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EDITORIAL

Plant-animal interactions: patterns and mechanisms in terrestrial ecosystems

From the early work of Darwin, then Ehrlich and Raven (1964), to the recent novel contribution of mutualistic networks developed by Jordano and colleagues (Bascompte and Jordano 2007, Nuismer et al. 2013, Guimarães et al. 2017), it is clear that plant-animal interactions—especially those involving herbivory, seed dispersal, seed predation and pollination—are significant drivers of ecological processes and the evolution and maintenance of biodiversity. The vast majority of plant species, for example, are influenced by animal-mediated seed dispersal and pollination. Hence, understanding the complexities of these interactions and their broader geographic patterns is central to better deciphering complex ecosystem processes and the potential fate of biodiversity in the advent of climate change. For this symposium, we assembled a group of leading authorities on plant-animal mutualisms and related interactions in an effort to highlight new findings, demonstrate subtle but significant interactions and to further identify and focus the direction of this important field of ecological research. Based on review of approximately 700 papers published in the past few years and our knowledge of research in this area, we identified invitees from around the globe to join us for a colloquium on plant-animal interactions at the 9th International Symposium of Integrative Zoology held on August, 27-31, 2017 in Xining, Qinghai Province, China.

We selected authors from a broad geographic range who could cover a diversity of topics. The final colloquium, represented by contributors from four continents, included ten papers that are briefly highlighted here. Although not our original intent, this volume offers a heavy focus on oak-animal interactions, due in part to the organizers’ research interests but also to the current intensive research on oak (Quercus)–animal interactions across the globe. As such, seven of these contributions dealt either directly or indirectly with the intricacies of animal-mediated dispersal of oak and/or acorn predation in oak ecosystems from Asia to North America. An eighth paper dealt with the community-wide processes of seed predation and dispersal in another system and the remaining two papers focused on avian pollination in New Zealand and the life history strategies of two provovigenic parasitoids of a phytophagous insect in North America.

Bartlow et al. (2018) experimentally demonstrated that varying degrees of cotyledon damage in oaks, as often results from partial-predation by a diverse group of insects and vertebrates, have minimal impact on oak seedling establishment. The authors argue that the acorn serves as much as a fruit for dispersal and tolerance of partial seed predation as it does a source of energy for the developing seedling. Working in a similar system, Sundaram et al. (2018) introduced a novel approach for evaluating seed choices by a scatter-hoarding rodent (Eastern gray squirrel; Sciurus carolinensis) when selecting seeds and nuts for both feeding and caching. Using a multivariate quantitative method, the investigators successfully quantified the simultaneous effects of numerous seed traits (e.g., caloric value, protein content, tannin and dormancy period) on behavioral decisions. This novel approach to quantifying decision making in these animals has the potential to revolutionize foraging studies, historically based exclusively on energetic costs and benefits.

In one of the first studies of its kind, Dittel and Vander Wall (2018) investigated the effects of both rodent abundance and rodent community richness on pilferage rates of artificial scatterhoards of native seed species and sunflower seeds in seven habitat types distributed across an elevational gradient in western Nevada, USA. They find that pilferage rates were most
directly related to the abundance of scatter-hoarding rodents but not the rodent community richness, leading the authors to conclude that in these systems “density-dependent competition” for cached seeds is a primary determinant of pilferage rates.

In a geographically extensive, 3-year field experiment in the Central Hardwoods region of North America, Sawaya et al. (2018) used a published model on conditional mutualism to predict and test the strength of this relationship between scatterhoarders and four species of hardwood trees. They demonstrated how both seed traits and environmental conditions interact to influence seedling establishment and the strength of these relationships. They found that the strength of this scatterhoarder-tree mutualism is strongest for red oaks (Q. rubra), weakest for white oaks (Q. alba) and intermediate for chestnut oak (Castanea dentata) and hybrid chestnut (C. dentata x Castanea mollissima). Canelo et al. (2018) employ a careful experimental approach to quantify the effects of herbivory by a leaf-feeding insect on the Mediterranean Holm oak’s (Q. ilex) physiological responses in individual shoots that ultimately results in a drop in acorn production. Unlike other correlational studies, this study demonstrated the causal physiological links between herbivory and reproductive loss.

Bogdziewicz et al. (2018), working in oak forests of Europe, demonstrated how the invasive red oak (Q. rubra) is nearly free of insect seed predators compared with a common native oak (Q. petrea) in Europe and, as a result, afforded significant dispersal and regeneration advantages over the native species. Urgoiti et al. (2018) explored the distribution of small-mammal communities and genetic isolation of two keystone seed dispersers in the Pyrenees Mountains of Europe. Their results hold important implications for how geographic variation in seed consumers influences native plant dispersal and how global warming will likely influence isolation of these keystone seed dispersers.

Working in NE Spain, Arias-Leclaire et al. (2018) examined how acorn phenology and sexual maturation of female seed weevils of four species (Curculio), as well as the trophic breadth of these seed predators and the distribution of their host trees (hazel [Corylus avellana] and two oak species [Q. ilex and Q. pubescens]), influenced the genetic structure of weevil populations. They find that one weevil species (C. nucum), in particular, that matures early and specializes on hazelnuts, exhibits recent genetic bottlenecks, as evidenced by rare local haplotypes likely to follow from the discontinuous stands of hazel trees in the region.

We then moved from the seed dispersal to pollination and to New Zealand, where Biddick and Burns (2018) explored the structure of a plant-bird pollination network. They demonstrated how the network interactions are largely defined by flower size and bill morphology and that the network is largely nested. They also described phenotypic trait matching as a reliable predictor of pollination networks. Finally, we capped the series with life history studies of two species of proovigenic parasitoid wasps (Anagrus spp.) that complete their entire development in the eggs of leafhoppers (Erythrocephalina spp. and Erasmosyneura spp.) and emerge with a full lifetime supply of mature eggs (Segoli et al. 2018). The authors sought to compare life history strategies of the parasitoids in two neighboring habitats: vineyards and riparian habitats where leafhopper hosts were abundant and rare, respectively and where parasitoids engage in different foraging strategies. Results suggest a more complex mechanism of energy allocation than predicted and one that appears independent of host size.

Although only a snapshot of some of the underlying ecological and evolutionary complexities of plant-animal interactions in a few systems, we believe these studies provide new perspectives and some novel approaches, and reveal complexities in these systems that have not previously been considered. We hope the reader agrees and is inspired to pursue research that follows from these studies.

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ORIGINAL ARTICLE

Invasive oaks escape pre-dispersal insect seed predation and trap enemies in their seeds

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Abstract

Species introduced to habitats outside their native range often escape control by their natural enemies. Besides competing with native species, an alien species might also affect the native herbivores by introducing a new source of different quality food. Here, we describe the case of northern red oak (Quercus rubra) invasion in Europe. We collected data on insect (moth Cydia spp. and weevil Curculio spp.) seed predation of northern red oak in its native (USA, North America) and invasive (Poland, Europe) range, as well as for sessile oaks (Quercus petrea) in Europe. We also evaluated the quality of acorns as hosts for weevil larvae by collecting infested acorns and measuring weevil developmental success, and quantifying acorn traits such as seed mass, tannins, lipids and protein concentration. We used DNA barcoding to identify insects to the species level. The predation by moths was similar and very low in both species and in both ranges. However, red oaks escape pre-dispersal seed predation by weevils in Europe. Weevil infestation rates of northern red oak acorns in their invasive range were 10 times lower than that of sessile oaks, and also 10 times lower than that of red oaks in North America. Furthermore, even when weevils oviposited into northern red oaks, the larvae failed to develop, suggesting that the exotic host created a trap for the insect. This phenomenon might gradually decrease the local abundance of the seed predator, and further aid the invasion.

Key words: biotic invasions, ecological trap, enemy release hypothesis, seed predation, weevils

INTRODUCTION

Species introduced to habitats outside their native range (i.e. alien species) frequently escape control by their natural enemies (Keane & Crawley 2002; Torchin et al. 2003; Liu & Stiling 2006). Capitalization on predation release often allows the survival rate, recruitment
and growth to be higher in the introduced than in the native range, which gives invaders an advantage over native competitors (Siemann & Rogers 2001; Wolfe 2002; Colautti et al. 2004; Muller-Scharer et al. 2004). These changes cause or contribute to the troublesome success of many alien species.

Furthermore, in some situations, natural enemies may not only fail to control the population of an invader but may suffer themselves in the interaction (Keeler & Chew 2008; Harvey et al. 2010; Yoon & Read 2016). For example, lepidopterans that oviposit on and consume host plants do not show strong preference towards native hosts as compared to exotics, despite lower performance on the latter (Yoon & Read 2016). In contrast, the presence of non-native prey may increase food availability, which ultimately translates into higher survival rates of the predator (Cattau et al. 2010; Brown et al. 2016; Cattau et al. 2016). Thus, depending on the quality of the exotic prey for the native enemy, it can reduce or boost the predator population (Pearse et al. 2013; Brown et al. 2016; Yoon & Read 2016). If the exotic species harms the populations of native predators or herbivores, it can further accelerate the invasion of the exotic species, as the natural enemy is gradually removed from the local habitat (Pearse et al. 2013). Yet there are still few studies that, beyond the existence of “predator release effects,” have provided an evaluation of the effects of the invasive plant on the local enemy (but see e.g. Keeler & Chew 2008; Harvey et al. 2010; Brown et al. 2016).

In this study, we describe a case of a northern red oak (Quercus rubra) invasion in Europe, in which the plant not only escapes seed predation but also may harm natural enemies by trapping them in its acorns. The northern red oak was introduced to European forests from North America in the 17th century as an ornamental species, and was first commercially planted at the end of the 18th century (Woziwoda et al. 2014b). Nowadays, it is among the most frequent foreign deciduous species in Central European forests (Woziwoda et al. 2014b). Northern red oaks are considered invasive and their abundance correlates negatively with abundance and richness of numerous native species (Chmura 2013; Woziwoda et al. 2014a). The invasion will likely further accelerate as northern red oaks have great recruitment potential in Central Europe: at some sites, their density of regeneration exceeds all other tree species combined (Major et al. 2013; Myczko et al. 2014). Dense seedling banks beneath the canopies suggest that low seed predation may contribute to the regeneration advantage of red oaks over other local oaks (Major et al. 2013). Nonetheless, whether northern red oaks escape seed predation by weevils in their invasive range is unknown (but see Myczko et al. 2017).

Weevil larvae (Coleoptera: Curculionidae) and moth caterpillars (Lepidoptera: Tortricidae) represent the most important groups of oak acorn predators, although weevils are responsible for the majority of seed loss (Crawley & Long 1995; Branco et al. 2002; Bonal et al. 2007; Espelta et al. 2009). Weevil pre-dispersal predation shapes recruitment potential of trees (Crawley & Long 1995; Espelta et al. 2009; Bogdziewicz et al. 2017), and severely destroys crops of northern red oaks in their native range (Lombardo & McCarthy 2008; Bogdziewicz et al. 2017). While less is known about moth feeding ecology, weevils are feeding specialists, with particular species best adapted to exploit particular hosts (Bonal et al. 2011; Govindan et al. 2012; Muñoz et al. 2014; Peguero et al. 2017). Concerning predation upon red and European white oaks, differences may arise because red oaks acorns have higher tannin concentration, as well as thicker shells than European white oaks (Shimada & Saitoh 2006; Steele 2008; but see Luczaj et al. 2014). In fact, differences in the communities of endosymbiotic bacteria associated with weevils are correlated with the use of different host plants (Toju & Fukatsu 2011; Mer ville et al. 2013). Therefore, because European weevils have a relatively short history of co-occurrence with red oaks, we could expect a lower adaptation to this alien host and, thus, reduced acorn predation rates compared to native white oaks. Moreover, European oaks belong to the white oaks (section Quercus), while northern red oaks belong to the section Lobatae. This has been observed in some previous studies to be a factor for the specialization of acorn weevils (Peguero et al. 2017; but see Bonal et al. 2016). Host specialization could, however, differ between native predators, thus conditioning their abilities to exploit alien plant species.

In this study, we evaluated insect pre-dispersal predation on northern red oak in North America and Europe as well as on sessile oak (Quercus petraea), which is the dominant native oak in Central European forests. We predicted that weevils will destroy a higher proportion of northern red oak acorns in North America than in Europe, and a higher proportion of sessile than northern red oak acorns in Europe. Based on the acorn quality data from past studies (Shimada & Saitoh 2006; Steele 2008), in particular on high tannin concentrations and thick shells of red oaks acorns, we predict that the red oak is a lower quality host for European weevils than is
the sessile oak, and this results in lower predation rates on the former species. To evaluate these predictions, we measured weevil prevalence in acorns of northern red oaks (in Europe and North America) and of sessile oaks. Moreover, we evaluated the quality of acorns as hosts for weevil larvae by collecting infested acorns and measuring weevil developmental success, and quantifying acorn traits such as seed mass and shell thickness, and tannins, lipids and protein concentration. Finally, we assessed whether the ability to exploit alien host seeds differed among pre-dispersal insect predators, using DNA barcoding to identify weevil larvae and moth caterpillars to the species level.

**MATERIALS AND METHODS**

**Study site**

The study was conducted over the course of 3 years in Europe (2014–2016) and North America (2013–2015). In Europe, we conducted research in Gorzowska Forest (52°47′1.71″N, 15°3′21.87″E), situated in western Poland at an altitude of 60–80 m. Gorzowska Forest is located in the temperate climate zone, with average annual precipitation of 523 mm and average annual temperature of 8 °C (Zwolak et al. 2016). For the study, we selected 2 sites in mixed sessile-northern red oak stands. In USA, 2 sites were established in northern red oak-dominated stands in Harvard Forest (42°31′53.28″N, 72°11′23.78″W), Massachusetts, which is located in the temperate climate zone, with average annual precipitation of 1100 mm and average annual temperature of 10°C. In both regions, the study sites were spaced over 1 km from each other.

**Field sampling and laboratory procedures**

Each year, we collected acorns under haphazardly chosen trees (i.e. first trees that we encountered once entering the stand that shed acorns): 13 red and 12 sessile oaks in Poland, and 30 northern red oaks in USA (half of that number of the trees per site, red oaks in Europe 6 and 7 trees per site). In Poland, we collected samples from the ground beneath each tree in 0.5 × 0.5-m squares placed 1 m from each tree trunk in the 4 cardinal directions, and in a 2-m-radius circle centered at each tree trunk in the USA. Acorns were collected at the beginning of October, bagged separately according to the tree of origin, transported to the laboratory and refrigerated (4 °C) until further processing. The phenology of acorn fall is similar in red and sessile oaks in Poland; therefore, bias due to any phenological difference is unlikely (Bogdziewicz et al., personal observation; see also Myczko et al. 2017). The number of acorns collected in the samples was used as an index of individual tree acorn production.

In the laboratory, we weighed, measured (width and length) and assessed insect infestation for individual acorns from each treatment (Poland: northern red oaks = 981 acorns, sessile oaks = 1203; USA: northern red oaks = 1219). Previous studies provided evidence for chemical gradients in acorns that promote consumption of the basal end of the acorns (i.e. avoidance of the apical end) by seed predators (Steele et al. 1993; Bogdziewicz et al. 2017). Therefore, we cut each acorn transversely into a top (basal) and bottom half (apical) and counted all insect larvae in each half (following earlier protocols; see Steele et al. 1993).

Another sample of acorns was collected to estimate the probability of successful weevil emergence, the total number of larvae per infested acorn, and the probability that the larvae predated an acorn embryo. This sample consisted only of acorns with oviposition scars but without emergence holes (i.e. acorns that still had all larvae inside). We collected the acorns from the soil at 20 haphazardly chosen northern red oaks in USA (in 2015), and 10 red and 10 sessile oaks in Poland (in 2016) at the same sites as described above (Poland: northern red oak = 237 acorns, sessile oak = 296; USA: northern red oak = 352). In the laboratory, we placed the acorns in separate open plastic vials. Each day we checked the acorns to register emerged larvae. Once all larvae had emerged (i.e. no emergence was recorded for 3 weeks), we dissected the acorns to further check whether the acorn embryo had been predated and whether there were non-emerged larvae inside (following earlier protocols [e.g. Bonal & Muñoz 2008; Muñoz et al. 2014]).

**DNA barcoding and larval identification**

Larval identification is necessary to establish unequivocal trophic relationships between insects and their host plants, and the lack of morphological keys makes the use of molecular techniques necessary. For DNA-based species identification we selected a random sample of larvae collected from northern red oak acorns (North America, N = 24, and Europe, N = 16) and from sessile oak acorns (Europe, N = 24). We did not pre-determine whether larvae were moths or beetles. DNA was from tissue and extracted following the salt extraction protocol (Aljanabi & Martínez 1997). In the case of weevil larvae, we sequenced a fragment (826 bp) of the mitochondrial cytochrome oxidase subunit 1 (cox1) us-
ing primers Pat and Jerry following methods described previously (Hughes & Vogler 2004a). We chose this fragment of cox1 due to the availability of many reference sequences of European and North American Curculio spp. to compare with (Hughes & Vogler 2004a). In the case of Cydia spp. caterpillars we amplified a different fragment (625 bp long) of the same mitochondrial gene (cytochrome oxidase I) using the universal primers pair LCO1490/HCO12198, common in DNA barcoding (see Folmer et al. 1994 for details on the primer sequences and PCR protocols). Sequencing was performed using Big-Dye (Perkin-Elmer) technology and an ABI3700 sequencer. Sequence chromatograms were assembled and edited using Sequencher 4.6 (Gene Codes, Ann Arbor, MI, USA).

Two alignments (one for weevil larvae and another for caterpillar moths) were created using CLUSTALW supplied through http://align.genome.jp, with default gap open and gap extension penalties. The alignment sets were collapsed into unique haplotypes and each were compared to the reference sequences available at GenBank (for weevil larvae) or the Barcoding of Life Datasystem (for moth caterpillars). We applied the most conservative limit used in DNA barcoding, which states a maximum genetic divergence of 1% with the reference sequence for an unambiguous identification to the species level (Ratnasingham & Herbert 2007). In the case of acorn weevils, we additionally built a neighbor joining gene tree based on Kimura 2 parameter model genetic distances (2KP, Kimura 1980). The objective was to further assess whether the haplotypes corresponding to the individuals classified as the same species clustered together forming discrete groups separated from the others by significant genetic discontinuities (branch lengths). This gene tree was built pooling together the haplotypes of the individuals sequenced at our study sites with reference sequences of the Holartic species of Curculio spp. available at GenBank from Hughes and Vogler (2004a). The gall-feeding Curculio pyrroceras was used as an outgroup.

### Lipid, tannin and protein concentration in acorns

Previous studies have provided evidence for chemical gradients in acorns that promote consumption of the basal end of the acorns (i.e. avoidance of the apical end) by seed predators (Steele et al. 1993). Therefore, we cut each acorn transversely into a top (basal) and bottom half (apical), and analyzed the halves separately. We used only non-infested acorns, and measured the concentration of each compound based on 10 replicates per oak species. Each sample consisted of 5–6g of chopped cotyledons originated from different trees. The detailed description of procedures is presented in the Supplementary Materials.

### Data analysis

We analyzed data in R using the generalized linear mixed models (GLMMs) implemented via lme4 package (Bates et al. 2015). Each below-described model was run separately for European and North American data. For the European comparison, we did not include seed mass as a covariate because acorn mass and species were strongly confounded: northern red oak acorns were on average twice heavier than sessile oak acorns (Table 1). We estimated the proportion of acorns infested with weevil and moth larvae, as well as the overall infestation rate, each with a separate model. We built

### Table 1 Traits of northern red oak (in Europe and North America) and sessile oak acorns

<table>
<thead>
<tr>
<th>Trait</th>
<th>Sessile oak</th>
<th>Red oak (Europe)</th>
<th>Red oak (North America)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Apical</td>
<td>Basal</td>
<td>Apical</td>
</tr>
<tr>
<td>Tannins</td>
<td>3.59 (1.05)</td>
<td>2.65 (0.85)</td>
<td>3.75 (0.81)</td>
</tr>
<tr>
<td>Lipids</td>
<td>5.14 (0.39)</td>
<td>5.60 (0.73)</td>
<td>14.10 (1.46)</td>
</tr>
<tr>
<td>Proteins</td>
<td>0.36 (0.02)</td>
<td>0.28 (0.02)</td>
<td>0.49 (0.03)</td>
</tr>
<tr>
<td>Mass (g)</td>
<td>1.26 (0.91)</td>
<td>2.85 (1.19)</td>
<td>4.81 (1.48)</td>
</tr>
<tr>
<td>Shell thickness (mm)</td>
<td>0.97 (0.64)</td>
<td>1.71 (0.60)</td>
<td>1.49 (0.54)</td>
</tr>
</tbody>
</table>

\(^1\)Data on tannin concentration in northern red oak acorns in North America following Shimada and Saitoh (2006), measured for the whole acorn. Tannins and lipids are expressed as a percentage of acorn dry mass, and proteins in grams per dry mass. Values in parentheses represent standard deviations from the mean. Proteins were only measured in the European sample.
a binomial family, logit link GLMMs (1 per continent) with tree ID as a random effect. Fixed effects included year, crop size index, oak species and oak species by crop size interaction term. Species and interaction terms were only included in the “European” model. We used the total number of acorns collected under each tree as an index of individual crop size.

We estimated the probability of successful emergence from the infested acorn with binomial family, logit link GLMM, with tree ID included as random effect. In this analysis, the response variable was binomial with failure (0), indicating an oviposition mark on the acorn but no larvae emergence, and success (1) indicated acorn with both an oviposition mark and emerged larvae. We used oak species as the fixed effect, again, only in the “European” model. We also tested for differences in probability of acorn embryo survival in weevil-infested acorns using binomial family, logit link GLMM, with tree ID as random effect. Species was included as a fixed effect in “European” models, while “American” models were fitted with the intercept only.

RESULTS

In the models fitted to European data, the estimated overall proportion of infested acorns was higher in sessile (33–67%, depending on the year) than in northern red oaks (3–13%, \( \chi^2 = 254.53, \text{df} = 1, P < 0.001; \) Fig. 1). The difference was driven by the decrease in weevil infestation in red oak (3–10%) as compared to sessile oak (31–64%, \( \chi^2 = 256.71, \text{df} = 1, P < 0.001 \)). Infestation by Cydia sp. did not differ between species (\( \chi^2 = 0.03, \text{df} = 1, P = 0.86 \)), and ranged from 1% to 5% depending on the year. In the “North American” model, the estimated overall proportion of infested northern red oak acorns ranged from 35% to 62% depending on the year (Fig. 1). Weevil infestation ranged from 37% to 48%, while moth infestation ranged from 2% to 12%. In all models, infestation rates also differed significantly among years (\( P < 0.001; \) Fig. 1), but individual tree acorn production did not have an effect (\( P < 0.40 \)).

In Europe, the probability of successful weevil larvae emergence from an infested acorn was over 20 times higher in sessile oaks than in northern red oaks (approximately 40% vs 2%, \( z = 6.13, P < 0.001 \); Fig. 2). In contrast, the probability of emergence of weevils from northern red oak acorns in USA was similar to the probability of emergence of weevils from sessile oak acorns in Europe (Fig. 2).

When the acorns infested by any type of insect (either weevil or moth caterpillar) were pooled, the probability of embryo survival after infestation also differed among oak species. In Europe, this probability was lower in sessile than in northern red oaks (65% vs 90%, \( z = -4.40, P < 0.001 \); Fig. 3), and was the lowest (50%) in northern red oaks in North America.
Using DNA barcoding we successfully identified 21 out of 24 larvae collected from red oaks in North America, 16 out of 16 collected from red oaks in Europe, and 17 out of 24 from sessile oaks. We identified 2 acorn weevil species in North America: *Curculio proboscideus* (12 sequences, 7 haplotypes) and *Curculio nasicus* (4 sequences, 2 haplotypes), all of them feeding on northern red oak acorns. In Europe we identified only 1; namely, *Curculio venosus* (8 sequences, 2 haplotypes) and weevil larvae were found both in red and white oaks. The comparison with reference sequences retrieved a divergence below 1% in all cases. Moreover, the neighbor joining tree showed that the distinct haplotypes clustered with their respective reference sequences, being separated from the rest of the species in the phylogeny by longer branches (Fig. 3). Although not identified in our study, the other potential species present at our site are *Curculio glandium*, *Curculio pellitus* and *Curculio elephas* (Burakowski et al. 1995). In the case of moth caterpillars, 2 species were identified in Europe: *Cydia amplana* (6 sequences, 1 haplotype) and *Cydia splendana* (4 sequences, 2 haplotypes); both species were collected from northern red oak and sessile oak acorns.

The concentration of tannins was similar in northern red and sessile oaks in Europe (Table 1), although red oak acorns in Europe had almost 2 times lower tannin concentration than that reported for North America populations. Lipid concentration was almost 3 times higher in northern red than in sessile oaks in Europe. Protein concentration was also higher in northern red than in sessile oaks (Table 1). The mass of northern red oak acorns in Europe was almost twice lower than in their native range. Nonetheless, acorns of northern red oaks in Europe were almost twice heavier than those of sessile oaks. Acorn shell was thicker in northern red oaks than in sessile oaks (Table 1).

**DISCUSSION**

Northern red oaks escape pre-dispersal seed predation by weevils in Europe. The predation by moths was similar in both species and in both ranges, but the proportion of seeds infested by *Cydia* sp. was low compared to...
to that predated by weevils (see Myczko et al. 2017 for more detailed analyses of *Cydia* infestation in northern red and sessile oaks in Poland). The lower prevalence of *Cydia* spp. compared to weevils is common in temperate oak forests (see e.g. Bonal et al. [2007] for a similar result). The weevil infestation rates of northern red oak acorns in their invasive range were 10 times lower than that of sessile oaks, and also 10 times lower than that of red oaks in North America. Plant species with large seeds, like oaks, are particularly likely to be seed-limited (Moles & Westoby 2002; Clark et al. 2007). Thus, the drastically reduced pre-dispersal seed predation of northern red oaks in their exotic range is likely to favor their recruitment and accelerate their invasion in European forests. Furthermore, even when weevils, the main pre-dispersal insect predators of oaks, oviposit into northern red oaks, the larvae fail to develop. Thus, the exotic host apparently creates a “trap” for the insect (Schlaepfer et al. 2002).

The enemy release hypothesis (ERH) makes 3 predictions about the effects of natural enemies on alien plants (Keane & Crawley 2002), and even though our study was not designed to test them, several lines of evidence suggest that the escape from seed predation in the exotic range may aid the spread of northern red oaks. First, ERH assumes that enemies are important regulators of plant populations. Although we have not determined whether oaks at our sites are seed-limited, seed predation commonly limits recruitment in oaks (Crawley & Long 1995; Haas & Heske 2005; Espelta et al. 2009; Bogdziwicz et al. 2017). Second, according to the ERH, enemies should have greater impact on native than on alien plants. Our results show that the magnitude of pre-dispersal seed predation by weevils is almost 10 times higher on sessile oaks than on northern red oaks, and a similar study by Myczko et al. (2017) reported even greater differences. Assuming that these populations are seed-limited, we can tentatively conclude that the impact of weevil predation on populations of sessile oaks is greater than on northern red oaks. Finally, according to the ERH, alien species should be able to capitalize on the enemy release and increase in abundance (Keane & Crawley 2002). Northern red oaks have a large recruitment advantage over native oaks in Central Europe (Major et al. 2013; Myczko et al. 2014) but the mechanism is unknown. Our results suggest that the release from pre-dispersal seed predation might be an important driver of the difference in recruitment success between these species.

Northern red oaks not only escape seed predation by weevils but also seem to effectively trap insects in their acorns. In Europe, the probability of successful larvae emergence from a northern red oak acorn with an oviposition scar was lower than 3%, compared to 48% in red oaks in North America and over 40% in sessile oaks. This low success could be the result of larval development failure due to the lack of trophic adaptation to feed on the alien host tree. Recent studies have stressed the importance of endosymbiotic bacteria associated with acorn weevils in the adaptation to different trophic sources. The community of these bacteria not only changes among weevil species but also within species between populations feeding on different host plants (Toju & Fukatsu 2011; Merville et al. 2013). The species feeding on red oak acorns in North America (*C. proboiscideus* and *C. nasica*) were different from that recorded in Europe (*C. venosus*). In fact, there is not a single acorn weevil species found in both continents (Hughes & Vogler 2004b). Thus, along its evolutionary history, European species like *C. venosus* have not faced any red oak species, as red oaks (section Lobatae) are exclusive to America. This might have prevented associations with endosymbiotic bacteria that would allow insects to feed on seeds with a different chemical composition.

One caveat to the argument of the lack of trophic adaptation in acorn weevils is that it is not possible to distinguish whether females laid eggs but the larval development stopped in the early stages, or that females drilled an oviposition hole but failed to lay eggs. Which scenario is more likely? On one hand, red oaks have a shell that is twice the thickness of that of sessile oaks, and the weevil rostrum size correlates positively with the size of the acorn host (Hughes & Vogler 2004b). Therefore, European weevils may be less successful at drilling oviposition holes in red oak acorns. In such a scenario, the oviposition scars we observed on red oak acorns may not always result in egg laying. However, recent studies suggest that the rostrum length of weevils is not critical for exploiting seeds (Bonal et al. 2011). Furthermore, *C. venosus* lays eggs very early in the season, before the acorn shell is fully developed (Venner et al. 2011; Pélisson et al. 2013).

The alternative scenario is that female weevils fail to perceive northern red oak acorns as lethal hosts for their larvae. Weevils generally seem to concentrate ovipositioning in seeds of higher quality, although the host-selection process is not clear (Desouhant 1998; Espelta et al. 2009; Bonal et al. 2011; Muñoz et al. 2014). In our population, northern red oaks have similar tannin con-
centration to sessile oaks (although the level of non-tannin phenolics is much higher in red than in sessile oaks in Poland: Łuczaj et al. 2014), but much higher lipid and protein concentration. Therefore, a mismatch between perceived and real quality of the host seems to take place, suggesting an ecological trap (Schlaepfer et al. 2002). If true, northern red oaks will function as sink habitats for weevils across the landscape (Govindan et al. 2012). The process would slowly result in gradual removal of insects from local habitats, which will further decrease predation and accelerate northern red oak invasion. As a caveat, we have not measured the preference of weevils to oviposit in red versus sessile oaks in Europe. Thus, further studies are required to test whether red oaks create true ecological traps for weevils (i.e. are preferred and are lethal hosts; see Schlaepfer et al. 2002) or are just lethal hosts that are, nevertheless, exploited.

To conclude, our study shows that northern red oaks almost completely avoid pre-dispersal seed predation in their exotic range in contrast to their native range, and in contrast to sessile oaks. Furthermore, European weevils oviposit in the red oaks, but the probability of larval emergence from acorns with oviposition scars is less than 3%. Moreover, even infested northern red oak acorns have a 90% chance that their embryo survives, and that these acorns will germinate and produce viable seedlings (Bonial et al. 2007; Xiao et al. 2007; Muñoz et al. 2014). We suggest that the mechanisms described in our study might contribute to the invasion of the northern red oak across Central European forests. The next important step towards testing this hypothesis would be to confirm the demographic impacts of reduced pre-dispersal seed predation in the northern red oak.

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**SUPPLEMENTARY MATERIALS**

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

S1 Lipid tannin, and protein concentration in acorns

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Cite this article as:

Seed loss before seed predation: experimental evidence of the negative effects of leaf feeding insects on acorn production

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Abstract

Insect herbivory decreases plant fitness by constraining plant growth, survival and reproductive output. Most studies on the effects of herbivory in trees rely on correlational inter-individual comparisons and could, thus, be affected by confounding factors linked to both herbivory and plant performance. Using the Mediterranean Holm oak (Quercus ilex) as a study model, we followed an experimental approach in which leaf-feeding insects (mainly Lepidoptera caterpillars) were excluded from some shoots in all study trees. Shoots subjected to herbivore exclusion exhibited lower defoliation rates and produced more acorns than control shoots. Defoliation constrained shoot growth throughout the study period, but had no effect on the number of female flowers produced per shoot. Acorn production was, however, lower in control shoots due to their higher abortion rates, and also to their greater mortality risk during summer drought, as shoots with fewer leaves were less likely to survive. Plant reaction to herbivory inhibits certain physiological pathways involved in plant growth, which, together with the effects of physical damage, reduces the amount and efficiency of the photosynthetic tissue. This increases their vulnerability to environmental stresses, such as water deficit, which limit resource assimilation. Defoliation is likely a key factor affecting oak regeneration, as it may be a significant source of seed loss prior to pre-dispersal acorn predation. Further experimental studies could help to elucidate its effects in contrasting environments. In Mediterranean regions, the harsher droughts predicted by climate change models could worsen the effects of insect herbivory on oak reproductive output.

Key words: drought stress, insect defoliation, oak, seed abortion, shoot mortality

INTRODUCTION

Herbivory constitutes one of the main antagonistic interactions between plants and insects, being the trigger of numerous coevolutionary processes (see review in Strauss & Zangerl 2002). The loss of photosynthetic tissue reduces plant productivity (Nabity et al. 2009), constraining their growth and threatening their survival (Rausher & Feeny 1980; Davidson et al. 1999; Piper...
& Fajardo 2014; Camarero et al. 2015). Herbivory may also reduce plant fecundity directly or indirectly. Direct effects include the consumption of flowers by herbivorous insects (review in Crawley 1989), whereas indirect effects result from the lower number of flowers produced due to the reduction of shoot growth (Samson & Werk 1986). In addition, seed set and growth is costly, and a decrease of available resources increases seed abortion rates (Rausher & Feeny 1980; Marquis 1992; Niesenbaum 1996; Espelta et al. 2008; Sánchez-Humanes & Espelta 2011). The negative effects of defoliation on plant reproductive output may be extended in time and persist for many years (Morrow & LaMarche 1978; McConnel 1988).

Most studies on the effects of insect herbivory on plant fitness have followed a correlational approach, especially in the case of woody species (Landsber & Ohmart 1989; Simmons et al. 2014; Camarero et al. 2015). Those including manipulation (i.e. removal or reduction of herbivory) have been mostly carried out on herbaceous plants (but see Crawley 1985) due to the ease of performing such experimental treatments (Hawkes & Sullivan 2001; see review in Massad 2013). However, correlational studies may sometimes mislead our conclusions when other factors related to herbivory and acorn production are involved. For example, recent reports have shown that, within the same population, genetic diversity and relatedness between trees affect caterpillar herbivory rates in oaks (see Fernandez-Conradi et al. 2017) and these factors might, also, be related to plant reproductive output.

Oaks *Quercus spp.* are among the most prevalent tree species in the temperate forests of the northern hemisphere and may suffer severe defoliations (Schultz & Baldwin 1982; Davidson et al. 1999), especially with the periodic outbreaks of the gypsy moth (*Lymantria dispar* Linnaeus, 1758) among the most dramatic ever recorded in forest ecosystems (Gottschalk et al. 1998; Liebhold et al. 2000; Asaro & Chamberlin 2015). Defoliation, however, has not usually been included among the main factors impacting the decrease of natural regeneration, which is one of the main problems of oak forests worldwide. Rather, studies on recruitment failure have focused their attention on other issues, such as pre-dispersal and post-dispersal acorn predation, seed dispersal or the lack of favorable microhabitats for seedling establishment (e.g. Callaway 1992; Gómez 2004; Bonal et al. 2007; Zhang et al. 2008; Bonal & Muñoz 2009; Pulido et al. 2010; Xia et al. 2016).

Pre-dispersal seed predation reduces the number of sound seeds available for recruitment. In some oak species the proportion of acorns infested by insects averages 50% (Bonal et al. 2007), but in certain trees and years the losses may be much higher (Bonal & Muñoz 2007; Espelta et al. 2008; Xia et al. 2016). Post-dispersal acorn consumption may also be noteworthy and reduce the number of seed seeds available for dispersal (Bonal & Muñoz 2007; Zhang et al. 2008; González-Rodríguez & Villar 2012). Nonetheless, and in spite of the relevance of all these factors and bottlenecks along the regeneration cycle, constraints on acorn production may start before pre-dispersal predation.

Some factors related with early acorn losses are abiotic, especially those related with pollination failure (Pearse et al. 2016). In addition, in areas with severe droughts, like the evergreen Mediterranean forests, seed abortion during the summer may provoke sharp decreases in the number of acorns produced (Espelta et al. 2008). At this early stage, insect defoliation can also reduce the size of the acorn crop significantly. Different studies have shown strong decreases after massive defoliation events (Davidson et al. 1999; Nakajima 2015). Nonetheless, reports on the effects of the average defoliation rates are less common and mostly correlational. Moreover, the few experimental studies that have approached this subject on individual trees have quantified the effects once the acorns have matured (e.g. Crawley 1985). They have analyzed neither the stage of flower or seed development at which defoliation has a stronger effect, nor the consequences of the interactions between defoliation and the environmental conditions on acorn production.

We carried out an experimental study to determine the effects of insect defoliation on acorn production using the Holm oak (*Quercus ilex*) as a model. The Holm oak is the most widespread tree in the Mediterranean forests of the Iberian Peninsula, where it inhabits areas with strong summer droughts and suffers defoliation mainly by moth caterpillars (*Lepidoptera*) (Ruiz-Carbajo et al. 2016). Contrary to previous studies with other oak species, in which defoliators were excluded from certain trees and compared with control ones (Crawley 1985), we randomly excluded caterpillars from a number of branches of all trees and compared them with control branches in the same oaks, thus controlling for potential individual effects. Moreover, we followed the effects of defoliation along the whole season: from early shoot elongation to the final stage of acorn growth after summer drought. In addition, analyzing whether the effects of defoliation differed through the season, we ap-
plied 2 different exclusion treatments: in some branches caterpillars were excluded from early new shoot elongation and in others, exclusion experiments were carried out 1 month after the caterpillars had started feeding.

Our concrete objectives were: (i) to analyze whether the exclusion of caterpillars increased the final acorn production; (ii) to assess whether defoliation reduces oak growth, shoot length and/or the number of leaves per shoot; and (iii) to investigate the mechanisms by which defoliation may reduce acorn production, analyzing the effect of shoot length and the number of leaves on female flower production and acorn set success.

MATERIALS AND METHODS

Study area

The study was performed at a Holm oak Q. ilex L. savannah located in Malpartida de Plasencia, in the province of Cáceres, western Spain (39°56′N, 5°58′O) between April and September 2016. Oak savannahs (so called dehesas in Spain) are agroecosystems resulting from the human use of Mediterranean forests over centuries. Tree clearing has produced landscapes with oaks interspersed within a grassland matrix, in which livestock raising is the main use of land (Moreno & Pulido 2009). Our study was carried out in a dehesa of 50 ha with a mean density of 14 trees/ha.

Study species

The broadleaved evergreen Holm oak (Q. ilex L.) spreads over the western Mediterranean basin, being the most widely distributed tree species in the Iberian Peninsula (Blanco et al. 1997). It inhabits a wide range of habitats but predominates in dry Mediterranean regions with strong summer droughts, which will presumably suffer the consequences of global warming more severely (Petit et al. 2005). In these areas, July and August are the warmest months, with temperatures that can reach 40°C and almost no precipitation except for some storms later in the season with an average rainfall of just 6 mm in July and 7 mm in August (AEMET, Spanish Meteorological Agency).

Holm oak acorns mature in 1 year and budburst takes place in early April. Flowering occurs in April–May and acorns grow throughout the summer until early autumn. Summer drought, with its extreme temperatures and water deficit, constitutes the main cause of premature acorn abortion (Espelta et al. 2008).

Fresh new oak shoots are defoliated by a number of insect species, mainly moth caterpillars (Lepidoptera) (Ruiz-Carbayo et al. 2016). According to a previous sampling at our study site the more abundant species feeding on Q. ilex are: Catocala nymphagoga (Esper, 1787), Dryobota labecula (Esper, 1788), Dryobotodes eremita (Fabricius, 1775), Dryobotodes monochroma (Esper, 1790), Phycita torrenti (Agenjo, 1972), Tortricodes alternella (Denis & Schiffermüller, 1775) and Tortrix viridana (Linnaeus, 1783) (unpublished results). Caterpillars start feeding from early budburst in early April and continue throughout the spring until early June, when the sclerified leaves become too tough to be eaten (Elkinton et al. 1996). This phenology is, however, subjected to variability due to the stochasticity of the Mediterranean climate (Gilioli et al. 2013). The species composition of caterpillar guilds also changes during the spring, with the family Tortricidae the first to feed on the incipient new shoots and the Geometridae species the last (Soria 1987). Their reproductive strategies also differ and go from short-cycle species, which oviposit before summer, to long-cycle ones that overwinter as pupae. Caterpillar damage in the Holm oak also exhibits interannual variability and severe defoliations, especially by Tortrix viridana L. (Lepidoptera: Tortricidae) (Schroeder & Degen 2008).

Experimental design and sampling methodology

Fifteen adult Holm oaks of similar size were selected for the experiment, excluding those with evident symptoms of decay. At each tree, 12 branches were assigned randomly to 4 different categories: Control (C, no treatment), Treatment 1 (T1, caterpillars excluded from the onset of new shoot elongation onwards), Treatment 2 (T2, excluded 1 month after caterpillars started feeding) and Negative Control (NC, treated with water). At each tree, 1 branch was assigned randomly to 1 treatment and, starting from it, the rest were allocated to the other treatments following a clockwise design (see Fig. 1a).

Ten shoots per branch were arbitrarily selected on 1 April (a total of 1800 shoots initially marked including all trees: 15 trees, ×12 branches/tree, ×10 shoots per branch). All shoots were individually identified by surrounding their base with a soft wire with a numbered label. The soft wire was adjusted just below the axillary bud, and was wide enough not to damage the shoot. Caterpillars were excluded by spraying insecticide dissolved in water (cypermethrin 0.005% vol.) over the treated branches 2 times per week. When it was raining, the spraying was repeated the day after the rain stopped. Shoots subjected to Treatment 1 (T1) were sprayed from...
the onset of shoot elongation (1 April) to 1 June. In the case of Treatment 2 (T2), spraying started 1 month later (1 May) and ended also on 1 June. In the Control group (C) shoots were not treated at all and suffered natural defoliation levels throughout the study period. We also established a Negative-Control (NC) in which branches were sprayed from the beginning just with regular water containing no insecticide at all (see Fig. 1b).

Cypermethrin is a pyrethroid insecticide ($\text{C}_{22}\text{H}_{19}\text{Cl}_{2}\text{NO}_3$) with an environmental persistence lower than 90 days. The European Commission and the United States Environmental Protection Agency (EPA) allow its use on plants (Pesticide Properties Database: http://sitem.herts.ac.uk/aeru/footprint/index2.htm). Its bioaccumulation has been detected on aquatic animals such as freshwater fish when used in high concentrations (Corcellas et al. 2015) but there were no rivers in our study area. This insecticide can be extensively used in croplands (Al-Sarar et al. 2006; Varikou et al. 2015) and is also recommended for gardening and horticulture, thus having no adverse effect on plant growth or productivity (what excludes a potential bias of insecticide application on our results). We used the dose provided by the commercial product Cythrin spray (0.005% vol.) approved by the Spanish Ministry of Agriculture (license number 25581). This product is aimed at phytophagous insects and our results showed that that concentration was, indeed, enough to reduce defoliation rates.

Control, T1 and T2 shoots were monitored 3 times: on 1 May, 1 June and 5 September. The Negative-Control shoots were measured at the end of the experiment on 5 September, to assess whether the application of the experimental treatment (water spraying) might have had any independent effect on the variables of interest, and so rule out any potential confounding effects (Fig. 1). At each revision we recorded whether the shoot was still present or it had been prematurely abscised, registered shoot length to the nearest millimeter and the number of leaves. The number of female flowers was recorded in June to compare it with the number of grown acorns in September, just after the summer drought. In September, we harvested all shoots and took them to the laboratory to record their length, the number of leaves and the number of acorns produced. We also calculated the final defoliation rates by dividing at each shoot the number of leaves with signs of herbivory by the total number of leaves.

**Statistical analyses**

We used the Tukey test of additivity to assess whether the experimental design adjusted to a complete randomized block, and so the effects of the experimental treatments on defoliation rates and acorn production per shoot in September did not differ among trees. The dif-

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**Figure 1** Experimental design. (a) Scheme depicting the distribution of all the experimental treatments and controls at the canopy of each tree (T1, T2, C and CN) corresponding to Treatment 1, Treatment 2, Control and Negative Control, respectively. (b) Chronology of the experimental treatments: filled black dots show the timing of insecticide spraying in T1 and T2. Empty triangles show the duration of the water spraying (no insecticide) on the Negative Controls. Control shoots received no treatment throughout the study period.
ferences between treatments (C, T1, T2 and NC) were analyzed using an ANOVA to assess the differences in defoliation rates and in the number of acorns produced. Post-hoc analyses for pairwise treatment comparisons were performed with Tukey tests.

We analyzed whether the temporal changes in shoot length differed between treatments using a linear mixed model (LMM) in which shoot identity was included as a random factor. For the analysis on the number of leaves per shoot, a generalized linear mixed model (GLMM) with a Poisson distribution of errors was conducted, including shoot length as a continuous covariate and shoot identity as a random factor. Pairwise post-hoc Tukey tests were performed in both cases (shoot length and number of leaves) to assess differences between treatments at different times of the study period.

The differences between treatments (C, T1 and T2) in the number of female flowers in June were evaluated using an ANOVA. To test whether acorn set success differed between treatments (C, T1 and T2) we used 2 GLMMs with a binomial distribution of errors and shoot identity as a random factor. In the first test, we compared the number of female flowers in June versus the number of grown acorns in September in all shoots that had flowers in June. In those that were not present in September (shoots prematurely abscised during the summer) acorn set success was zero. In the second test, we did the same but only including those shoots that had flowers in June and were alive in September. Doing this it is possible to estimate the relative impact of shoot mortality during the summer drought on acorn set success.

Shoot survival throughout the study period was analyzed using a Kaplan–Meier analysis. In addition, we investigated the factors affecting shoot mortality during summer drought with a generalized linear model (GLM). The dependent variable was shoot mortality risk (binomial distribution: variable takes the value 1 when the shoot died and zero when it did not); independent variables included the shoot length, the number of leaves and the number of flowers.

All data analyses were performed with the statistical analysis program R Core Team (2016) and using the following libraries: rJava (Urbanek 2016), xlsxjars (Dragulescu 2014a), xlsx (Dragulescu 2014b), asbio (Aho 2016), multcomp (Hothorn et al. 2008), nlme (Pinheiro et al. 2017), lme4 (Bates et al. 2015), Matrix (Bates & Maechler 2016), survival (Therneau 2015) and survMisc (Dardis 2016).

RESULTS

The Tukey test of additivity showed that the effect of the experimental treatments (C, T1, T2 and CN) did not differ between trees (block factor) either in the defoliation rates ($F = 0.029; \text{df} = 1461; P = 0.87$), nor in the number of acorns produced ($F = 0.122; \text{df} = 1461; P = 0.72$). We could, thus, perform an ANOVA to assess the effect of the experimental treatment on both defoliation rates and the number of leaves per shoot. Caterpillar exclusion (i.e. insecticide spraying) reduced the percentage of leaves defoliated (ANOVA $F_{3,146} = 47.595; P < 0.001$; Fig 2a). In both exclusion treatments (T1 and T2) the final defoliation rates recorded in September were significantly lower than in C shoots (T1 estimate: $−16.098; t = −7.697; P < 0.001$ and T2 estimate: $−10.879; t = −5.178; P = 0.001$) (Fig. 2a). There were

![Figure 2 Bar plot showing (a) the defoliation rates (number of leaves with signs of herbivory/total number of leaves; mean ± SE) and (b) the number of acorns per shoot (mean ± SE) measured at oak shoots subjected to all the different treatments and controls (T1, T2, C and NC) at the end of the study period (September). Different letters on the bars indicate statistically significant differences between groups ($P < 0.05$). The asterisks show the groups with the same letters among which the differences are marginally significant ($0.10 > P > 0.05$).]
not significant differences between C and NC, whereas defoliation rates were lower (marginally significant) in T1 compared to T2 shoots (in the latter caterpillars were excluded 1 month later) (estimate: 5.219; \( t = 2.575; P = 0.05 \)) (Fig. 2a). Defoliation decreased acorn production, as there were differences between treatments (ANOVA \( F_{3,1462} = 5.106; P = 0.002 \)) (Fig. 2b). The number of acorns in September was higher in shoots subjected to T1 than either in the C (estimate = 0.065; \( t = 2.689; P = 0.036 \)) or the NC shoots (estimate = 0.072; \( t = 3.156; P = 0.009 \)). The rest of the pairwise comparisons were not significant (\( P > 0.05 \) in all cases) (Fig. 2b).

Caterpillar defoliation constrained shoot development. The LMM showed a significant interaction between time and treatments in shoot length (\( F_{4,2333} = 9.467; P < 0.001 \); Fig. 3a) as T1 shoots grew more than the others throughout the study period. In June only a marginal significant result (comparison between T1 and C) was found (estimate = 5.726; \( Z = 2.804; P = 0.092 \)), but in September, T1 shoots were significantly longer than C shoots (estimate = 7.325; \( Z = 3.426; P = 0.014 \); Fig. 3a). Differences in the number of leaves per shoot were found between treatments as well. Longer shoots had more leaves (estimate = 0.0086; \( Z = 32.64; P < 0.001 \)) but, irrespective of their length, the number of leaves was higher in T1 compared with the C shoots in June (estimate = 0.1625; \( Z = 4.919; P < 0.001 \); Fig. 3b). The number of leaves was also higher in T2 compared to C shoots, but the result was only marginally significant (estimate = 0.10; \( Z = 2.979; P = 0.067 \)). In September the gap between treatments decreased, as even the differences between C and T1 did not reach significance (estimate = 0.097; \( Z = 3.008; P = 0.062 \); Fig. 3b).

Caterpillar feeding had no effect on the number of female flowers in June, which did not differ between treatments.
treatments (C, T1 and T2) \( (\text{ANOVA } F_{2,1273} = 2.107; P = 0.122) \). The number of acorns in September, however, did vary between them (Fig. 2b). The first GLMM including all the shoots bearing female flowers in June \( (n = 137) \) and setting to zero the number of acorns in those shoots that died and were abscised during the summer drought (i.e. not present in September), showed that acorn set success was significantly higher in T1 than in C (estimate = 1.380; \( Z = 2.478; P = 0.013 \); Table 1a). Acorn set rates were also higher in T2 compared to C, although the result was marginally significant (estimate = 1.055; \( Z = 1.945; P = 0.052 \)). To figure out the effect on acorn abortion, a second GLMM test was analyzed only with those shoots that had flowers in June and had survived the dry season \( (n = 112) \). In this case, the differences between treatments decreased (Table 1a); only the comparison between T1 and C was significant (estimate = 1.095; \( Z = 1.990; P = 0.045 \); Table 1b).

Shoot survival probability changed throughout the study period (Fig. 4). The Kaplan–Meier analyses showed that summer survival (from June to September) decreased less in those shoots sprayed with insecticide (T1 and T2) compared to C shoots subjected to natural defoliation rates \( (\chi^2 = 26.1; \text{df} = 2; P < 0.001; \text{Fig. 4}) \). A further general lineal model of the causes underpinning this higher mortality risk showed that those shoots with a lower number of leaves in June had a higher chance of dying (i.e. being prematurely abscised) during the summer drought period (Fig. 5) (estimate = -0.06; \( Z = -2.875; P = 0.004 \)), whereas neither shoot length (estimate = 0.004; \( Z = 1.259; P = 0.208 \)) nor the number of flowers (estimate = -0.015; \( Z = -0.128; P = 0.898 \)) had any significant effect.

### DISCUSSION

Defoliation significantly reduces acorn production in the Holm oak \( Q. ilex \); that is, experimental shoots sprayed with insecticide exhibited lower defoliation rates and produced more acorns. Defoliation decreased shoot growth throughout the study period, but the number of female flowers at the end of the spring did not differ between treatments. However, acorn abortion rates and premature shoot abscission during the summer drought were higher in the smaller and heavily defoliat-
ed control shoots than in the experimental shoots, which provoked a sharp reduction of the acorn crop.

With respect to the few previous experimental approaches that have used trees as study models to assess the effects of defoliation on seed production (e.g. Crawley 1985), ours comprises a number of novelties. We included a negative control group in the analyses, which ruled out any effect of the experimental procedure itself (i.e. liquid spraying) on the variables of interest, thus confirming the negative relationship of caterpillar defoliation with shoot length, number of leaves and acorn production. In addition, we monitored the effects of defoliation throughout the study period, which evidenced the progressive negative effects on shoot growth. More importantly, this temporal monitoring showed the impact of summer drought on shoot premature abscission (i.e. mortality). To the best of our knowledge, this negative effect had gone previously unnoticed in oaks, because measurements in previous studies of acorn production were made just once in autumn comparing control and treated trees (e.g. Crawley 1985). That sort of approach attributes acorn losses from defoliation just to seed abortion, while this work has shown that they are the consequence of both seed abortion and summer shoot abscission (i.e. mortality).

There are a number of mechanisms involved in plant responses to defoliation that may be underpinning the decreased shoot growth observed in our study. Plants are able to recognize herbivory damage thanks to fine-tuned sensors of the elicitors of the saliva (Kessler & Baldwin 2002; Darwish et al. 2008). The reaction may start even before the damage occurs, as oviposition can also be detected (Balbyshev & Lorenzen 1997; Doss et al. 2000; Meiners & Hilker 2000; Meiners et al. 2000). When non-lethal herbivory stress occurs, compounds like reactive oxygen species (ROS) trigger plant responses by modifying the transport of phytohormones and/or plant metabolites (Potters et al. 2007).

The physiological response to insect damage may reduce plant growth. Insect defoliation activates the synthesis of jasmonates (Filella et al. 2006; Nably et al. 2009; Erb et al. 2012), which interact with the pathways of other compounds such as abscisic acid, cytokinins, brassinosteroids, gibberellins and auxins (Erb 2012). Auxins (indole-3-acetic acid, IAA) deserve special attention, due to their importance for plant growth and development (Geldner et al. 2003; Teale et al. 2006). Jasmonates reduce auxin activity, which, in tum, decreases photosynthetic PSII efficiency and CO₂ fixation rates (Agtuca et al. 2014); in angiosperms auxin inhibition may also reduce vessel size (Uggla et al. 1996). Our results suggest, however, that the physiological pathways that constrain plant growth may be reverted if defoliation disappears or decreases its intensity. The shoots subjected to Treatment 2 suffered defoliation at the beginning of the study period, as insecticide spraying started 1 month later; however, these shoots could partially recover their normal growth rates and finally produced more acorns than control shoots (which suffered defoliation throughout the spring).

In the case of our study model, Q. ilex, experimental increase of jasmonates on leaves has been shown to decrease photosynthetic rates and stomata conductance, thus reducing CO₂ assimilation rates (Filella et al. 2006). These changes that alter photosynthetic efficiency and water balance make plants especially vulnerable to environmental stresses, such as water deficit (Quentin et al. 2011; Sánchez-Humanes & Espelta 2011). During drought periods, such as occurs in Mediterranean summers, plants face a trade-off between carbon gain and water loss (Cowan & Farquhar 1977) so that an optimal stomata functioning is essential to overcome severe water-stress conditions (Xu & Baldocchi 2003). The Holm oak Q. ilex has evolved physical and physiological adaptations to the Mediterranean climate, such as sclerotic leaves and the capability of closing its stomata to avoid excessive evaportranspiration. Stomatal closure limits the number of hours in which CO₂ may be assimilated during summer drought (Chaves et al. 2002), which may explain the high mortality rates observed in the more heavily defoliated shoots. To the natural environmental limitation for CO₂ assimilation during the summer, defoliated shoots would add a reduced photosynthetic tissue (i.e. fewer leaves), a diminished photosynthetic efficiency and a reduced stomata conductance. Moreover, the direct mechanical damage would increase their vulnerability to drought, as dehydration risk is higher in damaged leaves (Aldea et al. 2005). This higher risk could also explain the lower number of leaves in the control shoots (not only explained by their shorter length) as dehydration could lead to premature leaf abscission.

The negative effects of defoliation on resource assimilation may also underlie the lower final reproductive output in control shoots. The initial investment in reproduction (number of flowers in June) did not differ between treatments, suggesting that, contrary to other cases (Crawley 1989), caterpillars did not decrease oak reproductive output by eating flowers. However, the final production of acorns per shoot did differ. Seed
abortion was higher in control shoots (not sprayed with insecticide), probably because the investment in reproduction was programmed before spring and they could not adapt their inversion to the level of damage (Marquis 1992). Acorn growth is based on the photosynthates produced during the seeding season (Hoch et al. 2013; Fernández-Martínez et al. 2015) and the loss of leaf tissue would reduce the capability of storing resources available for acorn production. The lower resource availability may also account for the higher summer mortality (premature abscission) recorded in control shoots, which reduced acorn production significantly. Even though adult Holm oaks are well adapted to the dry Mediterranean climate and rarely die (López-Iglesias et al. 2014), shoot mortality during the summer may be very significant.

In accordance with previous studies, our results show that, due to the modular architecture of trees (Takenaka 1994), oaks did not mobilize resources from healthier shoots to more damaged shoots (Sánchez-Humanes et al. 2011; Hoch et al. 2013). Acorn abortion or shoot mortality could, thus, be barely counterbalanced by the shoots nearby. Treated and control shoots were in the same trees, but their mortality and reproductive output differed greatly. In addition, irrespective of the treatment, those shoots with a higher number of leaves in June had a higher probability of surviving summer drought. In September there were no differences in the number of leaves between control and experimental shoots, as the control shoots that were more heavily defoliated were prematurely abscised during the summer.

From a conservation perspective, these results stress the importance of defoliation as a limitation of natural oak recruitment. Defoliation may reduce the number of acorns available for regeneration even more than pre-dispersal acorn predation. In the Holm oak, infestation rates by acorn-boring insects average 50% of the crop, but a percentage of the infested acorns may still germinate and produce viable seedlings (Bonal et al. 2007). In comparison, acorn production in control shoots was almost half of that of the experimental shoots, and the difference could be wider considering that more severe defoliations rates have been reported in oaks (Schroeder & Degen 2008).

Within the global change context, the negative effects of defoliation on oak recruitment may worsen. Climate models predict an increase of air temperatures and water deficit in the Mediterranean areas, which will presumably favor leaf feeding insects (Hódar & Zamora 2004; Dymond et al. 2010; Carnicer et al. 2011; Jacquet et al. 2012). Considering that defoliated shoots are more vulnerable to die during the summer drought, such a climatic scenario will seriously hamper oak natural regeneration. The economic losses would also increase in that context, especially in the Spanish oak savannahs (dehesas), where acorns constitute a key food source for livestock in autumn (Moreno & Pulido 2009). However, the natural value of such agroecosystems, included in the Directive Habitats of the European Union, advise against any massive insecticide spraying to control caterpillar populations. Rather, management measures should promote their natural enemies, such as insectivorous birds (Sanz 2001) or parasitoids.

In summary, these results put forward that seed loss may start before pre-dispersal acorn predation. Defoliation should be included in future studies on oak natural regeneration, preferentially in contrasting ecological contexts, as its negative effects could vary depending on the environmental conditions. At least in Mediterranean oak forests, subjected to strong summer droughts, defoliation poses an important threat to oak reproductive output.

ACKNOWLEDGMENTS

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ORIGINAL ARTICLE

Acorn size and tolerance to seed predators: the multiple roles of acorns as food for seed predators, fruit for dispersal and fuel for growth

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Abstract

Fitness of parents and offspring is affected by offspring size. In oaks (Quercus spp.), acorns vary considerably in size across, and within, species. Seed size influences dispersal and establishment of oaks, but it is not known whether size imparts tolerance to seed predators. Here, we examine the relative extent to which cotyledon size serves as both a means for sustaining partial consumption and energy reserves for developing seedlings during early stages of establishment. Acorns of 6 oak species were damaged to simulate acorn predation by vertebrate and invertebrate seed predators. Seedling germination/emergence and growth rates were used to assess seedling performance. We predicted that if cotyledons are important for dispersal, acorns should show tolerance to partial seed consumption. Alternatively, if the cotyledon functions primarily as an energy reserve, damage should significantly influence seedling performance. Acorns of each species germinated and produced seedlings even after removing >50% of the cotyledon. Seed mass explained only some of the variation in performance. Within species, larger acorns performed better than smaller acorns when damaged. Undamaged acorns performed as well or better than damaged acorns. There was no pattern among individual species with increasing amounts of damage. In some species, simulated invertebrate damage resulted in the poorest performance, suggesting alternative strategies of oaks to sustain damage. Large cotyledons in acorns may be important for attracting seed dispersers and sustaining partial damage, while also providing energy to young seedlings. Success of oak establishment may follow from the resilience of acorns to sustain damage at an early stage.

Key words: acorns, damage tolerance, dispersal, Quercus, seed size
INTRODUCTION

Offspring size is a widely-studied life history trait with implications for dispersal, reproductive success, population dynamics and community structure (Bernardo 1996; Eriksson & Jakobsson 1998; Leishman 2001). A trade-off in resource allocation exists between the benefits of producing few relatively large offspring versus the production of many relatively small offspring (Smith & Fretwell 1974). This tradeoff concerns both the fitness of the parents and the offspring, which, under many circumstances, may conflict. From the offspring’s perspective, large size may be especially advantageous for early growth and survival. However, for parents, producing “many small offspring” rather than “few large offspring” may be an advantage, depending on conditions (Bernardo 1996; Alcántara & Ray 2003; Gómez 2004; Agosta 2008).

In plants, larger seeds generally produce larger seedlings with higher relative growth rates, presumably because of the greater metabolic energy available for establishment, growth and development (Tripathi & Khan 1990; Leishman & Westoby 1994; Seiwa 2000; Green & Juniper 2004; Khan 2004; Lehtila & Ehrlen 2005; Du & Huang 2008; Tilki 2010; Elwell et al. 2011). Yet, there is increasing evidence for conflicting advantages of large and small seeds during the processes of seed dispersal and seed predation. Larger seeds may have a dispersal advantage (see below) or higher tolerance of post-germination damage (Harms & Dalling 1997; Dalling & Harms 1999), whereas small seeds may have higher survival in the face of seed predation if seed predators prefer larger seeds (Alcántara & Ray 2003; Gómez 2004; however, see Moles et al. 2003).

Seed size may also play a role in tolerance to seed damage by seed predators. Although it is assumed that seed predators kill seeds in the process of seed consumption, some predators inflict nonlethal damage, allowing partially consumed nuts, fruits and seeds to survive and establish (Steele et al. 1993; Branco et al. 2002; Perea et al. 2011; Yang & Yi 2012). Previous studies suggest that, in some species, seed damage may have minimal effects on seedling performance (Armstrong & Westoby 1993; Dalling et al. 1997; Branco et al. 2002; Mendoza & Dirzo 2009; Hou et al. 2010; Yi & Yang 2010) and even increase germination rates (Branco et al. 2002; Giertych & Suszka 2011). In other species, seed damage may decrease overall seedling height (Robertson et al. 1990; Mack 1998; Yi & Zhang 2008; Giertych & Suszka 2011) and survival (Janzen 1976; Robertson et al. 1990; Yi & Zhang 2008; Sage et al. 2011), but, nevertheless, permit seed growth after nonlethal damage by a predator. In general, larger seeds may better tolerate damage because of larger initial energy reserves (Bonfil 1998; Mack 1998; Pizo et al. 2006; Xiao et al. 2007; Mendoza & Dirzo 2009; Yi & Yang 2010; Yang & Yi 2012). Hence, tolerance of such damage, often influenced by seed size, is a potentially significant selective advantage for plant species such as oaks.

With respect to resource allocation, the production of large seeds is expensive, which translates to fewer seeds. In the context of significant predation pressure, producing smaller seeds may be a better solution than producing fewer larger seeds. Moreover, in some circumstances, small seeds may be better dispersers (e.g. some wind dispersed species: Howe & Smallwood 1982) or may have higher survivorship due to the preferences of seed predators (e.g. Bekker et al. 1998; Espeleta et al. 2009; Bartlow et al. 2011).

In species that produce seeds with large cotyledons, such as oaks (Quercus spp.) and chestnuts (Castanea spp.), larger seeds with high energy content are afforded a distinct dispersal advantage, especially when facing rodent predation (Xiao et al. 2004, 2005; Moore et al. 2007; Chang et al. 2009; Lichte et al. 2017). Such selective pressures, coupled with the potential influence of seed size on the ability to tolerate partial damage (Steele et al. 1993) and enhance seedling performance (Gómez 2004), may account for larger acorn size than is needed for growth in many oak species. Numerous vertebrates (e.g. eastern gray squirrels [Sciurus carolinensis Gmelin, 1788], blue jays [Cyanocitta cristata Linnaeus, 1758] and grackles [Quiscalus quiscula Linnaeus, 1758], for example, selectively consume only the basal half of acorns (Steele et al. 1993), likely because of chemical gradients in the acorns that make the basal half more attractive (Steele et al. 1993; Bogdziewicz et al. 2017, 2018; Steele et al. unpubl. data). Predators such as weevils, which do not directly affect seed dispersal, may also place selective pressure on seed size, energy content and chemical gradients in seeds. Weevils lay eggs in acorns and the developing larvae consume the acorn from the inside. Weevils damage seeds and affect germination (Yi & Yang 2010). Weevils can also affect the interactions between vertebrate seed dispersers and acorns, such as when weevil infestation affects the choice to cache or consume the acorn (Steele et al. 1996). Here, we sought to understand how intraspecific and interspecific variation in acorn size influence both seedling performance and tolerance to seed damage in...
oaks.

We specifically hypothesized that in addition to their role in seed germination and early seedling establishment, acorns are highly robust propagules adapted to sustain significant damage following partial consumption by seed predators. To test this hypothesis, we simulated specific damage by seed predators and examined the functional relationship between seed size and germination/seeding performance during the critical stage of seedling establishment in 6 species of oaks. We predicted that if the cotyledon functions primarily as an energy source for germination and growth of young seedlings, we should observe: (i) a strong positive functional relationship between seed size and seedling performance; (ii) a strong positive functional relationship between damage and the degree to which performance is reduced relative to undamaged (control) acorns; and (iii) an advantage for large seeds (and large-seeded species) in response to damage. In contrast, we predicted that if the cotyledon serves also as a fruit with excess energy able to be lost to seed predators/dispersers, we should observe: (i) significant residual variation in the relationships between seed size and young seedling performance; (ii) a general ability to suffer significant cotyledon loss and still survive; and (iii) similar responses to damage in small-seeded acorn species compared with large-seeded species. Although not a set of mutually exclusive hypotheses, this comparison of the functional roles of the acorn cotyledon as an “energy-reserve” and a “fruit” provides a logical framework for understanding how acorn size relates to competing selective pressures that ultimately influence acorn germination and oak seedling survival and establishment.

MATERIALS AND METHODS

Study species and acorn collection

We studied the effect of acorn damage in 6 North American oak species: northern red oak (Quercus rubra), pin oak (Quercus palustris), black oak (Quercus velutina), chestnut oak (Quercus prinus = Quercus montana), bur oak (Quercus macrocarpa) and white oak (Q. alba). All 6 species are sympatric across much of eastern USA, with the exception of bur oak, which is found primarily in midwestern USA. These 6 species belong to 1 of 2 sections in the genus Quercus. Red, pin and black oak are all red oak species (section Lobatae). Acorns of this group have high levels of lipids (attractive to seed predators) and tannins (defense against seed predators), exhibit a delayed dormancy and overwinter before germinating (Smallwood et al. 2001). The other 3 species in our study (white, bur and chestnut oak) are all white oak species (section Quercus). Acorns of this group typically have lower levels of lipids and tannins, and germinate in autumn, during or soon after seed fall (Smallwood et al. 2001).

The acorns of bur oak, black oak and chestnut oak used in this study were purchased from Sheffield’s Seed Company (Locke, NY, USA) in late autumn 2010, and were reported to originate from the ground beneath multiple trees of each species. We collected acorns of red oak, white oak and pin oak from the ground beneath trees (n ≥ 8 of each species) in north-eastern Pennsylvania during the autumn of 2010 (41°14′N, 75°52′W). All acorns were stored in humid conditions at 4°C until the time of experiments. Multiple maternal sources for acorns of each oak species ensured variation in individual acorn size and, thus, allowed us to test the effects of both inter-specific and intra-specific variation in acorn size on performance and tolerance to damage.

Prior to experiments, composite samples of acorns of each species were created, from all source trees of the same species. Individual acorns of each species used in the experiment were then randomly selected from these composite samples. Acorns with any signs of damage (e.g. oviposition scars or exit holes of weevils [Curculio spp.], desiccation of the cotyledon or fungal growth) after close inspection were rejected. We chose not to rely on flotation of seeds to determine soundness because previous experience with this technique proved inadequate for detecting light weevil damage (M.A. Steele, personal observation). Instead, we relied on individual manipulation of the acorn by rolling it and applying pressure to the outer pericarp (shell), coupled with close visual inspection of the pericarp.

Experimental design

Based on the types of partial damage that acorns are reported to experience (primarily rodent, bird and insect damage; Steele et al. 1993, 1998; Perea et al. 2011), we chose 4 experimental treatments representing varying degrees and types of cotyledon damage: (i) 25% cotyledon removal from the basal end of the acorn; (ii) 50% cotyledon removal from the basal half of the acorn; (iii) 1 drilled hole (3 mm in diameter and across the width of the acorn); and (iv) 4 drilled holes (each of which was 3 mm in diameter and across the width of the acorn). The 25 and 50% treatments simulated rodent and bird damage as reported in the literature (Steele et al. 1993, 1998; Perea et al. 2011), while the drilled holes simulat-
ed weevil damage. Experimental acorns in which rodent and bird damage was simulated were cut transversely with a rodent guillotine (Harvard Apparatus, item number: 550020) to remove approximately 25% or 50% of the cotyledon from the basal half of the acorn. Acorns in which we sought to simulate weevil damage were drilled with a Dremel (Model 300 with 3-mm drill bit) at approximately the mid-point between apical and basal ends. This allowed us to standardize “weevil” damage among acorns. Based on a long-term dataset monitoring acorn weevils, the 4-hole treatment represented a high weevil infestation commonly seen in oak populations (M.A. Steele, unpublished data). Each drilled hole resulted in only a single opening in the acorn shell. Whole, intact acorns served as controls. The number of acorns in each treatment ranged from 40 to 46.

Prior to treatment, all acorns were weighed (±0.001 g). To reduce the risk of experimenter-induced microbial infection affecting germination, acorns were wiped clean with 70% isopropanol before being subjected to treatment. The blade of the cutting instrument was cleaned with an alcohol swab between each acorn, and the drill bit was washed in 70% ethanol prior to treatment of each acorn. These methods do not limit pathogens infecting acorns after processing that may arise throughout the growing period. Any experimental acorns showing signs of rot or damage that were revealed during treatment preparation were retained for the experiment because comparable damage in control acorns would have remained undetected. This prevented the introduction of bias between experimental and control acorns. All acorns were weighed after treatment (±0.001 g), and this final post-treatment mass was used to quantify the percent cotyledon loss due to each treatment.

To control for soil, temperature and light differences that may occur under field conditions, acorns were planted in the laboratory and were all subject to the same growing conditions. Acorns were planted immediately after damage treatments. All acorns were processed and planted from 9 to 15 February 2011 and were planted in individual cells within a seed tray consisting of 72 cells (5 × 5 cm) arranged in 2 × 4 blocks, with each block consisting of 9 cells (3 × 3 cells). Acorns of the same treatment, but different species, were planted in a pseudo-random manner within the same tray. The acorns were planted in a medium consisting of a 1:1 ratio of Pro-Mix (Premier Tech) to commercial topsoil to control for any differences related to soil chemistry that may influence acorn growth. After all acorns were planted, the blocks were detached and further randomized so that each tray consisted of 8 blocks of varying treatment types. Throughout the experiment, each tray was rotated every 2 weeks to account for variation in temperature, light and any other position effect in the growing room. Growing conditions included natural light and constant temperature (19–23°C). Each plant was watered with approximately the same amount of water at the same time every 2–3 days.

Data collection

Germination of acorns and the survival of seedlings throughout the growing period were recorded. Because we were interested primarily in the effects of damage on germination, emergence and performance of young seedlings, the seedlings were grown for 2 months and survival was assessed throughout the experiment. Previously we found that partially damaged acorns often survive a full growing season if they make it past the first 2 months of growth (Steele et al., unpublished data). At the end of the experiment, seedlings were harvested over a 3-day period (19 April 2011 to 21 April 2011). Seedlings were removed from the soil, with all debris carefully removed from the roots, and were weighed to the nearest ±0.001 g. Seedlings were dried for 4 days at 40°C and were again weighed to the nearest ±0.001 g. This final mass was then divided by the number of days grown to calculate the growth rate, hereafter referred to as the average daily increase in biomass (ADB).

Data analysis

All statistical analyses were performed using the program R (R Core Development Team 2012) and JMP Pro 10 (SAS Institute, Cary, NC, USA). Mean seed size (mass) and standard deviation were calculated for each species and compared across species. Kolmogorov–Smirnoff tests were used to determine if the masses of each species were normally distributed. Several species failed to meet assumptions of normality, so the acorn masses of all species were log10 transformed. An ANOVA, followed by Tukey’s post-hoc test, was used to assess interspecific differences in seed size.

To determine the relationship between seed size and early seedling performance, separate linear regressions of seed mass versus ADB were performed with the control acorns of each species. Initial seed mass was log10 transformed, and ADB was square root transformed to better approximate normal distributions (a linear model including all species with interaction terms showed an interactive effect of species and initial seed mass on ADB). In cases where relationships appeared curviline-
ear, a polynomial function was fit to the data using AIC scores to determine the best fit. In addition, separate logistic regressions were performed to determine relationships between seed size and survival (germination and emergence). Again, this was done separately for each species and for control acorns only. As above, initial seed mass was log10 transformed prior to analysis.

Before testing for damage effects, we confirmed that treatments represented discrete and independent levels of damage by comparing the cotyledon lost in all treatments using ANOVA, followed by Tukey’s post-hoc test. These data were first square root transformed to approximate normal distributions.

To test the hypothesis that seed size affects robustness to damage, we analyzed the data in 3 ways, each of which provided a complementary test of our predictions. First, linear and logistic analysis of covariance (ANCOVAs) was used to determine how initial seed mass and damage treatment predicted ADB and survival. In these models, initial seed mass was first log10 transformed. For the linear models, ADB was square root transformed. In these linear and logistic regression models, there were significant interactions between species and treatment. Therefore, ANCOVAs were run separately for each species using treatment as the factor and initial seed mass as the covariate. Because some treatments were planted on different days, the number of days each seedling was grown was included as a second covariate. If the relationship between performance and damage is a simple function of the amount of energy reserves, then we expected larger seeds to perform better at each level of damage. However, the relationship between performance and damage may be a more complex function of seed size, if, for example, there is a threshold energy level needed for successful germination and growth. In this case, larger seeds may be able to withstand proportionally more cotyledon loss because they have higher initial energy reserves above this threshold. If so, we predicted a significant interaction between initial seed mass and damage level in the ANCOVAs, and an increasing advantage (steeper slope) of seed size with a greater proportional loss of cotyledon (i.e. the strongest effect of seed size on performance should have occurred in the 50% damage treatment). Second, to determine if larger-seeded species performed better in each damage treatment, acorn masses (mean ± SE) of the 6 species were plotted against the ADB (mean ± SE) and percent survival. Linear regression was used to determine how well the initial seed mass of a species predicted the ADB and survival across damage treatments. Finally, to determine if small-seeded species suffered a greater relative reduction in performance, the relationship between damage and the degree to which performance was reduced relative to undamaged control acorns was examined. In each damage treatment, acorn masses (mean ± SE) of the 6 species were plotted against the corresponding mean ADB of each treatment divided by the mean for each control ADB. In addition, the percent survival of each treatment was divided by the percent survival of the control and plotted against mean acorn masses.

RESULTS

Variation in acorn mass within and among species is summarized in Table 1. Differences in acorn mass among species were significant (ANOVA, \( F_{5,128} = 1033, P < 0.0001; \) Table 1). All species significantly differed, except for pin oak and white oak acorns (Tukey post-hoc, \( P = 0.43 \)) In our study, there was a 4-fold difference between the largest chestnut oak acorns (mean ± SD; 5.90 ± 1.43 g) and the smallest white oak acorns (1.50 ± 0.74 g).

Table 1 The mean masses and standard deviations of acorns from the 6 oak species. All species were significantly different from one another, except Quercus palustris and Quercus alba (Tukey’s post-hoc, \( P = 0.43 \)).

<table>
<thead>
<tr>
<th>Species</th>
<th>Sample size</th>
<th>Range</th>
<th>Mean (g)</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chestnut oak (Quercus montana)</td>
<td>198</td>
<td>0.84–10.4</td>
<td>5.90</td>
<td>1.43</td>
</tr>
<tr>
<td>Bur oak (Quercus macrocarpa)</td>
<td>199</td>
<td>2.78–7.41</td>
<td>5.07</td>
<td>0.96</td>
</tr>
<tr>
<td>Red oak (Quercus rubra)</td>
<td>202</td>
<td>2.34–8.97</td>
<td>4.66</td>
<td>1.28</td>
</tr>
<tr>
<td>Black oak (Quercus velutina)</td>
<td>200</td>
<td>1.40–3.45</td>
<td>2.25</td>
<td>0.49</td>
</tr>
<tr>
<td>Pin oak (Quercus palustris)</td>
<td>203</td>
<td>1.06–3.14</td>
<td>1.68</td>
<td>0.35</td>
</tr>
<tr>
<td>White oak (Quercus alba)</td>
<td>202</td>
<td>0.54–4.31</td>
<td>1.50</td>
<td>0.74</td>
</tr>
</tbody>
</table>
For control acorns, seed mass was a significant positive predictor of ADB in each species except black oak (Fig. 1). Across species, $R^2$ ranged from reasonably high in black oak (0.74) and white oak (0.73), to moderate in pin oak (0.39), to 0.11 in bur oak. With respect to survival (germination and emergence) of the control acorns, logistic regression revealed a significant positive effect of initial seed mass in only pin oak. Black oak showed a significant negative relationship between seed mass and survival. Interestingly, there was no significant relationship between seed mass and survival in the other 4 species (Table 2).

Four distinct damage classes were created during the damage process based on the percentage of cotyledon removed (mean ± SE % of cotyledon loss; 1 hole, 2.08% ± 0.093%; 4 holes, 5.06% ± 0.25%; “25%,” 17.54% ± 0.28%; “50%,” 43.60% ± 0.48%; Fig. 2). These 4 classes of damage approximated the varying levels of damage we sought and were significantly different from one another (ANOVA, $F_{4,1198} = 6879, P < 0.001$; Fig. 2).

![Figure 1](image1.png)  
**Figure 1** The linear relationships between initial seed mass and average daily increase in biomass (ADB) of the control acorns of the 6 oak species: (a) red oak, (b) pin oak, (c) black oak, (d) chestnut oak, (e) bur oak and (f) white oak. The ADB was square root transformed and the acorn mass was log transformed. The ADB increased as seed mass increased in 5 of the species.

![Figure 2](image2.png)  
**Figure 2** The 4 experimental categories showing the amount of cotyledon removed after acorns were subject to damage. The amount removed was significantly different for each treatment.
Table 2 Summary of generalized linear model with binomial distribution of seed size versus survival (germination and emergence) for control acorns of each species using the initial seed masses as predictors

<table>
<thead>
<tr>
<th>Species</th>
<th>Coefficient</th>
<th>Degrees of freedom</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chestnut oak</td>
<td>9.592</td>
<td>1</td>
<td>0.058</td>
</tr>
<tr>
<td>Bur oak</td>
<td>1.865e-08</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Red oak</td>
<td>7.939</td>
<td>1</td>
<td>0.077</td>
</tr>
<tr>
<td>Black oak</td>
<td>-8.401</td>
<td>1</td>
<td>0.032*</td>
</tr>
<tr>
<td>Pin oak</td>
<td>11.028</td>
<td>1</td>
<td>0.036*</td>
</tr>
<tr>
<td>White oak</td>
<td>2.3926</td>
<td>1</td>
<td>0.15</td>
</tr>
</tbody>
</table>

* indicates significance at \( P = 0.05 \).

Acorns in all 4 of the damage classes germinated and survived until harvest (Fig. 3). Bur oak had the highest survival in every damage class, maintaining >60% survival even at 50% damage. Four species (red, pin, bur and white oak) showed higher survival when 25% of the cotyledon was removed than in either of the treatments simulating weevil damage (1 and 4 holes), even though far less cotyledon was removed in the weevil treatments.

To test for the effects of damage, initial acorn mass, and their potential interaction on ADBs, we conducted separate ANCOVAs for each species. There was no significant interaction between treatment and initial acorn mass for any species, indicating that within treatments, ADBs responded similarly to seed size (Table 3). There-

Figure 3 The survival of each species in response to the 4 experimental treatments and the control acorns, not subject to damage. The species are ordered smallest to largest by mass (see Table 1). Values indicate numbers of individuals surviving.

Figure 4 Least squares (LS) means of the average daily increase in biomass (ADB; mg/day) ± SE of the 6 species of oaks in each damage treatment. ADB was square root transformed. The higher the LS mean for a given species within a treatment, the faster the average daily increase in biomass. Performance generally decreased as damage increased, except for the 25% damage treatment.
For the 4 other species, there was a significant main effect of initial acorn mass on ADBs increased with an interaction between seed mass and treatment. * indicates significance at $P = 0.05$.

Average seed mass was a significant predictor of mean ADB across species for each treatment and the control (Fig. 5). The 3 largest oak species produced acorns that had the highest ADB in all 4 damage classes and the control treatment. However, acorn mass was a poor predictor of acorn survival in each damage treatment. For survival, there was no significant interaction between treatment and initial acorn mass for any species. This suggests that the relationship between mass and survival is similar across damage treatments. In all species but 1 (white oak), there were significant negative effects of treatment on acorn survival (Table 4). The initial seed mass successfully predicted the survival of 4 of the species; the seed masses of red oak and bur oak were not significant predictors of their survival.

Average seed mass was a significant predictor of mean ADB across species for each treatment and the control (Fig. 5). The 3 largest oak species produced acorns that had the highest ADB in all 4 damage classes and the control treatment. However, acorn mass was a poor predictor of acorn survival in each damage treat-
Figure 5 The mean seed size (± SE) and the mean absolute average daily increase in biomass (ADBs; ± SE) for the 6 species of oaks in the 4 damage treatments and the control treatment. In each treatment, the mean seed mass was a significant predictor of ADB among species; the largest species produced seedlings with the highest ADB. (a) 1 hole, (b) 4 holes, (c) 25% damage, (d) 50% damage, (e) control.

Table 4 Summary of generalized linear models with binomial distributions using survival (germination and emergence) as the response variable with 2 predictor variables and the interaction

<table>
<thead>
<tr>
<th>Species</th>
<th>Source factor</th>
<th>Degrees of freedom</th>
<th>$\chi^2$</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chestnut oak</td>
<td>Treatment</td>
<td>4</td>
<td>12.59</td>
<td>0.014*</td>
</tr>
<tr>
<td></td>
<td>Initial mass</td>
<td>1</td>
<td>11.61</td>
<td>0.001*</td>
</tr>
<tr>
<td></td>
<td>Initial mass × treatment</td>
<td>4</td>
<td>2.60</td>
<td>0.63</td>
</tr>
<tr>
<td>Bur oak</td>
<td>Treatment</td>
<td>4</td>
<td>23.45</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>Initial mass</td>
<td>1</td>
<td>&lt;0.001</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>Initial mass × treatment</td>
<td>4</td>
<td>2.53</td>
<td>0.64</td>
</tr>
<tr>
<td>Red oak</td>
<td>Treatment</td>
<td>4</td>
<td>33.62</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>Initial mass</td>
<td>1</td>
<td>1.83</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>Initial mass × treatment</td>
<td>4</td>
<td>5.91</td>
<td>0.21</td>
</tr>
<tr>
<td>Black oak</td>
<td>Treatment</td>
<td>4</td>
<td>20.10</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>Initial mass</td>
<td>1</td>
<td>24.03</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>Initial mass × treatment</td>
<td>4</td>
<td>0.77</td>
<td>0.94</td>
</tr>
<tr>
<td>Pin oak</td>
<td>Treatment</td>
<td>4</td>
<td>40.41</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>Initial mass</td>
<td>1</td>
<td>9.80</td>
<td>0.0017*</td>
</tr>
<tr>
<td></td>
<td>Initial mass × treatment</td>
<td>4</td>
<td>5.68</td>
<td>0.22</td>
</tr>
<tr>
<td>White oak</td>
<td>Treatment</td>
<td>4</td>
<td>4.86</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>Initial mass</td>
<td>1</td>
<td>10.64</td>
<td>0.001*</td>
</tr>
<tr>
<td></td>
<td>Initial mass × treatment</td>
<td>4</td>
<td>5.31</td>
<td>0.26</td>
</tr>
</tbody>
</table>

Each species was analyzed separately. Reported are the degrees of freedom, $\chi^2$-statistics, and the $P$-values for the main effects of initial mass, treatment, and their interaction. Asterisks indicate significance ($P < 0.05$).
Figure 6 The mean seed size of the 6 species (± SE) of oaks and the proportion surviving in each damage treatment and the control treatment: (a) 1 hole, (b) 4 holes, (c) 25% damage, (d) 50% damage and (e) control. Overall, seed size did a poor job predicting survival.

Figure 7 The mean seed size and the relative average daily increase in biomass (ADB) defined as the mean treatment ADB divided by the mean control ADB of each species in each treatment: (a) 1 hole, (b) 4 holes, (c) 25% damage and (d) 50% damage. When scaled relative to control acorns, there was no significant relationship between mean seed size and mean ADB in any damage treatment, except the 4 hole treatment.
Figure 8 The mean seed size and the relative survival (proportion of treatment surviving divided by the proportion of control surviving) of each species in each treatment (a) 1 hole, (b) 4 holes, (c) 25% damage and (d) 50% damage. Survival relative to the control acorns was not dependent on mean seed size. The relative reduction in survival was similar across all species regardless of size.

DISCUSSION

The acorn cotyledon, although certainly critical for seedling survival, establishment and growth, also serves a secondary role of manipulating the dispersal process (Steele & Smallwood 2002; Moore et al. 2007; Steele et al. 2014). However, as we demonstrate here, the acorn also serves yet another function: to tolerate partial damage by potential seed predators.

Our argument that the cotyledon in the acorn functions in part to promote tolerance of seed predators is further supported by studies suggesting that a suite of acorn characteristics may divert damage by avian and rodent acorn predators and dispersers, and even insect seed predators, away from the apical (embryo-containing) end of the acorn (Steele et al. 1993, 1998). When acorns are abundant, these predators often inflict partial damage to only the basal end of acorns (Steele et al. 1998; Perea et al. 2011), allowing these partially damaged seeds to still germinate and potentially establish (Steele et al. 1993; Steele et al. unpubl. data). Long-term studies, for example, indicate that weevil larvae are found significantly more often in the basal half of the acorn compared with the apical half closer to the seed (Steele et al. unpublished). Acorn characteristics considered central to influencing this partial acorn consumption are chemical gradients, including higher levels of phenolic defense compounds (tannins) in the apical end of the acorn, near the embryo; and higher levels of nutrients (some minerals and lipids; Steele et al. unpublished) at the basal end. We suggest that these chemical gradients direct partial seed damage away from the embryo, thereby allowing seed survival (germination and emergence) and young seedling establishment (Steele et
As we show here, at least 6 oak species can germinate and establish even when 50% of the cotyledon is removed from the basal half of the acorn. Although such damage may somewhat reduce seedling performance, we postulate that this may represent a critical strategy for tolerating seed predation. In the life of an oak (>150 years in some species), such a strategy only requires occasional establishment success to significantly influence lifetime reproductive success. Thus, our results, especially in the context of previous studies, support the hypothesis that the cotyledon acts as both a fruit and an energy reserve.

We predicted that if acorns, indeed, serve as a fruit for dispersal and also to tolerate damage then we should observe: (i) significant residual variation in the relationships between seed size and seedling performance; (ii) a general ability to suffer significant damage and still survive; and (iii) similar performance in response to damage between small and large-seeded species. We observed that numerous individuals of all species were able to germinate and grow into young seedlings with nearly half of the initial cotyledon removed. Second, among surviving acorns, anywhere from 26% to 89% of the variation in average daily increase in biomass (ABD) was unexplained by initial acorn mass. Thus, the expectation that larger acorns correspond to larger seedlings was only partially supported by these data. Moreover, in black oak, intermediate-sized acorns produced the largest seedlings and initial acorn mass was negatively related to acorn survival, a result that is unexpected. In all other species, “bigger” was “better,” but the explanatory power of initial acorn size ranged from high in pin oak and white oak to very low in bur oak. Seed mass was not a significant factor that explained survival in 4 of the 6 species (Table 2).

Results of the ANCOVAs indicated that larger seeds performed better in each damage treatment, with a significant main effect of initial mass on ADBs for 4 of the 6 species and a significant effect on survival in 4 of the 6 species. In chestnut oak and white oak, treatment was not significant in explaining ADB. Some seedlings performed better when 25% of the cotyledon was removed compared to when 4 holes were drilled (Fig. 3). Cutting acorns therefore may have functionally different effects than drilling into acorns. Observations of our damaged acorns suggested that those in which we simulated partial damage by birds and rodents (removal of 25% or 50% of the basal portion) seemed to show far less infestation of pathogens (fungi and mold) than those in which we simulated weevil damage, despite sanitizing prior to treatment. Sanitizing prior to treatment limits experimenter-induced pathogens, but does not limit pathogen infestation that may occur throughout the entire growing period. We suggest that this difference may result from the greater exposure of the cotyledon to air, which likely results in oxidation of tannins to more toxic quinones (Appel 1993). Such a reaction may explain why “half-eaten acorns” are not necessarily more susceptible to pathogens and should be tested in the future.

In addition, treatment had an effect on performance, with lower ADBs and survival in damage treatments (Fig. 4). However, there was no evidence of an increasing advantage for larger seeds with increased damage (i.e. there were no significant interactions between initial acorn mass and treatment) and therefore, no evidence for a threshold level of energy needed for growth and survival. Although it seems likely that some minimal amount of cotyledon is ultimately needed for successful germination, we did not detect it even after removing approximately 50% of the cotyledon from acorns over a 4-fold size range.

At the interspecific level, large-seeded species (red oak, bur oak and chestnut oak) had higher absolute ADBs than small-seeded species (white oak, pin oak and black oak) in all treatments (damage and control). In contrast, there were no significant interspecific relationships between seed size and survival in any treatment or the control. When scaled relative to control acorns, all species responded similarly to damage (growth: Fig. 7; survival: Fig. 8). Small-seeded species did not suffer a greater proportional reduction in performance compared to large-seeded species, except in the 4-hole treatment for relative ADB (Fig. 7).

Collectively, these results demonstrate that a significant portion of the energy packaged in acorns is not essential for early seedling development and survival. Based on these results, we suggest that acorns act in part as a fruit to attract potential dispersers and to sustain partial seed damage by seed predators. Up to 50% (and possibly more) of the cotyledon reserve is not essential for acorn germination and early seedling survival, even though performance may be reduced when this amount of cotyledon is lost.

Several oak species found worldwide are now known to tolerate insect and rodent damage and still germinate and produce seedlings (Steele et al. 1993; Bonfil 1998; Xiao et al. 2007; Hou et al. 2010; Yi & Yang 2010; Giertych & Suszka 2011; Perea et al. 2011; this study). Furthermore, at least 2 species of white oaks in North America (Quercus alba and Q. montana) can tol-
erate the complete removal of acorns from developing taproots, suggesting that the acorn is not essential for growth and survival once the taproot reaches a minimal threshold length (Yi et al. 2012, 2013). These observations, coupled with those reported herein, suggest that acorns are tremendously robust and well adapted for tolerance of seed and seedling damage.

In the current experiments, the larger seeded species had higher ADBs in each damage treatment. Therefore, a larger-seeded oak species would have an advantage over a smaller-seeded species when subject to similar damage, given the same growing conditions. However, this does not appear to be the case in terms of survival; large and small-seeded species showed a similar survival response to damage. The apparent advantage of large-seeded species, in terms of ADB, does not seem to follow from the phylogeny of these 6 species. Of the 3 large-seeded oaks, 2 are in the white oak section, and the other is a red oak. Although the white oak acorns in our studies were small, this species often produces substantially larger acorns in more southerly portions of its geographic range.

Whereas variation in acorn size may in part result from the need for acorns to tolerate damage and still survive, acorn size is also known to directly influence dispersal by rodents and corvids. Rodents, for example, are known to selectively cache larger seeds (including acorns) and disperse them significantly greater distances than smaller seeds of the same species (Jansen et al. 2004; Xiao et al. 2005; Moore et al. 2007; Wang & Chen 2009; Lichti et al. 2017). Recently it has also been shown that larger acorns are selectively dispersed by Eastern gray squirrels (Sciurus carolinensis) to more secure locations in open sites where the probability of cache pilferage is reduced (Steele et al. 2014), and the probability of seedling establishment, if not recovered by the cache owner, is higher than smaller acorns of both the same and different species (Steele et al. 2014).

If acorn size is favored for dispersal, growth and partial damage by seed predators, then why are smaller-seeded oak species still common? The answer to this may in part follow from the classic trade-off between offspring size and number. However, it may also in part follow from the size preferences of other dispersal agents, such as jays, which are gape-limited and prefer smaller-seeded acorns for dispersal (Moore & Swihart 2006; Bartlow et al. 2011). Such gape limitation also favors multiple-prey loading by jays in which small acorns are swallowed and placed in the crop. These multiple loads (up to 8 acorns for 1 dispersal event) are then dispersed greater distances and to presumably better cache sites than large acorns that must be dispersed individually (Bartlow et al. 2011). Such gape-limited corvids and smaller rodents may, therefore, exert disruptive selection on seed size, with the other factors discussed above favoring larger seed size. In the present study, all 6 oak species are consumed and dispersed by rodents, while the 3 small-seeded species (pin oak, black oak and white oak) are selectively consumed and dispersed by jays as well (Darley-Hill & Johnson 1981; Moore & Swihart 2006).

Partial acorn consumption and subsequent tolerance to damage by seed consumers may be more important than previously thought and may play a major role in the dispersal, establishment and regeneration of oaks. Why the 6 oak species in the current study had different responses to damage is unclear. Future studies that replicate field conditions, especially stressful conditions, should be considered to further elucidate the importance of cotyledon reserves to developing seedlings. In addition, future work should consider the functional roles of acorns as fruit and/or energy reserves to understand how seed size influences pre-dispersal and post-dispersal tolerance to damage, seed dispersal, and seedling establishment for other oak species worldwide.

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Role of seed size, phenology, oogenesis and host distribution in the specificity and genetic structure of seed weevils (*Curculio* spp.) in mixed forests

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Abstract

Synchrony between seed growth and oogenesis is suggested to largely shape trophic breadth of seed-feeding insects and ultimately to contribute to their co-existence by means of resource partitioning or in the time when infestation occurs. Here we investigated: (i) the role of seed phenology and sexual maturation of females in the host specificity of seed-feeding weevils (*Curculio* spp.) predating in hazel and oak mixed forests; and (ii) the consequences that trophic breadth and host distribution have in the genetic structure of the weevil populations. DNA analyses were used to establish unequivocally host specificity and to determine the population genetic structure. We identified 4 species with different specificity, namely *Curculio nucum* females matured earlier and infested a unique host (hazelnuts, *Corylus avellana*) while 3 species (*Curculio venosus, Curculio glandium* and *Curculio elephas*) predated upon the acorns of the 2 oaks (*Quercus ilex* and *Quercus pubescens*). The high specificity of *C. nucum* coupled with a more discontinuous distribution of hazel trees resulted in a significant genetic structure among sites. In addition, the presence of an excess of local rare haplotypes indicated that *C. nucum* populations went through genetic expansion after recent bottlenecks. Conversely, these effects were not observed in the more generalist *Curculio glandium* predating upon oaks. Ultimately, co-existence of weevil species in this multi-host-parasite system is influenced by both resource and time partitioning. To what extent the restriction in gene flow among *C. nucum* populations may have negative consequences for their persistence in a time of increasing disturbances (e.g. drought in Mediterranean areas) deserves further research.

Key words: *Corylus avellana*, *Curculio* spp., genetic structure, *Quercus* spp., trophic breadth

INTRODUCTION

Seed predation by insects may play a crucial role in plant population dynamics, by reducing the reproductive output (Bonal et al. 2007; Espelta et al. 2008) and con-
straining the regeneration process (Espelta et al. 2009b). Trophic breadth and specificity of seed-feeding insects is often explained by differences among plant hosts in chemical or morphological traits (Bernays & Graham 1988; Forister et al. 2015). Differences in phytochemistry (mainly nitrogen-based defensive compounds) have been observed to be highly relevant for the diversification of phytophagous insects and their diet breadth (Kergoat et al. 2005). Concerning other seed features, size is a trait claimed to influence ecomorphological diversification in many endophytic insects (e.g. body size and rostrum shape), promoting differences in their trophic niche, ecological adaptations and species radiation (Hughes & Voegler 2004a; Bonal et al. 2011; Peguero et al. 2017). In addition to chemical and morphological differences, seeding phenology and stochasticity in the availability of this resource have also been suggested as key factors influencing the guild of insect species predaing upon a particular plant host (Espelta et al. 2008, 2009b; Coyle et al. 2012; see also Pélisson et al. 2013a). As insects are short-living organisms, synchronization of their life-cycle with the resources upon which they depend is critical (Bale et al. 2002, 2007; Hood & Ott 2010). Therefore, processes such as adult emergence (Espelta et al. 2017) and oogenesis (Trudel et al. 2002; Son & Lewis 2005) have to be tightly connected with the presence of seeds for oviposition (Bonal et al. 2010). In particular, oogenesis (i.e. egg maturation in females) has been predicted to differ depending on the stochasticity of seeds availability. Thus, proovigenesis (i.e. females have already mature eggs at the onset of their adult life) would be favored in species predaing upon hosts that regularly produce seeds while synovigenesis (i.e. females start their adult life with immature eggs) would be advantageous for species exposed to more random fluctuations of seed production (Jervis et al. 2008; Richard & Casas 2009), as they can better adjust the amount of energy invested in reproduction to the amount of seeds (but see Pélisson 2013b). Ultimately, the co-existence of the different seed consumers in a multi-host community could be mediated by resource partitioning (e.g. insects predate preferentially upon different species according to different seed traits; see Espelta et al. 2009a), time-partitioning (e.g. insects exhibit differences in life span and the timing of seed predation; see Pélisson et al. 2012) or the trade-off among dispersal versus dormancy ability to cope with resource scarcity (Pélisson et al. 2012). Yet, the importance of the interplay among seed size, seeding phenology and oogenesis in driving the guild of insects predaing upon seeds of different hosts in multi-specific systems has been seldom explored.

The breadth of the trophic niche of seed-feeding insects (specialist vs generalist) may influence the number of species that predate upon different seeds and it has consequences for the dynamics of the community of hosts (Espelta et al. 2009b). However, beyond the effects on plant fitness, differences in the trophic niche may also influence the population dynamic of the seed consumers (Ylioja et al. 1999) depending on life-history traits such as dispersal ability and landscape attributes (i.e. abundance and spatial distribution of hosts). Spatial connectivity among plant-hosts has been shown to be especially relevant for insects with low dispersal ability (Thomas et al. 2001; Krueess 2003), resulting in a stronger genetic structure and reduced gene flow in the insect populations located on more isolated hosts. In the long run, host isolation may even result in colonization credits for some insect species, especially those with a narrower diet breadth (Ruiz-Carbayo et al. 2016) and poor dispersal ability (Pélisson et al. 2013b; Heineger et al. 2014). Conversely, generalist species may show a more continuous distribution in the landscape, benefiting from the spatial overlap of the different host plants upon which they feed (Newman & Pilson 1997), and show no genetic structure differences among populations owing to gene flow. Interestingly, for seed-feeding insects a comparison of the genetic structure of their populations and the spatial structure of their potential hosts could provide strong evidence about differences of the trophic niche breadthths. Moreover, the use of molecular techniques (DNA barcoding) may help to detect cryptic speciation and trophic niche segregation among morphologically similar species (Peguero et al. 2017), and also to establish species specificity in an unequivocal way in comparison to classifications based on the presence or absence of a species on a particular plant, especially when the lack of morphological differences at certain stages (e.g. larvae) make species identification impossible otherwise (Govindan et al. 2012). Unfortunately, this combination of landscape ecology (i.e. host connectivity) and population genetics when studying the breadth of the trophic niche and dispersal ability of phytophagous insects remains largely unexplored.

The main aims of this study have been to investigate in a multi-host and multi-seed-predator system the role of seed size, seed phenology and oogenesis in the host specificity of seed-parasite weevils (Curculio spp.) and to analyze the consequences that potential differences in trophic specialization and host distribution may have.
in the genetic structure of weevil populations. *Curculio* spp. (Coleoptera: Curculionidae) are seed parasites that differ in their dispersal ability (Venner et al. 2011), diapause duration (Pélisson et al. 2013a,b), oogenesis (with both proovigenic and synovigenic species; Pélisson et al. 2013a) and the breadth of their trophic niche (Muñoz et al. 2014; Bonal et al. 2015; Peguero et al. 2017). We conducted this study in Catalonia (northeast Spain) in mixed forests including oaks (*Quercus ilex*, *Quercus pubescens*) and common hazel trees (*Corylus avellana*) with 4 different weevil species present (*Curculio nucum* Linnaeus, *Curculio glandium* Marsham, *Curculio venosus* Gravenhorst and *Curculio elephas* Gyllenhaal). Interestingly, in this region oaks show a much more continuous distribution and later seeding, while hazels often appear in more discontinuous patches and have an earlier production of fruits (Gracia et al. 2004). Concerning weevils, the 4 species overwinter underground, but they differ in the duration of their diapause, the phenology of emergence, oogenesis and dispersal ability. Adults of *C. glandium*, *C. venosus* and *C. nucum* emerge in spring 2 years after larvae buried into the soil, while *C. elephas* exhibits variable diapause and adults emerge in early autumn (Bonal et al. 2010; Espelta et al. 2017) for up to 3 years (Pélisson et al. 2013b). Concerning oogenesis, in *C. glandium*, *C. venosus* and *C. nucum* females are reproductively immature (synovigenic) and ovarian development is accomplished after 1 or 2 months of the feeding period (Bel-Venner et al. 2009), while *C. elephas* females are proovigenic and food intake is not required for ovarian development (Pélisson et al. 2012). Regarding host selection, previous studies have suggested that *C. nucum* is highly specialized in hazelnuts (Bel-Venner et al. 2009), while the other weevils depredate upon several oak species (Muñoz et al. 2014). However, these results have not been confirmed by means of DNA analyses as no study has been conducted in mixed hazel–oak forests. Considering the traits of the species involved in this multi-host and multi-predator system and the spatial distribution of hosts, we hypothesize that: (i) seed size and the synchronization of seeding phenology and oogenesis will be responsible for the guild of weevils predating upon the different plants; and (ii) the narrower trophic breadth of *C. nucum* and the more patchy distribution of hazels in comparison to the more generalist habit of the other weevils and the continuous distribution of oaks will result in differences in the genetic structure of weevil populations of these species.

**MATERIALS AND METHODS**

**Study area and species**

The study was carried out in mixed forests with the presence of oaks (*Q. ilex* and *Q. pubescens*) and common hazel trees (*C. avellana*) in Catalonia (north-east Spain, Fig. 1). The evergreen *Q. ilex* and the winter-deciduous *Q. pubescens* are extensively distributed in pure and mixed forests in all the western rim of the Mediterranean basin (Espelta et al. 2008), while the common hazel (*C. avellana*) often appears in scattered groups in mixed deciduous forests or cultivated in monospecific stands (AliNiazee 1998). Acorns in *Quercus* spp. and hazelnuts in *C. avellana* mature in 1 year and both are subjected to intense pre-dispersal seed predation by weevils (*Curculio* spp.), a group of granivorous insects extensively distributed in the northern hemisphere (Hughes & Voegler 2004a). In Catalonia, the most common weevil species predating upon acorns are *C. glandium* and *C. elephas* (Espelta et al. 2009b), the latter also depredating upon chestnuts (*Castanea* spp.), while in hazelnuts the unique species described up to now has been *C. nucum*, a hypothesized highly specific seed parasite (Guidone et al. 2007; Bel-Venner et al. 2009b).

![Figure 1](image-url)
During early summer on hazelnuts (AliNiazee 1998) and early autumn on acorns (see Bonal & Muñoz 2009) female weevils perforate the seed cover with their snout and oviposit commonly a single egg so the larvae develop feeding on the seed kernel. At the middle of summer in C. nucum (Bel-Venner et al. 2009) or late autumn in C. elephas and C. glandium (Espelta et al. 2009a), larvae exit the seed and bury into the ground to overcome the diapause period and undergo full metamorphosis.

**Sampling design**

In 2013 we established a total of 23 sampling plots grouped into 5 geographical clusters (Sites) in a north to south latitudinal gradient (see Fig. 1). This sampling procedure was selected to account for the possible effects of latitude on the duration of the vegetative season and, thus, on the seedling phenology of oaks and hazelnuts, their overlap and the overlap among these host species and the weevils predating upon their seeds. Presumably a tighter vegetative season in northern and colder sites would lead to more similar patterns of seed production while these could be more relaxed and longer in southern and warmer places. Ultimately, this could lead to differences in the guild of weevils predating upon these plants. Plots were selected by searching for the presence of trees of Co. avellana and Q. ilex or Q. pubescens based on the Catalan Forest Inventory (Gracia et al. 2004) and field observations of their reproductive status (i.e. presence of seeds). From late July (end of hazel seeding season) to early October (end of the acorn crop) we carried out 3 sampling campaigns: (i) late July to early August; (ii) late August to early September; and (iii) late September to early October to account for possible differences in the phenology of seed infestation by the different weevil species present. In every plot and in each sampling period we randomly collected a minimum of 100 seeds from each species (Co. avellana and Quercus spp.) under the canopies of several randomly selected trees. Seeds were taken to the laboratory and classified as sound or infested to assess infestation rates per species and sampling period. Infested seeds are easily recognizable by the presence of female oviposition scars. We calculated the volume of both sound and infested seeds by measuring the length and width to the nearest 0.01 mm with a digital caliper (see Espelta et al. 2009a). Infested seeds were placed individually in plastic trays for individual monitoring. Each seed was checked daily to register the emergence of larvae, which were immediately transferred to 2 mL Eppendorf with 96% alcohol. Once larvae stop emerging (approximately 3 weeks after seeds were collected) seeds were dissected to check for the presence of non-emerged larvae. From hazelnuts only Curculio larvae emerged, while for acorns the 6% of larvae corresponded to the Cydia spp. moth.

In parallel, during the abovementioned field campaigns adult weevil were captured by shaking the canopy and collecting the fallen individuals in an inverted umbrella held beneath the foliage for species identification at the laboratory. To establish whether females were sexually mature, they were dissected under a microscope to observe abdominal segments and ovary maturity. We considered the presence of eggs as a sign of female ready for oviposition and the absence of eggs as females that were still immature or had already oviposited (Péisson et al. 2013a).

**DNA barcoding and larval species identification**

A total of 1657 Curculio larvae emerged from hazelnuts and acorns. In order to establish unequivocal trophic relationships between insects and their host plants we used molecular techniques (DNA barcoding) as larvae cannot be determined according to morphological characters. Therefore, from 342 larvae selected randomly among the ones emerged in the laboratory from the 3 hosts we extracted DNA from a small piece of larval tissue (approximately 2-mm long) using the NucleoSpin-Tissue kit according to the manufacturer’s instructions (MACHEREY-NAGEL GmbH, Düren, Germany; www.mn-net.com). We amplified a fragment (826 bp) of the mitochondrial cytochrome oxidase subunit 1 (cox1) using primers Pat and Jerry (please see Hughes & Vogler [2004b] for details on primer sequences and PCR protocols). We chose this fragment of cox1 due to the availability of many reference sequences from correctly determined adults of European Curculio spp. For comparison (Hughes & Vogler 2004b). Sequencing was performed using Big-Dye (Perkin-Elmer) technology and an ABI3700 sequencer. Sequence chromatograms were assembled and edited using Sequencher 4.6 (Gene Codes, Ann Arbor, MI, USA). For
species identification we discarded those sequences that after edition were shorter than 500 base pairs. Edited sequences were aligned using CLUSTALW supplied via http://align.genome.jp, with default gap open and gap extension penalties. The alignment sets were collapsed into unique haplotypes and each of this compared to the *Curculio* spp. reference sequences available at GenBank. We applied the most conservative limit used in DNA barcoding, which states a maximum genetic divergence (number of different nucleotides by the total number of compared nucleotides) of 1% with respect to the reference sequence for an unambiguous identification to the species level (Ratnasingham & Herbert 2007).

**Data analysis**

To evaluate the occurrence of the different weevil species in the 3 potential hosts *Co. avellana, Q. ilex* and *Q. pubescens* across the 5 study sites, we conducted a Pearson’s $\chi^2$-test. Similarly, we used $\chi^2$-tests for the comparison of the presence of male and female weevil proportion, as well as that of immature and mature females among sampling periods. A generalized linear mixed model (GLMM), following a binomial distribution, was used to test for the effects of the study site (RI, Ripoll; OL, Olot; MO, Montseny; MA, Maresme; PR, Prades), sampling period (1, 2, 3) and host species (*Co. avellana, Q. ilex* and *Q. pubescens*) on the seed predation rate by weevils. The factor “plot” was included as a random effect in the GLMM analyses to account for the repeated nature of the measurements and other unexplained variation. Analyses of deviance Type II Wald $\chi^2$-tests were performed to establish the significance of each different independent variable in the model. A general linear mixed model was applied to test for the effects of host species, sampling period and seed condition (sound or infested) on seed size (volume in mm$^3$) with the factor “plot” included as a random effect.

For population genetic analyses we chose those species in which there were a minimum of 10 individuals per population with sequences longer than 750 bp; namely, *Curculio glandium* and *Curculio nucum*. We used ARLEQUIN software (Excoffier et al. 2005) to calculate standard molecular diversity indices (gene diversity and nucleotide diversity) and to perform analyses of the molecular variance (AMOVAs). Signatures of population demographic changes (bottlenecks or expansions) were examined by Tajima’s $D$ (Tajima 1989) and Fu’s $F$ (Fu 1997) as implemented in ARLEQUIN software. We also tested whether there was any geographic pattern in the population genetic structure using SAMOVA 1.0 (Dupanloup et al. 2002). This method identifies the optimal grouping option ($K$) that maximizes the among-group component (FCT) of the overall genetic variance. We defined the number of populations ($K$) and ran 100 simulated annealing processes. We simulated different numbers of populations, ranging from $K = 2$ to $K = 4$, to determine the best population clustering option.

**RESULTS**

Molecular analyses allowed the identification of the larvae emerged from the seeds of the 3 host species (*Co. avellana, Q. ilex* and *Q. pubescens*) as all sequences showed a divergence below 1% with respect to *Curculio* spp. reference sequences from GenBank. This divergence was much lower than inter-specific differences, which in all cases exceeded 8%. All larvae corresponded to 4 species; namely, *C. elephas, C. glandium, C. nucum* and *C. venosus*. As shown in Figure 2, weevil species were not randomly distributed among hosts; that is, *C. nucum* was exclusively present in hazelnuts while the other 3 weevils emerged uniquely from acorns ($\chi^2_s = 263.9, P < 0.001$). *C. glandium* and *C. elephas* were more abundant in *Q. ilex* (respectively, $\chi^2_{14} = 91.8, P < 0.001$, and $\chi^2_{14} = 23.3, P < 0.001$) while there were not significant differences in the presence of *Curculio venosus* between the 2 oak species ($\chi^2_s = 7.47, P > 0.05$). The different presence of larvae of the 4 weevil species...
in the 3 hosts, especially among hazelnut and the 2 oaks, was not due to the lack of a particular species in a given site as we captured adult specimens of all weevil species along the entire gradient. Moreover, as hazelnuts were bigger than acorns during almost the entire seeding season (Table 1 and Fig. 3), the abovementioned differences in weevil specificity among these 2 groups of hosts does not seem to be due to the exclusion of certain weevil species from hazelnuts by a too small seed size.

Male and female weevils occurred in nearly the same frequency with no significant variation along the sampling periods ($\chi^2 = 2.28, P > 0.05$). Yet the proportion of females with presence of eggs and without eggs showed significant differences through the season ($\chi^2 = 33.7, P < 0.001$) and for the different weevil species. As shown in Figure 4, through the season the presence of females with eggs was earlier in C. nucum, followed by C. glandium and C. elephas. In the 2 synovigenic species, C. nucum had a decreasing pattern ($\chi^2 = 10.5, P < 0.01$) in the presence of females with eggs, while C. glandium exhibited an increasing pattern ($\chi^2 = 35.3, P < 0.001$). In the proovigenic C. elephas we did not find females on the very first sampling period but as soon as they appeared during the second and third sampling dates they were already sexually mature ($\chi^2 = 16.5, P < 0.001$). Consistently with the seasonal patterns of the presence of females ready to oviposit, we found that infestation rates showed significant variation among study sites ($\chi^2 = 16.5, P < 0.001$), sampling periods ($\chi^2 = 5.5, P < 0.05$) and host species ($\chi^2 = 6.4, P < 0.05$). Overall, infestation was higher in northern localities and it increased as the seeding season progressed (see coefficients for the different effects in Table 2). Concerning host species, infestation rates showed contrasting temporal patterns in hazelnut versus oaks (Table 2, Fig. 5), in agreement with host seeding phenology and oogene-

![Figure 3](image_url)

**Figure 3** Proportion of females with eggs (black column) and without eggs (white columns) for the 3 weevil species (*Corylus avellana, Quercus ilex* and *Quercus pubescens*) captured in the 3 sampling periods during the seeding season: Jul–Aug = from the end of July to the beginning of August; Aug–Sep = from the end of August to the beginning of September; Sep–Oct = from the end of September to the beginning of October.

<table>
<thead>
<tr>
<th>Effects</th>
<th>Estimate</th>
<th>Standard error</th>
<th>$t$-value</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>3.199</td>
<td>0.0278</td>
<td>153.912***</td>
<td>$&lt; 0.001$</td>
</tr>
<tr>
<td>Date</td>
<td>0.01695</td>
<td>0.004249</td>
<td>3.990***</td>
<td>$&lt; 0.01$</td>
</tr>
<tr>
<td>Host, Q. pubescens</td>
<td>-0.1876</td>
<td>0.008793</td>
<td>-21.336***</td>
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</tr>
<tr>
<td>Host, Q. ilex</td>
<td>-0.4808</td>
<td>0.009984</td>
<td>-48.159***</td>
<td>$&lt; 0.001$</td>
</tr>
<tr>
<td>Seed condition, sound</td>
<td>-0.03143</td>
<td>0.006962</td>
<td>-4.515***</td>
<td>$&lt; 0.01$</td>
</tr>
</tbody>
</table>

$*P < 0.05$, $**P < 0.01$ and $***P < 0.001$. 

---

**Table 1** Estimates for the significant effects of tree host, sampling period and seed condition (sound or infested) over seed size (volume in mm$^3$) according to the linear mixed model.
Causes of host specificity in seed weevils

The population genetic analyses showed marked differences between *C. nucum* and *C. glandium*. Mean genetic diversity was higher in *C. nucum* (Table 3), mainly due to the higher number of distinct haplotypes; that is, an ANOVA in which the population was included as

**Table 2** Estimates for the significant effects of study site, tree host and sampling period on weevil infestation rates according to the generalized linear mixed model

<table>
<thead>
<tr>
<th>Effects</th>
<th>Estimate</th>
<th>Standard error</th>
<th>z-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>-2.8280</td>
<td>0.5462</td>
<td>-5.177***</td>
</tr>
<tr>
<td>Site Olot</td>
<td>1.0830</td>
<td>0.4776</td>
<td>2.268*</td>
</tr>
<tr>
<td>Site Ripoll</td>
<td>1.5611</td>
<td>0.4788</td>
<td>3.260**</td>
</tr>
<tr>
<td>Host, <em>Q. pubescens</em></td>
<td>0.9615</td>
<td>0.3810</td>
<td>2.523*</td>
</tr>
<tr>
<td>Date</td>
<td>0.4634</td>
<td>0.1973</td>
<td>2.349*</td>
</tr>
</tbody>
</table>

*P* < 0.05, **P** < 0.01 and ***P*** < 0.001.

**Figure 4** Mean ± SE volume (mm$^3$) of sound (open dots) and infested (black dots) seeds of the 3 host species (*Corylus avellana*, *Quercus ilex* and *Quercus pubescens*) along the sampling dates. Jul–Aug = from the end of July to the beginning of August; Aug–Sep = from the end of August to the beginning of September; Sep–Oct = from the end of September to the beginning of October. Notice the difference in the scale of the y-axis for the 3 host species.

**Figure 5** Mean ± SE seed infestation rate of the 3 host species (*Corylus avellana*, *Quercus ilex* and *Quercus pubescens*) along the sampling dates. Jul–Aug = from the end of July to the beginning of August; Aug–Sep = from the end of August to the beginning of September; Sep–Oct = from the end of September to the beginning of October.

sis in females; that is, in hazelnuts infestation occurred earlier and slightly decreased through the season, while it was absent during the first sampling date in the 2 oak species, and progressively increased towards the end of the season (Fig. 5).
Table 3 Values of gene diversity, nucleotide diversity, Tajima’s D and Fu’s F recorded at each population for Curculio nucum (a) and Curculio glandium (b)

<table>
<thead>
<tr>
<th></th>
<th>Gene diversity</th>
<th>Nucleotide diversity</th>
<th>Tajima’s D</th>
<th>Fu’s F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ripoll</td>
<td>0.87</td>
<td>0.0015</td>
<td>-1.63*</td>
<td>-4.54***</td>
</tr>
<tr>
<td>Olot</td>
<td>0.75</td>
<td>0.0024</td>
<td>-1.96**</td>
<td>-8.34***</td>
</tr>
<tr>
<td>Montseny</td>
<td>0.89</td>
<td>0.0032</td>
<td>-1.40*</td>
<td>-6.87***</td>
</tr>
<tr>
<td>Maresme</td>
<td>0.88</td>
<td>0.0034</td>
<td>-0.41</td>
<td>-1.20</td>
</tr>
<tr>
<td>Prades</td>
<td>0.59</td>
<td>0.0012</td>
<td>-1.69**</td>
<td>-5.27***</td>
</tr>
<tr>
<td>(b) Curculio glandium</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ripoll</td>
<td>0.71</td>
<td>0.0012</td>
<td>-1.10</td>
<td>-2.61**</td>
</tr>
<tr>
<td>Olot</td>
<td>0.57</td>
<td>0.0009</td>
<td>-0.46</td>
<td>-0.84</td>
</tr>
<tr>
<td>Montseny</td>
<td>0.69</td>
<td>0.0013</td>
<td>-1.22</td>
<td>-2.61**</td>
</tr>
<tr>
<td>Maresme</td>
<td>0.69</td>
<td>0.0013</td>
<td>-0.75</td>
<td>-1.95</td>
</tr>
<tr>
<td>Prades</td>
<td>0.63</td>
<td>0.0034</td>
<td>-1.79</td>
<td>1.46</td>
</tr>
</tbody>
</table>

*P < 0.05, **P < 0.01 and ***P < 0.001.

a random factor showed that mean gene diversity was significantly higher in C. nucum ($F_{1,4} = 9.40; P = 0.03$). A total of 31 haplotypes were retrieved from the 118 sequences of C. nucum included in the analyses versus just 13 from 96 sequences in C. glandium (see Tables 4 and 5). In C. nucum, 48% of the individuals had the most common haplotype but there were many rare haplotypes sometimes found in just one individual and/or at a single population (Table S1). In the case of C. glandium, there were much fewer rare haplotypes and the two most frequent ones were found in 80% of the individuals (Table S2). The high proportion of rare haplotypes in C. nucum suggests population expansion after recent bottlenecks and, accordingly, both Tajima’s D and Fu’s F had negative and significant values in all populations except one. In the case of C. glandium only for Fu’s test were the values significant in two populations, thus showing that most populations were in equilibrium (Tajima 1989; Fu 1997). The AMOVA revealed a more restricted gene flow between populations in the case of C. nucum, in which differentiation among populations explained 5.02% of the total molecular variance (degrees of freedom = 4; $P < 0.01$), whereas in C. glandium inter-population differences were not significant. The results of the SAMOVA were marginally significant for C. nucum ($F_{CT} = 0.08$; degrees of freedom = 1; $P = 0.08$) and defined two clusters within the geographical range of our study, the first grouping the nearby populations of Montseny (MO) and Maresme (MA) and another one including the rest (see Fig. 1). No significant geographical pattern of molecular variance was found in C. glandium.

DISCUSSION

Seed infestation by weevils did not occur randomly but with 2 opposite breadths of host specificity; namely, the highly specialized C. nucum infested a unique host (hazelnuts), while up to 3 species (C. glandium, C. elephas and C. venosus) predated almost indistinguishably upon 2 oaks (Q. ilex and Q. pubescens). These differences in trophic specificity coupled with differences in the geographical distribution of the hosts resulted in 2 distinct patterns concerning the genetic characteristics of weevils’ populations; that is, we only found a significant genetic structure among the populations in the highly specialist C. nucum. Ultimately, the results of these genetic analyses confirmed the specialist or generalist trophic breadth of the different weevil species according to the identification of the larvae found in the seeds and they stress how molecular techniques may help to establish unequivocal trophic relations for seed feeding insects.

Previous studies have suggested that seed size has been a relevant trait promoting ecomorphological adaptations in the genus Curculio and driving species diversification (Hughes & Vogler 2004a; see also Peguero et al. 2017). In the end, a tight relationship between seed and weevils’ body size would result in differences in the ability of larger and smaller weevils to infest seeds of different size (differences in trophic breadth); that is, small species would be able to infest both small and large seeds while species with a larger body size would be restricted to larger seeds to obtain enough resources to complete larvae development (see Bonal & Muñoz 2008; Espelta et al. 2009a; Bonal & Muñoz 2011; Peguero et al. 2017). Yet, this does not seem to be the case in our study system where hazels, the species infested by a single species (C. nucum), showed the largest seeds during most of the season (Fig. 3) and experienced the lower infestation rate (see Table S3). Instead of an influence of seed size, our results suggest that the exclusive infestation of hazelnuts by C. nucum could be more related to a different pattern of sexual maturation of females among the 2 weevil species emerging from the soil in spring, specifically an earlier maturation in C. nucum in comparison to C. glandium (Fig. 4). These dif-
ferences could be due to differences between the 2 weevils in the requirements of resource acquisition as it has been demonstrated that sexual maturation in females of these synovigenic species critically requires some feeding at adulthood before reproductive development takes place (Bel-Venner et al. 2009; Pélisson et al. 2012). The early maturation in C. nucum would be advantageous to oviposit in hazelnuts before the hardening of the nutshell, as this is a fast process occurring during seed growth and the main mechanism in hazels to avoid infestation (Guidone et al. 2007). Moreover, oviposition of C. nucum would be expected to occur soon after mating as weevils do not adjust laying eggs to the moment of highest seed availability, but they oviposit as soon as females have mature eggs (Bonal et al. 2010). This behavior is probably linked to the temporal unpredictability of seed crop size (Bonal & Muñoz 2008; Espelta et al. 2008) and other constraints they have to cope with, such as the need of rainfall episodes to soften the soil and allow the emergence of adults (Bonal et al. 2010, 2015; Espelta et al. 2017). Only during the 2 earlier samplings, females of C. nucum seemed to preferentially choose bigger seeds, a behavior related with the need to select a minimum seed size to ensure larvae development and also owing to the availability of more seeds for oviposition (see Espelta et al. 2009a).

In comparison to the extreme host–parasite specificity of C. nucum, the other 3 weevils (C. venosus, C. glandium and C. elephas) predated indistinctively upon the 2 oaks with no evidence of a strategy in the partitioning of this resource according to the identity of the host species or to seed size. The avoidance of competitive exclusion among these species could be explained by several mechanisms contributing to stabilize their coexistence; that is, time partitioning (Pélisson et al. 2012; see also Espelta et al. 2009a) and/or diversification of dispersal-dormancy strategies (Pélisson et al. 2012). On the one hand, time partitioning in breeding activity can exist when 2 competing species differ in the speed of energy acquisition to be allocated to reproduction by females and the duration of their lifespan; that is, one species acquires resources faster and it is able to oviposit earlier on seeds, but it is exposed to a higher risk of seed abortion, while the other oviposits later but has a longer life span allowing it to lay eggs during a larger time frame (see Pelisson et al. 2012 for C. pellitus and C. glandium). On the other hand, stabilization can be reached by means of different dispersal versus dormancy strategies with some species relying on a high dispersal ability and others depending on dormancy strategies (e.g. variable diapause) to cope with seed scarcity. This seems to be the case for C. glandium and C. elephas; that is high dispersal ability (up to 11 km) in the former species and an extended diapause (up to 3 years) in the later (see Venner et al. 2011; Pélisson et al. 2012). Yet, other factors not covered in this study, such as the risk of parasitism or survival of larvae during diapause, may also help equalize their success to infest (Bonal et al. 2011). Similarly, future studies with more intense and appropriate sampling schemes should address the relationship between the number of adults of the different species and the number of larvae to disentangle the different predation rates upon each species and the influence of other environmental factors.

Ultimately, differences in the trophic breadth leave a contrasting genetic signature in the populations of the 2 species of weevils. A much higher number of local rare haplotypes were found in the monophagus C. nucum, along with a marginally significant genetic structure among populations, contrary to the more generalist C. glandium (see, for a similar example in aphids, Gaete-Eastman et al. 2004). Inter-specific differences in genetic characteristics of phytophagous insects could arise from differences in their dispersal ability or in the spatial distribution (isolated vs continuous) of the host (Peterson & Denno 1998; Kubish et al. 2014). Unfortunately, in comparison to the precise information about the dispersal ability of C. glandium (approximately 10 km in Pélisson et al. 2012), we lack detailed knowledge about the dispersal range of C. nucum, except some evidence of weevils moving away from local sites to feed during adulthood and prior to mating (Bel-Venner et al. 2009). Yet the fact that C. nucum and C. glandium are sister species (Hughes & Voegl 2004a) and they share many similar life-history traits, such as ecomorphological adaptations and body size, adult emergence in spring, synovigenic females and a fixed diapause of 2 years (see Hughes & Vogler 2004a; Bel-Venner et al. 2009; Pélisson et al. 2012a,b), make us consider that they may have a similar dispersal ability. Therefore, the differences we observed in their genetic characteristics would be probably due to their different diet breadth and the more patchy and discontinuous distribution of hazels in comparison to the more abundant and constant presence of oaks (Gracia et al. 2004; see also Fig. 1), along the geographical gradient sampled (approximately 225 km from Ripoll to Prades).

Connectivity may be critical for population survival (Fahrig & Merrian 1985; Fahrig & Paloheimo 1988) and metapopulation dynamics (Levins 1970), especially in...
CONCLUSION

The use of molecular analyses allowed us to precisely identify the weevil species depredating upon the various potential hosts in these mixed deciduous forests and to unequivocally confirm the high specificity of the hazelnut *C. nucum* and the more flexible and wider trophic breadth of the rest of the weevils (*C. venosus, C. glandium* and *C. elephas*) depredating upon acorns. In this multi-host and multi-parasite system, co-existence of the various weevil species seems to be mediated by a combination of extreme resource partitioning (i.e. among *C. nucum* and the rest of species) and a combination of time partitioning and differences in dispersal-dormancy strategies among the 3 species depredating upon oaks. Interestingly, although sometimes suggested, differences in seed size did not have any effect in driving host specificity or the trophic breadth of the weevil species present. Moreover, our results highlight that differences in specificity in trophic breadth and in the spatial distribution of hosts at a large geographical scale may result in the presence of genetic structure among the populations of highly specific parasites (*C. nucum*) depredating upon patchily distributed hosts (*Co. avellana*). To what extent this restriction in gene flow (dispersal) may have negative consequences for the persistence of the populations of these highly specialized seed-feeding pests in a time of increasing disturbances (e.g. drought events in Mediterranean areas) is a fascinating question that deserves further research.

ACKNOWLEDGMENTS

This research was supported by the projects FOR-ASSEMBLY (CGL2015-70558-P) and PLAGANADO (AGL2014-54739-R) of the Spanish Ministry of Economy, and the projects BEEMED (SGR913) (Generalitat de Catalunya) and PI1C09-0256-9052 (Regional Government of Castilla-La Mancha and the European Social Fund). R.B. was funded by a contract of the Program Atracción de Talento Investigador (Gobierno de Extremadura).

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SUPPLEMENTARY MATERIALS

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

Table S1 Number of Curculio nucum individuals bearing each haplotype in the 5 study populations

Table S2 Number of Curculio glandium individuals bearing each haplotype in the 5 study populations

Table S3 Mean ± SE density of host plants and the percentage of sound and infested seeds per location and host plant. Density of host plants was calculated as the mean of the nearest inventoried plots included in the Catalan Forest Inventory (Gracia et al. 2004).

Cite this article as:

Eastern gray squirrels are consistent shoppers of seed traits: insights from discrete choice experiments

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Abstract

Seeds of many hardwood trees are dispersed by scatter-hoarding rodents, and this process is often mediated by the traits of seeds. Although numerous studies have linked seed traits to seed preference by rodents, little is known about how rodents forage for seeds when multiple desirable and undesirable seed traits are available simultaneously. Here, we adopt a novel method of designing choice experiments to study how eastern gray squirrels (Sciurus carolinensis) select for 6 traits (caloric value, protein content, tannin concentration, kernel mass, dormancy period and toughness of shell) among seeds. From n = 426 seed-pair presentations, we found that squirrels preferentially consumed seeds with short dormancy or tougher shells, and preferentially cached seeds with larger kernel mass, tougher shells and higher tannin concentrations. By incorporating random effects, we found that squirrels exhibited consistent preferences for seed traits, which is likely due to the fitness consequences associated with maintaining cached resources. Furthermore, we found that squirrels were willing to trade between multiple traits when caching seeds, which likely results in more seed species being cached in the fall. Ultimately, our approach allowed us to compute the relative values of different seed traits to squirrels, despite covariance among studied traits across seed species. In addition, by investigating how squirrels trade among different seed traits, important insights can be gleaned into behavioral mechanisms underlying seed caching (and, thus, seed survival) dynamics as well as evolutionary strategies adopted by plants to attract seed dispersers. We describe how discrete choice experiments can be used to study resource selection in other ecological systems.

Key words: cached resources, discrete choice experiment, resource selection, trade-offs, trait-dependent

INTRODUCTION

Scatter-hoarding rodents influence tree regeneration processes in 2 ways that have opposing effects on a tree’s fitness (Vander Wall 1990). First, by consuming large proportions of seeds, rodents can reduce numbers
of seeds available for recruitment (Chambers & McMahon 1994). Alternatively, scatter-hoarding rodents may disperse seeds horizontally away from the parent plant and bury seeds in caches, thereby enhancing chances of germination for unrecovered seeds (Chambers & McMahon 1994). Both predation and dispersal behaviors of scatter-hoarding rodents are influenced by traits of seeds (Wang & Chen 2008; Wang et al. 2012; Sundaram et al. 2015). Therefore, understanding how seed traits affect the perceived value of seeds to granivores is important because of potential ramifications for seed mortality and dispersal (Wang & Smith 2002; Wang et al. 2012).

Multiple seed traits influence rodent foraging decisions (Xiao & Zhang 2006; Wang et al. 2012). On encountering a seed, scatter-hoarding rodents likely evaluate quality and condition (Preston & Jacobs 2009; Delgado et al. 2014). Seeds germinating immediately or seeds with a lower mass are of a low storage value and tend to be consumed immediately and carried shorter distances (Smallwood et al. 2001; Sundaram et al. 2015). In contrast, seeds with thick shells or seeds with large kernel masses are of higher storage value and tend to be transported and cached at greater distances (Sundaram et al. 2015). Given that multiple seed traits influence a rodent’s decision to cache or consume seed and that different clades of hardwood trees produce predictable combinations of seed traits (Sundaram et al. 2015), selection of a seed type may not result in maximization of all desired traits and minimization of less desirable traits. Furthermore, changes in rodent preference due to 1 seed trait may be offset by changes in another trait. Thus, the following question arises: How do rodents trade between multiple traits?

Consumer economic models routinely quantify “attractiveness” of product traits, and the tradeoffs made by consumers selecting for multiple product traits (Brown ing & Zupan 2015). Whenever selection occurs among discrete items (e.g. discrete prey items), economists typically use a class of methods called discrete choice models and experiments (DCMs and DCEs) to study trait-based selections (Louviere et al. 2010). These methods offer 2 major benefits. First, with DCMs traits are modeled together using a common currency of “utility,” defined as the attractiveness of a product (or satisfaction derived from consuming a product) to the decision-maker (Louviere et al. 2010). The utility is typically inferred from choices made by the consumer given all of the alternatives that are available (Louviere et al. 2010). Utility models such as DCMs describe how product attractiveness varies as a function of product traits (Louviere et al. 2010). Traits are linked together under a unitless currency of utility. Thus, it is possible to examine how variations in different traits alter the utility of a product, how variations in 1 trait can offset utility changes resulting from variations in a second trait, and, hence, how a decision-maker trades among traits (Louviere et al. 2010; Browning & Zupan 2015). A second benefit is the ability to maximize the amount of information captured from choice data through the use of DCEs (Louviere et al. 2010). DCEs involve construction of choice scenarios with the goal of quantitatively eliciting preferences for attractive and unattractive traits (Louviere et al. 2010). By designing trait levels of alternatives ahead of time and then presenting designed alternatives to consumers, DCEs can maximize the information captured in choice data (Louviere et al. 2010). More specifically, this is achieved by ensuring orthogonality and equal representation of trait levels in the design (Kuhfeld 2010). Both orthogonality and equal representation of trait levels allow for choices made by individuals to be tied to specific traits (Louviere et al. 2010). DCMs without DCEs have been used previously by ecologists to study prey selection and habitat use as well as tradeoffs made with respect to habitat and prey traits (McCracken et al. 1998; Cooper & Millspaugh 1999; McDonald et al. 2006; Cooper et al. 2007). While DCEs have been extensively employed in consumer economics to construct trait-based experiments (Louviere et al. 2010), to our knowledge they have not yet been used in ecology.

In this paper, we describe and combine a DCE and DCM approach to evaluate how preferences for seeds in eastern gray squirrels (Sciurus carolinensis Gmelin, 1788) are related to seed traits. We utilize a DCE to increase the number of unique combinations of traits presented to squirrels and then model the resulting choice data with a DCM to quantify squirrels’ willingness-to-trade 1 seed trait against another. We then discuss the implications of willingness-to-trade estimates from the perspective of the squirrel and the perspective of the tree.

MATERIALS AND METHODS

Overview of discrete choice experiments

Discrete choice experiments offer a means to maximize the statistical power associated with detection of trait-based selections whenever a consumer selects between 2 or more items. Subjects in DCEs select between alternatives in a set of pre-determined, designed choice scenarios (Kuhfeld et al. 1994; Louviere et al. 2010).
Figure 1  Four steps in designing and analysing data from discrete choice experiments (DCE). Steps outlined for: (a) a hypothetical discrete choice experiment with 2 traits and (b) the discrete choice experiment in this study with 4 traits. The 4 steps include: (1) select traits, (2) categorize traits, (3) determine all possible alternatives or combinations of traits, (4) pair trait combinations in choice scenarios, and (5) data collection and analysis. In a(1), traits X and Y are selected, which represent price and nutrient quantity in milk brands. In a(2), traits X and Y are categorized into low and high values. In a(3), all combinations of low and high for traits X and Y are listed, such that items are represented across rows and traits across columns. In a(4), pairings of trait combinations are considered in a candidate design. Multiple choice scenarios are designed so that each scenario presents 2 alternatives, and the group of scenarios allows for all possible contrasts among trait levels to be examined. In a(5), choice scenarios are presented to subjects and selections are analysed. In b(1), 4 seed traits are selected based on patterns of inheritance across phylogenetic clades (more details on phylogenetic analysis in Sundaram et al. 2015). In b(2), seed traits are categorized into low and high or low, medium and high. In b(3), examples of all possible combinations of low, medium or high for the 4 traits are listed. Some combinations are represented by naturally occurring seeds, including Fagus grandifolia, Corylus americana, Carya glabra and Castanea mollissima. Others do not occur in natural seeds and are eliminated from the list, shown as “No seed.” In b(4), pairs of seeds are arranged in a design that attempts to capture all possible contrasts with the minimal number of scenarios. In b(5), choice scenarios are presented to squirrels and selections are analyzed.
Individually, each scenario represents a choice between items, each of which contains a specific combination of traits. Provided that researchers are willing to discretize traits into levels, careful selection of the items presented in each scenario will allow them to construct a set of scenarios in which all possible combinations of traits are presented while maintaining a balanced and orthogonal statistical design (Kuhfeld et al. 1994). For example, consider a hypothetical experiment involving 2 traits (Fig. 1a, step 1). The 2 traits may represent the price and quantity of nutrients in several different brands of milk, where consumer preference for price relative to nutrients is unknown. After discretizing the 2 traits into high and low levels (akin to treatment levels in analysis of variance; Fig. 1a, step 2), all possible combinations of the trait levels are represented by 4 different alternatives (Fig. 1a, step 3). These combinations of traits, which are found in the different brands of milk, are then paired (Fig. 1a, step 4), presented to consumers, choices are recorded, and the selections are interpreted as a function of the milk-brand traits (price and nutrient content) using discrete choice models or DCMs (Fig. 1a, step 5; Louviere et al. 2010).

Importantly, discrete choice pairings (Fig. 1a, step 4) represent a balanced and orthogonal design. The design is balanced because the number of “lows” in the design equals number of “highs” represented for each trait. The design is also orthogonal because trait X and Y are not correlated to one another in the final design, which is important to decouple the effects of the traits on the choices made by subjects. In more complex experiments that involve multiple traits or trait levels (e.g. Fig. 1b), an orthogonal and balanced set of choice scenarios can be selected using design algorithms (Kuhfeld et al. 1994).

In contrast to standard, observational study designs, DCEs can more easily parse out the effects of correlated traits on preference by pairing items so that correlations among trait levels in the final design are minimized, ideally to zero (Hensher et al. 2005; Kuhfeld 2010). In addition, DCEs can take advantage of fractional factorial designs that maximize the proportion of information captured relative to the full factorial design while minimizing the number of unique scenarios presented. The proportion of information captured in a candidate design is typically measured using an optimality criterion such as D-efficiency (Hensher et al. 2005; Kuhfeld 2010; Louviere et al. 2010). Consequently, the designed approach avoids logistical difficulties of using full factorial designs where every trait level combination must be paired and tested (Kuhfeld 2010; Louviere et al. 2010).

Selection of seed traits and design of choice experiments

Four seed traits were used to design choice experiments and were selected from a suite of 11 seed traits for which we had estimates. Summaries of all 11 seed traits (including measurements and correlations) across 23 hardwood tree species are provided in Sundaram et al. (2015). Briefly, species seed trait estimates are highly correlated to one another and show distinct patterns of inheritance across phylogenetic clades. When a phylogenetic PCA was employed on all traits, 3 axes with varying degrees of phylogenetic autocorrelation were found to be important determinants of eastern gray squirrel behavior (Sundaram et al. 2015; Fig. 1b, step 1). The first phylogenetically correlated axis differentiated seeds with high lipid concentration and thick shells (such as Juglans and Carya) from species with high carbohydrate concentration and thin shells (such as Quercus). The second phylogenetically correlated axis differentiated high protein seeds (such as Juglans and Castanea seeds) from low protein and high tannin seeds (such as Quercus section Quercus). The third axis showed no correlation to phylogeny and differentiated seeds of higher kernel mass from seeds of lower kernel mass. All 3 axes significantly predicted squirrel foraging behaviors such as handling time and transport distance to cache site (Sundaram et al. 2015); thus, we selected and combined seed traits from these axes to obtain 4 seed traits for designing choice experiments (Fig. 1b, step 1): (1) total caloric value of a seed (calories, product of kernel mass and calories/gm); (2) shell toughness (Nm or Newton-meter, product of shell thickness and shell hardness); (3) protein content (product of kernel mass and protein concentration); and (4) Tannin content (product of kernel mass and tannin concentrations, measured as % tannic acid equivalent or %TAE in radial diffusion assays).

To design the DCE scenarios, we discretized trait values into categories of low and high (2 levels), or low, medium and high (3 levels) by identifying natural breaks in seed trait values across the candidate seed set (Fig. 1b, step 2; see Table S2 and Suppl. Fig. 1 in Supporting Information 1 for break points). Although traits may be divided into more than 3 categories, increasing the number of discretized levels significantly increases the number of combinations to be tested (Hensher et al. 2005). Therefore, we limited the number of levels investigated to preserve the tractability of the design. Note
that the discretization used here applied only to the selection of items for the experimental design; analysis is based on the actual, continuous trait values of the items presented.

Next, a list of all unique combinations of low or medium or high attribute levels of the 4 seed traits was compiled (Fig. 1b, step 3). Because 3 traits had 2 levels and 1 trait had 3 levels (Table S2 in Supplementary Materials 1), this step produced 24 \((2^3 \times 3^1)\) unique combinations of seed trait levels, each representing a particular type of seed. Many of these trait-level combinations are represented by naturally occurring seed species (e.g. *Fagus grandifolia* seeds have low kernel mass, low protein content, low tannin content and low toughness levels (Fig. 1b, step 3). However, many combinations of trait levels generated in the list do not exist in real seeds. Therefore, we eliminated 14 unrealistic combinations from the design (represented as “No seed” in Fig. 1b, step 3).

Finally, we used PROC PLAN and OPTEX in SAS 9.2 to identify an optimal set of choice scenarios using the remaining 10 seed types (Fig. 1b, step 4). PROC PLAN generates a full factorial design with all possible choice scenarios (Kuhfeld et al. 1994). In our study, this consisted of 45 \((10 \text{ choose } 2)\) choice scenarios. PROC OPTEX uses the modified Federov algorithm to drop and add candidate choice scenarios and identify those that maximize an optimality criterion (Kuhfeld et al. 1994). For our optimality metric, we used D-efficiency, a measure of the proportion of information cap-

### Table 1 Paired seeds (or choice scenarios) designed based on seed traits and presented to squirrels

<table>
<thead>
<tr>
<th>Seed pairs</th>
<th>Species 1</th>
<th>Protein mass</th>
<th>Tannin mass</th>
<th>Toughness</th>
<th>Seed type</th>
<th>Species 2</th>
<th>Protein mass</th>
<th>Tannin mass</th>
<th>Toughness</th>
<th>Seed type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
<td>Medium</td>
<td><em>Corylus americana</em></td>
<td>Low</td>
<td>High</td>
<td>Low</td>
<td>High</td>
<td><em>Carpinus carolinensis</em></td>
</tr>
<tr>
<td>2</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
<td><em>Fagus grandifolia</em>, <em>Quercus velutina</em>, <em>Quercus muehlenbergii</em>, <em>Quercus palustris</em>, chestnut hybrid, <em>Q. macrocarpa</em>, <em>Quercus alba</em> or <em>Quercus coccinea</em></td>
<td>Low</td>
<td>High</td>
<td>Low</td>
<td>High</td>
<td><em>Carya tomentosa</em></td>
</tr>
<tr>
<td>3</td>
<td>Low</td>
<td>Low</td>
<td>High</td>
<td>Medium</td>
<td><em>Carya ovata</em> or <em>Carya cordiformis</em></td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
<td>Medium</td>
<td><em>Corylus americana</em></td>
</tr>
<tr>
<td>4</td>
<td>Low</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
<td><em>Castanea dentata</em></td>
<td>Low</td>
<td>Low</td>
<td>High</td>
<td>Medium</td>
<td><em>Carya ovata</em> or <em>Carya cordiformis</em></td>
</tr>
<tr>
<td>5</td>
<td>Low</td>
<td>High</td>
<td>Low</td>
<td>High</td>
<td><em>Carya tomentosa</em></td>
<td>Low</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
<td><em>Castanea dentata</em></td>
</tr>
<tr>
<td>6</td>
<td>Low</td>
<td>High</td>
<td>Low</td>
<td>High</td>
<td><em>Carya tomentosa</em></td>
<td>High</td>
<td>High</td>
<td>Low</td>
<td>High</td>
<td><em>Juglans nigra</em>, <em>Juglans cinerea</em> or <em>Juglans regia</em></td>
</tr>
<tr>
<td>7</td>
<td>High</td>
<td>High</td>
<td>Low</td>
<td>High</td>
<td><em>J. nigra</em>, <em>J. cinerea</em>, or <em>J. regia</em></td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
<td><em>F. grandifolia</em>, <em>Q. velutina</em>, <em>Q. muehlenbergii</em>, <em>Q. palustris</em>, BC3 chestnut hybrid, <em>Q. macrocarpa</em>, <em>Q. alba</em> or <em>Q. coccinea</em></td>
</tr>
<tr>
<td>8</td>
<td>High</td>
<td>High</td>
<td>Low</td>
<td>High</td>
<td><em>J. nigra</em>, <em>J. cinerea</em>, or <em>J. regia</em></td>
<td>High</td>
<td>High</td>
<td>High</td>
<td>Medium</td>
<td><em>Quercus michauxii</em></td>
</tr>
<tr>
<td>9</td>
<td>High</td>
<td>High</td>
<td>Medium</td>
<td>Low</td>
<td><em>Q. michauxii</em></td>
<td>Low</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
<td><em>Carya tomentosa</em></td>
</tr>
<tr>
<td>10</td>
<td>High</td>
<td>High</td>
<td>High</td>
<td>Low</td>
<td><em>Q. rubra</em> or <em>Q. prinus</em></td>
<td>High</td>
<td>High</td>
<td>High</td>
<td>Low</td>
<td><em>Castanea mollissima</em></td>
</tr>
</tbody>
</table>

*These seeds were used in the first phase of data collection. Species 1 and 2 were presented to eastern gray squirrels (*Sciurus carolinensis*) by balancing seeds on golf tees. In the second phase of data collection, whenever multiple species could represent attribute combinations, one species was randomly selected from the list.
tured relative to a fully orthogonal and balanced design, where 100% signifies a completely orthogonal and balanced design (Kuhfeld et al. 1994). Our final design had a D-efficiency of 92% and consisted of only 10 choice scenarios, a 78% reduction in logistical requirements relative to the full factorial design (Table 1; Fig. 1b, step 4).

Designing the choice experiments on the basis of these 4 seed traits allowed us to maximize the amount of information captured, particularly with respect to the 4 traits. This step is especially important in this study given that traits of seeds are correlated to one another and redundant combinations of seed traits exist within a phylogenetic clade (Sundaram et al. 2015). Of course, other traits or variables can be considered post hoc in analysis. The results of these post hoc assessments then reflect an approach more like other observational DCMs (McCracken et al. 1998; Cooper & Millspaugh 1999; McDonald et al. 2006; Cooper et al. 2007). We used average trait values for each species in our analysis, which could produce misleading results if intraspecific variation in traits was large relative to interspecific variation. Fortunately, this was not the case; interspecific variation in seed trait estimates was significantly higher than intraspecific variation in seed trait estimates, based on 3 different tests (details in Supplementary Materials 1).

**Data collection and field methods**

To implement a DCE in consumer economic studies, subjects are individually presented with choice scenarios and their selections are recorded. Each scenario is replicated multiple times. Ideally, scenarios are replicated across multiple independent subjects, and each subject is presented with all scenarios in a randomized sequence. When scenarios cannot be presented to subjects in a completely controlled manner (a common situation in field studies), a random effects model may be used to search for evidence of non-independence post hoc (see section on “Random parameters logit model”).

We collected choice data for the 10 choice scenarios in our study during 2 phases. The first phase occurred between 1 September 2012 and 5 January 2013. Nine seed species were used to represent the 10 seed types (Table 1; Fig. 1b step 4). For each choice, a pair of seeds was presented to a free-ranging eastern gray squirrel on the Purdue University campus. Presentation locations were separated by at least 150 m to minimize the chance that data were collected from the same small group of squirrels. Exceptions to this proximity rule were made only if we could present seeds to different squirrels at the same time. For each presentation, an observer balanced a pair of seeds (Table 1) on golf tees spaced approximately 3 cm apart at the base of a tree and waited for a squirrel to approach the seed pair. Observers noted the seed species selected first and its fate (cached or eaten). The next seed pair was then presented on the golf tees, until the squirrel stopped making selections or until data for all 10 choice scenarios was collected from the squirrel. The order in which the 10 paired trait levels were presented was randomized for each location.

The second phase of data collection occurred between 4 November 2012 and 5 January 2013. During this phase, we replaced seed species for those seed types in the design that could be represented by more than 1 species (Table 1). When more than 2 species could represent a particular seed type, we randomized the species assignments across presentations. The final design included the same 10 choice scenarios as phase 1, but a total of 20 seed species were used to represent the 10 seed types in the design (Table 1). Phase 2 thus enabled us to verify that choices truly were dependent on seed traits rather than on seed species alone. Presentations followed the same procedure as phase 1. It was not possible to record data blind because our study involved focal animals in the field.

**Data analysis**

Seed selection by squirrels was analyzed using 2 different types of DCM. We initially fit simple multinomial logit models to the data. Then we fit a random parameters logit model to test for heterogeneity in preferences, a post hoc indicator of non-independence among the choice trials. Finally, we used the results from these models to calculate willingness-to-trade estimates. These estimates quantify the degree to which the squirrels in the study viewed different seed traits as tradable. The next 3 sections describe these models and metrics in greater detail.

*Multinomial logit model*

Choice experiments can be analyzed using DCMs based on random utility theory. In economics, utility is interpreted as satisfaction derived from consuming or using a product. Although utility cannot be measured directly, it can be inferred from choices made by individuals that select among a set of options. Random utility models assume a decision-maker (a squirrel in this study) always maximizes utility by selecting the alternative with the highest utility from all available options (i.e. 1 of the seed species in a presentation) (Manski 1977). Mathematically, utility for the i<sup>th</sup> selection event (i.e.
seed pair presentation) and jth alternative (i.e. jth seed species) is a random variable ($U_{ij}$) expressed as:

$$U_{ij} = V_{ij} + \varepsilon_{ij}. \quad (1)$$

Here, $V_{ij}$ is the systematic portion of utility and $\varepsilon_{ij}$ is an independently and identically distributed error term with a Gumbel distribution. In our current use, each alternative represents a collection of traits, and selection of a particular seed species implies selection of the trait values associated with that species. To determine the preferences associated with specific traits, we defined the systematic portion of utility ($V_{ij}$) as

$$V_{ij} = \beta_{0j} + \beta_1 x_{ij1} + \beta_2 x_{ij2} + \cdots + \beta_n x_{ijn}, \quad (2)$$

where $x_{ij1}, x_{ij2}, \ldots, x_{ijn}$ are the n trait values associated with the jth alternative in the ith choice scenario, $\beta_{0j}$ is an intercept explaining utility of the jth alternative as a result of factors other than the n traits included in the model, and $\beta_1, \beta_2, \ldots, \beta_n$ are preference coefficients associated with each of the n traits.

The DCM assumes that decision-makers select alternative k if $U_{ik} > U_{ij}, \forall k \neq j$. Therefore, the probability of a decision-maker selecting the kth alternative is given by (Louviere et al. 2000):

$$P_{ik} = P(V_{ik} + \varepsilon_{ik} > V_{ij} + \varepsilon_{ij}), \forall k \neq j. \quad (3)$$

With a Gumbel error distribution, equation (3) reduces to the multinomial logit model (Boxall & Adamowicz 2002):

$$P_{ik} = \frac{e^{V_{ik}}}{\sum_{j=1}^{n} e^{V_{ij}}}. \quad (4)$$

The model may be fit using the method of maximum likelihood, provided that one of the potential alternatives in the overall experiment (i.e. one of the seed types) is defined as a reference case and assigned a fixed utility, typically 0. The odds of selecting alternative k over a reference alternative r are (Louviere et al. 2000):

$$\frac{P_{ik}}{P_{ir}} = \frac{e^{V_{ik}}}{e^{V_{ir}}} = e^{V_{ik} - V_{ir}} \quad (5)$$

and the logarithm of the odds of choosing k over r is (Louviere et al. 2000):

$$\log \left[ \frac{P_{ik}}{P_{ir}} \right] = V_{ik} - V_{ir} = \sum_n \beta_n (X_{ikn} - X_{irn}). \quad (6)$$

Therefore, the model may be fit by setting the utility of the reference alternative r to 0 and solving $j - 1$ simultaneous linear equations for the log odds of selecting $j - 1$ alternatives over r in a choice scenario (Louviere et al. 2000). Derivations and further mathematical details can be found elsewhere (McCracken et al. 1998; Louviere et al. 2000).

Initially, we separated choice events into 2 datasets, those for which caching was observed and those for which consumption was observed. We then fit models to each dataset using as covariates the 4 seed trait variables on which the design was based. In addition, we created a third alternative of opting to ignore a seed pair in order to accurately model all possible alternatives available to a squirrel. This third alternative had a value of 0 for all seed traits and took a value of 1 for an indicator variable called “ignore” if the squirrel investigated the present seeds but left the area without taking either seed (if a seed was selected, “ignore” = 0). We interpret the utility of “ignore” as the value that the squirrel perceives in leaving to pursue other activities; in other words, the utility of ignore represents the missed opportunity cost associated with selecting a seed. We separated our 4 combined variables back into individual seed traits (e.g. calories in kernel was separated back into kernel mass and calories per gram in models; Fig. 1a) and iteratively added each trait to models (see Supplementary Materials 2 for a list of all models examined). At each stage, we used likelihood ratio tests to examine if the addition of a seed trait improved fit either as a fixed effect or as a random effect (Supplemental Materials 2).

Both caching and seed consumption choice events were influenced by similar traits; therefore, for our final models, we combined the caching and consumption datasets and used an indicator variable for caching (1 = cached, 0 = consumed) and interaction terms to estimate different coefficients for traits when seeds were cached versus when seeds were consumed. The final model included kernel mass (g), toughness of shell (Nm), tannin concentration of kernel (g per 100 g of kernel), dormancy period (measured by average number of cold stratification days required to germinate a given seed type, Sundaram et al. 2015) and the corresponding interaction terms with caching events. Therefore, the model can be expressed as:
Furthermore, we evaluated whether “unfamiliar” seeds influenced seed selections. We added an indicator variable to the model in Equation (7) to quantify how seed familiarity influenced utility of a seed ($1$ = unfamiliar seeds which included chestnut species, $0$ = familiar seeds). A seed species was considered familiar if the tree species occurred within the geographic extent of the study area. This additional parameter resulted in the following model structure:

$$V_{ij} = \beta_0 + \beta_1 \text{Kernel}_{ij} + \beta_2 \text{Tannin}_{ij} + \beta_3 \text{Tough}_{ij} + \beta_4 \text{Dormancy}_{ij} + \beta_5 \text{Kernel}_{ij} \cdot \text{cache}_{ij} + \beta_6 \text{Tannin}_{ij} \cdot \text{cache}_{ij} + \beta_7 \text{Tough}_{ij} \cdot \text{cache}_{ij} + \beta_8 \text{Dormancy}_{ij} \cdot \text{cache}_{ij} + \beta_9 \text{Novel}_{ij} + \beta_{10} \text{Ignore}_{ij}.$$  

Finally, we examined if seed selections by squirrels were different in the first and second phases of data collection. Given that seed types used to represent the design were different in the 2 phases of data collection, this question examines whether combinations of seed traits explain seed selection by squirrels rather than species identity. First, we compared mean $\beta \pm 1$ SE intervals of coefficients for traits from multinomial logit models fit separately to the first and second phases of data collection to verify that choices were driven by combinations of seed traits rather than seed types. If intervals from the 2 models overlap for each trait, no significant differences between models can be concluded at $\alpha = 0.05$ (Payton et al. 2003). Second, we added an indicator variable to quantify how the phase of data collection influenced seed selection (phase $= 1$ if the choice event belonged to the second phase of data collection, phase $= 0$ if the choice event occurred in the first phase of data collection). Likelihood ratio tests were then used to examine if including phase and interactions terms as covariates improved fit.

Random parameters logit model

If individuals in a population are heterogeneous in their preferences for modeled traits, then inferences made from the multinomial logit model can be incorrect (Olynk et al. 2010). Conversely, if no heterogeneity exists among individual choice events, then either a single squirrel was responsible for all choices (impossible, given our sampling design) or squirrels behave consistently with one another and individuality can, therefore, be ignored from a statistical perspective. Heterogeneity is incorporated into the model by including a random utility term for each trait in a choice set. Therefore, Equation (1) can be rewritten as (Tonsor et al. 2005):

$$U_{ijt} = V_{ij} + (u_{jt} + \varepsilon_{ij}).$$  

where $u_{jt}$ is the random utility estimate for the $t$th individual on the $j$th alternative distributed identically and independently across individuals and alternatives. The systematic portion of utility is still specified as a function of $n$ traits as in Equation (2). However, the addition of the random utility term across individuals allows for the estimation of variation around $\beta$ coefficients due to individual differences in preferences for each trait (Louvierre et al. 2000) (equivalently, $u_{jt} = g_{jt}X_{jt}$, so that $V_{ij}$ are fixed effects and $g_{jt} \sim N(0, \Sigma)$ are random, individual effects on preference). If the estimated standard deviation of $u_{jt}$ is close to 0, then variability across individuals is considered to be negligible and suggests homogeneity of preference for the trait. A standard deviation significantly different from 0 indicates heterogeneity of preferences for a specific trait (Hensher et al. 2005).

We fit a random parameters logit model for the traits in the best-fit model of the previous section, under the assumption that all selection events made at the same location were made by the same squirrel. Although we did not mark individual squirrels in this study, observers were able to visually track individuals between presentations at a given location. While we cannot be certain that the same individuals did not visit multiple locations, we believe this to be unlikely given our attempts to segregate locations geographically. Therefore, we estimated heterogeneity in squirrel preferences for traits across locations as a post hoc check for variability in individual preferences. The model was fit using programs NLOGIT 6 and LIMDEP 11 (Greene 2011).

Willingness-to-trade

An advantage of discrete choice models is the ability to estimate an animal’s willingness to exchange value derived from different traits using willingness-to-trade, also referred to as the marginal rate of substitution (Cooper & Millspaugh 1999). The marginal utility of a continuous trait $g$ is defined as the partial derivative of utility with respect to $g$, $\partial u/\partial g$. The exchange rate between $g$ and another trait, $h$, is obtained by scaling the marginal utility of $h$ so that it offsets a unit change in $g$ and maintains the same constant utility value. That is, we need to know the value $W_{g,h}$, where $W_{g,h} = -\partial u/\partial h \cdot \partial u/\partial g$ and is interpreted as the amount of trait $h$ that must be increased or
decreased to offset a unit increase in trait g in order to maintain a constant utility. In a multinomial logit model that lacks interaction terms, \( \partial U/\partial g = \beta_g \), so,

\[
W_{g,h} = -\frac{\beta_h}{\beta_g}
\]

(10)

For the multinomial logit models specified in Equations (7) and (8), the WTT estimate of kernel for tannins can be given as

\[
W_{\text{kernel,tannins}} = -\frac{(\beta_2+\beta_4*\text{Cache})}{(\beta_1+\beta_3*\text{Cache})}
\]

(11)

where “cache” is the indicator variable used to represent seed fate. Therefore, for a consumed seed the above equation becomes \(-\beta_2/\beta_1\). Note that these calculations are valid only if no heterogeneity exists in preferences. We estimated the 95% confidence intervals around mean marginal utilities and willingness-to-trade estimates for all seed traits from our best-fit multinomial logit model using the method of Krinsky and Robb (1986). The Krinsky and Robb method relies on random draws of \( \beta \) estimates using the mean \( \bar{\beta} \) estimate for each seed trait and the estimated variance accompanying each \( \beta \). For cached seeds, random draws for the interaction terms with caching were added to random draws for the beta estimates of consumed seeds to obtain marginal utilities. If the 95% CI around the marginal utility of a trait overlapped 0, then the trait was considered to have a utility that is not significant. We did not compute WTT estimates for such traits because ratios involving 0 would either yield 0 or approach infinity and generate uninformative estimates. Using the Krinsky and Robb (1986) method, we also calculated the 95% CIs around WTT estimates (Supplementary Materials 3). If 95% CI around a WTT estimate incorporates 0, then the WTT estimate is deemed not significant.

RESULTS

We presented a total of 426 seed pairs to eastern gray squirrels across 27 locations on the Purdue campus, of which 307 (71.8%) trials resulted in seeds being cached, 118 (27.7%) resulted in seeds being consumed, and 1 seed pair was ignored. The coefficient estimates were not significantly different between first and second phases of data collection (mean \( \beta \pm 1 \) SE intervals overlapped for all traits). Furthermore, including phase as an indicator variable did not improve models of squirrel selection of seed traits (\( P = 1 \), Table 2), providing verification that selection of seed traits by squirrels did not change when seed types represented in the design changed. Therefore, we pooled data from the 2 phases of data collection for purposes of fitting models.

Grams of protein, protein concentration, lipid concentration and energy concentration failed to explain seed selection by squirrels for either consumed or cached seeds. From likelihood ratio tests, grams of protein did not influence seed selection (\( P = 0.88 \), \( P = 0.51 \) for caching and consumption events; Supplementary Materials 2). In addition, when protein concentration re-

Table 2 Comparisons of models fit to seed selection events

<table>
<thead>
<tr>
<th>Model</th>
<th>AIC</th>
<th>ΔAIC</th>
<th>Pseudo R²</th>
<th>Log-likelihood</th>
<th>Number of parameters</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ignore + Tannin + Kernel + Tough + Dormancy + Tannin (*cache) + Kernel (*cache) + Tough (*cache) + Dormancy (*cache)</td>
<td>531.4</td>
<td>3.9</td>
<td>0.094</td>
<td>-256.72</td>
<td>9</td>
<td>—</td>
</tr>
<tr>
<td>Ignore + Phase<em>Tannin + Phase</em>Kernel + Phase<em>Tough + Phase</em>Dormancy + Phase*Tannin (<em>cache) + Phase</em>Kernel (<em>cache) + Phase</em>Tough (<em>cache) + Phase</em>Dormancy (*cache)</td>
<td>537.9</td>
<td>10.4</td>
<td>0.120</td>
<td>-251.97</td>
<td>17</td>
<td>1</td>
</tr>
<tr>
<td>Ignore + Tannin + Kernel + Tough + Dormancy + Tannin (*cache) + Kernel (*cache) + Tough (*cache) + Dormancy (*cache)</td>
<td>531.4</td>
<td>3.9</td>
<td>0.094</td>
<td>-256.72</td>
<td>9</td>
<td>—</td>
</tr>
<tr>
<td>Ignore + Unfamiliar + Tannin + Kernel + Tough + Dormancy + Tannin (*cache) + Kernel (*cache) + Tough (*cache) + Dormancy (*cache)</td>
<td>527.5</td>
<td>0</td>
<td>0.104</td>
<td>-253.74</td>
<td>10</td>
<td>0.014</td>
</tr>
</tbody>
</table>

The bold-faced model (Equation 8 in text) was selected as the best-fit model. For each model, AIC value, ΔAIC value, pseudo \( R^2 \) value, log-likelihood value and number of parameters is provided. The \( P \)-value associated with the likelihood ratio test statistic is provided for each pair of models to examine how model fit changes with added predictors (— in the \( P \)-value column represents the null model in a likelihood ratio test).
placed shell toughness in the model for cached seeds, fit of the model worsened (ΔAIC = 27; Supplementary Materials 2) and protein concentration was not a significant predictor of seed selection. Energy concentration (calories per g) did not improve fit for cached or consumed seeds ($P = 0.54$, $P = 0.21$ for caching and consumption events; Supplementary Materials 2). Percentage lipid concentration also did not improve fit for cached seeds ($P = 0.33$; Supplementary Materials 2). Kernel mass, tannin concentration and shell toughness consistently influenced seed selection and, therefore, all caching and consumption events were examined separate - ly, models incorporating random effects did not improve

Coefficients for interactions of seed traits with caching were significant. In particular, the utility of seeds chosen for consumption was positively associated with toughness of seed and negatively with species’ dormancy periods (Table 3). However, the utility of seeds being cached was positively related to tannin concentration, kernel size and dormancy period. Finally, a significant negative coefficient was found for the “ignore” variable and a significant positive coefficient was associated with seed unfamiliarity (Table 3). The random parameters logit model showed no significant variation in preferences among locations with respect to any trait ($P > 0.5$ for all trait standard deviations, suggesting that the estimated standard deviations of random trait coefficients are not significantly different from 0). When caching and consumption events were examined separately, models incorporating random effects did not improve

<table>
<thead>
<tr>
<th>Trait</th>
<th>Coefficient</th>
<th>Standard error</th>
<th>t-ratio</th>
<th>P-value</th>
<th>Lower CI</th>
<th>Upper CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tannin (%)</td>
<td>−0.106</td>
<td>0.197</td>
<td>−1.20</td>
<td>0.22</td>
<td>−0.38</td>
<td>0.14</td>
</tr>
<tr>
<td>Tannin*Caching</td>
<td>0.212**</td>
<td>0.067**</td>
<td>3.164**</td>
<td>0.002**</td>
<td>0.081**</td>
<td>0.344**</td>
</tr>
<tr>
<td>Kernel (g)</td>
<td>−0.104</td>
<td>0.139</td>
<td>−0.748</td>
<td>0.455</td>
<td>−0.377</td>
<td>0.169</td>
</tr>
<tr>
<td>Kernel*Caching</td>
<td>0.514**</td>
<td>0.169**</td>
<td>3.051**</td>
<td>0.002**</td>
<td>0.184**</td>
<td>0.845**</td>
</tr>
<tr>
<td>Toughness (Nm)</td>
<td>0.477**</td>
<td>0.160**</td>
<td>2.983**</td>
<td>0.003**</td>
<td>0.163**</td>
<td>0.790**</td>
</tr>
<tr>
<td>Toughness*Caching</td>
<td>−0.148</td>
<td>0.183</td>
<td>−0.809</td>
<td>0.419</td>
<td>−0.507</td>
<td>0.211</td>
</tr>
<tr>
<td>Ignore</td>
<td>−5.616**</td>
<td>1.172**</td>
<td>−4.791**</td>
<td>0.000**</td>
<td>−7.913**</td>
<td>−3.319**</td>
</tr>
<tr>
<td>Dormancy (days)</td>
<td>−0.021**</td>
<td>0.007**</td>
<td>−3.133**</td>
<td>0.002**</td>
<td>−0.035**</td>
<td>−0.008**</td>
</tr>
<tr>
<td>Dormancy*Caching</td>
<td>0.023**</td>
<td>0.008**</td>
<td>2.848**</td>
<td>0.004**</td>
<td>0.007**</td>
<td>0.040**</td>
</tr>
<tr>
<td>Unfamiliar</td>
<td>0.660**</td>
<td>0.272**</td>
<td>2.423**</td>
<td>0.015**</td>
<td>0.126**</td>
<td>1.194**</td>
</tr>
</tbody>
</table>

**Variables significant at $\alpha$ of 0.05. CI, confidence interval.

Figure 2 Utility contour lines plotted for varying seed toughness and dormancy period. Contours represent lines of indifference at utilities of $1$, $0.02$ and $-1$, assuming a familiar seed with 0.5% TAE tannin concentration and 1-g kernel mass that is consumed instead of cached. The lines of indifference represent combinations of toughness and dormancy viewed as tradable by eastern gray squirrels (Sciurus carolinensis) at the specified utility. Dormancy and toughness plotted for Quercus macrocarpa, Quercus prinus, Juglans nigra, Juglans cinerea and Castanea dentata. The 2 Quercus species and 2 Juglans species occur close to line of indifference at 0.02, indicating that these seeds are approximately equally likely to be consumed in pairwise trials. In contrast, C. dentata occurs below the $-1$ line of indifference, indicating that this species is less likely to be consumed when presented with one of the Quercus or Juglans species.

Table 3 Best-fit model (Equation 8) parameterized for choices made by eastern gray squirrels (Sciurus carolinensis)
Table 4 Marginal utilities and willingness-to-trade estimates for all seed traits

<table>
<thead>
<tr>
<th>Variable</th>
<th>Marginal utilities of consumed seeds</th>
<th>Marginal utilities of cached seeds</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lower CI</td>
<td>Mean</td>
</tr>
<tr>
<td>Kernel mass (g)</td>
<td>−0.366</td>
<td>−0.100</td>
</tr>
<tr>
<td>Toughness (Nm)</td>
<td>0.152</td>
<td>0.472</td>
</tr>
<tr>
<td>Dormancy (days)</td>
<td>−0.035</td>
<td>−0.021</td>
</tr>
<tr>
<td>Tannin concentration (%)</td>
<td>−0.183</td>
<td>−0.072</td>
</tr>
</tbody>
</table>

Mean marginal utilities and 95% confidence intervals for seed traits under conditions of caching and consumption. Mean willingness-to-trade estimates for all paired seed traits (— provided when WTT could not be computed because the marginal utility for a seed trait overlapped 0).

model fit \( P = 1.00, P = 0.76 \) for caching and consumption datasets; Supplementary Materials 2)

Willingness-to-trade (WTT) estimates were calculated separately for consumed and cached seeds, and varied for different combinations of traits (Table 4). Mean WTT estimates were not computed for 8 of 12 seed trait combinations because at least 1 of the marginal utilities in the combination was not significantly different from 0 (Table 4, confidence intervals for WTT estimates overlap 0 for 8 of 12 seed trait combinations in Supplementary Materials 3). For consumed seeds, WTT estimates between toughness and dormancy period were positive, indicating that for perceived value of a seed to remain constant, any change in dormancy period would need to be accompanied by a corresponding, parallel change in toughness, or vice versa. For cached seeds, negative WTT estimates were computed between kernel mass and toughness, kernel mass and tannins, and toughness and tannins. Therefore, for a seed to maintain a constant desirability for caching relative to other species, a decrease in kernel mass could be offset by increased toughness or increased tannin content, or a mixture of both. Similarly, an increase in kernel mass or tannin content could compensate for reduced toughness.

To aid in interpretation of WTT estimates, we plotted lines of indifference for consumed seeds using coefficient estimates from Equation (8) (Fig. 2). Lines of indifference represent combinations of 2 traits for which utility remains constant. The slope of the line is equal to the WTT estimate for the 2 traits. For consumed seeds we created 3 lines of indifference to represent tradeoffs between toughness and dormancy period at utilities of 1, 0.02 and −1. For all 3 lines, we set tannin concentration at 0.5% TAE, kernel mass at 1 g and assumed seed type was familiar. Overlaying the seed species with the highest estimated utility when consumed in Fig. 2, *Juglans cinerea, Juglans nigra, Quercus macrocarpa* and *Quercus prinus* fall close to the line of indifference at utility of 0.02, indicating that these seeds are approximately equivalent in terms of their desirability for consumption. All other seeds were of lower utility when selected for consumption and, if plotted, would occur below the −1 line of indifference (see e.g. *Castanea dentata* in Fig. 2).

For cached seeds, more complex trading scenarios occurred. In this case, finite WTT estimates for 3 traits
Seed selection by eastern gray squirrels

The methods and models outlined here allow some general inferences to be made about seed selection by eastern gray squirrels and also provide novel insights regarding the effects of seed traits on squirrel foraging and caching behaviors. Species identity, reflected by the different phases of data collection in this study, did not alter coefficient estimates predicting seed selection by squirrels. This result strongly suggests that seed trait combinations, as opposed to species identities, influenced selections made by the eastern gray squirrels in our experiment. Understanding the selection of seed traits by squirrels gives insight into the evolution of seed construction strategies in hardwood trees and may also allow us to anticipate the animals’ functional responses to changes in neighborhood or community composition. Furthermore, it emphasizes the argument that seed selection by rodents is context-dependent, where the combination of traits of available seeds influences seed selection as opposed to species identity.

The preferences for seed traits observed in this study corroborate previous findings. We found that the percentage of tannins in seed kernels significantly increased utility for caching and decreased utility for consumption. Steele et al. (1993) show that eastern gray squirrels are sensitive to tannins and reject portions of kernels that contain relatively high concentrations of tannins when consuming seeds, but also preferentially cache high-tannin seeds. The mass of a seed may be increased either by increased shell mass (and, thus, toughness) or by increased kernel mass, and our results show that both factors positively influence utility for caching. Seed coat toughness was a significant and positive predictor of seed selection for caching by eastern gray squirrels (Table 3), which supports previous assertions that selection by squirrels in the fall follows a time-minimization strategy rather than an energy-maximization strategy (Thompson & Thompson 1980). Specifically, seeds with tougher shells have a higher consumption time and are cached more often to reduce time spent handling and, hence, increase time available to cache additional seeds in the fall (Thompson & Thompson 1980; Jacobs 1992; Sundaram et al. 2015). It should be noted, however, that in our seed set toughness is highly correlated to lipid concentration and, thus, to energy content; seeds of Betulaceae and Juglandaceae (including walnuts and hazelnuts) have evolved both thicker shells and higher lipid concentrations when compared to seeds of Fagaceae.
(including oaks and chestnuts) (Sundaram et al. 2015). Thus, the selection by squirrels of seeds with tough shells in conjunction with long dormancy periods results in the selection of the highly energetically valuable walnuts (J. nigra and J. cinerea) for consumption (Table 3 and Fig. 2). Utility for caching was also influenced positively by kernel mass (Table 3). All else being equal, large kernels reflect high total caloric content, thus making seeds more attractive to rodents and increasing the probability of seeds being cached (Grubb & Burslem 1998; Yang et al. 2012).

Apart from corroborating existing findings, we tested and found additional seed trait trends that merit further attention. Total protein content of a seed was not a significant predictor of utility, in contrast to systems where rodents were found to maximize protein intake (Henderson 1990). Although several studies have found that seeds with higher protein content are selected more frequently (reviewed by Lichti et al. 2017), these studies generally have relied on observational, post hoc correlations and could not unambiguously attribute selection to protein content. Finally, we found a significant positive effect of unfamiliar seeds on utility, implying that squirrels were more likely to choose unfamiliar chestnut seeds regardless of seed fate. While the addition of this variable significantly improved the fit of the model (Table 2), we cannot determine from our results whether squirrels are more likely to select chestnuts due to an unobserved trait or if squirrels are more likely to select chestnuts because they are unfamiliar. Studies directly comparing familiar and unfamiliar seeds are required to address this question.

Squirrels in our study were strikingly consistent in their preferences for seed traits. Such consistency in selection of seed traits across individuals (see section on “random parameters logit model” and Supplementary Materials 2), and extreme preferences for a trait (WTT estimates approach 0 for 8 of 12 seed trait combinations; Supplementary Materials 3), is not well documented in the ecological literature. Consistency in seed preference of rodents has been noted in other studies (Lobo et al. 2009) as well as reviews that have compared preferences among rodent consumers (Vander Wall 2010; Lichti et al. 2017). In contrast to rodents, consumer economic discrete choice models evaluating the utility of a product with respect to product traits typically find heterogeneity in responses of human consumers (Ortega et al. 2011). Human heterogeneity is attributed to differences in taste of consumers (Olynk & Ortega 2013) and probably reflects the low fitness consequences of decisions typically studied in consumer economics. When it occurs, heterogeneity in responses of free-ranging animals is sometimes attributed to differences in experience or age and differences in effort expended by individuals (Mauk et al. 2012). Homogenous responses such as those we observed are likely a result of natural selection.

For free-ranging squirrels, creation and maintenance of caches during fall is critical to ensure survival during winter when food is scarce (Nixon et al. 1975). In a separate study, we determined that significant proportions of foraging behaviors of eastern gray squirrels were explained by hardwood tree phylogeny, and that the seed traits explored in this study are likely constrained by phylogeny to different degrees (Sundaram et al. 2015). The consistency in selection of seed traits that we observed also indicates that squirrels may exert selective pressures on hardwood trees by consistently caching seeds with large kernel sizes, tougher shells and higher tannin concentrations so long as the seed attributes are heritable. Some nut morphological characters, such as weight and thickness, show very high broad-sense heritability, indicating that the environment influences these traits to a minimal extent (Lan-Ying et al. 2009). To our knowledge, no studies have explored heritability of tannin concentrations of hardwood tree seeds, although a genetic basis has been established for seed coat tannins in other plant species (Caldas & Blair 2009).

Our analysis of squirrels’ marginal utilities and willingness-to-trade among different seed traits provides new insights into the interactions between trees and scatter-hoarding rodents. Previous theoretical and empirical work has shown that seed dispersal effectiveness in hoarded trees depends in part on the composition of seed crops available to rodents and the rodents’ perception of different species values for consumption or caching (Lichti et al. 2014; Sundaram et al. 2017). Trees that increase the consumption utility of their seeds relative to their neighbors while decreasing their relative caching utility could face significant negative selective pressure. In contrast, any tree that can decrease consumption utility and increase caching utility stands to benefit from hoarder behavior. For example, either a decrease in the length of overwinter dormancy or an increase in toughness (and energy content) will make seeds more attractive for consumption (Table 3). However, assuming that neighbors remain static, simultaneous increases in both toughness and dormancy as seen in the shift from white oaks (Quercus section Quercus) to walnuts (Juglans) could maintain a tree’s desirability for consumption at a constant level and avoid placing the tree at a disadvan-
tage to its neighbors.

In contrast to consumption, WTT estimates for cached seeds were negative (Table 4). As a result, increased kernel mass (e.g., *Q. macrocarpa*), increased toughness (e.g., *C. ovata*), increased tannin concentrations (e.g., *Q. velutina*) or increases in any combination of these traits would increase a seed’s desirability for caching (Fig. 3). Given such conditions, squirrel behavior might be expected to drive diversification of seed types as various species develop along alternative evolutionary pathways. For instance, nuts from the family Juglandaceae have evolved tough shells, whereas acorns from *Quercus* section Lobatae have evolved high tannin concentrations (Sundaram et al. 2015). Yet, our results indicate that these seeds are functionally similar (Fig. 2). From a squirrel’s perspective, the flexibility that results from trading off a larger number of traits may allow hoarders to reduce the time spent examining seeds in the fall when competition to cache is high and when there is a need to cache high numbers of seeds to ensure availability of food during winter (Thompson & Thompson 1980).

To further test the hypothesis that seed traits such as kernel size and tannin concentration are truly interchangeable from the squirrel’s point of view at the estimated WTT values, an artificial seed experiment could be devised wherein these traits are manipulated. For example, an artificial seed with 2.9% TAE tannin concentration could be paired with a second seed having a 0% TAE but a larger kernel mass (larger by 1 g). If the 2 constructed seeds have the same total perceived value, as is suggested by our model, then a squirrel should be equally likely to cache either of the constructed seeds, all else being equal. Furthermore, a discrete choice experimental framework may be useful to evaluate selection of traits in an artificial seed study to shed light on how squirrels perceive artificial seeds and assess whether results from artificial seed experiments can be extrapolated to hardwood tree seeds.

**Applying discrete choice experiments to an ecological system**

We believe our study is the first to present discrete choice experiments to free-ranging animals based on a design informed by pre-determined traits. In theory, a main advantage of DCEs is the ability to create balanced and orthogonal fractional designs with correlated traits. In practice, while we successfully parsed out the effects of moderately correlated traits, we faced some constraints in applying the approach to squirrel preferences for hardwood seeds. Developmental and evolutionary constraints have resulted in a high degree of correlation for some seed traits (Sundaram et al. 2015); consequently, some combinations of traits are not found in real seeds of any species. This issue of biologically missing trait combinations is likely to occur in many systems. Missing traits may also arise if traits are inadequately sampled, although this is not likely for the seeds used here (Sundaram et al. 2015). If only a few trait combinations are missing, a choice experiment may still be designed with existing traits. D-efficiency of the resulting design (or the amount of information captured by the resulting design relative to a fully orthogonal and balanced design), however, will be lower when compared to a design incorporating missing trait combinations.

The interpretation of coefficients and intercepts must be carefully considered when planning discrete choice experiments based on traits. Our experiment used a design that was optimized over combinations of trait values, not combinations of seed species. During data collection, each combination of trait values could be represented by any of a number of actual seed species. Consequently, structuring our models so that the intercept terms corresponded to one of the alternatives in a presentation (i.e. for alternative 1 or alternative 2) would not have yielded interpretable results because species representing the alternative changed across scenarios. Instead, we estimated an intercept for the third alternative available to squirrels (i.e. ignoring a seed pair). We interpret this coefficient as the perceived value of leaving to forage elsewhere or pursue other activities. It therefore provides an estimate of the missed opportunity cost associated with selecting seeds. Allowing for the option to ignore alternatives and not make a choice between available alternatives is important in certain decision-making frameworks, but is often overlooked in traditional ecological resource selection models.

We designed our experiment with algorithms that maximize D-efficiency and used a multinomial logit modeling approach, but alternatives may be appropriate in some instances. D-efficiency is computed from the geometric mean of the determinants of candidate designs. Although D-efficiency is considered to be a standard metric representing the total information captured by a design, other metrics have been proposed, including A-efficiency and G-efficiency (Kuhfeld et al. 1994). A-efficiency is computed from the arithmetic mean of determinants of candidate designs, while G-efficiency is derived from the maximum standard error for predic-
tion from a candidate design (Kuhfeld et al. 1994). Similarly, alternatives exist to the multinomial logit framework used here, including Bayesian choice models that offer additional flexibility when compared to traditional choice models. For example, Bayesian methods may include non-normal distributions for coefficients and allow for situations where the full choice set is unknown (Train 2003).

Overall, DCEs and DCMs offer a powerful tool for modeling and designing experiments to investigate resource selection by consumers. Traditional foraging studies generally model consumer preference for 1 currency at a time (e.g. caloric value of food item, Stephens et al. 2007) or examine preferences across artificial food items (e.g. clay “seeds” with manipulated attributes; Wang & Chen 2008). As previous studies have already described, DCMs are broadly applicable in studies of resource selection and allow consumer preference to be examined for multiple attributes and, thus, multiple currencies simultaneously (Buskirk & Millspaugh 2006). However, most DCM applications in ecology rely on observational data and lack clearly defined choice sets or the set of available resources that animals select from is generally unknown (Baasch et al. 2011). Designed choice experiments help to address both of these issues. By using multiple different resource pairs to construct the same orthogonal contrasts among levels of focal traits, the DCE controls the choice set used in modeling and averages over any other functional traits that resources may possess as well as the effects of environmental context on decisions. As a result, DCEs reduce the risk that spurious results might be generated by lurking variables and, therefore, support stronger inferences. Although our specific approach is most directly applicable to diet and some mate selection systems, it may be possible to construct similar approaches for other aspects of resource selection by conducting a post hoc classification of observed selection events, followed by a random subsampling of the classified events for analysis.

CONCLUSIONS

Our study illustrates a designed approach to studying seed trait selection by eastern gray squirrels. We suggest that the designed approach is useful for studying effects of traits on resource selection, especially when testing in a full factorial design is not feasible. We showed that eastern gray squirrels choosing between 2 seeds select for specific seed traits, including dormancy period, tannin concentration, kernel mass and toughness of a seed. Moreover, we found that selection for traits is remarkably consistent across squirrels. Our approach allowed us to compute tradeoffs between seed traits and show that squirrels may trade among multiple traits when caching seeds and between fewer traits when consuming seeds. Our results suggest that tradeoffs by squirrels during fall may have substantial implications for the evolution and diversification of nut-bearing trees.

ACKNOWLEDGMENTS

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REFERENCES


**SUPPLEMENTARY MATERIALS**

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

**Supplementary Material 1** Tests confirming interspecific variation in seed traits is higher than intraspecific variation in seed traits.

**Supplementary Material 2** Model selection and model fit summary for cached and consumed seed selection events.

**Supplementary Material 3** Willingness-to-trade (WTT) 95% confidence intervals for all seed trait combinations.

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ORIGINAL ARTICLE

Factors shaping life history traits of two proovigenic parasitoids

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Abstract

What shapes the relative investment in reproduction versus survival of organisms is among the key questions in life history. Proovigenic insects mature all their eggs prior to emergence and are short lived, providing a unique opportunity to quantify their lifetime investments in the different functions. We investigated the initial eggloads and longevity of 2 proovigenic parasitoid wasps: Anagrus erythroneurae and Anagrus daanei, (Hymenoptera: Mymaridae) that develop within leafhopper eggs in both agricultural vineyards and natural riparian habitats in Northern California. We collected Vitis spp. leaves containing developing parasitoids from 3 natural sites (Knight Landing, American River and Putah Creek) and 3 agricultural vineyards (Solano Farm, Davis Campus and Village Homes). We recorded eggloads at parasitoid emergence and female parasitoid longevity with or without honey-feeding. Theory predicts that parasitoids from vineyards (where hosts are abundant) would have higher initial eggloads and lower longevity compared with parasitoids from riparian habitats (where hosts are scarce). Although host density and parasitoid eggloads were, indeed, higher in vineyards than in riparian habitats, parasitoid longevity did not follow the predicted pattern. Longevity without feeding differed among field sites, but it was not affected by habitat type (natural vs agricultural), whereas longevity with feeding was not significantly affected by any of the examined factors. Moreover, longevity was positively, rather than negatively, correlated with eggloads at the individual level, even after correcting for parasitoid body size. The combined results suggest a more complex allocation mechanism than initially predicted, and the possibility of variation in host quality that is independent of size.

Key words: Anagrus parasitoids, eggload, leafhoppers, life-history tradeoffs, longevity

INTRODUCTION

Life history theory seeks to explain optimal allocation of resources to different developmental, physiological and reproductive functions of the organism. While the approach of “one solution per species” is tempting, it is widely acknowledged that environmental conditions are likely to alter optimal life-history traits, either at the phenotypic (i.e. plastic responses) or genotypic
(i.e. evolutionary responses) level (Stearns 1992; Roff 2002).

Parasitoids are insects (mostly wasps) that lay their eggs in or on the body of another organism (mostly another insect) and whose offspring develop while feeding on the host, eventually killing it. Parasitoids have long been used for life history studies due to their varied strategies, relative ease of rearing and manipulating in the laboratory, and high responsiveness to environmental conditions (Godfray 1994). The reproductive success of parasitoids is considered to be limited mostly by the availability of mature eggs, or by the availability of suitable hosts in the environment (Rosenheim 1999). Hence, what shapes the relative investment in egg production versus longevity is a key question in parasitoid life history.

Despite early opinions that female parasitoids rarely deplete their eggs in nature (van Alphen et al. 1992; Visser et al. 1992; Sevenster et al. 1998), recent empirical data combined with theoretical developments suggest that egg limitation in parasitoids, as well as in other insects, is likely to occur under field conditions (Heimpel & Rosenheim 1998; Casas et al. 2000; Rosenheim et al. 2008; Segoli & Rosenheim 2013; Phillips & Kean 2017). Moreover, even in cases where egg limitation is rare, it may impose strong selection on female reproductive traits, due the disproportionately high reproductive contribution of egg-limited females (i.e. those that lay all of their eggs) to the next generation (Rosenheim 2011). Hence, the risk of egg limitation may lead to higher investment in egg production in environments with many oviposition opportunities. In contrast, in environments with limited host availability, higher investment in longevity, at the expense of egg production, is expected, as parasitoids need an extended amount of time to search for scarce hosts (Rosenheim 1996; Ellers et al. 2000; Gandon et al. 2009).

The majority of parasitoids are synovigenic; that is, they continue to mature eggs throughout their adult life (Godfray 1994; Jervis et al. 2001). Hence, decisions regarding the relative investment in survival versus egg production may change during the lifetime of the parasitoid, making it difficult to quantify. In contrast, strictly proovigenic parasitoids emerge with their full egg complement and do not mature additional eggs following emergence. In this case, the relative investment in egg production is a “one-time decision” that is made during parasitoid development inside the host, prior to its emergence. This provides a unique opportunity to study intraspecific variation in lifetime investment of parasitoids. Nevertheless, very few studies have investigated life history responses in proovigenic parasitoids in relation to environmental conditions. Part of the reason could be difficulties in handling and dissecting proovigenic parasitoids due to their small size.

We studied egg load and longevity of 2 strictly proovigenic parasitoids of the genus Anagrus (A. erythronoeurae S. Trjapitzin & Chiappini, 1994 and A. daanei Triapitsyn, 1998). These parasitoids attack the eggs of leafhoppers that are considered major pests in vineyards in Northern California. Both the parasitoids and the leafhoppers occur also on wild grapes in riparian habitats in California. This provides an excellent opportunity to study the life history traits of parasitoids originating from contrasting habitats. Previous work demonstrated higher initial eggloads for parasitoids from vineyards (approximately 25 eggs) compared to their congener from riparian habitats (fewer than 20 eggs) (Segoli & Rosenheim 2013). This is consistent with theory, as leafhoppers are more abundant and may reach very high densities in agricultural vineyards. In this study we extended this work to compare parasitoid longevity among these different habitats. Based on theory, we predicted higher longevity for parasitoids from riparian habitats (where hosts are scarce) compared to those from vineyards (where hosts are abundant). Longevity was quantified either with or without feeding the parasitoids with a sugar source (honey). Sugar supplementation was previously shown to extend parasitoid lifespan from less than a day to few days (Segoli & Rosenheim 2013). We predicted that differences in longevity would be more pronounced under starvation conditions, where the parasitoid needs to rely on tenereal reserves (Jervis et al. 2008), than when provided with honey.

MATERIALS AND METHODS

Study species

Anagrus spp. parasitoid wasps are among the most important natural enemies of Erythronoeura and Erasmoneura spp. leafhoppers in California (Doult & Nakata 1973; Daane & Costello 2000; Bentley 2009). They complete their entire development (egg to adult) inside the leafhopper egg, consuming the egg as they develop. These parasitoids are solitary (develop singly inside the host), pro-ovigenic (emerge with their full lifetime complement of eggs already matured) and do not re-sorb eggs (Jepsen et al. 2007). Anagrus are short lived, even under the most benign laboratory conditions (English-Loeb et al. 2003).
Erythroneura and Erasmoneura spp. leafhoppers deposit eggs singly or in clusters under the epidermal tissue or along the veins of Vitis spp. leaves. Each egg is approximately 0.8 mm long. The freshly deposited egg is colorless and transparent. When eggs are parasitized, they become brown or red. The leafhoppers complete approximately 3 generations per growing season (June to September) and may reach very high densities in vineyards towards the end of the season (Daane & Costello 2000).

Collection of parasitoids

Grapevine leaves with apparent leafhopper damage were collected from 3 riparian habitats and 3 vineyards located in Yolo, Solano and Sacramento counties in California, during August 2016. Some of the leaves were put immediately in emergence cages (approximately 10 cages per agricultural site and 20 per natural site; approximately 40 leaves per cage) and kept at room temperature for one week. The cages were empty paper carton containers with a transparent funnel and a vial on top. Parasitoids emerging from leafhopper eggs on the leaves were attracted to the light and collected from the vials. The remaining leaves were kept in the refrigerator (4 °C) and were used to replenish the emergence cages throughout the week. Emergence cages were monitored hourly between 07:00 and 20:00 hours.

Longevity without feeding

Newly emerged parasitoids were put in individual glass vials, kept in an incubator (29 °C, 12:12 h light : dark cycle) and checked hourly during 12 h. Experimental temperature reflected temperatures in the field during this season. Following death, parasitoid sex was determined and females were used for egg load and body size estimates (see below). Longevity was calculated as the number of hours that passed from parasitoid emergence until its death. Only parasitoids that emerged between 0700 and 1000 hours were used for this estimate. This is because it was impossible to determine the timing of emergence for parasitoids that were already emerged at 0700 hours, and it was sometimes impossible to record the time of death for parasitoids emerging after 1000 hours (they sometimes survived beyond the observation period).

Longevity with sugar feeding

Newly emerged parasitoids were put into individual vials with a small piece of filter paper soaked with honey solution. Parasitoids were kept in an incubator (29 °C, 12:12 h light : dark cycle) and checked daily until all died. Longevity was calculated as the number of days from parasitoid emergence until its death. Parasitoids that were found stuck in honey were excluded from the sample as this might have expedited their death. Following death, each parasitoid was sexed, females were identified to species (Triapitsyn et al. 2010), and the length of a hind tibia was measured.

Eggload estimates

Females were then dissected to determine their eggloads. Dissection was done under a dissecting microscope using Minuten insect pins (Fine Science Tools, USA, Inc.). The number of eggs upon death was assumed to represent the number of eggs upon emergence. This is because these parasitoids do not resorb eggs, did not have oviposition opportunities in the vials and were never observed to lay eggs outside a host. Males were not used in this study.

Sample sizes

Sample sizes (Table 1) were highly unequal across sites because of strong inter-site variation in leafhopper and parasitoid abundance, and despite the higher intensity of sampling (larger number of emergence cages) in low-density sites.

Host density estimates

To test the assumption of higher host density in agricultural fields we examined 20 of the leaves collected from each site. The percentage of leaf area that was damaged by leafhopper feeding (visible as white stippling on the leaves) was estimated. Host density estimates differed significantly among field sites and were higher in agricultural sites (Fig. 1, GLM, habitat type, feeding treatment (feeding vs. no feeding), parasitoid species and tibia length as independent factors). Eggloads were compared using general linear models with habitat type, field site (nested within habitat type), feeding treatment (feeding vs. no feeding), parasitoid species and tibia length as independent factors. Because longevity with or without feeding was es-
RESULTS

Body size

Hind tibia length of female parasitoids did not differ among habitat types, field sites or parasitoid species (GLM, habitat type $F_{4,187} = 0.02, P = 0.88$; field site $F_{4,187} = 0.88, P = 0.47$; Species $F_{1,187} = 0.34, P = 0.56, N = 188$ females).

Eggloads

Mean eggloads of females varied among sites and were higher in agricultural vineyards (Fig. 2, GLM, field site $F_{4,187} = 2.39, P = 0.05$; habitat type $F_{4,187} = 20.46, P < 0.001$). In addition, eggload variation among females was positively correlated with tibia length ($R^2 = 0.29$ for *A. erythroneurae* and $R^2 = 0.21$ for *A. daanei*). Eggloads were not affected by either the parasitoid species ($F_{1,187} = 0.20, P = 0.66$) or by the feeding treatment ($F_{1,187} = 3.28, P = 0.07, N = 188$). Residual eggloads based on this model were used for the regression between longevity and eggload (see below).

Effect of feeding

As expected, feeding increased female lifespan from less than a day (mean = 5.44 ± 2.36 hours, $n = 115$) to several days (mean = 4.08 ± 0.33 days, $n = 72$).

<table>
<thead>
<tr>
<th>Habitat type</th>
<th>Field site</th>
<th>Eggloads</th>
<th>Longevity (hr) without feeding</th>
<th>Longevity (d) with feeding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>Anagrus daanei</em></td>
<td><em>Anagrus erythroneurae</em></td>
<td><em>Anagrus daanei</em></td>
</tr>
<tr>
<td>Agricultural</td>
<td>Village Homes</td>
<td>4</td>
<td>4</td>
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<td></td>
<td></td>
<td>29</td>
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<td></td>
<td>Davis Campus</td>
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<td>43</td>
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<td>Solano Farm</td>
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<td>Natural</td>
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<tr>
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<td>37</td>
<td>27</td>
<td>9</td>
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<tr>
<td></td>
<td></td>
<td>7</td>
<td>2</td>
<td>5</td>
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</tbody>
</table>

**Table 1** Sample sizes of females of the different field sites

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Figure 1 Means ± SE leafhopper density estimated as % leafhopper damage on the leaf at the different field sites ($n = 20$ leaves per site).
**Longevity without feeding**

Longevity without feeding differed significantly among field sites but was not affected by habitat type, by tibia length or by parasitoid species (Fig. 3, GLM, habitat type $F_{1,114} = 1.07, P = 0.30$; Field site- $F_{4,114} = 3.41, P = 0.01$; tibia length- $F_{1,114} = 0.01, P = 0.93$; Species- $F_{1,114} = 0.44, P = 0.51$). The main variation was
among the different natural sites, while the agricultural sites had similar values.

**Longevity with feeding**

Longevity with feeding was not significantly affected by any of the independent factors (Fig. 4, GLM, habitat type $F_{1,71} = 2.15, P = 0.09$; field site $F_{4,71} = 2.75, P = 0.10$; tibia length $F_{1,71} = 0.75, P = 0.39$; species $F_{1,71} = 0.27, P = 0.6$). This may partly be due to the small sample size for some of the species/site combinations.

**Relationship between eggload and longevity**

We found a weak positive, rather than a negative, relationship between longevity without feeding and residual eggload (controlling for tibia length, habitat type, field site and species [see above]; linear regression, $P = 0.01$, $R^2 = 0.06$; Fig. 5). Longevity with feeding was not related to residual eggload (linear regression, $P = 0.37$, $R^2 = 0.01$).

**DISCUSSION**

**Variation in eggloads**

We studied life history traits of 2 species of proovigenic parasitoid wasps originating from either natural riparian habitats or agricultural vineyards. Consistent with our predictions, parasitoids from agricultural habitats emerged with more eggs, and, hence, were prepared for the plentiful oviposition opportunities in their environment. This is consistent with previous results (Segoli & Rosenheim 2013), further confirming the robustness of this observation. To our knowledge, this is the only example of inter-population variation in initial eggloads in a strictly proovigenic parasitoid. Given that eggloads are determined prior to parasitoid emergence, variation in eggloads cannot be explained as an immediate plastic response to host availability experienced by adult females in the environment. Alternative explanations include variability in host quality among the different habitats, transgenerational phenotypic plasticity (maternal effects) or genetic differences among parasitoids of the different populations. We will next discuss these possibilities.

Host size is one of the most basic aspects of host quality and in other host-parasitoid systems has often been shown to affect both the number and quality of emerging parasitoids (Godfray 1994). For example, in the parasitoid *Uscana lariophaga*, which parasitizes eggs of bruchid beetles, females reared in small host eggs developed slower, were smaller and produced fewer eggs compared to those reared in large host eggs (Spitzen & van Huis 2005). Previous work in the *Anagrus*-leafhopper-grapes system found no consistent differences in leafhopper egg size on leaves collected from vineyards or from wild grapes, suggesting the lack of differential host quality between these habitats (Segoli & Rosenheim 2013). This conclusion is strengthened by the lack of difference in mean parasitoid body size between these habitats, as demonstrated in the current

![Graph](image-url) **Figure 5** Relationship between longevity of unfed female parasitoids and their eggload.
study. Nevertheless, other aspects of host quality may also be of importance. For example, fertilization and irrigation in vineyards may affect the nutritional composition of leafhopper eggs, independent of their size, subsequently affecting parasitoid traits. This hypothesis is yet to be examined.

Transgenerational plasticity of reproductive traits in relation to environmental conditions experienced by ovipositing females has been demonstrated in many insects (Mousseau & Dingle 1991; Mousseau & Fox 1998), including parasitoids (Morag et al. 2011). The possibility of transgenerational response to host availability in Anagrus parasitoids was previously tested by exposing A. erythroneurae females to either high or low host densities and examining the eggloads of their daughters (Andreazza & Rosenheim 2015). However, no differences across host density treatments were found, arguing against the operation of transgenerational phenotypic plasticity.

Initial fecundity has been found to be inheritable in many insects, including Anagrus parasitoids (Cronin & Strong 1996; Andreazza & Rosenheim 2015). Although the observed inter-population variation in Anagrus is likely to be at least partially genetic, the relative contribution of genetic variation is currently unknown. Future work will aim to conduct common garden experiments (in which parasitoids of different origins are reared on the same host cohort), transplant experiments (in which parasitoids originating of one habitat are reared on hosts of another habitat and vice versa) and evolutionary experiments (in which parasitoids are reared at high or low host densities over multiple generations) to address this question.

Tradeoff between eggload and longevity

Higher investment in egg production is likely to come at the expense of other functions. Indeed, studies have demonstrated both phenotypic and genetic tradeoffs between reproduction and longevity in many insects (Miyatake 1997; Tatar 2001; Roff 2002; Edward & Chapman 2011). In parasitoids, such tradeoffs were demonstrated at the species level (i.e. long-lived species emerge with relatively fewer eggs; Jervis et al. 2001, 2003) and the individual level (i.e. individuals investing more in egg production die sooner; Ellers et al. 2000; Zhang et al. 2011; Segoli & Rosenheim 2013). Tradeoffs at the population level were also suggested, but these were either confounded with parasitoid reproductive mode (sexual vs asexual; Pelosse et al. 2007; Ameri et al. 2015), or were more likely to represent differences in the timing of allocation than in total investment (Ellers & van Alphen 1997).

Here we examined the potential tradeoff between eggload and longevity for proovigenic species, at both the population and individual level. Because longevity of parasitoids that do not feed is dependent exclusively on their teneral reserves (Jervis et al. 2008), we expected that variation in longevity will be more pronounced in food deprived females. Indeed, the longevity of females fed with honey was substantially greater (up to 10-fold) and was not dependent on the specific site or habitat of origin. The longevity of starved females, however, differed significantly among field sites, suggesting inter-population variation in parasitoid energy reserves. Nevertheless, in contrast to our predictions, longevity was not consistently higher for parasitoids originating from natural habitats. Moreover, longevity was positively, rather than negatively, correlated with eggload at the individual level, even after correcting for parasitoid body size and site of origin. This contradicted previous results that instead suggested a negative relationship between female eggload and longevity at the individual level (Segoli & Rosenheim 2013).

There are several general explanations for the absence of an expected tradeoff in life history traits (Stearns 1992; Agrawal et al. 2010). One possible explanation is that a tradeoff may not be expressed under certain environmental conditions. For example, parasitoids from natural habitats could perhaps have the ability to survive longer in the field, but this ability might not be expressed under the artificial conditions experienced in the lab. In particular, confining parasitoids in a vial could substantially alter their movement behavior (van den Assem 1996), thereby affecting their energy expenditure. However, documenting the realized longevity of such small insects in the field, or quantifying their energy reserves at emergence (Ellers 1996), may be extremely challenging.

Another potential reason is that allocation patterns and selection pressures may be more complex or simply different than initially assumed. For example, higher investment in egg production could come at the expense of energy reserves, but could also potentially trade off with egg quality, female mobility, immune function or stress tolerance. Hence, certain functions may not always directly compete for resources (Agrawal et al. 2010). In particular, the tradeoff between egg number and size was suggested to be of high importance (Berrigan 1991; Rosenheim 1996). Indeed, previous results suggest that Anagrus eggs in agricultural sites in Cali-
California are smaller (Segoli & Rosenheim 2013). Hence, the tradeoff between egg number and size may be more pronounced than that of eggload and longevity in this system. Moreover, additional environmental factors, such as food availability, habitat complexity, predation and competition, may impose stronger or even opposing selection on longevity, leading to inter-site variation that is independent of host density. For example, higher competition among females at higher densities may compromise their oviposition rates, as was shown for another Anagrus species (Cronin & Strong 1993).

Finally, the expectation for a tradeoff is based on the assumption of a resource pool whose size is constant across individuals. If instead there is individual variation in total resource availability, individuals with larger total resource pools may have both greater fecundity and longevity, thus masking the occurrence of a tradeoff. This does not mean that these functions do not trade off; rather, that in order to detect the tradeoff there is a need to control for individual quality, either statistically or experimentally (Stearns 1992). Although we statistically controlled for female body size, as mentioned above, this may not be a reliable measure of the quality of resource available for parasitoid development. In particular in egg parasitoids, body size may be constrained by the size of the host egg, which could be relatively constant. Hence, parasitoid traits may be dependent on the composition of nutrients inside the host, which, in turn, may depend on the nutritional value of the plant (Turlings & Benrey 1998; Sarfraz et al. 2009). Such effects may lead to positive correlations between different life history components as observed in this study. This may also explain the previously documented tradeoff (Segoli & Rosenheim 2013) that was observed in a small sample of parasitoids of a uniform origin (parasitoids of the same site, emerging on the same day).

In conclusion, parasitoid life history traits varied among field sites, and although we confirmed the greater fecundity of parasitoids originating in agricultural sites, we found no evidence of a tradeoff with parasitoid longevity at either the population or individual level. The lack of apparent tradeoff between eggload and longevity may be due to inappropriate experimental conditions, complex allocation patterns or variation in host quality that is independent of host size.

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ORIGINAL ARTICLE

Distribution and space use of seed-dispersing rodents in central Pyrenees: implications for genetic diversity, conservation and plant recruitment

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Abstract

The function and conservation of many forest ecosystems depend on the distribution and diversity of the community of rodents that consume and disperse seeds. The habitat preferences and interactions are especially relevant in alpine systems where such granivorous rodents reach the southernmost limit of their distribution and are especially sensitive to global warming. We analyzed the community of granivorous rodents in the Pyrenees, one of the southernmost mountain ranges of Europe. Rodent species were identified by DNA with particular attention to the Apodemus species, which are prominent seed-dispersing rodents in Europe. We confirmed for the first time the presence of the yellow-necked mouse, Apodemus flavicollis, in central Pyrenees, a typical Eurosiberian species that reaches its southernmost distribution limit in this area. We also found the wood mouse, Apodemus sylvaticus, a related species more tolerant to Mediterranean environments. Both rodents were spatially segregated by altitude. A. sylvaticus was rare at high altitudes, which might cause the genetic differentiation between populations of the different valleys reported here. We also found other seed consumers like dormice, Elyomis quercinus, and voles, Myodes glareolus, with marked habitat preferences. We suggest that population isolation among valleys may increase the genetic diversity of rodents, like A. sylvaticus. We also highlight the potential threat that global warming may represent for species linked to high-altitude refuges at the southern edge of its distribution, like Apodemus flavicollis. Finally, we discuss how this threat may have a dimension in the conservation of alpine forests dispersed by these rodent populations.

Key words: DNA analysis, Pyrenees, rodents, seed dispersal, vegetation structure

INTRODUCTION

Granivorous rodents have a strong influence on plant regeneration patterns as prominent seed consumers and dispersers, thus playing an essential role in ecosystem function (Vander Wall 1990; Sunyer et al. 2015). Many authors have suggested their potential as ecological bio-
indicators of sustainable forest management and conservation (Pearce & Venier 2005; Avenant 2011). Hence, analysis of their diversity and distribution may help to better understand and preserve the structure and functioning of many forest ecosystems.

The distribution, patterns of habitat selection and population dynamics of granivorous rodents are strongly conditioned by climate, predation risk and food availability (Mazurkiewicz 1994; McCain 2005) and can be approached from different spatial scales, ranging from macrohabitat and a landscape context, to microhabitat (Bailey et al. 1996; Boyce et al. 2003). A given rodent species will basically fit a given area depending on whether: (i) the environmental requirements are met; (ii) there is shelter against predators; and (iii) food availability is sufficient. As most habitats often hold more than a single rodent species, their coexistence and community assemblage may also depend on the partitioning of food resources (e.g. seeds), variation in daily seasonal activity and microhabitat segregation (Kotler 1984; Mitchell et al. 1990; Kotler et al. 1991; Østfeld 1992; Canova 1993; Hille & Mortelliti 2010). According to the concept of phylogenetic niche conservatism (Wiens & Graham 2005; Losos 2008; Wiens et al. 2010), close relative species are likely to possess similar niches, which theoretically would result in stronger competition, whereas species more separated phylogenetically are more likely to coexist by exploiting different resources.

Alpine and sub-alpine ecosystems are attractive scenarios for analyzing small rodent distributions, as the steep gradients of physical characteristics and climatic variables result in contrasting habitats that may lead to changes in species richness of small mammals over short distances (Heaney 2001; McCain 2004, 2005). This heterogeneity of alpine and sub-alpine habitats may also promote species diversity (Tews et al. 2004; Hortal et al. 2009) and shape population genetic structure at the intra-specific level (Trizio et al. 2005). Moreover, high mountains are among the most sensitive regions to climate change, where predictions suggest a higher warming than the global average (Kohler & Maselli 2009).

The Pyrenees is among the most emblematic alpine systems of southern Europe that has acted as refuge for species of northern latitudes and as a place of speciation after the end of the last cold period of Pleistocene (Blondel & Aronson 1999). Thus, the Pyrenees represent the southern distributional limit for many mammals with biogeographical origins in northern latitudes, and the point of intersection with species from other geographic areas. This is especially the case for 2 scatter-hoarding rodents belonging to the Apodemus genus: the yellow-necked mouse, Apodemus flavicollis (Melchior, 1834), a typical Eurosiberian species that has been found in the western Pyrenees (Castièn & Gosàlbez 1994), although its presence in central and oriental Pyrenees is yet to be confirmed. This species is considered to overlap, in terms of ecological niche, with the wood mouse, Apodemus sylvaticus (Linnaeus, 1758), a more Mediterranean species with which it shares morphological similarities, rendering their identification by means of molecular techniques (DNA sequencing) the most reliable method for species determination (Arrizabalaga et al. 1999; Michaux et al. 2001; Ancillotto et al. 2017).

DNA sequencing provides further advantages that go beyond species determination. At the intra-specific level, it allows identifying landscape and environmental features that affect inter-population gene flow, thus shaping genetic structure (Giordano et al. 2007). In the case of rodents, some mitochondrial markers, such as the control-region (d-loop), show a high mutation rate that permits detection of population structure at a scale of just a few kilometers (Hirotani et al. 2004). With these markers it is possible to estimate the degree of connection between populations located in different habitats, something that would not otherwise be possible.

The present study aims to: (i) assess the community structure and the patterns of space use by granivorous rodents in different forest systems of the central Pyrenees, from the macrohabitat context to a microhabitat perspective; and (ii) test the genetic population structure and thereby estimate the degree of connectivity and movement between populations at a landscape scale for scatter-hoarding rodents of Apodemus spp. Finally, we use these results to evaluate how the distribution of seed-eating rodents in these high mountain ranges may influence forest regeneration in a scenario of global warming.

MATERIALS AND METHODS

Study area

The study was conducted in the National Park of Aigüestortes i Estany de Sant Maurici (PNAESM), in north-east Spain (Catalan Pyrenees; 42°34′N, 1°0′E), which holds the most representative sub-alpine and alpine habitats of the central Pyrenees. The climate is montane and characterized by a variable number of microclimates, influenced by the altitude (between 1200 and 3000 m) and topography of the valleys and slopes. Annual rainfall (also in the form of snow) is approxi-
mately 1500 mm (Torbidoni et al. 2005) and the mean annual temperature is 4.6 °C (mean maximum of 9.6 °C and mean minimum of −0.5 °C; Arnán et al. 2014). Most sub-alpine systems in the Park are dominated by coniferous forests of black pine (Pinus uncinata) and silver fir (Abies alba), but Scots pine (P. sylvestris), birch (Betula pendula) and beech (Fagus sylvatica) are also present at lower altitudes. Besides closed forests, the Park holds open areas as meadows with different tree species and screes due to the typical process of frost weathering that occurs in alpine and sub-alpine systems.

**Sampling of granivorous rodents**

The main trapping campaigns were performed along 3 sampling points of the “Valley of Espot” in the summers of 2013 and 2014. In summer 2013, we additionally sampled 3 sites in the “Valley of Boi,” located 5 km from the “Valley of Espot,” to analyze the potential genetic differences between the rodents captured in each valley (Fig. 1). We sampled 4 common habitats of PNA-ESM located at 4 different altitudes: (i) pine forests of *Pinus sylvestris* at 1600–1700 m (Boí and Espot); (ii) beech (*Fagus sylvatica*) and oak (*Quercus robur*) forests at 1700–1800 m (Boí); (iii) meadows with hazels (*Corylus avellana*) and birches (*Betula pendula*) at 1700–1800 m (Espot); and (iv) silver-fir forests (*Abies alba*) at 1800–1900 m (Boí and Espot).

In each habitat type, we established 10 trapping stations, each one consisting of 10 Sherman live traps (23.5 × 8 × 9 cm; H.B. Sherman Traps, Tallahassee, Florida, USA) distributed along 2 lines of 5 traps with a distance of 10 m among them (i.e. 100 traps per habitat type). Each trapping station was separated at least 50 m from other trapping stations of the same habitat, so that trap distribution allowed us to make an assessment at 3 spatial scales: (i) at the scale of habitat (macrohabitat) to analyze the distribution of small rodents along the different habitat types and the changes in community structure at a wider spatial scale; (ii) at the scale of the trapping station (mesohabitat) to analyze the extent of spatial segregation between different species within a shared habitat type; and (iii) at the scale of individual trap (microhabitat) to find out preferences among different vegetation structures.

Traps were baited with a mixture of flavor and tuna in oil, and an apple piece to avoid dehydration. All the traps also contained a handful of hydrophobic cotton so that the captured rodents could make a nest to keep warm until they were marked and released (Muñoz et al. 2009). At each sampling point, each trapping campaign had a duration of 4 nights, and in each year the traps were placed in the exact same places, as they were geo-referenced by GPS. Each captured rodent was marked with a subcutaneous microchip inserted with a specialized syringe. This marking method allowed us to identify every recaptured individual in order to calculate the total abundance of rodents in each habitat type (number of different individuals captured) and also to estimate the survival rate from the first summer to the second summer (Sunyer et al. 2015, 2016).

Once marked, all individuals were released at the exact point where they had been captured. Microhabitat structure was measured in a 1-m radius around each Sherman trap. Following previous studies (Muñoz et al. 2009), we carried out a visual estimation of cover values (% projection on the ground) of: (i) tree cover; (ii)

![Figure 1](image_url) Study area in central Pyrenees with the location of the 2 valleys and the 3 trapping points in each valley.
high (>30) and low (<30 cm) shrub cover; (iii) high (>30 cm) and low (<30 cm) herbaceous cover; (iv) leaf litter; (v) rock cover; and (vi) bare soil.

Rodent identification

Most granivorous rodents found at the study area were easily identifiable on the basis of morphological traits. However, due to the difficulties in identifying the 2 potential Apodemus species (A. sylvaticus and A. flavicollis) with the naked eye (Arrizabalaga et al. 1999; Reutter et al. 1999), we performed genetic analyses (Michaux et al. 2001). DNA was extracted from rodent tissue (tail tip or ear skin) according to the Aljanabi and Martinez (1997) salt extraction protocol. We amplified the mitochondrial control region (d-loop) by polymerase chain reaction (PCR) using the primers M15997 and H16401 (Stacy et al. 1997; for the details of the PCR conditions see Hirota et al. 2004). Sequencing was run on a 3730XL DNA analyzer and sequences were edited using SEQUEENCER 4.1 (Gene Codes, Ann Arbor, MI, USA). The sequences were trimmed to 297 base pairs to reduce the proportion of missing data. We chose this region of the mitochondrial DNA (d-loop) because its mutation rate is very high and, at the same time, allowed us to distinguish the 2 species of Apodemus, and it permitted estimation of the patterns of inter-population gene flow and genetic structure at the intraspecific level (Hirota et al. 2004). First, we identified all Apodemus to the species level by blasting the sequence of each rodent against GenBank to assess the percentage of coin-cidence with the sequences of A. sylvaticus and A. flavicollis available at that database (number of different nucleotides by the total number of compared nucleotides).

We confirmed the identification by performing a phylogenetic analysis with all the sequences of mice tentatively assigned to 1 species or the other. We pooled all the sequences and aligned them with CLUSTALW supplied via http://align.genome.jp (gap open and gap extension penalties were those provided by default by the software: 15 and 6.66, respectively). This alignment was collapsed into its unique haplotypes using Perl Collapse (Chesters 2013) and these unique sequences were further pooled with mitochondrial d-loop sequences of A. flavicollis and A. sylvaticus, downloaded from GenBank, which were used as external reference sequences (accession numbers: AJ410624, AJ410626, AJ410628, EU220767, EU220768, EF396216 and EF396220) along with that of Apodemus mystacinus (accession numbers AY623065 and AY623066). This new set of sequences was aligned using CLUSTALW with the same gap penalty and extension parameters as before, and used to build a phylogenetic tree in which A. mystacinus was the outgroup, as it is the closet outer species to A. flavicollis and A. sylvaticus (Michaux et al. 2002). The phylogeny was built following Bayesian inference using MR BAYES 3.2 (Ronquist et al. 2012) applying a HKY+Gamma substitution model, according to the results of PARTITION FINDER 1.1.1 (Lanfear et al. 2012). Two parallel runs of 2 million generations each were conducted using one cold and 2 incremental-ly heated Markov chains (L = 0.2), sampling every 1000 steps. We first checked the standard convergence diagnostics implemented in MRBAYES and then assessed the average standard deviation of the split frequencies to deduce that the Markov chain had become stationary. After 500 000 generations, the average standard deviation of the split frequencies stabilized at values close to zero (0.001). Hence, phylogenetic trees were summarized using the all-compatible consensus command with 25% burn-in. These analyses enabled species to be identified by exploring the clusters in the phylogenetic tree.

Data analyses

The effects of habitat type on rodent abundance were analyzed by means of a generalized linear model (GLZ) with the error modeled as a Poisson distribution. We tested the effects of habitat type and microhabitat structural characteristics on the occurrence of small rodents by means of GLZ with error modeled as a binomial distribution. We followed Akaike’s information criterion (AIC) for model selection. To test whether the different groups of rodents were segregated in space within each shared habitat type we used multiple linear regression models with a stepwise procedure. We also tested at the scale of trapping station the effects of vegetation structure on the use of each group of rodents within each habitat, using the average values of the microhabitat variables measured in all traps of each trapping station.

Regarding the Apodemus species, we performed a GLZ with the error modeled as a Poisson distribution testing the effects of habitat type and mouse species (A. sylvaticus and A. flavicollis) on the total abundance of mice. Finally, we analyzed the effects of habitat type and microhabitat structural characteristics on the occurrence of both Apodemus spp separately by means of GLZs with the error modeled as a binomial distribution. We used AIC for selecting the proper model for the 2 mouse species. For both A. sylvaticus and A. flavicollis, we additionally analyzed inter-population genetic differ-
RESULTS

The density of small mammals at the study area was very low. We captured 58 small rodents with a trapping effort of 3600 traps-nights, including mice, dormice and voles. We did not recapture any rodent captured in the first year during trap sessions in the second year. Mice were the most abundant group, representing 59% of captures and occurring in all habitat types. The genetic analysis of mice tissues identified the wood mouse (*Apodemus sylvaticus*) and the yellow-necked mouse (*A. flavicollis*). After blasting the sequences of unidentified specimens against GenBank, all of them could be assigned to 1 species or the other according to their genetic similarity (greater than 97% in all cases) with reference sequences available from that database. Con-cordant with theories of DNA taxonomy, individuals identified as *A. sylvaticus* and *A. flavicollis* formed discrete clusters, separated by substantial branch lengths (Fig. 2), representing genetic discontinuities that allowed us to distinguish each species from the other (Herbert and Gregory 2005), thus confirming our initial species determination based on raw genetic similarity and GenBank blasting. The phylogenetic tree showed that *A. sylvaticus* and *A. flavicollis* were more closely related than with *A. mystacinus* or with those from other parts of Europe downloaded from GenBank and used as references (Poland and Greece for *A. sylvaticus* and *A. flavicollis* respectively; Fig. 2). In both species these non-Iberian sequences were grouped in clusters with high node support (over 0.8 Bayesian posterior probability; Fig 2).

Our results showed that *A. sylvaticus* and *A. flavicollis* shared 3 forest types: pinewoods (1600–1700 m), oak forests (1700–1800 m) and silver firs (1800–1900 m). *A. sylvaticus* was significantly more abundant than *A. flavicollis* (χ²₁ = 5.3, *P* = 0.021), except in silver firs, where their abundance did not differ significantly (χ²₁

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**Figure 2** DNA phylogeny of 1 mitochondrial gene (control region d-loop). Tree topology was inferred using Bayesian inference (HKY + gamma substitution model). Support for each node is represented by the Bayesian probability value. The tips correspond to the unique haplotype of either *Apodemus sylvaticus* or *Apodemus flavicollis* sequenced at our study site and the reference sequences downloaded from GenBank. The species membership and the geographical origin of each cluster are indicated.
Both species showed a segregation at the scale of habitat type ($\chi^2 = 8.4, P = 0.015$), as the abundance of each species was inversely related to the abundance of the other species (Fig. 3), and in meadows *A. sylvaticus* was the only mouse species. This segregation coincided with changes in altitude so that *A. sylvaticus* was comparatively well represented in the lowlands and *A. flavicollis* in the highlands. At a “meso-scale” level (i.e. trapping station) our results pointed to a strong spatial segregation, because both species were always captured in different trapping stations within the shared habitats. The analysis of microhabitat preferences showed that the probability of occurrence of *A. sylvaticus* was marginally associated with both shrub cover (positively) and rock cover (negatively), whereas no association was found between the microhabitat variables and *A. flavicollis* (Table 1).

The intra-specific population analyses at a landscape scale revealed a total of 13 haplotypes in *A. sylvaticus* and 5 in *A. flavicollis*, with a high number of single ones. Due to the low number of individuals per population, we could not perform any population genetic analyses on *A. flavicollis*. For *A. sylvaticus*, we compared population genetics between the valleys of Espot and Boí (Fig. 1), by pooling all individuals captured in the 3 populations of each valley. According to the high number of single haplotypes (Table 2), the gene and nucleotide diversity of *A. sylvaticus* was high in both sites (0.83 ± 0.08 and 0.010 ± 0.006 in Espot; 0.97 ± 0.06 and 0.012 ± 0.008 in Boí). In addition, the exact test of sample differentiation based on haplotype frequencies (20 000 Markov steps) showed that there was a significant genetic differentiation between the *A. sylvaticus* populations inhabiting the 2 valleys ($P = 0.014$).

**Figure 3** Mean (± SE) values of capture success of *Apodemus flavicollis* and *Apodemus sylvaticus* captured in shared habitat types.

**Figure 4** Mean (± SE) capture success of mice, dormice and voles in different habitat types of central Pyrenees.

**Table 1** Summary statistics for the generalized linear models with binomial distribution applied to analyze the effects of microhabitat variables on the probability of occurrence of *Apodemus sylvaticus* (Model AIC: 198.5) and *Apodemus flavicollis* (Model AIC: 99.7)

<table>
<thead>
<tr>
<th>Variable</th>
<th><em>Apodemus sylvaticus</em></th>
<th></th>
<th></th>
<th><em>Apodemus flavicollis</em></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Parameter SE</td>
<td>Z</td>
<td>P</td>
<td>Parameter SE</td>
<td>Z</td>
<td>P</td>
</tr>
<tr>
<td>Shrub</td>
<td>0.015 0.008</td>
<td>1.875</td>
<td>0.054</td>
<td>0.007 0.024</td>
<td>0.294</td>
<td>0.779</td>
</tr>
<tr>
<td>Herb</td>
<td>— —</td>
<td>—</td>
<td>—</td>
<td>0.004 0.023</td>
<td>0.172</td>
<td>0.848</td>
</tr>
<tr>
<td>Tree</td>
<td>0.004 0.006</td>
<td>0.667</td>
<td>0.477</td>
<td>−0.002 0.011</td>
<td>−0.175</td>
<td>0.842</td>
</tr>
<tr>
<td>Litter</td>
<td>— —</td>
<td>—</td>
<td>—</td>
<td>0.009 0.022</td>
<td>0.409</td>
<td>0.693</td>
</tr>
<tr>
<td>Rock</td>
<td>−0.020 0.011</td>
<td>−0.185</td>
<td>0.063</td>
<td>0.005 0.020</td>
<td>0.245</td>
<td>0.811</td>
</tr>
</tbody>
</table>

—, These variables are not included in the final models.
Besides these 2 typical scatter-hoarding rodents, we also captured other seed-eating rodents such as dormice, *Elyomis quercinus* (Linnaeus, 1766) (24% of all captures) and voles, *Myodes glareolus* (Schreber, 1780) (17%), which showed a marked habitat selection. Dormice were not present in beech-oak forests and were nearly absent in meadows. Conversely, voles were not present in beech-oak forests and pinewoods but were well represented in both meadows and silver firs (Fig 4). Even so, all habitats were shared by at least 2 different groups of rodents except beech-oak forests, where mice were the only rodent captured. Pinewoods were shared by mice and dormice, whereas in silver firs and meadows we found mice, dormice and voles.

At the mesohabitat scale (i.e. trapping station within shared habitat types), mice were not spatially aggregated or segregated with voles or dormice; they were randomly captured across the different trapping stations of the same habitat. Instead, the abundance of voles and dormice were significantly associated with the trapping stations in silver firs (*t*<sub>1,27</sub> = 3.54, *P* = 0.01), suggesting that, at the mesohabitat scale, they preferred the same environments within this habitat type. However, we found no associations between the abundance of each rodent group and the average of vegetation variables at the scale of the trapping station. In contrast, at the microhabitat scale (i.e. individual trap), mice and vole abundance were significantly associated with shrub cover, whereas dormice were associated with litter and rock cover (Table 3).

**DISCUSSION**

Our DNA analysis confirms, for the first time, the presence of the yellow-necked mouse (*A. flavicollis*) in the central Pyrenees, suggesting that this area covers part of its southern distribution, potentially connecting it with other populations found in the Catalan coastal mountain ranges and the Montseny massif in the east (see Arrizabalaga et al. 1999; Arrizabalaga & Torre 2002; Torre et al. 2015). *A. flavicollis* records based on DNA identification were available only for the western part of the southern slope of the Pyrenees (Michaux et al. 2005), but its presence in the central and oriental sectors of this mountain range is not clear yet, due to the limited studies in this zone (Gosálbez & López-Fuster 1985; Arnán et al. 2014) and the difficulty of differentiating it from the wood mouse (*A. sylvaticus*) using morphological traits (Arrizabalaga & Torre 2002; Ancillotto 2017).

The phylogenetic tree confirmed that *A. flavicollis* and *A. sylvaticus* are closely related species, and coin-

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**Table 2** Frequency of each *Apodemus sylvaticus* haplotype in the valleys of Espot and Boí

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>13</td>
<td>1</td>
</tr>
</tbody>
</table>

**Table 3** Summary statistics of the generalized linear models with binomial distribution applied to determine the effects of habitat type and microhabitat structural characteristics on the probability of occurrence of small mammal species

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mice</th>
<th>Dormice</th>
<th>Vole</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Parameter</td>
<td>SE</td>
<td>Z</td>
</tr>
<tr>
<td>Habitat</td>
<td>−4.257</td>
<td>0.774</td>
<td>−5.50</td>
</tr>
<tr>
<td>Shrub</td>
<td>0.014</td>
<td>0.007</td>
<td>2.059</td>
</tr>
<tr>
<td>Herb</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Tree</td>
<td>0.004</td>
<td>0.047</td>
<td>0.851</td>
</tr>
<tr>
<td>Litter</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Rock</td>
<td>0.015</td>
<td>0.008</td>
<td>1.786</td>
</tr>
</tbody>
</table>

—, These variables are not included in the final models.
cident with phylogenies based on nuclear and other mitochondrial markers. In fact, both species are included in the same subgenus (Sylvaemus) within the Apodemus genus (Michaux et al. 2002). The tree also revealed the genetic distinctiveness of the Iberian haplotypes in both species with respect to those from other areas of Europe (Greece and Poland for A. flavicollis and A. sylvaticus, respectively). A. flavicollis had a glacial refuge in the Balkans and, from there, it recolonized the remainder of Europe, including the Iberian Peninsula. These differences between Iberia and Greece have been observed in phylogeographic studies based on other mitochondrial markers (cytochrome b: Michaux et al. 2005). In the case of A. sylvaticus, Iberia was the refuge from which it recolonized north-central Europe after glaciation (Michaux et al. 2005). According to this, the differences between the Iberian haplotypes and those from Poland, included as reference sequences, should not be as high. However, despite their higher genetic proximity, Iberian haplotypes are more closely related than with those from north-central Europe, which is also reflected in phylogenies based on cytochrome b (Michaux et al. 2003). Moreover, in our study, based on a mitochondrial marker with a higher mutation rate (the control region d-loop), these differences are expected to be higher between geographical regions.

Our study points identify a spatial segregation between A. sylvaticus and A. flavicollis at the scale of forest type, as an increase in the abundance of one species was associated with a decrease in the other. This segregation becomes more dramatic at a “mesoscale” (i.e. trapping station), because both species were always captured in different trapping stations despite the shared habitat types. Part of this segregation may be explained by altitude (see Saint-Girons 1973), as A. sylvaticus abundance decreased with altitude.

Interestingly, in the valley of Espot all captures of A. flavicollis came from silver-fir forests (the habitat located at the highest altitude), where A. sylvaticus was absent. A. sylvaticus is a generalist mouse that has been found along a significant variety of habitat types in the Iberian Peninsula, from coastal dunes to northern mature forests (Torre et al. 2002; Muñoz et al. 2009; Sunyer et al. 2016), although our results suggest its worst performance may be at high altitudes. In contrast, A. flavicollis shows more strict forest requirements and is more linked to Euro-Siberian environments (Arrizabalga & Torre 2002), which seem to be fulfilled by most sub-alpine habitats of the Central Pyrenees, the southern limit of its range in Europe.

The rarity and arguably lower performance of A. sylvaticus at high altitudes may be due to the extreme environmental conditions in winter, with abundant snow cover and extremely low temperatures, which limit food availability and may account for the high rodent mortality. Thus, the mortality of small mammals in general is probably high during winter in this area, as suggests the lack of recaptures from the first to the second year of our study. In fact, this harsh alpine environment may also explain the generally low population densities compared to other nearby temperate ecosystems at lower altitudes (Sunyer et al. 2016). Similar low densities of small mammals have been documented in this and other alpine areas (e.g. Bertolino et al. 2001; Arnán et al. 2014). These severe environmental conditions due to altitude may be, in fact, the cause of the genetic differentiation between the populations of A. sylvaticus inhabiting different valleys, because to move from Boí to Espot it is necessary to cross high alpine habitats. The intra-specific population genetic analyses showed that some haplotypes that were relatively frequent in Espot were not present in Boí. The high number of haplotypes (including many single haplotypes) is common in studies using the mitochondrial control region as a genetic marker (see Trizio et al. 2005). The control region is not expressed functionally so that mutations are not eliminated by natural selection and with rodent short generation time, mutations are likely to accumulate in the mitochondrial genome (Li et al. 1996). When movements between populations are constrained, these new haplotypes resulting from local mutations are not spread resulting in inter-population genetic differentiation (Hirotà et al. 2004). In our study areas, this occurred at a relatively small spatial scale (the distance between the 2 valleys is only 5 km). Accordingly, other studies have demonstrated that such a genetic differentiation can take place at similar distances when the habitat between 2 populations is not suitable for dispersal (Hirotà et al. 2004). However, we need to be cautious with these results because mitochondrial DNA is only inherited maternally (Gyllensten et al. 1985) and, thus, just reflects female interchange between the 2 valleys. Regardless, we do not believe that male crossings from Boí to Espot are likely, because we did not observe a sex-biased altitudinal distribution in A. sylvaticus.

We found significant changes in the distribution of other small rodents among sub-alpine habitat types. The garden dormouse (E. quercinus) was present in all sub-alpine habitats of the central Pyrenees except in beech-oak forests, consistent with its widespread distri-
Seed-dispersing rodents in central Pyrenees

The habitats monitored in this study are dominated by tree species producing seeds (pine nuts, hazels, beechnuts, acorns) that are regularly consumed by the 4 rodent species reported. Two of these species are strict seed predators, the bank vole (Myodes glareolus) and the garden dormouse (Elyomis quercinus), with seeds representing >90% of their diet (Kuipers et al. 2012; Bonachi et al. 2017). They consume an important proportion of the seed crops in Western Europe (Ouden et al. 2005), especially in oak forests, but seed losses by these species are not expected in beech-oak forests in the Pyrenees because they were not found in these habitats.

However, they may cause a reduction in tree reproduction in habitats with hazel trees (i.e. meadows) where both species are well represented and where large numbers of hazels are usually found partially consumed or damaged by rodents (pers. obs.).

Most importantly, the 2 species of mice captured (A. sylvaticus and A. flavicollis) are prominent seed dispersers that may influence the forest recruitment dynamics, especially in the case of oaks, hazels and beech (Jensen & Nielsen 1986; Kollman & Schill 1996; Ouden et al. 2005; Sunyer et al. 2015; Zwolak et al. 2016). Given that patterns of seed selection differ between the 2 Apodemus species (García-Castaño et al. 2006), the increase of global temperatures, favoring the presence of A. sylvaticus at the detriment of A. flavicollis, may have cascading effects on the patterns of seed dispersal and recruitment in the forest habitats of the Pyrenees.

Thus, these alpine ecosystems, hosting low densities of small mammals that have an important role in forest tree recruitment, can be especially sensitive to the effects of global warming. Further research, carried out at a wider scale, should be focused on rodent population dynamics and genetics in the long term, as well as analyzing the patterns of seed dispersal by different rodents. This knowledge will improve our understanding on the structure and functioning of alpine and sub-alpine ecosystems, and help to preserve biodiversity in the context of global warming.

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Environmental variation shifts the relationship between trees and scatterhoarders along the continuum from mutualism to antagonism

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Abstract

The conditional mutualism between scatterhoarders and trees varies on a continuum from mutualism to antagonism and can change across time and space, and among species. We examined 4 tree species (red oak [Quercus rubra], white oak [Quercus alba], American chestnut [Castanea dentata] and hybrid chestnut [C. dentata × Castanea mollissima]) across 5 sites and 3 years to quantify the variability in this conditional mutualism. We used a published model to compare the rates of seed emergence with and without burial to the probability that seeds will be cached and left uneaten by scatterhoarders to quantify variation in the conditional mutualism that can be explained by environmental variation among sites, years, species, and seed provenance within species. All species tested had increased emergence when buried. However, comparing benefits of burial to the probability of caching by scatterhoarders indicated a mutualism in red oak, while white oak was nearly always antagonistic. Chestnut was variable around the boundary between mutualism and antagonism, indicating a high degree of context dependence in the relationship with scatterhoarders. We found that different seed provenances did not vary in their potential for mutualism. Temperature did not explain microsite differences in seed emergence in any of the species tested. In hybrid chestnut only, emergence on the surface declined with soil moisture in the fall. By quantifying the variation in the conditional mutualism that was not caused by changes in scatterhoarder behavior, we show that environmental conditions and seed traits are an important and underappreciated component of the variation in the relationship between trees and scatterhoarders.

Key words: Castanea dentata, conditional mutualism, Quercus alba, Quercus rubra, temperate forest

INTRODUCTION

The relationship between scatterhoarders and hardwood trees varies along a continuum from mutualism to antagonism (Theimer 2005; Zwolak & Crone 2012). In this interaction, scatterhoarders benefit from having a non-perishable food source throughout periods of food
scarcity, whereas trees potentially benefit from seed dispersal and seedling establishment when caches are not recovered. However, the stability of this relationship varies considerably. High relative numbers of scatterhoarders can lead to low seedling recruitment as a result of high rates of seed predation; however, in contrast, scatterhoarders can also benefit trees through dispersal and subsequent abandonment of the caches when seed abundance is high. Dispersal is critical for allowing seeds to escape the shadow of the parent tree, where density-dependent mortality is highest due to a variety of factors (Janzen 1970; Connell 1971; Howe & Miritti 2000; Liu et al. 2012). Therefore, understanding how the scatterhoarder–tree relationship shifts between a mutualistic and antagonistic outcome is central to understanding the process of animal-mediated seed dispersal.

Theimer (2005) describes scatterhoarders as “conditional mutualists,” whose effect on trees can vary over time and space as the ratio of seeds to scatterhoarders varies. Zwolak and Crone (2012) propose a simple mathematical model to quantify the nature of this relationship between scatterhoarders and trees using only 3 variables. The first, \( Es \), is the rate of emergence of seeds from the soil surface (i.e. those that are not cached by scatterhoarders). The second, \( Ec \), is the emergence rate of seeds when cached. The last variable, \( Pc \), is the probability that cached seeds remain uneaten (i.e. the probability that seeds will be cached and left uneaten). When the probability is higher than the ratio of seedling emergence on the surface to emergence in a cache, the seed–scatterhoarder relationship is mutualistic. If the ratio of the emergence of the seeds from the surface to the emergence of the seeds in the cache is higher than the probability that seeds will be cached and left uneaten, the relationship is antagonistic (Fig. 1).

Most studies on the scatterhoarder–tree relationship have focused on understanding factors that influence \( Pc \), which is directly related to the ratio of seeds to scatterhoarders. When there is a low seed : scatterhoarder ratio (e.g. few seeds are produced in a year), virtually all of the seeds will be handled by a scatterhoarder, and the vast majority of seeds will be eaten and \( Pc \) will be low. When there is a high seed to scatterhoarder ratio (e.g. in a year of high seed production) the scatterhoarders cache more seeds than they require, which results in a higher \( Pc \) (Garcia et al. 2002; Jansen et al. 2004; Zwolak & Crone 2012; Lichti et al. 2015; Zwolak et al. 2016; but see Xiao et al. 2013). The number of seeds produced in a population of trees is strongly influenced by “masting,” the synchronous production of seeds by most trees in a population in some years followed by years of very low seed production (Sork 1993). Hence, seed numbers can vary considerably, causing the seed : scatterhoarder ratio to fluctuate substantially. The Zwolak and Crone (2012) model illustrates how the abiotic environment and the characteristics of the seeds also influence this interaction. Yet, we have little information on the role of environmental and seed characteristics in shaping this relationship.

In this study, we investigated 2 potential sources of variation in the \( Es/Ec \) ratio: (i) the provenance, or source, of the seed; and (ii) different abiotic conditions to which seeds were exposed during germination and establishment. Different seed origins may have different responses to abiotic conditions because of local adaptation to various conditions. Evidence of local adaptation of oak seeds and seedlings to varying environmental conditions (e.g. frost) has been documented in several sites in Europe (Deans & Harvey 1996; Jensen 2000), although a similar study in North America produced more ambiguous results (Huang et al. 2016)

The second source of variation in the \( Es/Ec \) ratio that we investigated includes abiotic conditions such as soil moisture, leaf litter and temperature. Soil moisture increases seed survival and germination rates (Garcia et al.

![Figure 1 Graphical model of the relationship between scatterhoarders and trees from Zwolak and Crone (2012).](image-url)
al. 2002) by preventing desiccation (Lopez-Barrera & Gonzalez-Espinosa 2001), improving frost resistance (Esteso-Martinez & Gil-Pelegrín 2004) and potentially increasing “germinative energy” (number of days required to reach 50% germination), although this latter effect is still unclear (Huerta-Paniagua & Rodríguez-Trejo 2010). Indeed, leaf litter and burial have been shown to induce germination in acorns of oak (Houle 2002) and in pine seeds (Briggs et al. 2009). We therefore hypothesize that warmer temperatures in burial sites may explain a key benefit of caching.

The goal of this study was to quantify the variation in the hardwood tree seed: the scatterhoarder relationship using the model presented by Zwolak and Crone (2012). Specifically, we focus on 2 sources of variation in the $Es/Ec$ ratio: seed provenance and abiotic conditions, both in and outside the cache. To test these, we established seed plots with predator exclusion cages. We established plots in 5 different sites across the Central Hardwoods Region to expose the seeds to varying environmental conditions, and at 1 site (Virginia) we also varied seed source to test its effect on seed survival. We use seeds from red oak ($Quercus rubra$), white oak ($Quercus alba$), American chestnut ($Castanea dentata$), and a hybrid American-Chinese chestnut ($C. dentata \times C. mollissima$).

We tested 3 predictions. First, we predicted that $Es/Ec$ values will correspond to seed dormancy requirements because the microsite characteristics within a cache will prevent desiccation and enhance survival during dormancy. Acorns of red oak, for example, should have lower $Es/Ec$ ratios (higher potential for mutualism) because of their longer dormancy period; white oaks acorns will have a higher $Es/Ec$ ratio (higher potential for antagonism) because they show no dormancy; and chestnuts are somewhere in between due to their intermediate dormancy period. Second, we predict that abiotic conditions will have a stronger effect on $Es/Ec$ than seed source. We predict that we will see more variation in $Es/Ec$ across sites and years (differing abiotic conditions), than when comparing seed sources within the same site and year. Finally, we predict that temperature will explain much of the variation in the $Es/Ec$ ratio. We expect sites with lower temperatures to have lower $Es/Ec$ ratios (more potential for mutualism) because these seeds will benefit more from the extra protection and warmth within a cache. We predict that seeds planted in northern sites will have a more mutualistic relationship (lower $Es/Ec$) with scatterhoarders than those planted in the south.

**MATERIALS AND METHODS**

**Study species**

Study species include red oak ($Q. rubra$), white oak, ($Q. alba$), American chestnut ($C. dentata$) and a hybrid American/Chinese chestnut ($C. dentata \times C. mollissima$). The oak species occur throughout the eastern US deciduous forests with ranges that extend as far south as Texas and as far north as Canada (Bourdeau 1954). The primary seed disperser for these tree species is the eastern gray squirrel ($Sciurus carolinensis$), with eastern chipmunk ($Tamias striatus$) and mice ($Peromyscus$) also dispersing seeds.

Red oak acorns require 2 growing seasons to mature (Straub 2016) and a dormancy period between seed fall and germination the following spring (Lichti et al. 2014). Red oak acorns in eastern deciduous forests typically have high concentrations of tannins (Smallwood et al. 2001): chemical deterrents that can have negative physiological effects on granivores (Chung-MacCoubrey 1997). In addition, red oak acorns have higher fat levels, which serves as the primary source of energy in the acorn. Because red oak seeds have a long dormancy period, granivores prefer to cache red oak seeds because of their low perishability (Hadj-Chikh et al. 1996; Smallwood et al. 2001).

Acorns of white oak have lower fat and tannin content, and they germinate in the autumn shortly after seed fall (Hadj-Chikh et al. 1996; Smallwood et al. 2001). Squirrels tend to eat white oak acorns and immediately cache red oaks because of these differences in perishability; when they do cache white oak acorns they often excise the embryos, preventing germination (Hadj-Chikh et al. 1996).

The historic range of the American chestnut extends from Maine to Georgia (Paillet 2002). Between 1900 and 1925, chestnut populations plummeted due to the introduced fungal pathogen, chestnut blight ($Cryptonectria parasitica$). The social and economic importance of the tree prompted breeding efforts to create a blight resistant tree (Jacobs et al. 2013). When presented with chestnuts, and red oak and white oak acorns, squirrels prefer to eat white oak acorns; likewise, in the presence of only chestnuts and white oak acorns, white oak acorns are selectively eaten (Lichti et al. 2014; Blythe et al. 2015).

**Study sites**

We conducted experiments over 3 years in 5 hard-
wood forests: Atkinson Grove, Maine; Harvard Forest, Massachusetts; Martell Forest, Indiana; Dorrance Township, Pennsylvania; and the College Woods, Virginia (Tables 1 and 2). Maine has warm summers, with temperatures reaching 26 °C, followed by 4 to 8 months of temperatures as low as −12 °C. Similarly, Massachusetts has an equally short summer, capped at 26 °C, with equally cold winters: −11 °C in Barre. The Martell Forest site near West Lafayette, Indiana has a winter low of −8 °C and a summer high of 29 °C, but with moderate temperatures from April to October. Northeastern Pennsylvania has an even milder winter, with a low of −6 °C, followed by 5 to 7 months of summer, peaking at 30 °C. The College of William and Mary’s College Woods of Williamsburg, Virginia experiences minimal winter conditions, with a winter low of −1 °C and a summer high of 30 °C.

Table 1 Summary of the provenance study design in Virginia

<table>
<thead>
<tr>
<th>Year</th>
<th>Species</th>
<th>Provenances planted in Virginia</th>
</tr>
</thead>
<tbody>
<tr>
<td>2012</td>
<td>Red Oak</td>
<td>Virginia, Pennsylvania, Maine</td>
</tr>
<tr>
<td>2013</td>
<td>White Oak</td>
<td>Virginia</td>
</tr>
<tr>
<td>2015</td>
<td>Hybrid</td>
<td>Maine</td>
</tr>
<tr>
<td>2012</td>
<td>Chestnut</td>
<td>Massachusetts, Maine</td>
</tr>
<tr>
<td>2013</td>
<td>(B, F2)</td>
<td>Maine</td>
</tr>
<tr>
<td>2012</td>
<td>American Chestnut</td>
<td>Indiana, Maine</td>
</tr>
<tr>
<td>2013</td>
<td>Chestnut</td>
<td>Indiana, Virginia</td>
</tr>
</tbody>
</table>

Table 2 Summary of the cross-site study design

<table>
<thead>
<tr>
<th>Site</th>
<th>Coordinates</th>
<th>Mean Annual Temperature</th>
<th>Mean Annual Precipitation</th>
<th>Dominant canopy</th>
<th>Years as study site</th>
<th>Species planted at this site (and their provenance)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atkinson Grove, ME</td>
<td>45°9′N, 69°3′W</td>
<td>4.6°C</td>
<td>9.4 cm</td>
<td>Castanea dentata, Quercus rubra, Abies balsamea</td>
<td>2013</td>
<td>Red Oak (VA), White Oak (VA), B3F2 (ME)</td>
</tr>
<tr>
<td>College Woods, VA</td>
<td>37°16′N, 76°42′W</td>
<td>14.8°C</td>
<td>10.4 cm</td>
<td>Quercus alba, Liriodendron tulipifera, Pinus taeda, Fagus grandifolia, Quercus falcata, Acer rubrum, Cornus florida</td>
<td>2013, 2014, 2015</td>
<td>Red Oak (VA, IL), White Oak (VA, IL), B3F2 (ME, MA, MD)</td>
</tr>
<tr>
<td>Harvard Forest, MA</td>
<td>42°31′N, 72°11′W</td>
<td>7.2°C</td>
<td>9.7 cm</td>
<td>Acer rubrum, Quercus rubra, Pinus strobus, Tsuga sieboldii, Picea abies, Pinus resinosa</td>
<td>2013, 2015</td>
<td>Red Oak (VA), White Oak (VA), B3F2 (ME, MD)</td>
</tr>
<tr>
<td>Martell Forest, IN</td>
<td>40°26′N, 86°54′W</td>
<td>11.1°C</td>
<td>7.9 cm</td>
<td>Quercus alba, Carya glabra, Quercus velutina, Quercus rubra, Acer saccharum</td>
<td>2013</td>
<td>Red Oak (VA), White Oak (VA), B3F2 (ME)</td>
</tr>
<tr>
<td>Dorrance Township, PA</td>
<td>41°13′N, 75°98′W</td>
<td>9.6°C</td>
<td>97.2 cm</td>
<td>Quercus rubra, Acer rubrum, Quercus alba</td>
<td>2014, 2015</td>
<td>Red Oak (VA, IL), White Oak (VA, IL), B3F2 (MA, MD)</td>
</tr>
</tbody>
</table>

IL, Illinois; ME, Maine; PA, Pennsylvania; VA, Virginia.
**Seed collection and field methods**

We hand-collected seeds from a variety of locations in all the years of study except for 2014 when a mast failure in Virginia prompted us to purchase red oak seeds from Illinois. Upon collection, each seed was visually inspected and float-tested to eliminate seeds with insect infestations. Virginia and Pennsylvania seed sources were collected from the same sources in different years. Hybrid chestnuts were donated from 3 different provenances (Table 1).

Each plot contained a 9 × 10 array in which each column of the array contained the seeds of a single species, and included a total of 3 rows of each species per enclosure (30 seeds/species; 90 seeds/cage). All rows in the plot alternated between cached and non-cached seeds, for a total of 10 rows. Buried seeds were cached so that the bottom of the seed was 2 cm below the soil surface. Non-cached seeds remained on the surface of the soil. We pushed the leaf litter aside at each seed site and then replaced the leaf litter immediately after placing the seed. Along with the seeds, each plot had 2 iButton temperature loggers: 1 placed on the surface and 1 buried like the seeds, recording temperatures every 4 h. Plots were then covered with cages made of hardware cloth that measured 1.2 m × 1.2 m × 17.8 cm and were attached to wire aprons measuring 15.2 cm in width.

The cages were secured with landscaping staples in the apron and on the corners of the cages to minimize pilferage by small mammals (Barnett 1977). At each site, each year, we relied on 15 replicate plots (1350 seeds per plot).

To investigate the effect of provenance on the Es/Ec ratio, we tested seeds from several sources in Virginia in 2 different years (Table 1). Each provenance was replicated in 15 plots for a total of 450 seeds per provenance per year. To investigate the effects of cache sites (i.e. abiotic conditions) on the Es/Ec ratio, we planted a single provenance of red oak, white oak and chestnut across 2–4 different sites over 3 years (Table 2). Each species was replicated in 15 plots/site for a total of 450 seeds per species per site per year.

**Analysis**

We found high rates of pilferage at some of our cages and these cages were removed from the analysis. We calculated the percent emergence from the surface and the percent emergence from the cache for each plot. Most plots had an individual value for the Es/Ec ratio and we used these plot-level values to test for differences between species and provenances using ANOVA (R core development Team). However, some plots at some sites did not have sufficient seeds remaining on the sur-

![Figure 2](image_url) Comparison of the Es/Ec ratio in 3 species. All 3 species were significantly different from each other with red oak having the lowest Es/Ec ratio (mutualistic), white oak having the highest (antagonistic) and hybrid American chestnut intermediate.
face due to pilferage. We therefore took an average of the emergence from the surface and the emergence from the cache over all plots within a site and used these averages to calculate the $Es/Ec$ ratio for each species/site/year combination.

We quantified the potential for a mutualistic versus antagonistic relationship between each of our 3 tree species and scatterhoarders using the model of Zwolak and Crone (2012). To do this, we needed estimates of $Pc$, the proportion of cached seeds that remain uneaten, for all 3 of our species. We took these values from Lichti et al. (2014), who tracked the fate of marked seeds for red oak, white oak and American chestnut across 2 years and 3 sites. In addition, each seed species was paired with both of the other 2 species in different trials. Thus, we had 8 estimates of $Pc$ for each seed species (see survival/removal in Fig. 2 in Lichti et al. 2014). We calculated the mean, minimum and maximum $Pc$ values reported by Lichti et al. (2014) across both years and all sites. We plotted all $Es/Ec$ ratios against the $Pc$ values from Lichti et al. (2014). We analyzed the relationship between $Es/Ec$ values and temperature as well as the relationship between surface emergence rates and temperature using Pearson’s correlation.

**RESULTS**

**Differences among species**

We tested for species differences using the data from Virginia only as this site had all 3 tree species planted in the same location in each of the 3 years. All 3 species had higher emergence rates when cached than when left on the surface. Red oak acorns had the lowest emergence on the surface (0.008 ± 0.03, mean ± SD) with white oak acorns the highest (0.10 ± 0.14, mean ± SD) and hybrid chestnuts intermediate (0.06 ± 0.07, mean ± SD). In contrast, hybrid chestnuts had the highest emergence from a cache (0.40 ± 0.25, mean ± SD) with red oak and white oak only slightly less when cached (0.31 ± 0.25, 0.30 ± 0.16, respectively, mean ± SD).

Despite the apparent benefit that all seeds experience when cached (i.e. all seeds increased emergence when cached), comparisons between the $Es/Ec$ ratio and $Pc$ values illustrate that in some situations it might not be enough to make the interaction mutualistic. The $Es/Ec$ ratio was significantly lower for red oak than white oak, supporting our hypothesis that red oak acorns have a higher propensity for mutualism with scatterhoarders than white oak ($F_{2,51} = 8.435$, $P = 0.0005$, Fig. 2). American chestnuts were intermediate.

**How does seed provenance affect the $Es/Ec$ ratio?**

Multiple provenances of all 3 species were tested within the same site within a single year. Comparing provenances of the same species within a single year showed no differences among provenances of the same species ($P > 0.10$, Fig. 3). However, when comparing the same provenance across 2 years, differences emerged. For white oak, American chestnut and B3F2 hybrid chestnut provenances, the $Es/Ec$ ratio differed significantly between years (Fig. 4, $P < 0.02$). In contrast, neither red oak provenance differed between years (Figs 3 and 5, $P > 0.5$).

**How do abiotic conditions affect the $Es/Ec$ ratio?**

**Across sites and years**

We found that red oak had the least amount of variation in the $Es/Ec$ ratio across all sites and years. Red oak also fell exclusively within the mutualistic range across all sites and years given average survival when cached (Fig. 5a). The 2 site-year combinations with the highest $Es/Ec$ ratio for red oak could be within the antagonistic range during years with low survival when cached ($Pc$, Fig. 5a).

White oak and hybrid chestnut were much more variable than red oak, and both these species had some years that fell within the mutualistic range and others that fell within the antagonistic range (Fig. 5b,c). For hybrid chestnut, 5 of the 8 points fell within the mutualistic range as the 95% confidence interval for the probability of survival when cached on these points all overlap the 1:1 line (Fig. 5b). The 3 points with the highest $Es/Ec$ ratios would be near the mutualistic range only in years of exceptionally high cache survival.

For white oak, only 3 of the 8 points fell within the mutualistic range, with at least 1 of these points falling in the antagonistic range in a year of lower than average seed survival when cached ($Pc$, Fig. 5c). The other 5 points were not in the mutualistic range given the 95% confidence intervals of $Pc$ values. Indeed, even the highest observed survival within a cache ($Pc$), would place only 2 of these 3 points within the mutualistic range (Fig. 5c).

**Effects of temperature and soil moisture**

Across all sites, caching increased the average tem-
Temperature experienced by seeds during cold months. From November to February, the average temperatures in a cache were up to $2^\circ$C warmer than temperatures on the soil surface at all 5 sites (Appendix I). In April and May, surface temperatures were on average $1^\circ$C warmer than the cache temperature. On average, there was no difference between cache and surface temperature in March.

Despite these consistent effects of caching on the thermal environment experienced by the seeds, we found no correlation between average temperature and the $E_s/E_c$ ratio at the microclimate scale (temperatures measured at each individual plot) in red oak ($P = 0.69$), white oak ($P = 0.54$), or American chestnut ($P = 0.70$) (Appendix I, Fig. 3). Similarly, we found no relationship between average or minimum temperatures and the $E_s/E_c$ ratio at the site level. However, warmer sites had higher frequency of surface emergence in white oak:

Figure 3 The $E_s/E_c$ ratio did not differ among provenances planted in the same location within the same year for any of the species tested. American chestnut had significant differences between years, while red oak was more consistent. (a) American chestnut, (b) hybrid chestnut, (c) red oak and (d) white oak.
57% emergence from the surface in Virginia, 36% in Massachusetts and 15% in Maine. We did not see this pattern in red oak, which had very low frequency of surface emergence in Virginia (5%) and Maine (8%), but higher surface emergence in Massachusetts (40%). In American chestnut, we saw a higher, and more similar, frequency of surface emergence at all sites: 55% in Virginia, 64% in Massachusetts and 36% in Maine.

Soil moisture measured in the fall was not correlated with the $Es/Ec$ ratio for any species ($P > 0.3$). In hybrid American chestnut, emergence from the soil declined with increasing fall soil moisture ($\rho = -0.30$, $P = 0.01$). A similar pattern was observed in the $Es/Ec$ ratio for hybrid American chestnut, although marginally significant.
(ρ = −0.23, P = 0.07). We found no relationship between hybrid chestnut emergence when buried and soil moisture. In addition, there was no relationship between soil moisture and surface or buried emergence in either red oak or white oak.

**DISCUSSION**

Although seed burial increased emergence in all 3 species, the Zwolak and Crone (2012) model, combined with multiple environmental measures across sites and years, allowed us to tease apart significant differences among our 3 species in the potential for the benefit of caching to create a mutualistic relationship between trees and scatterhoarders. Es/Ec ratios appeared related to dormancy requirements, which supports our first prediction. By comparing the Es/Ec ratio to the Pc values, we found that buried red oak acorns nearly always resulted in mutualism, whereas acorns of white oak consistently fell in the antagonistic range, across nearly all sites and years. Hybrid chestnuts were intermediate, suggesting that environmental context could have a much larger role in shaping the outcome of the interaction. Our second prediction was also supported: abiotic conditions had a stronger effect on Es/Ec ratios than seed provenance. However, our final prediction was not well supported: we found that temperature explained little of the variation we observed in the Es/Ec ratio. Although white oak experienced the more antagonistic relationship among the 3 tree species studied, we found that it benefited the most from the warmer temperatures at the most southern site, Virginia. White oak experienced a steady increase in the frequency of surface emergence from north to south. No such trend was observed for either red oak or chestnut.

Our finding that burial increased seed emergence in all 3 species is consistent with literature on many other species and is often taken as evidence that scatterhoarders are mutualists (Theimer 2005 and references therein). However, the presence of a measurable benefit alone cannot determine the net effect of the interaction. In a “conditional mutualism” such as scatterhoarding, the costs of seed predation as well as the relative benefit of caching compared to the benefit obtained in the absence of scatterhoarders is, indeed, key (Theimer 2005; Zwolak & Crone 2012; Xiao & Krebs 2015). However, simply comparing the increase in emergence as a result of seed burial would lead us to conclude that American chestnut experienced the greatest benefit from scatterhoarding, as it had the greatest difference in emergence rate when on the surface versus buried. The Zwolak and Crone (2012) model provides the added insight that American chestnut, rather than benefiting the most from the interaction as suggested by the differences in emergence rates, actually has the potential for the environment to easily shift the interaction outcome because its Es/Ec ratios are so near the border between antagonism and mutualism.

Although all species that we studied exhibited variation in their Es/Ec ratios, none of that variation was attributable to differences in seed provenance. When we tested different provenances in the same location in the same year, we found no evidence for differences among seed provenances in the Es/Ec ratio, suggesting that seeds from different provenances have similar potential for mutualism and antagonism under the same environmental conditions. These results are similar to those of Huang et al. (2016), who found that the emergence rates of white oak acorns were more strongly explained by planting site than seed provenance. They did, however, find significant differences among provenances of white oak in seedling height and diameter (Huang et al. 2016).

To date, conceptual models and explanations of the conditional nature of the seed : scatterhoarder relationship have focused on the impact of variable seed production (i.e. mast years) and the seed : scatterhoarder ratio (Theimer 2005; Lichti et al. 2015; Xiao & Krebs 2015). Theimer (2005) notes that in addition to the per capita seed abundance, environmental conditions (i.e. microsites) are not fixed and that the variation across space and years could also shift the relationship. Similarly, Jorge and Howe (2009) found that forest fragmentation altered seed removal and dispersal. Our study is among the first to quantify the role of environmental variation by focusing on the Es/Ec ratio. While the seed : scatterhoarder ratio will influence the amount of variation observed in the probability that seeds will be cached and left uneaten (Pc), how a species responds to differing conditions of environmental microsites across space and time also influences the amount of variation observed in the Es/Ec ratio. Zwolak and Cone (2012) present an analysis of 15 studies from the literature that report values of surface (Es) or cache (Ec) emergence. They found that most species tended toward mutualism, but most studies did not quantify environmental impact on the Es/Ec ratio. In our study, both American chestnut and white oak had wide-ranging Es/Ec values. Indeed, the amount of variation among Es/Ec ratios for these species was larger than the 95% confidence intervals of Pc reported by Lichti et al. (2014). Although masting al-
ters the seed : scatterhoarder ratio and can strongly increase the probability of seed survival within caches, our data illustrate that the traits of the seed, combined with abiotic conditions, can be equally important in determining where species fall on the continuum from mutualism to antagonism.

Our data support that caching can provide an insulating effect; buried seeds were 2 °C warmer during cold months and 1–2 °C cooler in the spring. The cooler temperatures in spring may prevent seeds from germinating too early or succumbing to late spring frosts. Despite these measurable effects of caching on temperature, this variable was not significantly related to the Es/Ec ratio or to emergence rates of seeds placed on the soil surface (i.e. seeds ignored by scatterhoarders). Many studies indicate that burial and leaf litter can increase seed survival due to an insulation effect of the soil and leaf (Garcia et al. 2002; Briggs & Vander Wall 2004). Garcia et al. (2002) found that buried red oak acorns and those covered by litter had greater moisture content and were significantly less likely to be eaten by deer (Odocoileus virginianus), suggesting that moisture and protection could be the driving factors in the benefits of burial instead of temperature.

Our study focuses on what Xiao and Krebs (2015) call the “burial effect” of scatterhoarders. They argue that the “dispersal effect” may be more important for understanding the benefits of scatterhoarders. To measure this, one compares the emergence of buried and surface seeds underneath the parent tree and away from the parent tree. Because we did not take into account whether seeds in our experiment were placed beneath trees of the same or a different species, we cannot fully tease apart a burial versus dispersal effect in our data. In addition, we are only focusing on 1 stage of the plant life cycle (Zwolak & Crone 2012; Xiao & Krebs 2015). Our interpretation of the potential for mutualism versus antagonism also could vary if seedling growth and survival were enhanced with burial more than is measured by simple seedling emergence. By using artificial caches and exclusion cages, we removed other potential effects of scatterhoarder behavior, such as escape from distance-dependent or density-dependent mortality (Hirsch et al. 2012) or providing directed dispersal into particularly suitable microsites (Yi et al. 2013).

Although the ratio of seeds to scatterhoarders is important for quantifying the mutualism between seeds and scatterhoarders, our data indicate the importance of variable Es/Ec ratios as a factor to quantify the continuum. Our results illustrate that the abiotic conditions play a significant and underappreciated role in shifting the seed : scatterhoarder relationship along the mutualism–antagonism continuum. Understanding the variation in both the seed : scatterhoarder ratio and the Es/Ec ratio is necessary to quantify the mutualism between seeds and scatterhoarders.

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REFERENCES


**SUPPLEMENTARY MATERIALS**

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

**Appendix 1**

Cite this article as:

Effects of rodent abundance and richness on cache pilfering

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Abstract

Scatterhoarding is a common behavioral strategy to conserve food during periods of scarcity, but this type of food storage is vulnerable to theft or pilferage. A variety of environmental factors and cache characteristics influence the rate of pilferage. Here we investigate 2 environmental factors, which heretofore have not received much attention: the abundance and species richness of scatterhoarding animals in the vicinity of scatterhoarded seeds. We measured the rate of cache pilferage at 7 sites that differed in the number and species composition of granivorous rodents in western Nevada using local native seeds and sunflower seeds. We found that there was no difference between the pilferage rate of native seeds and sunflower seeds, but that sites with different rodent abundances had different pilferage rates. Pilferage rates were proportional to the abundance of scatterhoarding rodents. Scatterhoarding rodents removed seeds at the rate of 1.3%/day/rodent individual. Species richness of scatterhoarding rodents was not correlated with rates of pilferage. These results suggest that density-dependent competition for scatterhoarded seeds is a strong determinant of pilferage rates.

Key words: cache pilfering, competition, food hoarding, heteromyids, rodent abundance, scatter hoarding, sciurids

INTRODUCTION

Many species of mammals (mostly rodents) and many species of birds (mostly corvids) scatterhoard food to conserve it for future use (Vander Wall 1990). Scatterhoarding is the preferred means of food storage when the hoarder is not able to defend a large collection of food items (i.e. larder) and when the foraging method is not conducive to spending most time near the larder. Hoarded food can play a critical role in maintaining body condition and improving survival and/or reproductive success (Price & Jenkins 1986; Wauters \textit{et al.} 1995; Kuhn & Vander Wall 2008). However, these undefended food stores are subject to pilfering from a variety of competitors. For animals that store seeds to be consumed weeks or months later, pilfering rates are typically 2–30% per day (Vander Wall & Jenkins 2003). These high rates of pilferage occur despite the fact that animals have evolved a variety of behavioral strategies to reduce pilferage (Dally \textit{et al.} 2006; Zhang \textit{et al.} 2013, 2014; Steele \textit{et al.} 2013, 2015). Managing pilferage is a key component of the adaptive strategy of scatterhoarding animals.

An aspect of scatterhoarding that makes this means
of food storage work is that pilfered items are not immediately eaten but recached, often repeatedly, until consumption (Vander Wall & Joyner 1998; Jansen et al. 2004, 2012; Perea et al. 2011) or germination (Vander Wall 2008; Jansen et al. 2012). In one intensive study of cache pilfering (Jansen et al. 2012), 57% of caches were recovered or pilfered within 1 week, with 99% of the caches being recovered or pilfered within the study period of 1 year. Seeds were often recached multiple times (up to 36 times), sometimes being moved several times in a single day. Most removals (84%) were pilferage events (i.e. by an animal other than the cacher) and despite being frequently handled most seeds were recached rather than eaten (Jansen et al. 2012).

There is a variety of factors that influence how quickly scatterhoarded items are pilfered, including those arising from the characteristics of the cache and those that are features of the environment. Important cache characteristics include the strength of odor emanating from seeds. Some species of seeds have stronger odors than others. From a seed’s perspective, once cached it is best not to be found, so there is strong natural selection to minimize odors emanating from seeds. Native seeds that are adapted for dispersal by scatterhoarding animals are pilfered at a slower rate (suggesting weaker odor) than non-native seeds (Hollander et al. 2012) and seeds with indurate seed coats (a coat that appears to restrict passage of odor molecules) are pilfered more slowly than seeds that lack indurate seed coats (Paulsen et al. 2013, 2014). Attractiveness of seeds may be important, with more attractive (e.g. large with thin seed coats) seeds being removed at a faster rate that less attractive (e.g. small with chemical defenses) seeds. Rates of pilferage are also influenced by the size and depth of a cache (Vander Wall 2000; Vander Wall et al. 2003). Finally, spacing of caches is important; widely spaced caches discourage area-restricted search and reduce cache pilferage (Stapanian & Smith 1978; Clarkson et al. 1986).

One of the most important environmental influences of cache pilfering is moisture. Wet soils and even high humidity can increase pilferage rates (Vander Wall 1998; Downs & Vander Wall 2009). This means that the rate of cache pilferage can change with the weather. Alternative food, either hidden in the soil (i.e. cached) or available on the ground surface or on plants can influence how diligently animals search for stored seeds. This appears to be one of the causes of masting (the synchronous production of large seed crops) observed in many species of plants that are dispersed by scatterhoarding animals (Vander Wall 2002; Jansen et al. 2004). In mast years, animals store more seeds than they can consume and many cached seeds are neglected and eventually germinate. Finally, rates of pilferage are likely to rise with increases in the number of animals that are foraging for hidden food (i.e. increased competition among granivores). This latter potential factor does not appear to have been studied.

Given that pilferage of caches is a universal and important feature of this food storing strategy, we need to understand it thoroughly. The goal of this study was to investigate the effect of rodent abundance and richness on pilfering rates. We predict that increased rodent abundance will increase the rate of cache pilferage. With more individuals, the demand for a limited food resource will increase. Most work has focused on the behavior of individuals, and by not fully examining pilfering as a community process (Dittel et al. 2017), we are likely missing important aspects of the mechanisms that determine rates of pilferage.

MATERIALS AND METHODS

Study areas

This study was conducted in 7 habitat types across an elevation gradient in western Nevada: low desert shrub, sand dune, juniper forest, piñon forest, montane shrub, Jeffrey pine forest and an alpine shrub (see Table 1 for more details). Different habitats had differences in rodent abundance, species richness and species composition, which were important elements of the study design. Sites were chosen near each other for logistical reasons, and in the cases where sites were within the same geographic area (e.g. Pine Nut Range and Whittell Forest and Wildlife Area) sites were chosen that were of obvious different habitat and not transition zones. Within the Pine Nut range, the juniper forest site was at a location dominated by Utah juniper (Juniperus osteosperma) and the piñon forest site was at a location dominated by piñon pine (Pinus monophylla). The montane shrub site was located on the eastern slope of the Carson range in an area that burned in 1982 and was dominated by greenleaf manzanita (Arctostaphyllos patula) and tobacco brush (Ceanothus velutinus). The Jeffrey pine site was located within an unburned forest community dominated by Jeffrey pine and lodgepole pine (Pinus contorta).
## Table 1 Description of the seven sites used for this study. All seven sites were located within the state of Nevada, USA

<table>
<thead>
<tr>
<th>Site</th>
<th>Location</th>
<th>Coordinates</th>
<th>Elevation (m)</th>
<th>Substrate description</th>
<th>Native seed used</th>
<th>Month sampled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low desert shrub</td>
<td>Nightingale Flats</td>
<td>39°42'55&quot;N, 119°1'56&quot;W</td>
<td>1261</td>
<td>Mix of fine sand and gravel</td>
<td>Achnatherum hymenoides</td>
<td>May</td>
</tr>
<tr>
<td>Sand dune</td>
<td>Hot Springs Mountains</td>
<td>39°42'22&quot;N, 119°3'34&quot;W</td>
<td>1270</td>
<td>Loose, sandy</td>
<td>Achnatherum hymenoides</td>
<td>May</td>
</tr>
<tr>
<td>Juniper forest</td>
<td>Pine Nut Range</td>
<td>39°5'10&quot;N, 119°39'0&quot;W</td>
<td>1920</td>
<td>Sandy</td>
<td>Pinus monophylla</td>
<td>May</td>
</tr>
<tr>
<td>Piñon forest</td>
<td>Pine Nut Range</td>
<td>39°5'10&quot;N, 119°39'0&quot;W</td>
<td>1920</td>
<td>Sandy</td>
<td>Pinus monophylla</td>
<td>May</td>
</tr>
<tr>
<td>Montane shrub</td>
<td>Whittell Forest and Wildlife Area</td>
<td>39°15'10&quot;N, 119°52'35&quot;W</td>
<td>1975</td>
<td>Decomposed granite</td>
<td>Purshia tridentata</td>
<td>September</td>
</tr>
<tr>
<td>Jeffrey pine forest</td>
<td>Whittell Forest and Wildlife Area</td>
<td>39°15'10&quot;N, 119°52'35&quot;W</td>
<td>1975</td>
<td>Decomposed granite</td>
<td>Purshia tridentata</td>
<td>September</td>
</tr>
<tr>
<td>Alpine shrub</td>
<td>Mount Rose</td>
<td>39°18'31&quot;N, 119°55'17&quot;W</td>
<td>2800</td>
<td>Decomposed granite</td>
<td>Pinus jeffreyi</td>
<td>September</td>
</tr>
</tbody>
</table>

## Study design

We determined rodent abundance and species richness by live-trapping. We placed 50 Sherman live animal traps (H. B. Sherman) in a 5 × 10 trapping grid with 15-m spacing between traps at each site. Traps were left open for 5 days and 5 nights for a total of 250 trapping days and nights, and checked twice daily (morning and evening). A single trap was placed at each point on the grid and the trap was baited with a mixture of sunflower and millet. We identified rodents to species and marked individuals with a serially numbered ear tag to prevent recounting. We classified rodent species as scatterhoarding because there is not a native seed species found across all 7 habitat types. A non-native seed type was necessary because there is not a native seed species found across all study sites. Native seeds that we used were Indian ricegrass (low desert shrub and sand dune sites), piñon pine (piñon and juniper forest sites), antelope bitterbrush (montane shrub and Jeffrey pine forest sites) and Jeffrey pine (alpine shrub). The number of seeds per cache was chosen based on the average size of rodent-created caches and adjusted so that all seed caches across sites were approximately equal in value (edible mass). Indian ricegrass caches contained 50 seeds (McMurray et al. 1997), piñon caches contained 3 seeds (Vander Wall 1997), antelope bitterbrush caches contained 10 seeds (Vander Wall 1994), Jeffrey pine caches contained 5 seeds (Tombback 1982) and sunflower seed caches contained 5 seeds. Cache depth was constant across sites at 10-mm deep at the top, which is within the range of depths that most rodents and corvids cache seeds (Tombback 1982; Vander Wall & Joyner 1998; Vander Wall 2000; Longland et al. 2001). We made caches using a metal spoon to minimize contaminating seeds with human scent, and groomed the soil after cache placement to remove signs of digging. We marked caches with natural objects (e.g. sticks, cones and rocks) in various arrangements to avoid rodents learning visual cues of cache locations.
Seed cache transects were checked daily for 5 days starting the day after establishment. If signs of digging were observed at cache sites, we excavated those sites to verify that seeds were removed. If seeds were not removed, they were reburied. Because rodents have difficulties detecting caches under dry conditions (Vander Wall 2000), all trials followed significant rain events. To ensure soil moisture conditions were conducive to cache detection, 5 soil samples were collected at day 0 and day 5 in Nasco Whirl-pack plastic bags. Plastic bags were sealed and kept in a cool location until returned to the laboratory. In the laboratory, soil samples were weighed, dried in an oven at 100°C for 48 h, and then reweighed to determine water content.

**Data Analysis**

All data were analyzed using R (R Development Core Team 2017). Rodent capture data (number of unique individuals) were used to determine species richness and abundance. Differences in the rates of cache removal were analyzed using survival analysis (package survival; Therneau & Grambsch 2000; Therneau 2013). Because seeds were only monitored for 5 days and we do not know the precise time of removal for seeds taken by rodents, we used right censoring in models. All models were fitted with a Weibull distribution, known for its robustness when modeling survival data (Mudholkar et al. 1996). Each site was analyzed separately comparing rates of removal among the 3 transects and seed types (native vs non-native). In addition, native and non-native caches (sunflower seeds) were analyzed to determine if there were differences in removal rates across sites using parametric survival regressions. Rodent abundance and species richness were regressed using linear mixed models with mean removal rate to determine if there were any effects of abundance or richness on removal rates. Models were run using the lmm function within the lme4 package (Bates et al. 2015). Because this R package does not offer any P-values we performed Type II Wald $\chi^2$-tests using the Anova function in the car package to obtain P-values. For all models, the dependent variable was the cache pilferage rate, and the independent variables were seed type with either rodent abundance, rodent richness or scatterhoarding rodent abundance. In each model, we used transect (1–3) at each site as random effects. Because we were interested in determining the strength of the relationship between our variables we calculated and are reporting $R^2$ values for mixed models following Nakagawa and Schielzeth (2013) using the sem.model.fits function within the piecewiseSEM package (Lefcheck 2016).

**RESULTS**

The total abundance of rodents over 5 days and nights of trapping ranged from 3 (alpine shrub site) to 38 (low desert shrub and Jeffrey pine forest sites; Table 2). Species richness across sites ranged from 2 (alpine shrub site) to 5 (low desert shrub, juniper forest and piñon forest sites), with an averaged of 4 species per site. We caught 2 species of cricetids (Peromyscus

![Figure 1](image_url) Removal rates of native and non-native (sunflower) seeds at each site over the 5-day trial period. The x-axis represents time (days) and the y-axis represents the percentage of caches remaining. Dashed lines represent native seeds, whereas solid lines represent sunflower seeds. Letters correspond to site location; (a) is low desert shrub, (b) is sand dune, (c) is juniper forest, (d) is piñon forest, (e) is montane shrub, (f) is Jeffrey pine first, and (g) is alpine shrub. There is no difference in the rates of removal in 4 of the 7 sites. At the juniper (c) and piñon forest sites (d), native piñon pine seeds were removed faster than sunflower seeds ($\chi^2 = 25.6, df = 1, P < 0.001$ and $\chi^2 = 15.36, df = 1, P < 0.001$, respectively). At the low desert shrub site (a), sunflower seeds were removed faster ($\chi^2 = 11.85, df = 1, P < 0.001$) than the native Indian ricegrass seeds.
Table 2  Abundance (number of individuals and number of scatterhoarding individuals) and richness (number of species) at each study site arranged from low to high elevation

| Species richness | Total rodent abundance | Scatterhoarding rodent abundance |  |
|------------------|------------------------|----------------------------------|--|---|---|---|---|---|---|
| 5                | 38                     | 17                               | 9 | 16 | 21 | 38 | 3 |
| 4                | 30                     | 17                               | 9 | 16 | 19 | 38 | 3 |
| 5                | 5                      | 4                                | 5 | 5  | 3  | 4  | 2 |

Figure 2  Predicted values for the linear mixed models of mean daily removal rates for sunflower (solid line) and native seeds (dashed line) as a function of scatterhoarding rodent abundance ($\chi^2 = 55.34$, df = 1, $P < 0.001$). Gray polygons surrounding the lines represent the 95% confidence intervals. The effect size of scatterhoarding rodent abundance was 1.3% caches pilfered/individual/day.

Seed removal rates were significantly positively correlated with total rodent abundance ($\chi^2 = 32.93$, df = 1, $P < 0.001$). The mean removal rate per individual was 1.1% per day. Mean rates of removal were not correlated with rodent species richness ($\chi^2 = 1.6$, df = 1, $P = 0.43$). When re-running the analysis including only the effect of scatterhoarding rodent abundance (i.e. excluding larder-hoarding ground squirrels) the correlation between pilfering and rodent abundance was improved ($\chi^2 = 55.34$, df = 1, $P < 0.001$, Fig. 2; $R^2_{\text{All}} = 0.54$, $R^2_{\text{scatterhoarders}} = 0.65$) and there was a significant interactive effect between scatterhoarder abundance and seed type ($\chi^2 = 4.53$, df = 1, $P < 0.03$). Confining the analysis to scatterhoarder abundance increased the daily pilferage rates slightly (1.3%/day/individual). The abundance of sciurid rodents (the only family of scatterhoarding rodents found at every site) did not have a significant effect on mean daily pilferage rate ($\chi^2 = 0.33$, df = 1, $P = 0.56$).
DISCUSSION

Pilferage rates did not significantly differ between sunflower seeds and native seeds across the 7 sites. Rodent abundance was the only factor that had a significant effect on seed pilferage rates in this study. The sites with the highest rodent abundance experienced the highest mean daily pilferage rates over the 5-day trials. Excluding non-scatterhoarding rodents from the analysis improved the correlation between rodent abundance and pilfering rates from explaining 54% of the variation in pilferage rates with all rodents to explaining 65% of the variation when considering only scatterhoarding rodents. The non-scatterhoarding rodents (golden-mantled ground squirrels and California ground squirrels) only accounted for 7% of the total individuals captured and occurred at only 2 sites. Our results suggest that on average, a single seed-pilfering rodent contributes 1.3% to the daily pilferage rates. The larger effect size of rodent abundance probably occurred because rodents that do not scatterhoard seeds appear not to be very good pilferers (Vander Wall et al. 2009). This is not surprising as scatterhoarding species are proficient pilferers (Vander Wall et al. 2009). Non-scatterhoarding rodents likely pilfer infrequently, if at all, and their presence in a community has little effect on pilferage rates.

Rodent species richness had no effect on mean daily pilferage rates. The lack of a correlation between rodent richness and seed removal rates suggests that the number of pilfering rodents has a larger impact on removal rates than the species mix (e.g., ratio of chipmunks to kangaroo rats). This is due, in part, to the fact that there was little variation in species richness across sites (max = 5, minimum = 2) with only 2 of the sites (montane shrub and alpine shrub) having fewer than 4 species (Table 2). The notion that cache pilfering in rodent communities is often reciprocal (Price & Mittler 2003, 2006; Vander Wall & Jenkins 2003) suggests that the identity of the species might not matter as long as the rodent caches seeds. If any 1 species of pilferer were able to disproportionately pilfer from heterospecifics, they would likely extirpate that species because they would lose seeds faster than they could replace them. At the Jeffrey pine forest site, Dittel et al. (2017) showed that pilfering was reciprocal among the most common scatterhoarding species and this is likely the case at the other study sites.

Natural rainfall events were sufficient to promote cache pilfering during this study. Of course, not all rain events were equal so some sites experienced higher soil moisture during the study period than others. Despite differences in rainfall, the minimum soil moisture required to promote pilfering was met for all 7 sites. The relationship between soil moisture and seed detection is a step function, with a threshold at approximately 0.5% soil moisture (Vander Wall 2000, 2003). Above the threshold seeds become detectable to rodents, and the amount of moisture above that level (within limits) does not really matter. We were also unable to quantify the amount of fruit and seed available to rodents at our sites. Seed abundance may have influenced pilfering rates through density-dependent factors, but our results were well within recorded pilfering rates (Vander Wall & Jenkins 2003) and none of the sites were experiencing mast years during the study (personal observations). Thus, we contend that differences seen in pilfering rates were primarily due to differences in rodent abundance.

The alpine shrub site happened to overlap a caching area used by Clark’s nutcrackers (Nucifraga columbiana). These birds cache pine seeds on open slopes at higher elevations, often many kilometers away from and over 1000 m above the forests where these seeds had been collected (Vander Wall & Balda 1977; Hutchins & Lanner 1982; Tomback 1982; Tomback & Linhart 1990; Lorenz et al. 2011). Our results help to explain why Clark’s nutcrackers invest so much energy to transport seeds to these high elevation sites. Pilferage rates at the alpine shrub site were low. In fact, nutcrackers often select caching areas more sparsely vegetated than our site, including cliff faces and rock ledges where seed-caching rodents are virtually absent. In the forests where the seeds were collected (i.e., Jeffrey pine and pinyon pine forests), pilferage rates are more than an order of magnitude greater. Furthermore, snow covers the ground for much longer periods (1–2 months longer) at higher elevations sites, and during periods of snow cover, pilferage rates would approach 0% per day. Therefore, caches at higher elevation sites with few rodents would be available to pilferers for shorter periods of time than at lower elevation sites where the seeds are harvested. Greatly reduced rates of pilferage may explain why nutcrackers invest so much energy transporting seeds to these remote sites.

This study is, to our knowledge, the first to show that there is a positive relationship between rodent abundance and cache pilfering rates. On average, the addition of a single scatterhoarding individual increases pilfering by 1.3% per day. Pilfering rates are not influenced by species richness as long as those species are scatterhoarders. Per capita rates of pilferage are similar across
habitat types and elevations. As long as the rodents scatterhoard seeds, the composition of the rodent community did not seem to matter much. With regard to pilferage, these results suggest that different scatterhoarding rodent species play a similar role in different ecological communities.

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Phenotypic trait matching predicts the topology of an insular plant–bird pollination network

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Abstract
Conceptualizing species interactions as networks has broadened our understanding of ecological communities. However, the factors shaping interaction patterns among species and, therefore, network structure remain unclear. One potentially important factor is the matching of phenotypic traits. Here, we tested for trait matching in a bird–flower visitation network from New Zealand. We first quantified the overall network structure and tested whether flower size could account for differences in the visitation rates of flowering plants. We then explored the relationship between the flower size and bill size. The results showed that the interaction network is nested. Plant species with large flowers received more visits from birds than plant species with small flowers. Moreover, plant species with large flowers were visited more frequently by birds with large bills, while species with smaller flowers were visited more frequently by birds with small bills. Overall, the interaction patterns between birds and flowering plants could be predicted by their morphology, suggesting that phenotypic trait matching is an important predictor of network structure.

Key words: bird–flower visitation network, flower morphology, plant–animal interactions, size coupling

INTRODUCTION
The development of network theory has been crucial to our understanding of ecological communities. Conceptualizing the myriad of species interactions that occur in nature as networks has provided ecologists with a more holistic approach to understanding ecological communities (Bascompte 2009). However, the mechanisms shaping species interaction patterns and, therefore, overall network structure, remain unclear.

One potential mechanism shaping network topology is the matching of phenotypic traits. Species morphology modulates network structure by both permitting and restricting possible interactions (Krishna et al. 2008; Olesen et al. 2010; Sankamethawee et al. 2011; Zhang et al. 2015). For example, the body size of predators can determine the size of prey they consume (Cohen et al. 1993; Emmerson & Raffaelli 2004; Owen-Smith & Mills 2008). Similarly, the gape size of frugivores determines the size of fruits they consume and predicts the interaction patterns of frugivores with fruit-
ing plants (Wheelwright 1985; Rey et al. 1997; Burns 2006, 2013). Phenotypic trait matching can also shape the topology of plant–insect pollination networks (Stang et al. 2006, 2007, 2009; Ibanez 2012). However, less is known about plant–bird pollination networks (although see Maruyama et al. 2014; Maglianesi et al. 2015a,b).

We quantified a bird–flower visitation network by observing birds visit flowers in a temperate rainforest on the North Island of New Zealand for 8 consecutive years. We then evaluated whether species morphology (i.e. bill and flower size) could predict network structure by testing 3 hypotheses: (1) plant species with large flowers receive greater total number of visits from birds than plant species with small flowers; (2) plant species with large flowers are visited more frequently by birds with large bills; and (3) plant species with small flowers are visited more frequently by birds with small bills.

MATERIALS AND METHODS

All observations were made in Zealandia, a managed forest reserve on the North Island of New Zealand (41°18.3′S, 174°44.8′E). Zealandia experiences a mild, temperate climate and supports evergreen forest composed of trees, tree ferns and lianas. The 225-ha reserve is surrounded by a unique fence designed to exclude introduced mammalian predators, which have led to the extinction of nearly half of New Zealand’s avifauna (Wilson 2004). In the absence of intensive mammal control, New Zealand forests typically support marginal populations of native bird species (Innes et al. 2010).

Many rare bird species have been translocated into the reserve following the construction of the fence (Table 1), including the bellbird (Anthornis melanura Sparman, 1786), the kaka (Nestor meridionalis Gmelin, 1788), the saddleback (Philesturnus carunculatus Gmelin, 1789), the hihi (Notiomystis cincta Du bus de Gessignies, 1839) and the whitehead (Mohoua albicilla Lesson, 1830). Many native species that were already present within the reserve prior to the construction of the fence have also benefited from its construction (e.g. the tui, Prosthemadera novaeseelandiae Gmelin, 1788). The Waxeye (Zosterops lateralis Latham, 1802) is a frugivore originally native to Australia that colonized New Zealand in the recent past (<200 years ago), whose numbers have also increased.

Bird–flower visits were quantified by observing wild birds foraging for nectar along a 4.6-km reserve trail that took approximately 1 h to traverse. Observations were made approximately every 5 days from January

### Table 1 List of species comprising a bird–flower interaction network in New Zealand

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Stamen length (mm)</th>
<th>Relative abundance</th>
<th>Flower color</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Fuchsia excorticata</td>
<td>22.99</td>
<td>29</td>
<td>Purple</td>
</tr>
<tr>
<td>B Pseudopanax arboreus</td>
<td>4.11</td>
<td>32</td>
<td>White</td>
</tr>
<tr>
<td>C Phormium tenax</td>
<td>50.97</td>
<td>7</td>
<td>Red</td>
</tr>
<tr>
<td>D Sophora microphylla</td>
<td>34.72</td>
<td>3</td>
<td>Yellow</td>
</tr>
<tr>
<td>E Cytisus proliferus</td>
<td>14.81</td>
<td>4</td>
<td>White</td>
</tr>
<tr>
<td>F Geniostoma lingustrifolium</td>
<td>0.81</td>
<td>51</td>
<td>Green</td>
</tr>
<tr>
<td>G Schefflera digitata</td>
<td>3.4</td>
<td>13</td>
<td>Green</td>
</tr>
<tr>
<td>H Berberis darwinii</td>
<td>5.57</td>
<td>1</td>
<td>Yellow</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bird species</th>
<th>Bill length (mm)</th>
<th>Relative abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Prosthemadera novaeseelandiae</td>
<td>23.93</td>
<td>49.2</td>
</tr>
<tr>
<td>B Zosterops lateralis</td>
<td>10.35</td>
<td>12.5</td>
</tr>
<tr>
<td>C Anthornis melanura</td>
<td>14.29</td>
<td>11.8</td>
</tr>
<tr>
<td>D Notiomystis cincta</td>
<td>15.97</td>
<td>23.4</td>
</tr>
<tr>
<td>E Nestor meridionalis</td>
<td>51.15</td>
<td>32</td>
</tr>
<tr>
<td>F Mohoua albicilla</td>
<td>10.22</td>
<td>103.2</td>
</tr>
</tbody>
</table>

Species labels correspond to those of Figure 1. Stamen and bill lengths are species means. Relative species abundances for birds were gleaned from Bell (2015). Flower color is described as it appears to the human eye.
2006 to December 2013. Given the erratic movements of foraging birds in the cover of dense foliage, accurate estimates of the total number of interactions made in a foraging bout could not be made. Consequently, we quantified bird–plant interactions more generally following Snow and Snow (1988) and Burns (2006). A visit was scored when a bird approached a plant and fed from at least 1 flower. To minimize pseudoreplication, observations were ceased after each recorded visit, and then resumed 10 m down the trail. Note that, throughout, birds are referred to sparingly as pollinators regardless of their effectiveness.

To quantify network structure, we calculated connectance, nestedness and negative co-occurrences using the R package bipartite v. 2.08 (Dormann et al. 2008). Because connectance values alone are sensitive to sampling effort (and, therefore, network species richness), weighted connectance is more appropriate for making comparisons between habitats. Weighted connectance is calculated by dividing linkage density by network species richness (Tylianakis et al. 2007).

The Brualdi and Sanderson (1999) discrepancy index (hereinafter ‘BR’) (Brualdi & Sanderson 1999) counts the number of discrepancies (absence or presence) that must be erased to produce a perfectly nested matrix. This is then standardized by dividing by the total number of occurrences in the matrix (the matrix fill) (Greve & Chown 2006). A null model is then used to generate a distribution of expected values, against which the observed value is tested. The network was assessed against 100 null model simulations with fixed row and column totals (as suggested by Ulrich & Gotelli 2007) and the independent swap algorithm to fill matrices (Gotelli 2000, 2001). That is, row and column totals in null model simulation never exceeded that of the observed network.

Negative co-occurrences were quantified using the $\chi^2$-score index. This is calculated by finding the (normalized) mean checkerboard units for each species pair: $cu = (n_i - N_{ij})(n_j - N_{ij})$, where $n_i$ and $n_j$ are the row totals (number of occurrences) of species $i$ and $j$, and $N_{ij}$ is the number of co-occurrences of both species (Stone & Roberts 1990). The observed $\chi^2$-score is then compared to a distribution of expected $\chi^2$-scores under chance alone. That is, the positions of entries within the matrix are randomized by computer simulation. Again, the network was assessed against 100 simulations with fixed row and column totals and the independent swap algorithm for matrix fill. A $\chi^2$-score greater than what is expected under the null model would support negative co-occurrences within the network.

To ensure that the mean size bill that foraged on flowering species was estimated accurately, we omitted bird species that were observed less than 10 times across the observation period. Likewise, plant species that registered fewer than 10 visits across the observation period were also omitted for their effect on the mean size of flowers visited by birds. Most omitted species were only observed a handful of times and are, therefore, unlikely to have an appreciable effect on results.

To estimate the mean bill size of each bird species, we measured specimens housed in the National Museum of New Zealand (Te Papa Tongarewa, Wellington, New Zealand). The distance from the tip of the bill to the anterior edge of the skull was measured using a pair of digital callipers. Between 11 and 20 specimens for each species were measured from the collection of skins available, including both males and females (see Table 2).

To characterize flower size, 4 aspects of flower morphology were measured. Stamen length was measured as the distance from the base of the nectary to the distal tip of the anther. Pistil length was measured as the dis-

<table>
<thead>
<tr>
<th>Species</th>
<th>Bill length (mm)</th>
<th>Mean (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Prosthemadera</em></td>
<td>♀ = 25.22 (n = 10)</td>
<td>23.94</td>
</tr>
<tr>
<td><em>novaseelandiae</em></td>
<td>♀ = 22.86 (n = 10)</td>
<td></td>
</tr>
<tr>
<td><em>Zosterops lateralis</em></td>
<td>♀ = 10.55 (n = 10)</td>
<td>10.35</td>
</tr>
<tr>
<td>*♀ = 10.15 (n = 10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Anthornis melanura</em></td>
<td>♀ = 15.35 (n = 9)</td>
<td>14.29</td>
</tr>
<tr>
<td>*♀ = 13.22 (n = 10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Notiomystis cincta</em></td>
<td>♀ = 16.30 (n = 10)</td>
<td>15.97</td>
</tr>
<tr>
<td>*♀ = 15.64 (n = 7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Nestor meridionalis</em></td>
<td>♀ = 51.63 (n = 8)</td>
<td>51.15</td>
</tr>
<tr>
<td>*♀ = 50.67 (n = 3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Mohoua albicilla</em></td>
<td>♀ = 10.35 (n = 10)</td>
<td>10.22</td>
</tr>
<tr>
<td>*♀ = 10.08 (n = 6)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Measurements are broken down by sex and sample size (in parentheses).
tance from the base of the nectary to the distal tip of the style. Tube length was measured from base of the nectary to the distal point of the corolla. Tube width was measured as the widest internal distance transecting the corolla. One flower from each of 30 individuals per plant species was measured. Species means for stamen length, pistil length, tube length and width were then correlated against each other in a 4-way correlation matrix and the respective correlation and Pearson $P$-values were calculated using the “Hmisc” package in the R-environment. Stamen size was used as a proxy for overall flower size because of its known scaling relationship with other flower traits (see Table 3), and because it is easy to measure consistently. Flower color was also recorded as it appears to the human eye.

To estimate relative local abundances of plant species, a 2-m pole was placed at regular intervals, alternating left and right either side of the trail. All vascular plant species immediately touching the pole or its vertically projected plane were recorded. This was repeated 100 times along the trail. Relative abundance was recorded as the number of observations out of 100. Relative abundance estimates for birds were gleaned from Bell (2015).

To explore the relationship between flower size and the number of visits received from birds, we ran linear model regression of stamen length against visitation rate. Because we were interested in the effect of morphology, and not the effect of abundance, the visitation rate for a species was calculated as the total number of visits divided by its relative abundance. This way, differences in visitation rates could be assessed without the confounding effect of differences in relative abundance. Both axes were log-transformed to conform to model assumptions.

To explore the relationship between bill and flower size, we ran 2 linear model regressions: 1 of flowering species stamen length against the mean bill length of visitors, and 1 of bird species’ bill length against the mean stamen size of flowers they visited. Bill length was log-transformed to conform to model assumptions. By using mean stamen and bill size, we are able to visualize the extent to which interactions are skewed. Regression analyses were conducted in the R environment (R Core Team 2017).

**RESULTS**

A total of 1216 bird–flower visits were observed over an 8-year period involving 8 plant species and 6 bird species (Fig. 1). After excluding seldom observed species, total interactions (visits) for plants ranged from 15–640 and from 11–882 for birds. Of 48 possible species interactions, 32 were realized, giving a network connectivity value of 67%, significantly less than expected under null model simulation (81.5% ± 0.7% mean ± SD; $T = 40.357$, $P < 0.01$). When weighted by network species richness, this value drops to 20.6%, again less than expected under null model simulation (24.5% ± 0.03% mean ± SD; $T = 532.9$, $P < 0.01$).

Network-level analysis of interactions found significant evidence for nestedness. The observed discrepancy index ($BR = 2$) was statistically greater than the distribution of expected values generated under null model simulation ($BR = 1.22 ± 0.188$, mean ± SD; $T = −8.12$, $P < 0.01$). However, the results indicated no support for negative co-occurrence patterns. The observed $\chi^2$-score

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**Table 3** A correlation matrix of morphological flower traits of 8 plant species comprising a bird–flower visitation network in New Zealand

<table>
<thead>
<tr>
<th></th>
<th>Tube length</th>
<th>Tube width</th>
<th>Pistil length</th>
<th>Stamen length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tube length</td>
<td>$r = 0.97$</td>
<td>$r = 0.95$</td>
<td>$r = 0.95$</td>
<td>$P = 0.001$</td>
</tr>
<tr>
<td></td>
<td>$P = 0.003$</td>
<td>$P = 0.003$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tube width</td>
<td>$r = 0.91$</td>
<td>$r = 0.91$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pistil length</td>
<td>$P = 0.011$</td>
<td>$P = 0.013$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stamen length</td>
<td></td>
<td></td>
<td>$r = 0.99$</td>
<td>$P &lt; 0.001$</td>
</tr>
</tbody>
</table>

Each cell denotes the relative ($r$) value and Pearson $P$-value between 2 morphological traits. Analysis was run in the R-environment using the “Hmisc” package.
(C = 0.171) was statistically indistinguishable from scores expected under null model simulation (C = 0.171 ± 0.032; mean ± SD; T = −0.006, P = 0.995).

Flower visitation rates were positively associated with stamen length (Fig. 2). After accounting for differences in relative abundance, species with larger stamen sizes and red or yellow color consistently showed greater visitation rates (T = 6.220, P < 0.001, r² = 0.843).

Moreover, small-billed birds tended to visit small flowers and large billed birds tended to visit large flowers. Species of greater stamen lengths received significantly more visits from birds of larger bill sizes (T = 4.178, P = 0.006, r² = 0.702) (Fig. 3). Likewise, larger-billed birds were more frequently observed interacting with spe-

Figure 1 Matrix of interactions between 8 flowering plant species (x-axis) and 6 bird species (y-axis) located on the North Island of New Zealand. Species are ranked by their total interaction frequencies, which are denoted in bold and represented by histograms above and right.

Figure 2 Stamen length is associated with the amount of visits flowering species receive from birds. Visitation rate is calculated as the total number of observed bird visits divided by the plant species’ relative abundance. Plant species with long stamens (i.e. big flowers) consistently receive more visits from birds than plant species with small stamens, irrespective of their relative abundance in the community.

Figure 3 There is a positive size coupling relationship between bill and flower size. (a) The mean length of bills of observed visiting flowering species is plotted against their stamen length. (b) Likewise, the mean stamen length of flowers visited by birds is plotted against their bill length. Birds disproportionately visit flowers of similar size to their bills. Open circles represent single species.
cies with greater stamen lengths \( (T = 3.068, P = 0.037, r^2 = 0.627) \). These results suggest that trait matching (i.e. flower and bill size) is a significant predictor of network topology in plant–bird pollination networks.

**DISCUSSION**

Observed interactions between birds and flowers were non-random. The interaction network was hierarchically nested. Plant species with large, showy flowers received greater total visits from birds than plant species with small, inconspicuous flowers. Significant pairwise size coupling was also observed, with birds foraging disproportionately on flowers that were similar in size to their bills. Overall, results suggest that trait matching is an important predictor of plant–bird pollination network topology.

Many ecological networks in nature are nested. In nested networks, the partners of specialists form proper subsets of the partners of generalists. Animal food webs are often nested (i.e. “hierarchical trophic guilds” Paine 1966; Pauly et al. 1998; Frederiksen et al. 2006). Most plant–animal interaction networks are also nested (Bascompte et al. 2003; Bascompte & Jordano 2013). The apparent ubiquity of nestedness among communities suggests that it might be a common attribute of ecological networks. However, it is unclear whether the mechanisms underpinning nestedness and other attributes of network topology are consistent across community types.

Morphological constraints (i.e. “forbidden links”) and size coupling between interacting species pairs are often associated with network topology (Krishna et al. 2008; Olesen et al. 2010; Sankamethawee et al. 2011; Zhang et al. 2015). For example, body size limits the number of prey species that predators can exploit in predator–prey webs (Cohen et al. 1993; Memmott et al. 2000; Woodward & Hildrew 2002; Emmerson & Raffaelli 2004; Woodward et al. 2005; Owen-Smith & Mills 2008). Similarly, “gape limitation” has been invoked to explain the phenotypic matching between fruits and frugivores. Much like body size in animal predators, gape width restricts the size of fruits that can be consumed by frugivores. This creates a nested hierarchy that is ordered by gape width and fruit size (Wheelwright 1985; Rey et al. 1997; Burns 2006, 2013). Size coupling has also been documented in a variety of plant-pollinator groups, including solitary bees (Armbruster & Guinn 1989), bumblebees (Harder 1985), long-proboscid flies (Goldblatt & Manning 2000), butterflies (Corbet 2000) and moths (Haber & Frankie 1989; Agosta & Janzen 2005). Little is known about the role species traits play in plant–bird pollinator network topology. However, bill and flower size might also shape interaction patterns within these communities (Maruyama et al. 2014; Maglianesi et al. 2015a,b).

Birds in this study foraged disproportionately on large, red or yellow flowers. Flower size is usually correlated with nectar volume (Thompson & Plowright 1980; Harder et al. 1985; Harder & Cruzan 1990; Fenster et al. 2006), which, in turn, elicits higher visitation rates (Galen & Plowright 1985; Mitchell & Wass 1992). By selectively foraging on large flowers, birds might reduce the number of visits needed to meet their energy expenditure. Alternatively, species with large flowers might receive more visits if they were simply more abundant in the community. However, in this study, large-flowered species received more visits than small-flowered species regardless of their abundance.

Size coupling is typically interpreted as a deterministic process whereby species actively seek out partners that have matching traits. Alternatively, it could also be viewed as a passive process whereby birds forage randomly on flowers that their bill morphology enables them to. For example, Burns (2013) found that a simple null model that included gape limitation predicted the topology of a fruit–frugivore network at the same study site. In other words, although gape width constrained which fruits birds ate, birds foraged otherwise randomly on the fruits available to them. This study found that birds interact similarly with flowering plants.

Bird-flowers are typically rare on islands (Crawford et al. 2011, and references therein). While large, colorful, tubular flowers are commonplace on continents, flowers in Hawaii (Carlquist 1974), Juan Fernandez Islands (Bernal dell et al. 2001) and New Zealand (Goddley 1979; Lloyd 1985; Webb & Kelly 1993) are predominantly small, dull and shallow. However, birds are often dominant vertebrates on islands (Holdaway 1989) and are unappreciated pollinators (Olesen & Valido 2004). Results from this study indicate that the dearth of bird-flowers in New Zealand does not result from a lack of ecological selection by birds.

Flower color in this study was qualitatively described as it appears to the human eye (see Table 1). Other studies have utilized more quantitative methods of measuring plant pigments (see Fadzley et al. 2009). Future work exploring the relationship between flower color and pollinator habits should employ such methods.
Overall, this study documented a nested plant–bird pollination network in New Zealand. Network structure was associated with species traits. We observed significant size coupling between birds and flowers. Therefore, phenotypic trait matching appears to be an important driver of nested bird–plant interactions.

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