

Effects of nitrogen deposition on reproduction in a masting tree: benefits of higher seed production are trumped by negative biotic interactions

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Summary

1. Relatively little is known about the effects of anthropogenic environmental changes on reproductive ecology of trees. Yet, recruitment is a primary determinant of the long-term dynamics of plant populations in changing environments.

2. We used the Long-Term Ecological Research site at Harvard Forest to evaluate the effects of chronic (over 25 years) nitrogen fertilization on reproductive ecology of red oaks (*Quercus rubra*).

3. Oaks growing in fertilized plots produced 4–9 times more acorns than control trees. However, nitrogen deposition simultaneously affected oaks' biotic interactions. It increased pre-dispersal seed predation by insects (primarily weevils, *Curculio* spp.) on fertilized plots, most likely as the result of the disruption of predator satiation. In addition, infestation by weevils was more likely to result in embryo destruction in fertilized than in control acorns. Furthermore, the proportion of acorns dispersed and cached by rodents decreased on fertilized plots. Finally, germination of fertilized acorns was lower than control acorns, even after controlling for the effects of weevils and rodents.

4. Inclusion of the altered biotic interactions reversed the final picture of the effects of long-term nitrogen fertilization on oak reproduction: the positive effects on acorn quantity were trumped by the nitrogen-mediated changes in biotic interactions.

5. *Synthesis.* Our results stress the importance of considering indirect effects and consumer interactions when evaluating the effects of environmental change on plant population dynamics. Long-term nitrogen fertilization has a strong potential to decrease the recruitment of masting trees. Given the ubiquitous increase in the anthropogenic nitrogen deposition, processes similar to those found in our system might operate in others, resulting in a widespread alteration in trees' recruitment dynamics.

Key-words: global change, mast seeding, nitrogen fertilization, predator satiation, recruitment, reproductive ecology, seed dispersal, seed predation, seed production, weevils

Introduction

Ongoing environmental changes exert increasing pressure on forests world-wide, and studies of their effects have become a priority for forest ecologists (Bonan 2008). To date, most research on the effects of environmental change on trees have focused on tree growth, carbon sequestration, seedling and tree survival, or phenology in the context of global warming or changes in atmospheric chemistry (CO₂ and other pollutants) (Wallace *et al.* 2007; Thomas *et al.* 2010; Rapp *et al.* 2012; Talhelm *et al.* 2013; Keenan *et al.* 2014; Murdiyarto

et al. 2015; Fernández-de-Uña *et al.* 2016). Studies of large-scale ecosystem alterations often overlook reproductive ecology of trees, yet long-term changes in reproduction determine whether tree populations can track moving windows of appropriate climate or soil conditions (Richardson *et al.* 2005; Pérez-Ramos *et al.* 2010). Therefore, insufficient information on changes in tree reproduction undermines our ability to predict responses of forests to global changes.

Anthropogenic nitrogen deposition is one of the main dimensions of global change (Vitousek *et al.* 1997). Anthropogenic emission of reactive nitrogen and its deposition in soils have doubled since the 1890s and are projected to double again by 2050 (Galloway *et al.* 2004, 2008). Currently, the amount of anthropogenic nitrogen added to terrestrial ecosystems exceeds the amount provided by all natural

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sources combined (Galloway *et al.* 2004). This sharp increase in nitrogen availability affects organisms and their interactions (Aber *et al.* 1989; Tylianakis *et al.* 2008). For example, N-fertilization increases tree growth and seed production (Callahan *et al.* 2008; Smaill *et al.* 2011) and can benefit seed dispersal by indirectly affecting behaviour of animal dispersers (Yi *et al.* 2016). In contrast, N-fertilization decreases seedling emergence and survival for a range of tree species (Catovsky & Bazzaz 2002; Patterson *et al.* 2012; Talhelm *et al.* 2013; but see Sefcik, Zak & Ellsworth 2007). Moreover, it alters tissue production and concentration of secondary compounds in plant tissues, which enhances herbivory (Tylianakis *et al.* 2008). Therefore, to understand the final effects of nitrogen deposition on tree reproduction, we need to integrate both direct effects (e.g. on seed quantity and quality) and indirect changes in plant–animal interactions on seed survival and establishment (Tylianakis *et al.* 2008; Kiers *et al.* 2010).

In this study, we evaluated the effects of long-term (>25 years) N-fertilization on reproductive ecology of red oaks (*Quercus rubra*), a tree species that is important economically and provides food resources to dozens of species (Ostfeld & Keesing 2000; McShea *et al.* 2007; Bogdziewicz, Zwolak & Crone 2016). Previous studies suggested that N-fertilization enhances reproduction of large-seeded, masting plants, such as oaks, by boosting their growth, seed production and seed size (Callahan *et al.* 2008; Smaill *et al.* 2011). However, the influence of nitrogen addition on oak reproduction depends not only on its direct impacts on seed production, but also on indirect consequences of changes in the quantity and quality of acorns on interactions with granivores. In this study, we focused on pre-dispersal seed predation by weevils (Coleoptera: Curculionidae) and seed dispersal by rodents. Both groups of organisms exert strong influence on oak reproduction: weevils can destroy up to 70–90% of the entire acorn crop of an individual tree (Crawley & Long 1995; Espelta *et al.* 2008; Lombardo & McCarthy 2008) and small mammals act as key seed dispersers of oak acorns (Steele 2008; Lichti *et al.* 2014) but can easily shift from mutualists to seed predators depending on various environmental conditions (Theimer 2005; Zwolak & Crone 2012).

Past research suggests that N-fertilization should affect both the quantity of seeds produced and their quality as food for granivores. For example, high availability of nitrogen should increase the amount of plant internal resources, leading to higher reproductive output and lower interannual variation of seed production in masting plants (e.g. Kelly & Sork 2002; Tanentzap, Lee & Coomes 2012; Crone & Rapp 2014). Higher resource availability may also lead to increased seed size (see Callahan *et al.* 2008). On the other hand, N-fertilization could lead to a decrease in tannin concentration in red oak acorns, for example, according to the carbon–nutrient balance hypothesis, which maintains that the concentration of carbon-based secondary compounds in plant tissues should decrease in response to increase in nitrogen availability (Stamp 2003).

Similarly, past studies indicate that changes in quantity and quality of acorns are likely to alter oak–animal interactions. Oaks produce large numbers of seeds at irregular intervals (mast seeding: Kelly 1994). One of the best-supported evolutionary explanations of this reproductive strategy is predator satiation (e.g. Janzen 1971; Norton & Kelly 1988; Crawley & Long 1995; Koenig & Knops 2002; Linhart *et al.* 2014); that is, during low mast years low seed production reduces the population of seed predators and high seed production during mast years exceeds the consumption capacity of consumer populations. Increased acorn production might disrupt this mechanism by providing food in non-mast years (McKone, Kelly & Lee 1998). Changes in seed chemical composition would also be likely to affect plant–consumer interactions. Weevil larvae frequently damage only a portion of the seed cotyledon and leave the embryo undamaged, and the infested acorn is still able to germinate (Steele *et al.* 1993; Bonal, Muñoz & Díaz 2007; Muñoz, Bonal & Espelta 2014). Bigger acorns are generally more likely to survive infestation (Bonal, Muñoz & Díaz 2007; Xiao, Harris & Zhang 2007; Muñoz, Bonal & Espelta 2014). Moreover, a higher tannin concentration around the embryo promotes consumption of the acorn basal end, and acorns with lower tannin content would be less likely to survive infestation (predator satiation at the seed level, cf. Bonal, Muñoz & Díaz 2007). Finally, rodents transport and bury seeds in topsoil for later consumption (scatterhoarding), but some cached seeds are never recovered and can germinate (Vander Wall 2010). Buried seeds are protected from other seed predators and escape high density-dependent mortality under the mother tree which benefits plant recruitment (Jansen *et al.* 2012; Johnson *et al.* 2012; Zwolak & Crone 2012). The fate of scatter-hoarded seeds is often determined by one or more seed traits (reviewed in Lichti, Steele & Swihart 2016). For example, larger seeds are cached more often than smaller ones (Theimer 2003; Jansen, Bongers & Hemerik 2004; Steele *et al.* 2014), and high-tannin seeds are often preferred for storage (Wang & Chen 2008; Vander Wall 2010; but see Zhang, Steele & Yi 2013). Thus, depending on the magnitude and the direction of N-effects on acorns traits, nitrogen addition might result in a higher (e.g. due to increased acorn size) or lower proportion (e.g. due to varied tannin concentration) of cached acorns.

Specifically, we measured the effects of chronic N-fertilization on the quantity and quality (tannin concentration and acorn volume) of acorns. We also estimated weevil infestation and probability of embryo survival of weevil-infested seeds. Finally, we measured preference of granivorous rodents for removing, consuming or caching seeds from N-fertilized trees, and the seedling establishment of these seeds. We combine the net effects of these various interactions in a simple algebraic model to predict the net number of established seedlings from N-fertilized and unfertilized trees. Together, these experiments show the potential for the effects of environmental change on oak reproduction, the relative importance of direct effects on acorn production, and indirect effects mediated by seed consumers.

Materials and methods

STUDY LOCATION

Long-term ecological research

The Chronic Nitrogen Amendment Experiment is located at Harvard Forest in Petersham, Massachusetts, USA. This ongoing experiment is located at a site dominated by ~60-year-old red oaks and divided into four experimental (30 × 30 m) treatments that are further divided into 36, 5 × 5 m subplots (Aber *et al.* 1989; Frey *et al.* 2014). Since 1988, six monthly applications of NH₄NO₃ have been applied to the plots during the growing season (Aber *et al.* 1989). Two plots receive 50 kg ha⁻¹ year⁻¹ ('low-N' plots), and one receives 150 kg ha⁻¹ year⁻¹ ('high-N' plot). The low-nitrogen treatment is applied at two plots because sulphur (74 kg ha⁻¹ year⁻¹) was added on one of them for the first 10 years of the experiment. The addition of sulphur was discontinued because no effects were observed (Magill *et al.* 2004).

Acorn tracking and seedling establishment experiments

For acorn tracking and establishment experiments (conducted in 2013–2014, see below), we established four sites in other parts of Harvard Forest. The sites were located in red oak-dominated hardwood forests and spaced 1.25–5 km apart. Rodent live-trapping revealed relatively low abundance of rodents that was similar among the plots (mean no. of captured individuals ± SE: 4 ± 1.27); acorn rain was estimated with seed traps as 8 ± 2 acorns m⁻² (mean per plot ± SE) (Appendix S1 in Supporting Information). Small mammal communities at the study sites were dominated by mice of genus *Peromyscus*. Although not captured, Eastern grey squirrels (*Sciurus carolinensis*) and Eastern chipmunks (*Tamias striatus*) were frequently seen at the sites and recorded with camera traps.

MEASUREMENTS AND EXPERIMENTS

Estimation of acorn production

We estimated acorn production of individual trees in years 2013–2015 by collecting and counting acorns in the interior of the treatment plots (excluding outer subplots) in a 2-m-radius circle centred at each tree trunk at the beginning of October (30 trees in control, 20 in low-N, and 16 in high-N treatments). Acorns were bagged separately according to the tree of origin, transported to the laboratory and refrigerated (4 °C) until further processing.

Acorn traits and weevil predation

In the laboratory, we weighed, measured (width and length) and assessed insect infestation for individual acorns from each treatment group (years 2013–2015, control = 1448 acorns, low N = 674, high N = 875). Based on acorn dimensions, we calculated acorn volume (following procedure of Bonal, Muñoz & Díaz 2007; see Appendix S1 for details). We also calculated the ratio between acorn length and width (as indicator of acorn shape) as acorn shape might influence rodent foraging decisions (Muñoz, Bonal & Espelta 2012). We cut each acorn transversely into a top (basal) and bottom half (apical) and counted all insect larvae in each half (following earlier protocols, for example Steele *et al.* 1993; Bonal, Muñoz & Díaz 2007). In the article, we only report data for *Curculio* larvae because

larvae of other insects (mostly Diptera; Gibson 1971) were present in less than 10% of all acorns. In 2014, we recorded whether the infestation resulted in embryo depredation and scored the estimated amount of cotyledon consumed.

Tannin concentration

A sample of sound acorns ($n = 5–10$) from each tree were combined to make one composite sample per tree for tannin analyses. 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). We therefore cut each acorn transversely into a top (basal) and bottom half (apical), removed the outer shell and pericarp and analysed the basal and apical halves separately. Two combined samples (each one consisting of either basal or apical halves of the acorns) were then immediately cut into smaller pieces, quick-frozen by immersion in liquid nitrogen, freeze-dried for 96 h and then ground to a fine powder. We analysed tannin concentration using the radial diffusion assay (Hagerman 1987) by calculating tannic acid equivalent (TAE, A. Hangerman, pers. comm.) for two replicate samples for each acorn sample. We analysed tannin concentration in 2013 and 2014 because acorn production in 2015 was too low to obtain sufficient material.

Acorn tracking experiments

To evaluate the effects of nitrogen fertilization on rodent dispersal behaviour, we conducted an acorn tracking experiment. We tracked acorns using the tin tag method (Xiao, Jansen & Zhang 2006). Preparation of acorns consisted of drilling 0.8 mm diameter holes through the basal end of the acorn, inserting and tying a stainless steel wire (120 mm length, 0.2 mm diameter) to the acorn, and attaching a red, uniquely numbered plastic tag (30 × 40 mm) to the terminal end of the wire. All acorns were weighed before the experiments. We placed acorns in enclosures (1 m × 1 m × 0.5 m, 1-cm mesh hardware cloth) with openings on each side to allow rodent access, but exclude other seed predators such as black bears (*Ursus americanus*) (Moore *et al.* 2007). Acorns were floated and visually examined for oviposition holes to determine weevil infestation; only non-infested acorns were used in experiments.

At each site, we deployed four enclosures spaced 100 m from each other. In each enclosure, we presented 15 acorns of each treatment (control, low-N and high-N; 720 acorns in total). We deployed acorns in October 2013 and checked and recorded acorn fates every day for first 10 days and then on the 15th, 30th, just after snowmelt in 2014, and again in July 2014 to determine their final fate. At each check, we recorded acorns that were eaten at the seed station or removed. If removed, we searched around the seed depots in ~20 m radius centred at the depot, with haphazard searches conducted at longer distances. For the acorns we found, we measured the distance from the depot and categorized the fate of the acorn as: (i) consumed, (ii) left on surface, (iii) buried in soil, or (iv) missing. Acorns found on the surface or cached were marked with a stick and their fate was further monitored at each check.

Seedling establishment experiment

To evaluate the effects of nitrogen fertilization on acorn germination, we conducted seed sowing experiments. We sowed non-infested acorns in 18 × 18 cm wire mesh enclosures (mesh size 13 mm). Acorns were buried 2 cm below the soil surface. At each site, we set

out six sets of four enclosures (24 cages per site). Enclosures within each set were spaced ~2 m apart forming a square. The sets were located in 30-m intervals on two transects that were spaced 30 m from each other. We sowed acorns (five per cage) in October 2013. In each set, we sowed control acorns in two enclosures, and in the other two, we sowed randomly low-N or high-N acorns (control = 240 acorns, low N = 120, high N = 120). Established seedlings were determined the following October (i.e. 2014).

The above treatment was crossed with a treatment for rodent exclusion: in half of the enclosures, rodents were excluded and in other half we cut 70 × 70 mm holes to allow access by small mammals. This allowed us to separate the effects of nitrogen-induced changes in seed traits *per se* on the probability of establishment (e.g. establishment lowered by the decline in tannin concentration in acorns; closed cages), from the potential effects of nitrogen fertilization resulting from trait-mediated changes in pilferage rates (e.g. greater retrieval of larger acorns by rodents (Perea *et al.* 2016)).

DATA ANALYSIS

We analysed data using generalized linear mixed models (GLMMs) implemented via 'LME4' package (Bates *et al.* 2015) in R (R Development Core Team 2015). We tested for statistical significance of fixed factors with Wald type II chi-square test. Unless stated otherwise, the degrees of freedom for fixed effects equalled 1. In all models, we arrived to final model structure by removing non-significant interaction terms. The data were deposited in Dryad repository (Bogdziewicz *et al.* 2016).

EFFECTS OF N-FERTILIZATION ON QUANTITY AND QUALITY OF ACORNS

We tested the effects of N-fertilization on the number of acorns produced using a Poisson family, log-link model with tree-level acorn count as the response variable, and nitrogen treatment (control, low-N, high-N), year and treatment × year interaction as fixed effects. We used tree ID as a random effect. We tested whether acorns differed in size among treatments using a Gaussian family, identity link model with acorn volume as the response variable, the treatment and year as fixed effects, and tree ID as the random effect. We also tested for differences in acorn shape (height to width ratio) using a similar model. We tested whether tannin concentration in acorns differed among treatments using a Gaussian family, identity link model with log-transformed TAE as the response variable. This model included nitrogen treatment, acorn part (basal/apical), year, treatment × acorn part and treatment × acorn part × year interactions as fixed effects, and tree ID as a random effect.

EFFECTS OF N-FERTILIZATION ON SEED-CONSUMER INTERACTIONS

We modelled the prevalence of larvae in acorns using a binomial family, logit link model with weevil infestation (0/1) as the response variable, treatment, individual tree crop size, acorn volume, year and interaction terms: treatment × individual tree crop size, treatment × year as fixed effects, and tree ID as a random effect. We also analysed the multi-infestation (i.e. the number of larvae per infested acorn), but it did not differ among treatments (see Appendix S1). We analysed the proportion of cotyledon depredated by weevil larvae using a binomial family, logit link model with the proportion of cotyledon consumed (Zuur *et al.* 2009) (using only acorns infested by

weevils) as the response variable, nitrogen treatment, weevil larvae count, acorn volume, year, and interaction of treatment × volume, treatment × larvae count, and treatment × year as fixed factors, and tree ID as a random effect. To analyse embryo survival of infested seeds, we used a binomial family, logit link model with embryo survival (0/1) as response variable, and the same set of fixed and random effects as in the cotyledon depredation model.

In the rodent-caching experiment, we tested whether the fate of acorns differed among treatments (control, low N, high N) with regard to (i) probability of removal, (ii) probability of consumption, (iii) probability of caching, and (iv) removal distance using four models. For response variables (i)–(iii), we used binomial family, logit link models. For response (iv), removal distance, we used a Gaussian family with log-transformed removal distance and an identity link. Each model included seed station nested within study plot as random effects. Fixed effects included treatment and acorn mass.

To evaluate the effects of N-fertilization on seedling establishment, we tested whether the establishment from experimental caches differed among treatments using a binomial family, logit link model. We compared the establishment of control and N acorns by building a GLMM with treatment (control, low N, high N), rodent access (open vs. closed cages) and the interaction term as fixed effects. Random effects included block (i.e. cages set) nested within study site.

THE NET EFFECT OF NITROGEN FERTILIZATION ON RED OAK REPRODUCTION

As a final step, we combined the results of experiments and calculated the net effect of N-fertilization on the reproductive output of red oaks by building a model:

$$\text{Net} = A_p \times (1 - P_i \times (1 - S_e)) \times P_c \times G_p.$$

In the model, the net reproductive output (Net) equals the number of acorns produced (A_p) reduced by the proportion of acorns infested (P_i), multiplied by the probability that infested acorns do not survive ($1 - S_e$). Of the non-destroyed acorns, a proportion will be dispersed and cached by small mammals (P_c) and finally establish with probability G_p . We used parameter estimates from the above-described GLMMs and obtained standard errors with parametric bootstrapping, that is sampling from the distributions defined by the mean and standard error of each coefficient to obtain a joint distribution for the derived variables.

Results

EFFECTS OF N-FERTILIZATION ON QUANTITY AND QUALITY OF ACORNS

Nitrogen fertilization significantly increased acorn production in red oaks ($\chi^2 = 179.16$, d.f. = 2, $P < 0.001$, Fig. 1). Acorn production was highest at the high-N plot (from 4 to 9 times higher than at the control plot, depending on year; control vs. high N: $\chi^2 = 113.32$, $P < 0.001$; low N vs. high N: $\chi^2 = 76.80$, $P < 0.001$), followed by the low-N plots (2–7 times higher than at the control; control vs. low N: $\chi^2 = 19.59$, $P < 0.001$). The effect of nitrogen fertilization varied by year ($\chi^2 = 35.18$, d.f. = 4, $P < 0.001$): the increase in acorn production in N-fertilized plots relative to the control plot was similar in 2013 and 2014 and weaker in 2015. Acorns produced by oaks growing at fertilized sites were

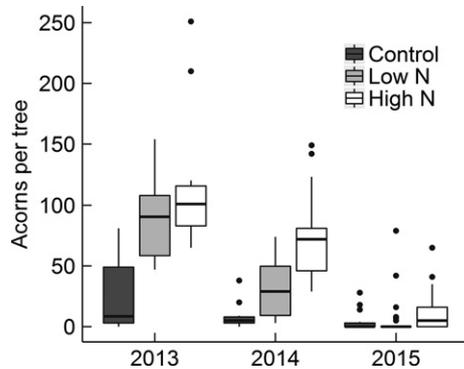


Fig. 1. Acorn production by red oaks in years 2013–2015. ‘Low N’ refers to acorns produced by red oaks growing on plots that were fertilized with $50 \text{ kg ha}^{-1} \text{ year}^{-1}$ of reactive nitrogen per year, and ‘high N’ with $150 \text{ kg ha}^{-1} \text{ year}^{-1}$. ‘Control’ refers to oaks growing on plots receiving ambient levels of nitrogen. Boxes denote 25th, 50th and 75th percentiles, whiskers denote the farthest data points within 1.5 interquartile range, and dots denote outliers beyond the 1.5 interquartile range.

smaller than acorns from the control plot (mean \pm SD: control: $75.90 \pm 18.55 \text{ cm}^2$; low N: $57.68 \pm 14.66 \text{ cm}^2$; high N: $65.56 \pm 22.52 \text{ cm}^2$), although the difference between control and high N was non-significant (control vs. low N: $\chi^2 = 22.50$, $P < 0.001$; control vs. high N: $\chi^2 = 2.89$, $P = 0.09$). Acorns of low N were smaller than high-N treatment ($\chi^2 = 21.20$, $P < 0.001$). Moreover, acorns produced by oaks growing on N-fertilized plots were more spherical than control acorns (i.e. had smaller length to width ratio, $\chi^2 = 17.73$, d.f. = 2, $P < 0.001$, Fig. S1).

Overall, nitrogen treatment did not have a significant effect on tannin concentration in acorns ($\chi^2 = 0.84$, d.f. = 2, $P = 0.65$). However, the effect of nitrogen fertilization on tannin concentration differed with acorn part and year (treatment \times acorn part \times year interaction: $\chi^2 = 9.66$, d.f. = 2, $P = 0.007$). When years were analysed separately, fertilization affected tannin concentration in 2013 ($\chi^2 = 7.32$, d.f. = 2, $P = 0.02$), and this effect differed between acorn parts

($\chi^2 = 20.46$, d.f. = 2, $P < 0.001$, Fig. S2). Nonetheless, general effects of nitrogen treatments were similar in basal and apical halves of acorns: low-N acorns had a lower tannin concentration than control acorns in both acorn parts (basal half: $\chi^2 = 10.04$, $P = 0.001$; apical half: $\chi^2 = 3.79$, $P = 0.05$), whereas the difference between control and high-N acorns was not significant (basal half: $\chi^2 = 2.31$, $P = 0.12$; apical half: $\chi^2 = 0.26$, $P = 0.60$). In 2014, we found no effect of nitrogen treatment on tannin concentration ($\chi^2 = 0.03$, d.f. = 2, $P = 0.98$). Generally, tannin concentration was higher in apical than in basal part of the acorns ($\chi^2 = 29.53$, $P < 0.001$).

EFFECTS OF N-FERTILIZATION ON SEED-CONSUMER INTERACTIONS

The overall prevalence of weevil larvae in acorns was 38% in control, 49% in low-N and 59% in high-N acorns (Fig. 2). The probability of acorn infestation increased with acorn volume ($\chi^2 = 16.58$, $P < 0.001$). When controlling for acorn volume, the odds of infestations by weevils were 1.61 times greater for low-N (95% CI: 1.1–2.4) and 2.66 times greater for high-N acorns (95% CI: 1.9–3.8) in comparison with control acorns. These differences were statistically significant (control vs. low N: $\chi^2 = 6.56$, $P = 0.01$; control vs. high N: $\chi^2 = 24.72$, $P < 0.001$; low N vs. high N: $\chi^2 = 6.83$, $P = 0.008$). The prevalence of weevils decreased with increasing acorn production of individual trees ($\chi^2 = 15.14$, $P < 0.001$; Fig. 2). The prevalence did not differ among years ($\chi^2 = 4.88$, d.f. = 2, $P = 0.08$). All interaction terms were non-significant ($P > 0.2$).

The proportion of cotyledon consumed by weevil larvae was ~ 1.5 times higher in N-fertilized acorns (both low and high N) than in control acorns, and decreased with acorn volume ($\chi^2 = 3.40$, $P = 0.05$). When controlling for acorn volume, the proportion of cotyledon consumed was lower in control acorns than in both low-N ($\chi^2 = 9.05$, $P = 0.002$) and high-N acorns ($\chi^2 = 18.22$, $P < 0.001$). It did not differ between low-N and high-N acorns ($\chi^2 = 0.23$, $P = 0.63$).

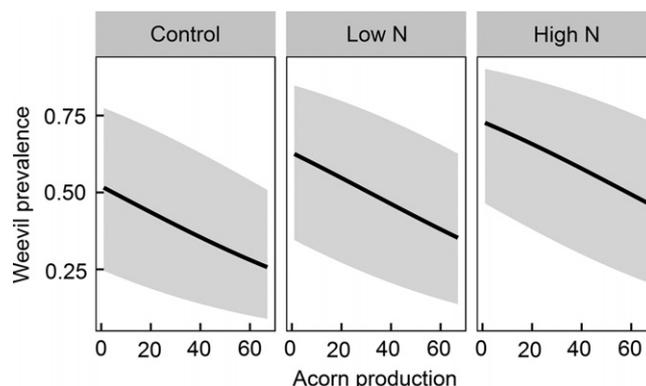


Fig. 2. The proportion of acorns infested by weevil larvae (prevalence) in relation to the tree-level index of acorn production and nitrogen treatment. The estimates are pooled for 2013–2015, because no significant difference was found in infestation among years. Trend lines are reported with 95% confidence intervals and are based on predictions from generalized linear mixed model (see Methods section for details). ‘Low N’ refers to acorns produced by red oaks growing on plots that were fertilized with $50 \text{ kg ha}^{-1} \text{ year}^{-1}$ of reactive nitrogen per year, and ‘high-N’ trees were fertilized with $150 \text{ kg ha}^{-1} \text{ year}^{-1}$. Control oaks were grown on plots receiving ambient levels of nitrogen.

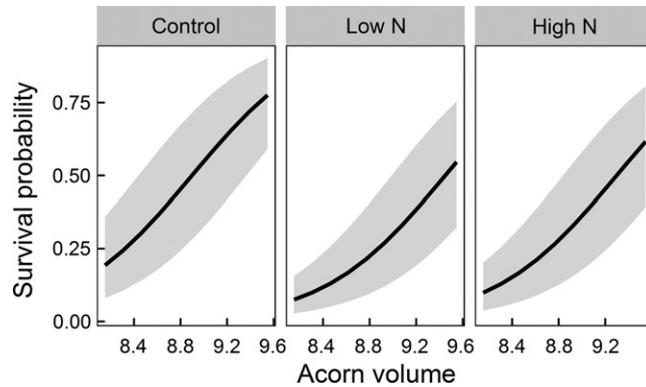


Fig. 3. Survival of embryos of weevil-infested acorns in relation to log-transformed acorn volume and nitrogen treatment. Trend lines are reported with 95% confidence intervals and are based on predictions from generalized linear mixed model (see Methods section for details). ‘Low N’ refers to acorns produced by red oaks growing on plots that were fertilized with 50 kg ha⁻¹ year⁻¹ of reactive nitrogen per year, and ‘high-N’ trees were fertilized with 150 kg ha⁻¹ year⁻¹. Control oaks were grown on plots receiving ambient levels of nitrogen.

None of the interaction terms were significant ($P > 0.40$). Probability of embryo survival in infested acorns increased with acorn volume ($\chi^2 = 10.84$, $P < 0.001$, Fig. 3). After controlling for acorn volume, the odds of acorn survival were 3.03 times higher in control acorns than in low-N acorns ($\chi^2 = 6.80$, $P = 0.009$), and 2.22 times higher in controls than in high-N acorns ($\chi^2 = 4.59$, $P = 0.03$, Fig. 3). The odds of acorn survival did not differ between low-N and high-N acorns ($\chi^2 = 0.32$, $P = 0.56$). None of the interaction terms were significant ($P > 0.15$), and we removed them from the final model.

In the rodent-caching experiment, 46% of all acorns were eaten *in situ*. Predation of control acorns was higher than that of both low-N ($\chi^2 = 6.70$, $P = 0.009$; Fig. S3) and high-N acorns ($\chi^2 = 31.24$, $P < 0.001$). A higher proportion of low-N acorns was consumed *in situ* than that of high-N acorns ($\chi^2 = 11.18$, $P < 0.001$). The probability of *in situ* predation was slightly higher for smaller acorns ($\chi^2 = 3.29$, $P = 0.07$). Eighteen percent of experimental acorns were removed by small mammals. The proportion of removed acorns differed among treatments ($\chi^2 = 19.10$, d.f. = 2, $P < 0.001$) and was positively affected by acorn mass ($\chi^2 = 5.90$, $P = 0.01$). Removal of control acorns (25%) was higher than that of both low-N (17%, $\chi^2 = 4.80$, $P < 0.001$) and high-N acorns (12%, $\chi^2 = 22.14$, $P < 0.001$). High-N and low-N acorns did not differ in removal rates ($\chi^2 = 3.05$, $P = 0.08$).

Fifty-six percent of removed acorns were found consumed and 19% were found cached. Acorn retrieval did not differ between treatments ($\chi^2 = 1.17$, d.f. = 2, $P = 0.56$). The proportion of removed acorns that were consumed did not differ between treatments ($\chi^2 = 1.47$, d.f. = 2, $P = 0.47$) and was not influenced by acorn mass ($\chi^2 = 0.04$, $P = 0.83$). Similarly, caching probability of removed acorns did not differ between treatments ($\chi^2 = 3.74$, d.f. = 2, $P = 0.15$) and was not influenced by acorn mass ($\chi^2 = 0.04$, $P = 0.83$). However, differences in acorn removal resulted in differences in the absolute proportion of cached and non-retrieved acorns ($\chi^2 = 11.37$, d.f. = 2, $P = 0.003$; Fig. S3). A higher proportion of control acorns than that of both low-N and high-N

acorns was cached (control vs. low N: $\chi^2 = 5.76$, $P = 0.016$, control vs. high N: $\chi^2 = 7.45$, $P = 0.006$). Low-N and high-N acorns did not differ in caching probability ($\chi^2 = 0.65$, d.f. = 1, $P = 0.41$). Dispersal distance did not differ among treatments ($\chi^2 = 3.85$, d.f. = 2, $P = 0.14$; Table 1) and was not influenced by acorn mass ($\chi^2 = 0.87$, $P = 0.37$).

In the germination experiment, seedling establishment was higher in control than in both low-N and high-N treatments, both in open and closed cages ($\chi^2 = 29.34$, d.f. = 2, $P < 0.001$; Fig. 4). This effect was stronger when rodents were excluded (nitrogen \times rodent access interaction: $\chi^2 = 8.64$, $P = 0.01$; see Table S1 for all *post hoc* tests). This interaction was driven by a stronger effect of rodent predation on establishment of control acorns in comparison with those from N-treatments (Fig. 4).

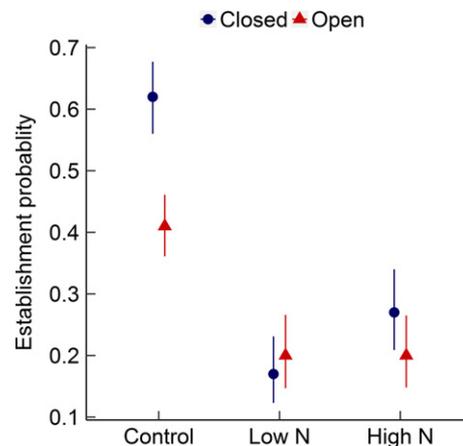


Fig. 4. Probability of seedling establishment of buried acorns. ‘Low N’ refers to acorns produced by red oaks growing on plots that were fertilized with 50 kg ha⁻¹ year⁻¹ of reactive nitrogen per year, and ‘high N’ with 150 kg ha⁻¹ year⁻¹. Control refers to oaks growing on plots receiving ambient levels of nitrogen. *Closed* denotes emergence when rodents were excluded, and *Open* refers to emergence when rodent access was allowed. Dots represent GLMM estimates, and whiskers represent standard errors (for detailed description of the model, see Methods) [Colour figure can be viewed at wileyonlinelibrary.com].

THE NET EFFECT OF NITROGEN FERTILIZATION ON RED OAK REPRODUCTION

Strong positive effects of nitrogen fertilization on acorn production were trumped by the negative biotic effects (Fig. 5). Depending on the year, N-fertilization reduced the mean net recruitment by ca. 13–29% (low-N) and by 3–16% (high-N) in comparison with control. The relative reduction in recruitment was more pronounced in the low-N treatment than in the high-N treatment because it was compensated by the stronger increase in acorn production in the latter.

Discussion

We found strong, multilevel effects of long-term N-fertilization on reproductive ecology of red oaks. Nitrogen addition increased acorn production up to nine fold, but simultaneously resulted in higher pre-dispersal seed predation by weevils, decreased caching by rodents and decreased seedling establishment. All these combined effects led to decrease in the net recruitment rate (Table 1). These results point to the importance of considering indirect effects and consumer interactions when evaluating the effects of global changes on tree reproductive ecology; inferences based on acorn quantity alone would lead to the opposite conclusions.

N-FERTILIZATION INCREASES PRE-DISPERSAL ACORN PREDATION

Despite substantial differences in acorn production among years, the proportion of acorns infested by weevils did not change over time (i.e. no effect of predator satiation at the population level). This result contrasts with studies on other species of oaks (e.g. Crawley & Long 1995; Espelta *et al.* 2008), but is consistent with our observations in the north-eastern USA (relatively constant levels of weevil infestation

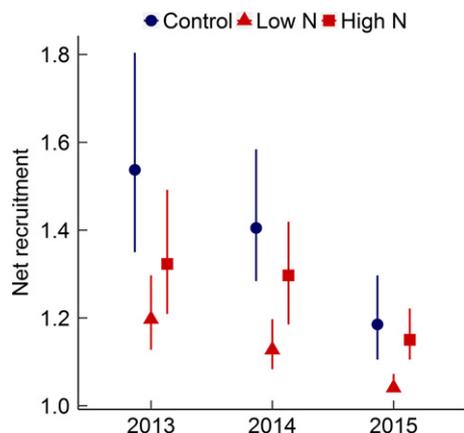


Fig. 5. Estimated net recruitment of red oaks: the number of acorns produced multiplied by weevil pre-dispersal acorn predation, probability of rodent dispersal and acorn germination. Whiskers indicate 95% confidence intervals. 'Low N' refers to acorns produced by red oaks growing on plots that were fertilized with 50 kg ha⁻¹ year⁻¹ of reactive nitrogen per year, and 'high-N' trees were fertilized with 150 kg ha⁻¹ year⁻¹. Control oaks were grown on plots receiving ambient levels of nitrogen. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 1. Summary of the effects of long-term N-fertilization on the reproductive ecology of red oaks

	Fertilization effect
<i>Quantity and quality of acorns</i>	
Acorn production	Twofold to nine fold increase
Tannin concentration	Decrease in 'low N', in 2013
Acorn size	Decrease
<i>Oak seed–consumers interactions</i>	
Proportion of acorn crop infested by weevils	10–20% increase
Proportion of cotyledon consumed by weevil larvae	20–30% increase
Survival of weevil-infested acorns	Twofold to threefold decrease
Proportion of acorns cached by rodents	Decrease from 6.3% to 1.7% (low N) or 0.008% (high N)
Germination probability	Twofold to threefold decrease
The net effect	3–29% decrease in recruitment

of red oak acorns in a 16-year data set; M. Steele, unpubl. data). Weevils are known to undergo diapause that lasts 2–4 years (Menu & Debouzie 1993; Venner *et al.* 2011). The diapause may allow weevils to track acorn production and buffer their populations against the negative effects of low mast years (Kelly *et al.* 2000; Maeto & Ozaki 2003). The tracking is most effective when masting occurs at a predictable cycle, for example in alternate years, and out-of-synchrony lean years severely decrease weevil numbers (Shibata *et al.* 2002; Maeto & Ozaki 2003). Similarly, two or more lean years in a row severely reduce weevil abundance (Higaki 2016). Thus, the higher proportion of acorn crop destroyed by weevils at the fertilized sites is likely to be the result of a more stable acorn supply, consequently a higher average survival of weevil cohorts, and a localized build-up of the weevil population (McKone, Kelly & Lee 1998; Maeto & Ozaki 2003; Higaki 2016). Enhanced seed production induced by global changes was reported for other masting plants (e.g. Richardson *et al.* 2005; Övergaard, Gemmel & Karlsson 2007; Buechling *et al.* 2016), but the apparent positive effects were treated cautiously because of possible consequences for seed predation (McKone, Kelly & Lee 1998; Richardson *et al.* 2005; Koenig *et al.* 2015). Our study shows that the enhanced seed production indeed comes with elevated costs.

N-FERTILIZATION REDUCES ACORN DISPERSAL

Reproduction of many large-seeded plant species relies on seeds that are dispersed and buried by scatter-hoarding birds and mammals, but are not recovered under certain conditions (Theimer 2005; Vander Wall 2010; Jansen *et al.* 2012; Lichti, Steele & Swihart 2016; Pesendorfer *et al.* 2016; Zwolak, Bogdziewicz & Rychlik 2016; Zwolak *et al.* 2016). Nitrogen fertilization decreased the proportion of cached and non-retrieved acorns from 6.3% of controls to 1.7% of low-N acorns, and to only 0.008% of high-N acorns. The differences in caching rates followed from the first step of the seed-handling process (Wang *et al.* 2013): rodents removed a lower proportion of nitrogen-fertilized than control acorns, but once

acorns were removed, there was no difference in the fates of acorns of different origin. Generally, rodents maximize their energy intake by removing higher energy, larger and lower tannin seeds (Lichti, Steele & Swihart 2016). Acorns of larger size are more likely to be removed and cached (Ivan & Swihart 2000; Steele *et al.* 2014; Lichti, Steele & Swihart 2016), which is consistent with our observation that smaller acorns at N-sites were less likely to be dispersed. Acorns from the 'low-N' site had also lower tannin concentration which also leads to higher rejection rate by rodents (Schmidt, Brown & Morgan 1998; Wang, Wang & Chen 2012; Wang *et al.* 2013). Moreover, acorns at N-sites were more spherical than control acorns, and the shape of acorns affects rodent behaviour as well, likely by affecting the mechanical cost of transport (Muñoz, Bonal & Espelta 2012). Nevertheless, we do not exclude the possibility that the change in rodent behaviour was caused by a nitrogen-induced alternation in chemical composition of acorn cotyledons that remained undetected in this study. In our other experiments, N-fertilization decreased the rate of cotyledon consumption by weevils and decreased seedling establishment of non-infested acorns, suggesting other changes in acorn chemical composition.

Contrary to our results, one other study that examined the effects of N-fertilization on the rodent seed dispersal in Korean pine (*Pinus koraiensis*) found an increase in energy content of seeds and alternation in volatile compounds that led to decreased predation and increased caching of seeds (Yi *et al.* 2016). Thus, the effects of N-fertilization on seed traits are likely to be species specific, which agrees with past studies examining the effects of N-addition on chemical composition of plant tissues (Stamp 2003; Endara & Coley 2011). Seed traits largely determine rodent predation and dispersal that act as strong ecological filters in plant communities (Howe & Brown 2001; Pérez-Ramos *et al.* 2008; Bricker & Maron 2012). Thus, nitrogen-mediated changes in seeds quality might alter preferences of seed predators potentially leading to shifts in composition of plant communities. Studies examining such effects will be a valuable area of future research.

N-FERTILIZATION REDUCES SEEDLING GERMINATION

The decrease in establishment of oak seedlings found in our study is consistent with results from a limited number of previous experiments that found decreases in establishment and survival of tree seedlings in response to N-fertilization (Catovsky & Bazzaz 2002; Patterson *et al.* 2012; BassiriRad *et al.* 2015; but see Sefcik, Zak & Ellsworth 2007). The exact mechanisms of the lowered establishment are currently poorly known (Catovsky & Bazzaz 2002; BassiriRad *et al.* 2015), but in small-seeded trees, such as maples, the lowered establishment follows from increased litter accumulation caused by increased N-deposition (Patterson *et al.* 2012). In other species, including oaks, the lowered establishment and survival is probably caused by the imbalance of above- and below-ground resource allocation (Catovsky & Bazzaz 2002; BassiriRad *et al.* 2015). These effects are unlikely to be compensated at later life stages because N-fertilization increases

browsing damage of oak saplings (Cha *et al.* 2010). Moreover, in adult trees, increased N-deposition increases growth but simultaneously also mortality, leading to a net loss of living basal area (Wallace *et al.* 2007).

CAVEATS AND OTHER POTENTIAL EFFECTS OF N-FERTILIZATION ON OAKS' REPRODUCTIVE ECOLOGY

Some estimates of the N-driven reduction in oak recruitment are likely conservative. For example, we assumed that germination of weevil-infested but not destroyed (i.e. with intact embryos) acorns did not differ among treatments. However, weevils consumed a higher proportion of cotyledons in N-treatments than in control treatments, which will further lower establishment and survival of seedlings because a smaller pool of resources is available for seedling development (Bonal, Muñoz & Díaz 2007; Xiao, Harris & Zhang 2007; Muñoz, Bonal & Espelta 2014). Secondly, we assumed that the dispersal of sound and infested but not destroyed acorns is similar. However, infested acorns are less preferred for caching (Steele, Hadj-Chikh & Hazeltine 1996; Perea, San Miguel & Gil 2011), and the number of infested but non-destroyed acorns was higher in N-treatments. Thirdly, more stable seed production might alter interactions of plants with other consumers. For example, seed production of masting trees determines population dynamics of small mammals and birds and causes aggregation of large-bodied seed predators in mast-rich habitats (Bogdziewicz, Zwolak & Crone 2016). Thus, N-mediated increases in seed production could result in not only more intense seed predation by weevils, but also increased predation by vertebrates.

On the other hand, the number of acorns not harvested by rodents was higher in N-treatment. We assumed uncached acorns do not germinate, which might underestimate the regeneration potential of fertilized oaks. However, the establishment of seedlings of red oaks is extremely low without burial (e.g. surface: 0.3% vs. buried: 86%; Haas & Heske 2005), and in the eastern North American hardwood forests acorns are dispersed and buried almost exclusively by rodents (Steele 2008). Moreover, seed dispersal allows seeds to escape the distance- and density-dependent mortality near the parent plant (Hirsch *et al.* 2012), and increases the likelihood of colonization of habitats favourable for germination and establishment for a number of plant species, including oaks (Steele *et al.* 2014; Yi *et al.* 2013). Therefore, the drop in acorn predation near parent plants caused by nitrogen fertilization is unlikely to counterbalance the decrease in acorn dispersal.

The effects of N-fertilization on plant reproduction are likely to be scale dependent. Contrasting nitrogen with control acorns in our experiment allowed rodents to harvest mostly preferred acorns (here, control). In a world polluted with nitrogen, all plants will be affected, but not all will respond in the same manner (Stamp 2003). Thus, nitrogen-induced changes in seed traits will be species specific (see Yi *et al.* 2016), which might alter preferences of seed consumers and potentially lead to shifts in plant communities (Lichti *et al.*

2014). This effect will likely interact with the effects of N-pollution on understorey vegetation; these have been shown to vary among systems from decline, through no effect, to increase in cover (Thomas *et al.* 1999; Gilliam 2006; Gilliam, Hockenberry & Adams 2006). Decrease in plant cover is likely to decrease rodent abundance (e.g. Malo *et al.* 2012; Zwolak, Bogdziewicz & Rychlik 2016), which could increase caching by increasing *per capita* seed availability (Theimer 2005). Furthermore, rodents prefer to cache seeds in open habitats where the pilferage risk is lower (Muñoz & Bonal 2007, 2011; Steele *et al.* 2015). Both effects could decrease or increase caching rates depending on the scale and the magnitude of the effect. Thus, it would be useful to study these effects in different systems in order to find out whether these could mitigate negative effects of N-fertilization on tree reproduction. Similarly, the LTER experiment is surrounded by a non-treated matrix, which could lead to redistribution of weevils in space. For example, if elevated weevil predation was mostly driven by aggregation of weevils in a N-rich habitat, then larger scale pollution might moderate the increase in predispersal predation rate.

Conclusions

Our research shows that N-fertilization has a strong potential to decrease the recruitment of masting trees. Any positive effects of fertilization on acorn production were eliminated by the N-mediated changes in biotic interactions. Given the ubiquitous increase in the anthropogenic nitrogen deposition (Galloway *et al.* 2004), processes similar to those found in our system might operate in others, resulting in a widespread alteration in trees' recruitment dynamics. For example, oak decline and replacement by other tree species is a well-recognized problem and received significant scientific attention in both North America (Abrams 2003; McShea *et al.* 2007) and Europe (Thomas, Blank & Hartmann 2002). When investigating causes of this decline, researchers focus mostly on changes in climate conditions, disturbance regime and introductions of exotic pathogens and pests that decrease growth and survival of seedling and adult trees (Thomas, Blank & Hartmann 2002; Abrams 2003; McShea *et al.* 2007). Our study documents a different mechanism that can play an equally significant role in this phenomenon: anthropogenic nitrogen addition reduces oak reproduction through its effects on oak–granivore interactions.

Author's contributions

M.B., R.Z., M.S. and E.C. conceived the ideas and designed methodology; M.B. and M.S. collected the data; M.B., R.Z. and E.C. analysed the data; and M.B. led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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Data accessibility

Data available from the Dryad Digital Repository <http://dx.doi.org/10.5061/dryad.2vk77> (Bogdziewicz *et al.* 2016).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Supporting information on data collection, analysis, figures, and table.

Table S1. The results of post-hoc comparisons of probability of seedling establishment among treatments.

Fig. S1. Shape of acorns expressed as length to width ratio produced by red oaks receiving different fertilizer treatments.

Fig. S2. Tannin acid concentration expressed as tannin acid equivalent in acorns produced by red oaks receiving different fertilizer treatments in 2013.

Fig. S3. Diagram of acorn fates in seed tracking experiment.