much more fertile than soils located beneath the plants. Thus, in the northern region of Caurel, the predictions of the NEH for the seedling survival component of recruitment were confirmed. These results are congruent with

some emerging biogeographical patterns in NEH validation, which suggest some adaptive importance for nutrient enrichment at nests in northern temperate forests (Culver and Beattie 1978, Beattie and Culver 1983, Hanzanwa et al. 1988, Gorb and Gorb 2003), but not in arid, semiarid and/or Mediterranean-type communities (Rice and Westoby 1986, Bond and Stock 1989).

An innovative level of exploration in this study is the species specificity of the NEH validation. The association between soil fertility at ant nests and seedling survival in Cazorla was negative or neutral for most ant species, but positive for one ant species. Lower fertility in nests, relative to the soil beneath plants, was associated with lower survival in nests for *A. iberica* and *C. cruentatus*, while lower relative soil fertility but similar seedling survival were observed in *C. velox* and *P. pallidula* (Fig. 2A, 4). In contrast, the soil at *C. vagus* nests was more fertile and rendered higher seedling survival than that beneath plants. Interestingly, nests of this ant species showed the highest seedling survival of all ant nests (approximately 50% seedling survival, Fig. 2A), which corresponds to a 3-fold advantage over the average survival beneath maternal plants (approximately 15% in this region). To some extent, this pattern translated to the final recruitment pattern because recruitment was highest overall at *C. vagus* nests (and significantly higher than in nests of *A. iberica* and *C. cruentatus*) and higher than beneath plants (Fig. 2B). Unlike other species in this region, *C. vagus* is a strict forest species that accumulates nutrients and organic matter in patent and persistent refuse piles at its nests and has large and long-lived colonies. Importantly, *C. vagus* is a legitimate and major disperser of *H. foetidus* in Cazorla; consequently, enhanced survival at its nest may have disproportionate consequences on the demography of the species in this region.

Concluding remarks

The predictions of the NEH were only partially corroborated by our analyses, which considered both geographical and inter-taxa variation. The enhanced soil fertility and seedling performance at ant nests postulated by the NEH were conditional. Three interesting patterns of conditionality for the NEH validation have emerged from our study. First, there was a geographical pattern, which seems to be linked to climate variation. We have previously provided evidence that in a Mediterranean climate, nutrient availability may be a minor limiting factor for seedling establishment of *H. foetidus* relative to water availability (Garrido 2003) and that the ant nest itself plays a secondary role in myrmecochore plant recruitment. It has recently been reported that the water content at ant nests is more important than nutrient content for seedling recruitment (Farji-Brener and Ghermandi 2004). This fact may explain why the NEH was corroborated in the humid northern region but not in the southern or central Iberian Peninsula, which experiences a much more severe summer drought. Second, there was a life-stage conditionality that, as expected from seed reserve depletion, shows that seedling survival relies more heavily to nutrient enrichment than does seedling emergence. Third, an ant species-specific conditionality

was found, i.e. that nutrient enrichment may be important in the nests of some ant species but not of others. The inter-relationships between ant behavior, life history traits and the waste-midden structure of each particular ant species may be crucial for a positive association between nutrient enrichment at nests and enhanced seedling performance (e.g. *C. vagus* in Cazorla).

This three-level conditionality may illuminate the ecological and evolutionary consequences of dispersal by ants in this and other systems. The evolution of this interaction may be driven by nutrient enrichment at ant nests (a qualitative component of seed dispersal efficiency) under two very different scenarios: 1) there is an overall benefit on seedling survival at nests in the region (e.g. Caurel in this study) and 2) in the absence of an overall survival benefit at ant nests, a legitimate ant disperser (e.g. *C. vagus*) is present whose nests are much more appropriate microsites for seedling survival because of their particular soil qualities than any other microsite in the same habitat. In order for this second scenario to work, such ant species should be a major disperser, i.e. both a nutrient enrichment advantage and quantitative seed dispersal efficiency are needed. Noticeably, this was the case for *C. vagus* in Cazorla.

In short, our findings illustrate the need to re-evaluate the NEH and other adaptive advantages of this dispersal system in the context of the geographic variation and considering the multiple ant species that may act as dispersers of the same plant throughout the species range.

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Supplementary material (Appendix E6923 at <www.oikosoffice.lu.se/appendix>). Appendix 1–6.