Embryo size as a tolerance trait against seed predation: Contribution of embryo-damaged seeds to plant regeneration

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ABSTRACT

Embryo damage in seed predation is a common occurrence and has been generally considered equivalent to seed death. We hypothesize that seeds with proportionally larger embryos (radicle plus plumule) provide greater tolerance to seed damage by rodents, allowing successful germination. To test this hypothesis, we examined germination and estimated the contribution of embryo-damaged seeds to plant regeneration by comparing the dispersal patterns of intact and embryo-damaged seeds and the ecophysiological responses of their emerged seedlings in two oak species of contrasting embryo size.

Our results show that embryo size was positively correlated with seed size in both oak species, but one (*Quercus lobata*) had proportionally longer embryos than the other (*Q. agrifolia*), revealing inter-specific differences in embryo size. Probability of embryo excision behavior (partial seed damage to extirpate the embryo) was positively associated with seed size but intensity of damage was relatively constant across all sizes. The species with proportionally longer embryos showed a stronger capacity to regenerate from embryo damage because longer embryos experienced reduced probability of total (lethal) embryo damage by rodents. Seed size and thus intraspecific embryo size only increased germination success in the species with shorter embryos, allowing larger seeds to escape full embryo damage.

Seedlings from intact seeds performed better (greater plant biomass and higher chlorophyll and nitrogen index) than those from embryo-damaged seeds. However, seedlings from embryo-damaged seeds showed higher anthocyanin content, a possible response to seed damage. Intact and embryo-damaged acorns showed similar dispersal patterns by rodents (e.g. seed caching behavior and dispersal distances). Importantly, seed retrieval by rodents was significantly lower for embryo-damaged seeds, allowing greater seed survival and supporting the expectation that embryo excision behavior is a strategy to store seeds for longer periods. We conclude that tolerance to embryo damage is an important reproductive strategy that enables plant recruitment. Embryo size could play a crucial role in the evolutionary and ecological responses of seeds to animal predation.

1. Introduction

Seed predation is an important selective pressure driving the evolution of seed traits (Janzen, 1969, 1971). Coevolution between seeds and their foragers through adaptation to reciprocating selection pressures may determine the development of mutualisms from antagonisms (Thompson, 1982). Seed-animal interactions can be particularly complex given the dual role of some seed foragers (e.g. rodents) as seed dispersers and predators (McEuen and Steele, 2005). Changes in seed traits (e.g., size, shape or chemistry) or in seed foraging behavior (e.g., seed selection, seed handling or hoarding behavior) can modify the sign and strength of the interaction and, hence, its position along the mutualism–antagonism continuum (Karst et al., 2008; Chamberlain and Holland, 2009; Perea et al., 2013). Therefore, animal-dispersed seeds are expected to evolve traits that will enhance effective seed dispersal (i.e., seedling establishment away from parents) while minimizing seed predation (McEuene and Steele, 2005). Simultaneously, seed foragers will develop behavioral strategies to maximize rewards from seeds (Vander Wall, 1990; Jansen et al., 2006; Lichti et al., 2015), setting up the potential for a coevolutionary arms race.

Embryo excision is a well-known strategy performed by several rodent species (mostly squirrels; Sciuridae), which consists of extirpating (or damaging) the embryonic parts (radicle and plumule) of the seed to prevent germination and ensure long-term seed storage

(Wood, 1938; Fox, 1982; McEuen and Steele, 2005; Smallwood et al., 2001; Steele et al., 2001a, 2006; Xiao et al., 2009, 2010; Xiao and Zhang, 2012a, 2012b; Yi et al., 2013). This embryo damage is common in nut-bearing trees, particularly in oak (genus *Quercus*) seeds, and has been, in general, considered equivalent to seed death (Andersson and Frost, 1996; Steele et al., 2001a; Branco et al., 2002; Steele, 2008; Xiao et al., 2009; Mendoza and Dirzo, 2009; Hou et al., 2010; Perea et al., 2011a; Perea et al., 2012a; Yang and Yi, 2012). However, other studies have documented the survival and germination of embryo-damaged acorns, with significantly lower germination rates as compared to intact acorns (Wood, 1938; McEuen and Steele, 2005; Xiao et al., 2009; Cao et al., 2011; Xiao and Zhang, 2012a, 2012b; Zhang et al., 2014). Interestingly, McEuen and Steele (2005) showed that although germination and radicle production are possible in embryo-damaged acorns, no seedlings successfully produced stems and leaves and, thus, their contribution to recruitment seems unlikely. In contrast, Cao et al. (2011) showed relatively high germination rates, and even successful seedling establishment, for artificially-damaged embryo acorns, which suggests a possible tolerance mechanism against embryo damage. However, potentially adaptive tolerance traits in seeds have been poorly investigated in the context of seed predation (but see Mendoza and Dirzo, 2009). As a result, our understanding of the possible adaptive traits responsible for embryo damage tolerance is still very limited. Research aimed at filling this lacuna may shed some light on the coevolutionary dynamics of seed predation.

Critical to the understanding of the consequences of partial embryo damage are the size of the embryo and the proportional magnitude of damage. However, to our knowledge, no study has explored the quantitative and qualitative contribution of embryo-damaged seeds to recruitment throughout the whole dispersal process, from seed removal to seedling establishment. A more comprehensive approach with experimental and field data is needed to help disentangle the ecological and evolutionary implications of embryo damage for plant regeneration.

Our hypothesis is that seeds with proportionally larger embryos will provide greater tolerance to embryo damage, increasing germination and establishment rates. We also hypothesise that, regardless of embryo size, intact seeds will produce more vigorous seedlings (larger and healthier) than embryo-damaged seeds. Additionally, we aim to quantify the embryo damage behavior of four rodent species on two California oak species that differ in embryo size. We specifically examine several factors potentially involved in embryo damage behavior, including seed size, acorn species and rodent behavior. Beyond the analyses of such factors, we examine the ecological consequences thereof, by comparing the dispersal patterns of intact and embryo-damaged seeds (dispersal distance, microsite of deposition, seed caching and recovery, and final seed fate). By integrating all these aspects, from seed removal to seedling establishment, with contrasting seed predators and oak species, we attempt to provide novel insights into the ecological and evolutionary implications of embryo damage for plant reproductive success.

2. Materials and methods

2.1. Study area and species

The study was conducted at the Jasper Ridge Biological Preserve (JRBP), located in the interior foothills of the Santa Cruz Mountains, Northern coastal California, USA (37.40°N, 122.23°W). JRBP is a 480-ha research preserve where elevation ranges from 66 to 207 m a.s.l., under a coastal Mediterranean climate, with warm, dry summers and cool, wet winters. Mean annual precipitation is 605 mm and average daily temperatures range from 2 to 4 °C (winter) to 25–27 °C (summer) (Zavaleta and Kettley, 2006). JRBP supports diverse vegetation types, including grassland, woodland, chaparral and forest. Oak-dominated systems (woodlands and savannas) are the most common vegetation

type in JRBP. Oak systems are dominated by coast live oak (*Quercus agrifolia* Née), valley oak (*Q. lobata* Née), and blue oak (*Q. douglasii* Hook. and Arn.). *Quercus agrifolia* is an evergreen red oak (sect. *Erythrobalanus*) whereas *Q. lobata* and *Q. douglasii* are closely-related, deciduous white oaks (sect. *Leucobalanus*). Compared to live oak, the latter two have lower tannin content and larger acorns (Koenig, 1991; Koenig and Faeth, 1998). *Quercus lobata* and *Q. agrifolia* are both considered slow-germinating species, germinating in November-February, approximately 2–5 months after seed drop (Matsuda and Mcbride, 1989).

Livestock (mostly cattle) was removed from JRBP in 1973, and black-tailed deer (*Odocoileus hemionus*) is the only ungulate present. Rodents are common in the area with a variety of species foraging on acorns (*Peromyscus* spp., *Sciurus* spp., *Neotoma* spp., *Otospermophilus* spp.). Among them, tree squirrels (*Sciurus* spp.) in North America are considered important acorn foragers and dispersers that perform embryo excision behavior (Fox, 1982; Steele et al., 2006). Three species of tree squirrels are present in Jasper Ridge (Jasper Ridge camera-trap monitoring of mammals; www.jrbp.stanford.edu): the native Western gray squirrel (*Sciurus griseus*) with few individuals in the dense forests of Jasper Ridge, and two introduced tree squirrels, the Eastern fox squirrel (*S. niger*) and the Eastern gray squirrel (*S. carolinensis*). Fox squirrels are by far the most abundant tree squirrel in the study area (oak savannas and open woodlands).

2.2. Acorn collection and characterization

Acorns of one deciduous (*Q. lobata*) and the evergreen oak (*Q. agrifolia*) were collected in the study area during September–October 2014 and 2015, from at least 14 different trees. We classified acorns into two categories: 1) Intact acorns (i.e., sound acorns with no damage), and 2) Embryo-damaged acorns, with the apical part of the acorn showing clear damage by rodents (gnawed; Fig. A1). Intact acorns were also tested for viability by a flotation method, following Perea et al. (2012b). Acorns were stored at 4 °C in plastic bags for approximately 15 days, and they were weighed (precision 0.01 g) before use.

To characterize intact acorns we randomly selected 100 sound acorns (fifty of each species), and measured length and maximum width (precision of 0.01 mm). Then, we cut the acorns longitudinally to measure the embryo length (radicle plus plumule), using a digital caliper with a 0.01 mm precision. We then obtained the embryo:seed length ratio (r), as shown in Fig. A1 (Supporting Information). To estimate average rodent damage on the embryo, we carefully selected 50 embryo-damaged acorns (25 of each species) where the apical edge of the pericarp (acorn apex) remained undamaged (see Fig. A2, Supporting Information). Then, we measured the length of the apical damage on the acorn (distance from the pericarp apex to the first undamaged point of the cotyledons; Fig. A2). These acorns were weighed and planted in the greenhouse to examine potential ability of naturally embryo-damaged acorns by rodents to produce seedlings.

2.3. Acorn germination and seedling emergence

Two plantation experiments were conducted. First, we planted 50 naturally embryo-damaged acorns by rodents (25 of each species; Fig. A2) to examine germination success. We also simulated embryo damage by rodents for another 80 intact acorns (40 of each oak species) by cutting 12% of the acorn length from the apical part, following the average rodent damage found in the naturally embryo-damaged acorns. We planted these 130 embryo-damaged acorns (50 naturally damaged and 80 simulated), together with 80 control seeds (intact acorns; 40 of each species) in individual containers 10 cm long x 10 cm diameter, filled with a substrate of 70% peat and 30% perlite. Acorns were partially covered in the substrate (1–2 cm) and kept under the same environmental conditions in Stanford University Plant Growth Facilities

(12 h of light per day; 18–23 °C). Containers were watered and checked, three times a week, for a maximum period of eight months (November 2015-June 2016). Successful emergence was considered when seedlings germinated and produced ≥ 1 cm of green stem above the substrate level. At this stage, seedlings are able to produce adequate photosynthate to survive by producing their own supply (Perea et al., 2012a). Thus, we considered that seeds were able to produce seedlings when seedling emergence occurred. Seedlings that did not emerge at the end of the experiment were checked for possible germination. If a radicle longer than 5 cm was found the acorn was considered as germinated. All acorn sizes were used for the naturally-damaged embryo acorns by rodents (3–7 g for *Q. agrifolia*; 4–11 g for *Q. lobata*). To compare germination, emergence and performance of embryo-damaged and intact acorns (simulation experiments) we selected acorns of similar weight within each species: *Q. lobata* with Mean ± SE = 7.94 ± 0.10 g (range ~ 7-9.5 g) and *Q. agrifolia* with Mean ± SE = 4.41 ± 0.05 g (range 4–5 g). Acorns of each oak species were assigned randomly to each treatment.

2.4. Seedling performance and physiology

Each emerged plant (≥ 1 cm of green stem above the substrate level) was harvested 45 days after its emergence (average plant height of 64.4 ± 2.6 mm). We chose 45 days after emergence to ensure successful seedling establishment and full development of stems and leaves. We separated the aboveground part (shoots and leaves) from the roots, washed the roots and dried both parts in an oven at 80 °C for 96 h. We weighed the samples to obtain root and aboveground dry biomass. Root to shoot ratio (R/S) was calculated as root dry biomass divided by aboveground dry biomass. Right before harvesting the plants, several measures of oak seedling physiology were obtained using the Dualex Scientific + ™, a leaf-holding device that provides indices of chlorophyll, flavonol and anthocyanin content in leaves (Agati et al., 2016). In addition, the Dualex device provides the NBI® (Nitrogen Balance Index) which is the Chlorophyll/Flavonols ratio (related to Nitrogen/Carbon allocation), that can be used as a proxy of the nitrogen status of plants (Cartelat et al., 2005; Agati et al., 2016). More details of Dualex indexes can be found in Casa et al. (2015) and Agati et al. (2016). Chlorophyll content and NBI were used to determine oak seedlings health. In order to obtain representative measures, Dualex data were collected from five leaves of each seedling.

2.5. Acorn dispersal and embryo damage by rodents

We offered intact acorns of *Q. lobata* and *Q. agrifolia* to the main four rodent species in the study area: Dusky-footed woodrat (*Neotoma fuscipes*), California ground squirrel (*Otospermophilus beecheyi*), fox tree squirrel (*Sciurus niger*) and California deer mice (*Peromyscus californicus*). In early fall 2014, we performed a preliminary survey with 12 cameras (Bushnell 6 MP Trophy cam with night vision and motion detection) to identify areas dominated by each rodent species (total of 36 camera points for five days; 180 trap-days). Video-recordings of 10 s length were taken for each animal contact. At least three supply stations were assigned specifically to each rodent species, ensuring that only one animal species was acting in each station with the use of video-recordings. We also built stations for rodents with a wire (7 cm mesh; 80 cm high × 40 cm diameter) to exclude birds and deer. Digital video cameras were used in all stations during all trials to ensure the identification of the animal and its behavior. Stations with two rodent species (checked by video-recordings) were discarded. Excluding the discarded acorns, a total of 1040 tagged acorns were offered to the 4 rodent species in fall, 2014 and 2015 (100–160 acorns per animal species and year). All acorns were weighed and each group of acorns that were offered simultaneously contained 20–40 acorns of all possible sizes (half *Q. agrifolia*, half *Q. lobata*) to examine the effect of seed size on acorn embryo damage. For acorn tagging, a wire (3 cm long and 0.6 mm

thick) was attached to the acorns by drilling a hole with a needle into the approximate center of the acorn following Perea et al. (2011b). To individually identify acorns, a 7 × 1.2 cm plastic tag was attached to the wire and numbered with waterproof permanent ink. We used this wire tagging method because it does not significantly alter acorn dispersal patterns and yields a high recovery rate (Xiao et al., 2006; Suselbeek et al., 2013). We searched for dispersed acorns daily during the first 3 days after seed offer and on day 7 and 15 of each trial. The locations of the dispersed acorns were marked with wooden sticks (40 cm × 0.5 cm) to allow easy relocation. Marked sticks are known not to have a significant effect on seed removal (Xiao et al., 2005). We also covered the plastic tags with litter or sand to avoid attracting/deterring foragers. For each dispersed acorn, we recorded the following data: tag number (acorn weight and species), damage category (intact, predated, partially consumed), distance to the supply station and whether they were cached or not. For each acorn we also recorded the embryo status (damaged or not). A final visit was made at the end of fall-early winter (December) to check for the survival of acorns. The following spring (March-April) we revisited the sites to record whether the seeds had successfully emerged or not.

2.6. Data analysis

All analyses were performed with R 3.3.1 software. To compare embryo length and the embryo length to acorn length ratio (r) across species we used Linear Models (LM) where the response variable was embryo length and ratio (r), and the explanatory variable was oak species. In all LMs we used Shapiro tests to ensure normality of residuals and Levene's tests to check for homoscedasticity. When normality was not achieved, we performed log, square root or Box Cox transformations, or used the non-parametric Wilcoxon test. To analyze seed germination and seedling emergence we used Generalized Linear Models (GLMs). Response variables were binary (germinated or not; emerged or not). Explanatory variables were oak species (acorns of *Q. agrifolia* and *Q. lobata*), acorn category (embryo-damaged vs. intact), seed size and their interactions. Models were fitted by the Laplace approximation with a logit link function according to our data properties (Bolker et al., 2009), using the "glmer" function within the "lme4" package. We used time-to-event analysis to examine seedling emergence times, following McNair et al. (2012). We used the function "survfit" within the "survival" package of R to fit the Kaplan-Meier survivor function (McNair et al., 2012). To assess the effect of embryo damage on seedling emergence time, we built two Kaplan-Meier curves (one for intact and one for embryo-damaged acorns) for each oak species. Then, we used the function "survdiff" to test for differences in the curves using the Gρ (G-rho) family of tests. We analyzed seedling performance and their ecophysiological responses using Linear Models. Response variables were root dry biomass, aboveground biomass, root/shoot ratio (R/S), chlorophyll content, NBI, flavonol and anthocyanin content. Predictors were oak species, acorn category (embryo damaged vs. intact) and their interaction. Acorn weight was also included as a covariate. Data transformation and model checking was performed following the same procedures as in the abovementioned LMs.

To analyze embryo damage by rodents in the field we used another GLM. The response variable was binary (embryo damaged or not). Explanatory variables were oak species, acorn weight and rodent species and their two-way interactions. The model was fitted as in the previous GLM (Laplace approximation with a logit link function). To compare dispersal attributes between embryo-damaged and intact acorns we restricted the analyses to tree squirrels since they were the only rodent species clearly performing embryo damage behavior. We analyzed seed caching (acorn cached or not) and final seed survival (survived or not) using GLMs (binomial distribution, logit link function) with the same properties as the models above. We also analyzed dispersal distances using a Linear Model where the response variable (distance to supply station) was fitted to a normal distribution by a log-

a)

b)
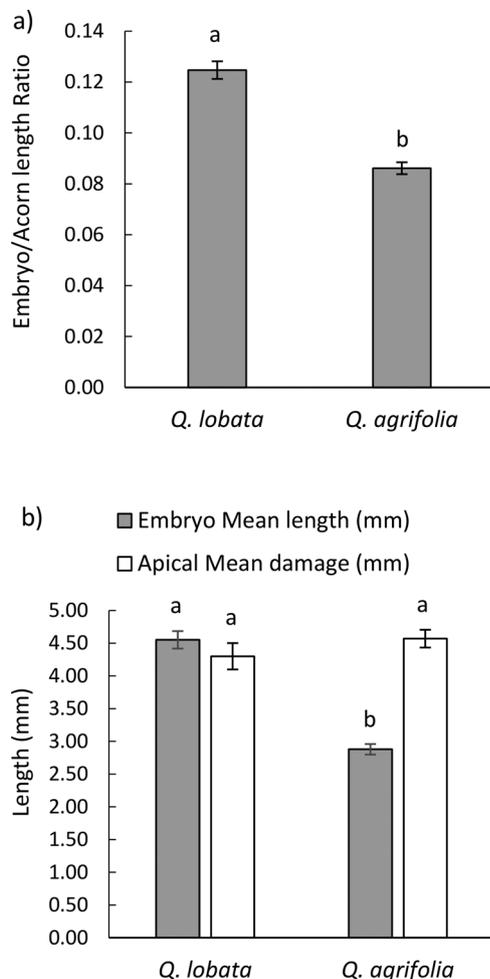
Fig. 1. a) Embryo length to acorn length ratio (r) for *Q. lobata* and *Q. agrifolia*. b) Mean embryo length and mean natural apical damage by rodents for both oak species. Values are Mean ± SE. Different letters above the bars indicate significant differences (*P* < 0.05).

transformation. All values shown are Mean ± SE unless otherwise indicated.

3. Results

3.1. Embryo length and damage

Embryo length and seed length were positively correlated in both oak species (*Q. lobata*: $t = 2.65$; $d.f. = 48$; $P = .011$; $r = 0.36$; *Q. agrifolia*: $t = 2.25$; $d.f. = 48$; $P = .029$; $r = 0.31$). We found significant differences in the embryo length/acorn length ratio (r) between both oak species. *Quercus lobata* showed a 45% greater ratio than *Q. agrifolia* ($t = 9.84$; $P < .001$; Fig. 1a). Overall, *Q. lobata* had a significantly longer embryo than *Q. agrifolia* ($t = 11.42$; $P < .001$; Fig. 1b). Average natural acorn damage (length) by rodents showed low variation (4.44 ± 0.13 mm; range 2.7–6.6 mm), with no significant effect of acorn size ($t = 0.52$; $P = .607$) or oak species ($t = -1.08$; $P = .285$; Fig. 1b). Thus, average damage by rodents was above mean embryo length in *Q. agrifolia* ($t = 10.49$; $P < .001$; Fig. 1b), usually resulting in full embryo damage whereas, for *Q. lobata*, average embryo damage was shorter than its average embryo length ($t = -1.01$; $P = .3185$; Fig. 1b), resulting in partial damage to the embryo.

3.2. Germination of embryo-damaged seeds by rodents

Germination rate of naturally embryo-damaged acorns of *Q. lobata*

a) *Q. agrifolia*
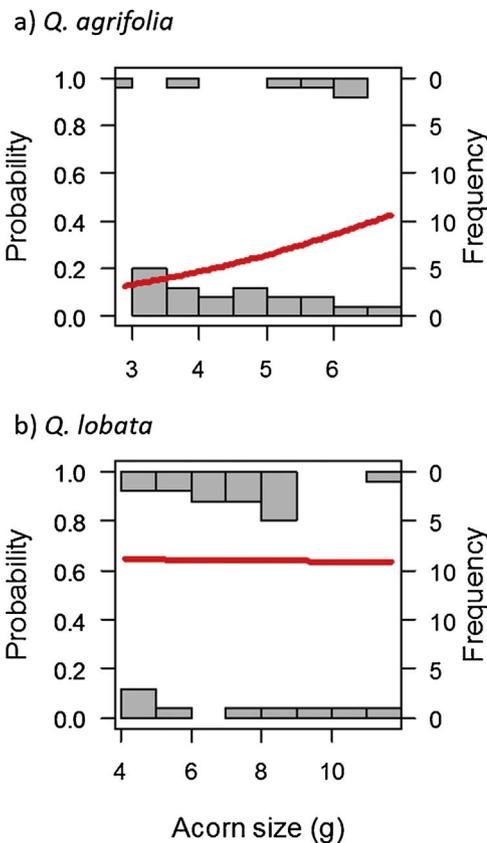
b) *Q. lobata*

Fig. 2. Predicted probability of germination of *Q. agrifolia* (a) and *Q. lobata* (b) as a function of acorn size for naturally embryo-damaged seeds by rodents. The histograms represent the observed data (1 = Germinated; 0 = Not germinated) and the line shows the predicted probability.

was 0.64, approximately 2.7-fold greater than that of *Q. agrifolia* (0.24), a highly significant difference ($\chi^2_1 = 8.37$; $P = 0.004$; Fig. 2). Probability of germination was higher for larger embryo-damaged acorns (i.e., larger embryos) of *Q. agrifolia* (Fig. 2a), but remained constant across all sizes of embryo-damaged *Q. lobata* acorns (Fig. 2b).

3.3. Germination and seedling emergence of embryo-damaged vs. intact seeds

Similarly, germination rate of simulated embryo-damaged acorns of *Q. lobata* was significantly greater (0.85) than that of *Q. agrifolia* (0.60; $\chi^2_1 = 9.02$; $P = 0.003$; Fig. 3a). Germination success of intact acorns was significantly greater than that of embryo-damaged acorns for *Q. agrifolia* but not for *Q. lobata* (Table 1; Fig. 3a). Thus, *Q. lobata* acorns showed similar germination rate between intact and embryo-damaged acorns (only 5% difference) as compared to *Q. agrifolia* acorns (35% greater germination for intact acorns; Fig. 3a). Acorn size only increased germination success in embryo-damaged seeds of *Q. agrifolia* (Fig. 4a) as reflected by the significant interaction Acorn size × Oak Species (Table 1). Embryo-damaged and intact acorns of *Q. lobata* showed no differences in germination success for all acorn sizes (Fig. 4b)

Successful emergence of embryo-damaged acorns was also significantly higher in *Q. lobata* (proportion of emergence success = 0.60) than in *Q. agrifolia* (proportion of 0.32; $\chi^2_1 = 8.21$; $P = .004$; Fig. 3b). For both species, intact acorns showed significantly greater emergence success than embryo-damaged acorns (Table 1; Fig. 3b). Similar to germination results, the difference in emergence between intact and embryo-damaged acorns was much larger in *Q. agrifolia* (58% difference) than in *Q. lobata* (a 15% difference; Fig. 3b), with a significant treatment-oak species interaction term (Table 1). Acorn size only

a)

between oak species ($\chi_1^2 = 2.61$; $P = .107$; Fig. A4).

3.4. Seedling performance and physiology

Production of root dry biomass in seedlings was significantly affected by oak species and embryo damage (Table A1). Overall, seedlings of *Q. lobata* produced significantly greater root biomass than *Q. agrifolia* seedlings (Fig. 5a). However, there was no difference between oak species in aboveground biomass (Table A1; Fig. 5b), which results in a much greater (3-fold) root to shoot ratio (R/S) of *Q. lobata* seedlings as compared to *Q. agrifolia* seedlings (Fig. 5c).

For both oak species, embryo-damaged acorns produced significantly less root biomass than intact acorns (Fig. 5a). Similarly, aboveground dry biomass was lower for embryo-damaged seedlings of both species (Table A1; Fig. 5b). Interestingly, and common for both oak species, no significant differences were found in root to shoot ratio (R/S) between seedlings from embryo-damaged and intact acorns (Table A1; Fig. 5c).

Chlorophyll, nitrogen (NBI) and anthocyanin content on leaves significantly varied between seedlings emerging from embryo-damaged and intact acorns (Table A2; Fig. 6). Both indexes of plant health (Chlorophyll and NBI) were significantly greater in seedlings emerging from intact acorns as compared to embryo-damaged acorns, for both oak species (Table A2; Figs. 6a and 6b). However, anthocyanin content was significantly greater in embryo-damaged seedlings of both oak species (Fig. 6d). Flavonol content did not significantly differ between seedlings from intact and embryo-damaged acorns (Table A2; Fig. 6c). No significant differences were found in any physiological measurements between both oak species (Table A2; Fig. 6).

3.5. Acorn dispersal and embryo damage by rodents

Tree squirrels were, by far, the main rodent species performing embryo damage behavior (62% of the acorns showed embryo damage) followed by deer mice (9%), woodrats (4%) and ground squirrels (0%; Fig. 7) with significant differences across animal species (LR-$\chi_1^2 = 69.61$; $P < .001$). However, no significant differences were found between oak species regarding embryo damage probability (LR-$\chi_1^2 = 0.04$; $P = .833$). Interestingly, probability of embryo damage was significantly affected by acorn weight (LR-$\chi_1^2 = 4.25$; $P = .039$). Thus, heavier acorns were associated with greater probability of embryo damage (Fig. 8).

Embryo-damaged acorns did not significantly differ from intact acorns in the probability of being cached by tree squirrels (LR-$\chi_1^2 = 2.27$; $P = .132$). However, seed recovery (retrieval of cached acorns) was significantly greater for intact acorns (81.5% retrieved) than for embryo-damaged acorns (40.9% retrieved; LR-$\chi_1^2 = 19.03$; $P < .001$). Thus, in late fall (end of experiments), most intact acorns were retrieved and predated (only 5 survived), whereas 27 embryo-damaged acorns were not retrieved and survived (i.e., they were not fully predated). Successful emergence in next spring only occurred in 6 acorns for both years (1.87% of all acorns offered to tree squirrels), with

Fig. 3. Predicted probability of germination (a) and emergence (b) for both oak species and for embryo-damaged and control (intact) acorns. Error lines are 95% confidence intervals. Different letters above the bars indicate significant differences (*P* < 0.05).

increased emergence success in embryo-damaged seeds of *Q. agrifolia* (Fig. A3) as reflected by the interaction Acorn size × Oak Species (Table 1), following the same pattern as in the germination experiments. Likewise, embryo-damaged and intact acorns of *Q. lobata* showed no differences in establishment success for all acorn sizes (Fig. A3)

In addition, the Kaplan-Meier curves revealed significant differences in the temporal patterns of seedling emergence between embryo-damaged and intact acorns of *Q. agrifolia* ($\chi_1^2 = 24.80$; $P < .001$; Fig. A4), but not in those of *Q. lobata* ($\chi_1^2 = 3.80$; $P = .052$; Fig. A4). Thus, for *Q. agrifolia*, time to emergence was significantly shorter (median 2 days earlier) for embryo-damaged acorns compared to intact acorns (Fig. A4). Finally, there was no significant difference in emergence time

Table 1
Summary of the GLMs to analyze acorn germination (model M1) and seedling emergence (model M2) of embryo-damaged acorns and intact acorns of similar seed size (7–9 g for *Q. lobata* and 4–5 g for *Q. agrifolia*).

Predictors	M1. Acorn germination			M2. Seedling emergence		
	L-R χ^2	d.f.	P-value	L-R χ^2	d.f.	P-value
Oak species	0.854	1	0.3553	1.648	1	0.1992
Treatment (damaged vs. intact)	11.951	1	**.0005**	**24.505**	**1**	**< .0001**
Oak Species × Treatment	4.527	1	**.0334**	**9.215**	**1**	**.0024**
Acorn size	1.529	1	.2163	1.718	1	.1899
Acorn size × Oak species	5.647	1	**.0175**	4.149	1	**.0417**

Bold type indicates statistical significance (*P* < 0.05). M1: Dispersion parameter = 0.85; Deviance explained = 0.13; M2: Dispersion parameter = 1.10; Deviance explained = 0.17.
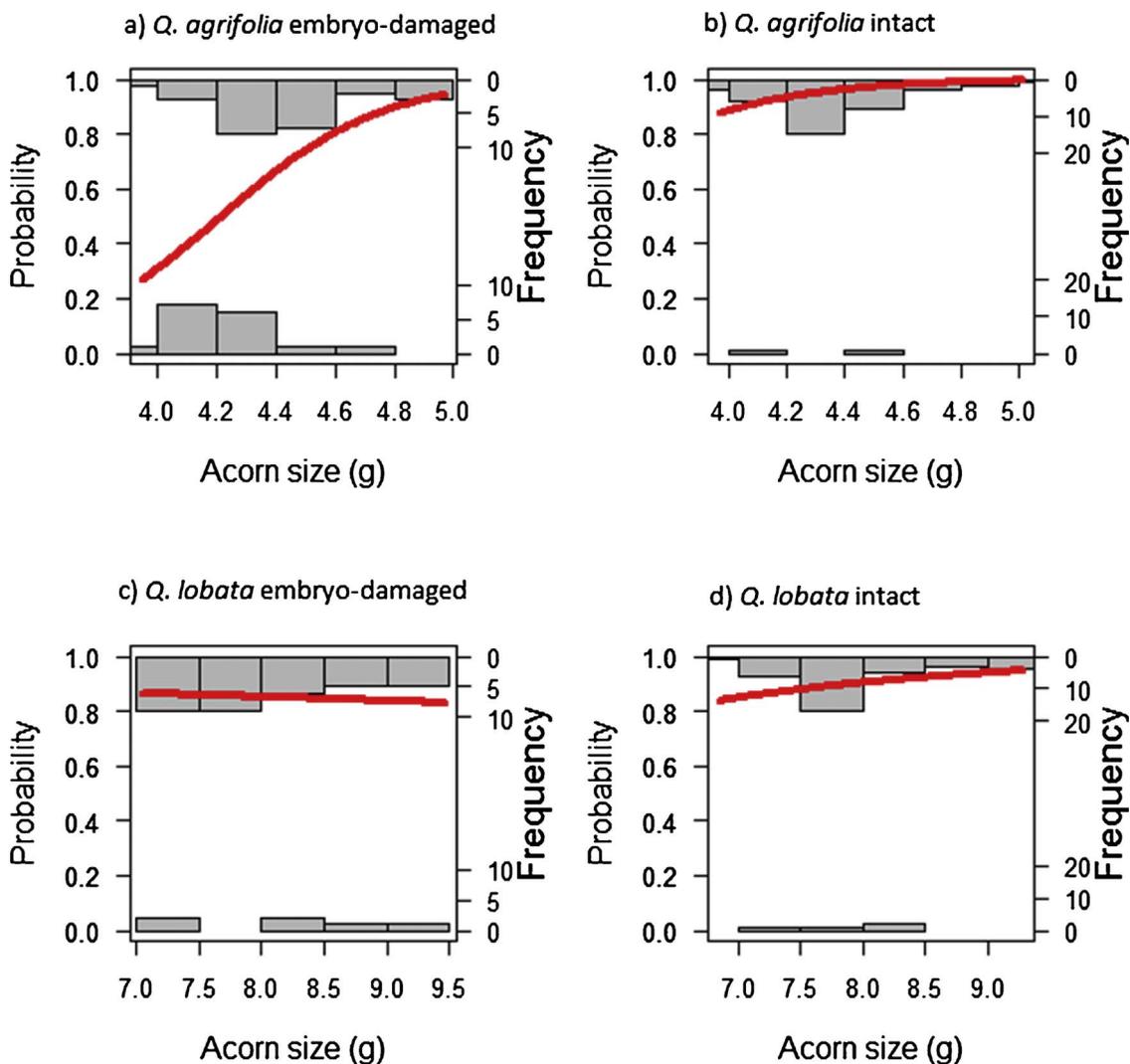
Fig. 4. Predicted probability of germination as a function of acorn size for intact and simulated embryo-damaged acorns of *Q. agrifolia* and *Q. lobata*. The histograms represent the observed data (1 = Germinated; 0 = Not germinated) and the line shows the predicted probability.

one seedling emerging from intact acorns (20% of success rate; 1 out of 5 intact acorns) and five from embryo-damaged acorns (18% of success rate; 5 out of 27 embryo-damaged acorns). Dispersal distances were significantly greater ($t = 2.67$; $d.f. = 112$; $P = .025$) for embryo-damaged acorns (Mean distance = 7.34 m; Median = 5.60 m; max = 33.10 m) than for intact acorns (Mean distance = 5.46 m; Median = 4.85 m; max = 20.01 m; Fig. 9a), but the strong correlation between acorn weight and embryo-damage probability should be considered (Fig. 8). Thus, heavy acorns were dispersed or consumed at significantly farther distances ($t = 2.11$; $d.f. = 280$; $P = .036$; Fig. 9b).

4. Discussion

Our results revealed that embryo size was positively correlated with seed size in both oak species despite strong inter-specific differences in embryo size. The difference between embryo length and embryo damage by rodents, albeit small, allowed parts of the embryonic tissue (plumule and radicle) to remain undamaged and, thus, seeds remained potentially viable. Importantly, the oak species with proportionally longer embryos showed stronger capacity to regenerate from embryo damage because longer embryos reduced the probability of total and, hence, lethal embryo damage. These results also support the idea that partial embryo damage is not equivalent to seed death and, thus, may allow germination and emergence, in line with Cao et al. (2011). Only

total or nearly total embryo damage (radicle and plumule) was found to limit seed viability.

This study also confirms that seed size is not particularly relevant for acorn germination since almost all intact seeds successfully germinated regardless of seed size, which agrees with the notion that acorns are very easy-to-germinate seeds (Matsuda and Mcbride, 1989; Branco et al., 2002). However, seed size and thus intraspecific embryo size did increase germination success of embryo-damaged acorns in the species with shorter embryos, allowing larger seeds to escape full embryo damage. In addition, establishment rates were very different depending on the oak species, with only a 15% difference in establishment success between intact and embryo-damaged acorns of the species with proportionally longer embryos, but 58% difference in the species with shorter embryos. All this suggests that embryo size may represent an important trait in seed predation tolerance. Zhang et al. (2014) recently found that embryos located deeper in the cotyledons prevented embryo excision behavior (no damage at all) and considered embryo migration a possible adaptation to avoid embryo excision by rodents although they also argue that it might be related to embryo protection against extreme cold temperatures in NE China (−40 °C). Here, we highlight a tolerance trait to actual damage by rodents on the apical end of the seed where the embryo is located in most oak species. Previous studies have shown the benefit of an embryo being located in the apical end, such as easier embryo protrusion from the seed coat or lower probability of
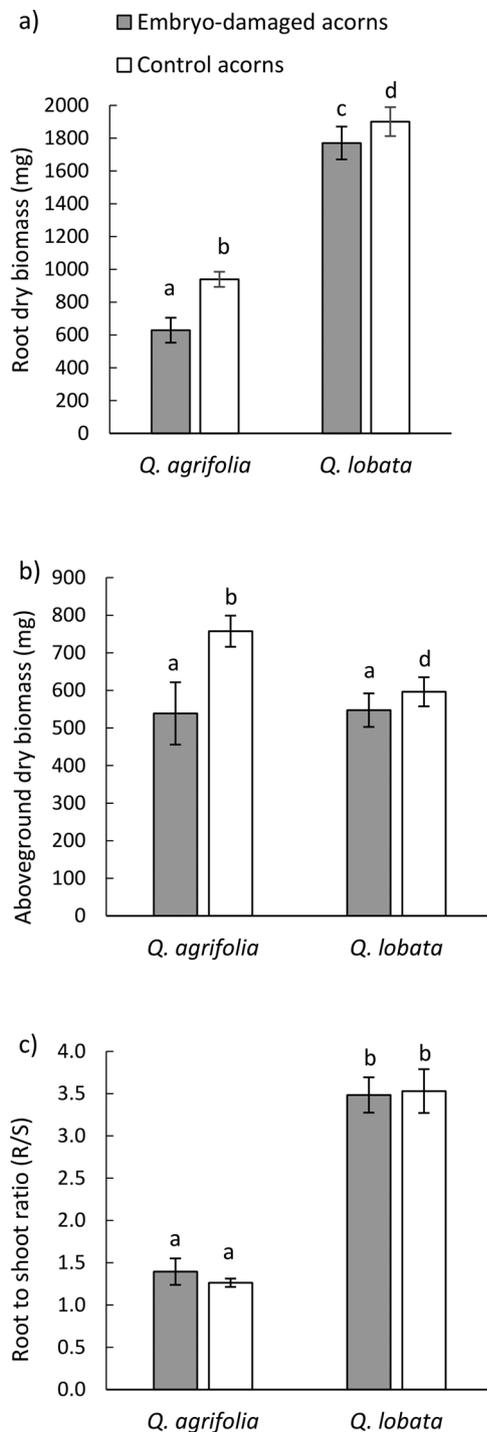
Fig. 5. Root dry biomass (a), aboveground dry biomass (b) and root/shoot biomass ratio (c) for embryo-damaged and control (intact) acorns of both oak species. Different letters above the bars indicate significant differences ($P < 0.05$).

embryo damage by invertebrates, such as weevils which mostly consume the basal part of the acorns (Steele et al., 1993; Yi and Yang, 2010; Perea et al., 2012a). Thus, embryo size, unlike embryo migration, may allow the seed to benefit from both the apical position of the embryo and the reduction in the probability of full embryo excision.

Interestingly, only embryo-damaged acorns of *Q. agrifolia* emerged earlier than intact acorns, suggesting that embryo damage might not necessarily affect germination time similarly across all oak species. Consistent with our predictions, intact acorns produced seedlings with greater dry biomass (both below- and above-ground) than embryo-

damaged acorns. However, we found no differences between both acorn categories in the root to shoot ratio, which supports the idea that seedling growth and plant architecture remain proportioned after embryo damage. Additionally, seedlings from intact acorns had greater chlorophyll and nitrogen indices, two key indicators of plant health. All this demonstrates that embryo-damaged plants performed more poorly than those coming from intact seeds. Further studies should investigate whether such poorer performance in embryo-damaged plants is related to the need of seeds to regenerate the embryo or, conversely, is mostly due to the small loss of cotyledon mass ($< 10\%$ in this study). Surprisingly, anthocyanin content was significantly higher in embryo-damaged plants. Anthocyanins have been proposed to function as protection against herbivores (Lev-Yadun and Gould, 2008) and, thus, greater anthocyanin content in embryo-damaged plants might represent a possible response to herbivore damage (i.e., partial seed predation) but further studies should specifically address this hypothesis.

Field experiments revealed that tree squirrels (*Sciurus niger* in this study) were, by far, the main rodent species performing embryo damage as expected from previous studies (Steele et al., 2001b, 2006). The introduced eastern gray squirrel (*Sciurus carolinensis*) and the western gray squirrel (*S. griseus*) were also seen (and some recorded) excising the acorns in nearby peri-urban and forest areas, respectively (R. Perea, pers. obs.). Thus, this study provides evidence of the widespread embryo excision behavior of other *Sciurus* species across North America. Deer mice (*Peromyscus californicus*) showed slightly greater excision rates (apical damage) than other mice species in other regions (e.g., *Apodemus sylvaticus*; Perea et al., 2011a). These low excision rates (9%), even lower for woodrats and null for ground squirrels, confirm that only some squirrels in the Sciuridae family perform clear and direct embryo-damage behavior (Steele et al., 2001a; Xiao et al., 2009, 2010; Xiao and Zhang, 2012a, 2012b), although further studies should not disregard the ecological and evolutionary implications of this seemingly incidental behavior in *Peromyscus* and other rodent species. It would be important to confirm, in future studies, whether this anecdotal embryo damage by mice was direct embryo excision behavior or, in contrast, collateral embryo damage due to light predation on the apical part of the seed.

Interestingly, the probability of embryo damage by both tree squirrels and deer mice was positively correlated with acorn size. Previous studies have shown that seed size plays an important role in natural regeneration since heavier seeds are preferred and dispersed farther (Xiao et al., 2005; Wang and Chen, 2009), leading to greater successful establishment (Bonal et al., 2007; Perea et al., 2011a). However, larger seeds are also more frequently recovered and pilfered by rodents, increasing predation (Perea et al., 2016). The relationship between embryo damage probability and seed size suggests that larger seeds may be an adaptation not only to increase plant growth and health but also to avoid full damage, enabling plants to regrow from partial embryo damage. In addition, our results show that the probability of seed caching (dispersal quality) was not different between embryo-damaged and intact acorns, and even dispersal distances were greater for embryo-damaged acorns, probably as a result of being larger and, therefore, highly preferred (more valuable food items). This means that embryo-damaged acorns had similar dispersal quality to intact acorns and, thus, may contribute similarly to natural regeneration although germination and establishment success has been proved to be significantly lower, particularly for oak species with shorter embryos. The differential aspect in the dispersal process was seed recovery probability (i.e., seed retrieval by rodents), which was significantly greater for intact acorns in comparison to embryo-damaged acorns. This novel finding could be associated with the evolutionary interpretation of embryo removal behavior as a strategy to store seeds for longer periods (Fox, 1982; Steele et al., 2001a, 2006; Xiao et al., 2010; Xiao and Zhang, 2012a, 2012b). In that way, squirrels may save these long-term caches, containing embryo-damaged seeds, for later in the season, increasing the probability of not being recovered. Contrary to
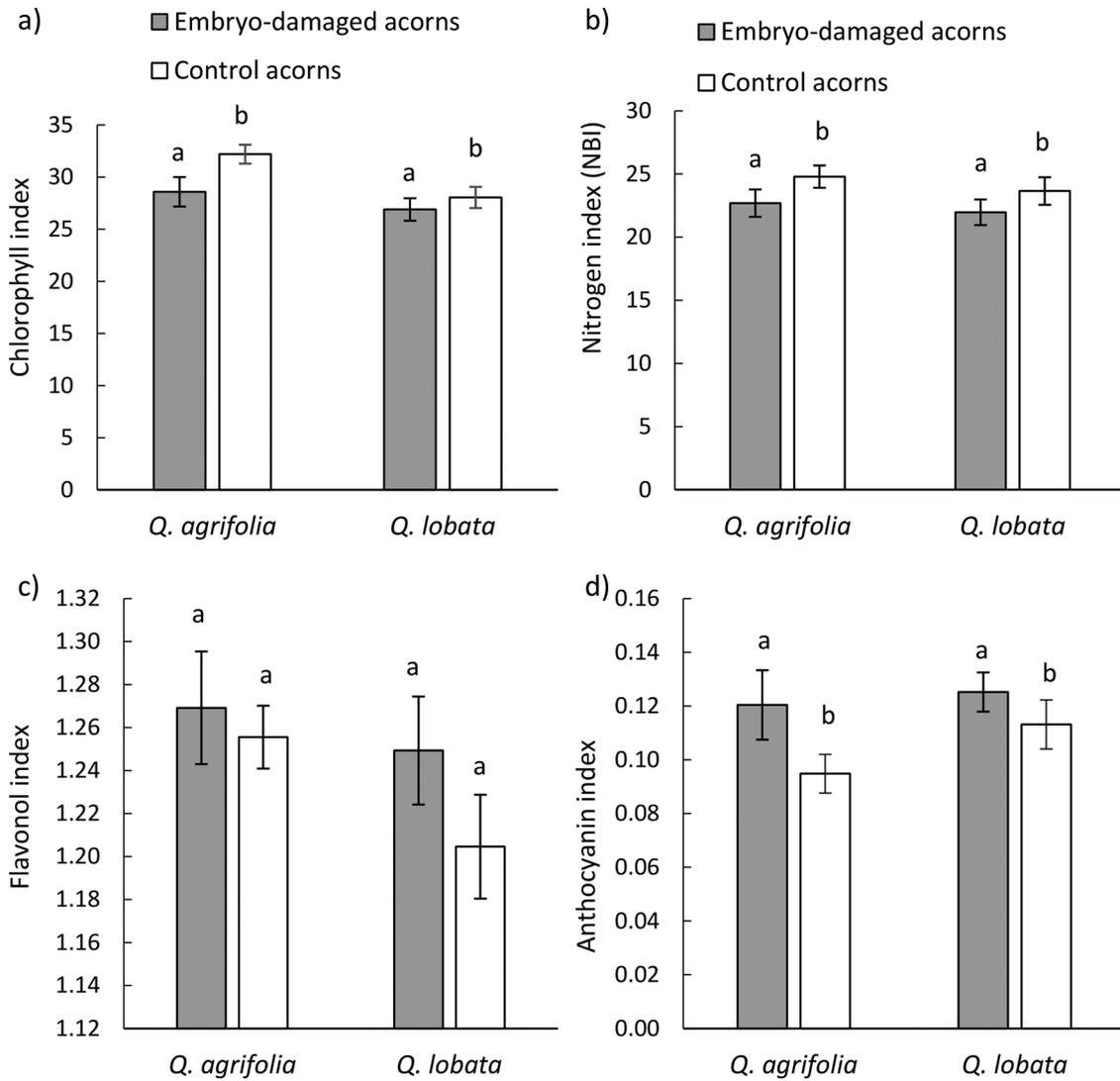
Fig. 6. Physiological indices (chlorophyll, nitrogen, flavonols and anthocyanins) for seedlings from embryo-damaged and intact acorns of *Q. agrifolia* and *Q. lobata* 45 days after above-ground emergence. Different letters above the bars indicate significant differences ($P < 0.05$).
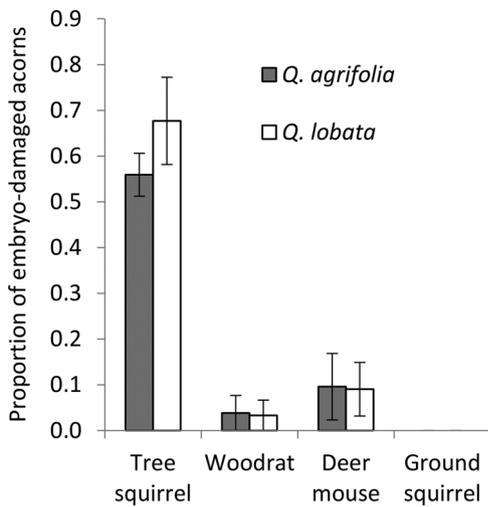
Fig. 7. Proportion of embryo-damaged acorns (resulting from embryo excision behavior) for each rodent species. No significant differences were obtained between oak species.
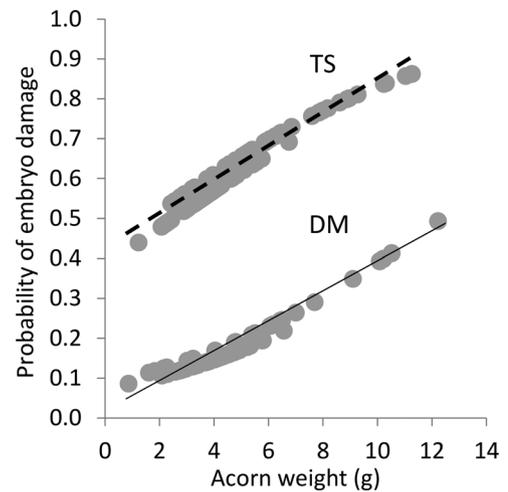
Fig. 8. Predicted probability of embryo damage for acorns handled by tree squirrels (TS) and deer mice (DM) in relation to acorn weight. TS-equation: $y = 0.044x + 0.409$ ($R^2 = 0.96$); DM-equation: $y = 0.035x + 0.018$ ($R^2 = 0.94$).

a)

b)
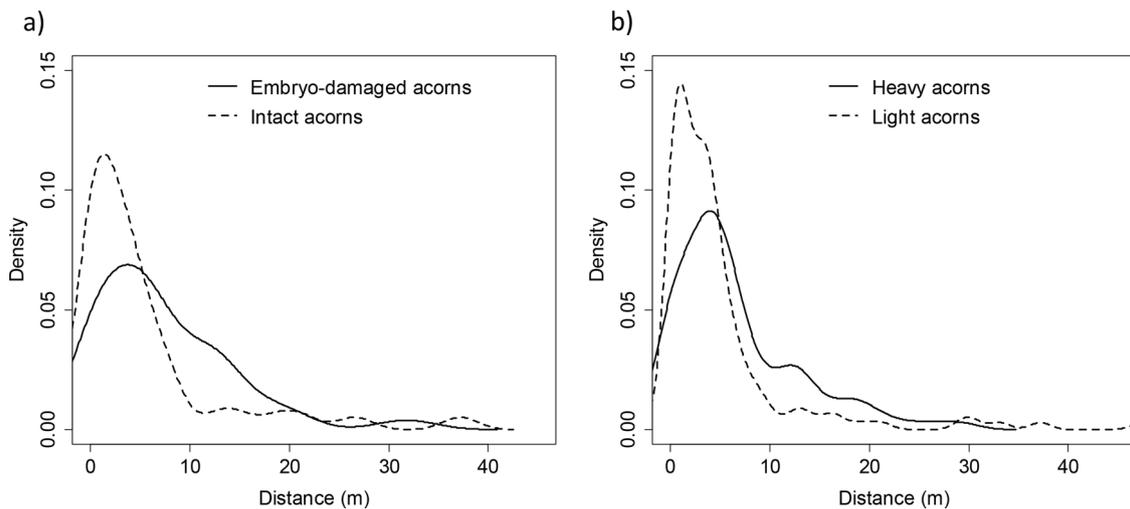
Fig. 9. Seed dispersal kernel for intact and embryo-damaged acorns (a) and for heavy and light acorns (b). Acorn weight categories were established based on the median acorn weight (heavy acorns > 4.40 g; light acorns < 4.40 g).

our expectations, embryo excision behavior could enhance the survival of seeds with longer embryos since they can better tolerate partial predation while remaining cached for longer periods. Our data reveal that in late fall (end of experiments) most intact acorns were retrieved and predated whereas embryo-damaged acorns were not retrieved and survived (i.e., they were not fully predated). Future seed dispersal studies should consider that embryo-damaged seeds, at least in highly nutritious nuts, can significantly contribute to plant regeneration.

We conclude that tolerance to embryo damage is an important reproductive strategy that enables recruitment in oaks. Large and nutritious seeds such as acorns may be an adaptation to avoid full predation of the seed, enabling plants to regrow and produce seedlings even after embryonic damage. Embryo size could play a crucial role in the ecological and evolutionary response of acorns to sophisticated animal behavioral strategies. Combined studies on seed traits, animal behavior and plant recruitment are warranted, and will help increase our understanding of the ecology and evolution of seed-animal interactions.

Author contribution

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ppees.2017.12.001.

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