

SEED TOLERANCE TO PREDATION: EVIDENCE FROM THE TOXIC SEEDS OF THE BUCKEYE TREE (*AESCULUS CALIFORNICA*; SAPINDACEAE)¹

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Tolerance, the capacity of plants to withstand attack by animals, as opposed to resistance, has been poorly examined in the context of seed predation. We investigated the role that the seed mass of the large-seeded endemic tree *Aesculus californica* plays as a tolerance trait to rodent attack by comparing, under greenhouse conditions, patterns of germination, and subsequent seedling growth, of seeds with a wide range of natural damage. Germination percentage was reduced by 50% and time to germination by 64% in attacked compared to intact seeds, and germination probability was negatively correlated with damage. Seedlings that emerged from intact seeds were taller and bore more leaves than those from damaged seeds. This species' large seed mass favors tolerance to damage because heavily damaged seeds are able to germinate and produce seedlings. This finding is significant given that seeds of this species are known to contain chemical compounds toxic to vertebrates, a resistance trait. We posit that this combination of tolerance and resistance traits might be a particularly effective antipredation strategy when seeds are exposed to a variety of vertebrate predators.

Key words: *Aesculus californicus*; California buckeye tree; Jasper Ridge; large-seeded plant; resistance traits; rodent seed predation; Sapindaceae; tolerance traits.

The greater nutritional value (e.g., in terms of nitrogen content) of seeds, in comparison to other plant structures, makes them a highly profitable resource for the vertebrates and invertebrates that prey upon them (Janzen, 1971). As a consequence, intense seed predation (frequently greater than 50% and sometimes affecting the individual's entire seed crop) occurs over a wide range of plant species and habitats (Crawley, 1992; Hulme, 1998). Seed predation has the capacity to strongly impact plant population dynamics by affecting plant regeneration, colonization ability, and spatial distribution (Louda, 1982; Hulme, 1997; Picó and Retana, 2000). Ultimately, seed predators have the potential to act as agents of natural selection that influence seed traits (Hulme and Benkman, 2002). Accordingly, plants deploy a variety of mechanisms to lessen the impact of predation on a plant's seed crop or on an individual seed.

Some plants prevent or reduce predation via traits in their fruits and seeds that can function as predator deterrents. Examples of such traits include thick, spiny fruits, hard seed coats, or defensive, toxic chemical compounds in the seed's endosperm (Janzen, 1969; Rosenthal and Bell, 1979; Grubb et al., 1998). Defense mechanisms involving any trait that reduces consumption of plant tissue and/or negatively affects herbivore performance are referred to as resistance traits (Strauss and Agrawal, 1999). However, given the abundance, diversity, and

efficiency of predators, seeds are unlikely to be able to prevent or deter attack by deploying only one or a few defensive traits.

An alternative set of antipredation mechanisms involves traits that allow seeds to tolerate damage from predators (Vallejo-Marín et al., 2006). Plant tolerance traits have been studied much less than resistance traits, particularly in seed–predator interactions (Rosenthal and Kotanen, 1994; Xiao et al., 2007). However, growing evidence suggests that tolerance traits in seeds might play an important role in the likelihood of seeds becoming seedlings in the face of predation (Mack, 1998; Dalling and Harms, 1999; Vallejo-Marín et al., 2006; Xiao et al., 2007). Tolerance traits, such as the capacity to germinate after some degree of damage (i.e., mass loss) or the ability to resprout after extensive damage to the young seedling's stem, have been shown for seeds with relatively large reserve mass, either in absolute terms or relative to the size of its predators. In these species, an oversized package of reserves can be a proximate trait that reduces the impacts of predation and has been assumed to be, at least in part, an adaptive response to intense seed attack (Dalling and Harms, 1999).

Tolerance has been studied by comparing the patterns of germination of intact seeds (and the growth of the seedlings they produce) against the response of seeds naturally infested by insects or of seeds in which contrasting levels of damage have been experimentally simulated by removing a portion of endosperm (Dalling et al., 1997; Harms and Dalling, 1997; Dalling and Harms, 1999; Fukumoto and Kajimura, 2000; Branco et al., 2002; Edwards and Gadek, 2002; Leiva and Fernández-Alés, 2005; Vallejo-Marín et al., 2006). However, with very few exceptions, seed tolerance to damage caused by vertebrates has not been evaluated under naturally varying levels of damage (but see Steele et al., 1993), despite the fact that vertebrates (e.g., rodents) cause major losses to individual seeds or plant seed crops (Crawley, 1992; Hulme, 1998). Evidence of seed tolerance comes from studies carried out with a small subset of species, predominantly from tropical forests (e.g., Janzen, 1976; Harms and Dalling, 1997; Dalling and Harms, 1999; Vallejo-Marín et al., 2006). The scant information from temperate

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zones comes largely from studies performed with species of the genus *Quercus* (Steele et al., 1993; Meiners and Handel, 2000; Fukumoto and Kajimura, 2000; Branco et al., 2002; Xiao et al., 2007). Therefore, studies evaluating mechanisms of seed resistance/tolerance in other temperate forest plants can further our understanding of the relative importance of these strategies among the suite of mechanisms plants deploy to lessen the effects of seed predators. Likewise, a more realistic understanding of the degree of seed tolerance/resistance may be gained by using natural levels of damage (as opposed to artificial damage) caused by predators, particularly rodents, whose impact has been rarely assessed in this context.

In this study, we experimentally examined the tolerance that seeds of the endemic buckeye tree, *Aesculus californica* (Spach) Nutt., may have to damage caused by rodents in a Mediterranean ecosystem in California. This species is particularly interesting because its seeds contain compounds that are toxic to a variety of animals, including vertebrates (Mooney and Bartholomew, 1974; Kubo and Ying, 1992), and at the same time, it produces very large seeds (with large reserve mass), which can be attractive to vertebrate predators. Therefore, this species seems to combine traits that can potentially operate as tolerance (large mass) and resistance (toxic metabolites) mechanisms. The way tolerance, resistance, and other traits interact to reduce the impact of predators on plants is poorly understood (Xiao et al., 2007).

We assess the nature of antipredation mechanisms by specifically examining the impact that variation in natural levels of seed damage has on (1) seed germination, (2) seedling sprouting, and (3) seedling growth of the buckeye tree under controlled experimental conditions. We hypothesized: (1) If resistance is the primary mechanism that *A. californica* seeds deploy to deal with the attack of vertebrate predators (as the reported presence of defensive compounds suggests; discussed later) seeds would have no damage or very low levels of damage in the field; moreover, if damage were present, germination, sprouting, and seedling growth should be negatively and strongly impacted. In contrast, (2) if *A. californica* seeds are using tolerance as a mechanism to deal with predation, intact seeds should be rare, while relatively high damage should be common and even heavily damaged seeds should still be able to germinate and produce seedlings that perform well. Finally, (3) if we find that intact seeds as well as seeds with a wide range in levels of damage in the field, and even heavily damaged seeds, are able to germinate, sprout, and produce seedlings, then the toxic seeds of *A. californica* might be using a combination of resistance and tolerance traits to deal with the attack by predators.

MATERIALS AND METHODS

Study species—*Aesculus californica* is a large shrub or tree (4–12 m) in the family Sapindaceae. Its distribution is restricted to the coastal and Sierran foothill regions of California with mediterranean-type climate, although some populations have been reported in Oregon (Mooney and Bartholomew, 1974; Hickman, 1993; Callahan, 2005). *Aesculus californica* trees are associated with mesic habitats such as those found near streams. In our study site, the Jasper Ridge Biological Preserve of Stanford University, *A. californica* occurs predominantly in mesic microenvironments, but it can also be found in dryer microenvironments. It is one of the most common trees in our study site, with the second highest value of importance, following oaks (*Quercus* spp.) (R. Dirzo, unpublished data). The leaves of this tree abscise and fall during the middle of the summer drought, before fruit development from late summer to fall/early winter (see Mooney and Bartholomew, 1974). Fruits of *A. californica* are spherical or slightly three-lobed, leathery capsules, borne at the tip of panicle-

like inflorescences. Fruits generally enclose one glossy brown seed 2–5 cm long, with a fresh mass of about 50 g (Hickman, 1993; Mooney and Bartholomew, 1974). All parts of the plant are considered toxic to humans and livestock because of the presence of glycosidal compounds (Kubo and Ying, 1992; Callahan, 2005). Flowers are poisonous to honey bees, but native insects such as the checkerspot (*Euphydryas chalcedona*) and the pale swallowtail (*Papilio eurymedon*) butterflies consume its nectar with no apparent deleterious effects (Murphy et al., 1984; Hickman, 1993; Callahan, 2005). Seeds of this species were used by Native Americans to make flour after removing the toxic compounds in a lengthy leaching process, and preparations from seeds or bark were used to treat several diseases and even to facilitate fish capture by forcing them to swim toward the water surface (Mooney and Bartholomew, 1974; Kubo and Ying, 1992; Anderson and Roderick, 2006).

Seed collection—In December 2006, we collected 137 seeds: of these 89 were intact, and 48 were naturally attacked. To prevent a significant removal of resources from the ground at the Jasper Ridge Biological Preserve, we were restricted to collecting nuts along trails running through woodland areas. Although our sample included seeds from several maternal trees, our collection protocol, coupled with the limited sample size, prevented us from analyzing our results in terms of genetic family effects. Our results represent, therefore, a random collection of several genetic families.

Collected seeds were weighed and measured (length). Rodent attack is readily recognizable by incisor marks (Fig. 1), and is caused mainly by tree squirrels (*Sciurus* sp.) (E. Mendoza, personal observation). Percentage of seed mass lost to rodent consumption was estimated visually by comparison with intact seeds of similar size. In a greenhouse at 25°C at Stanford University, each seed was placed on the surface of soil in pots (11.3 L) and watered daily. Seeds were monitored for germination, sprouting, and, after establishment, for leaf production and growth in height. We recorded the time to germination and the percentage of germination over time for intact and damaged seeds (regardless of the magnitude of damage). We also assessed the probability of germination as a function of the degree of seed damage. For these analyses, a seed was defined as germinated when the root was clearly visible. We also quantified the time until sprouting (i.e., the number of days it took for germinated seeds to produce a main stem with its first leaf).

We compared the growth of seedlings that emerged from intact and attacked seeds in terms of (1) the temporal pattern of leaf production measured since sprouting and (2) the increment in height growth, measured as the distance from the soil to the insertion point of the newest, fully developed leaf at a date that roughly corresponded to day 40 since germination.

Statistical analysis—We evaluated the impact of damage on seed germination with a *G*-test of independence (using Williams' correction; Sokal and Rohlf, 2003). The same analysis was used to examine differences in the number of seeds that sprouted after germination. We compared time to germination and to sprouting (once germination occurred) between intact and attacked seeds with a Wilcoxon two-sample test and its associated *U*-statistic (with correction for tied ranks and sample sizes >20; Sokal and Rohlf, 2003). The impact of degree of attack on the probability of germination was evaluated with a logistic regression analysis, using percentage of mass lost to predation as the explanatory variable, and whether the seed germinated (1) or not (0) as the response variable. Analyses were carried out using the program JMP version 4.0.2 (SAS Institute, Cary, North Carolina, USA).

Using the *lme* function of the R programming language (R Development Core Team, 2008), we fitted a mixed-effects model to analyze leaf production accounting for correlation of measurements within individuals (Crawley, 2002). The fixed effect was seed condition (intact, attacked), the random effect was time nested within individual seeds, and the response variable was number of leaves per plant. The variables time and number of leaves were square-root transformed to meet model assumptions of continuity and linearity. Residuals of the model were inspected for any trend in a plot of fitted values vs. standardized residuals. We compared growth rates (height divided by the number of days since sprouting) between seedlings from intact and attacked seeds with a Wilcoxon two-sample test in the same way we compared time required to germinate and sprout (Sokal and Rohlf, 2003).

We examined the relationship between mass of intact seeds and number of leaves produced at three different times over the course of the experiment with linear regression analysis using JMP version 4.0.2. Seed mass was the explanatory variable, and number of leaves was the response variable. We combined seedling size data for the periods 6–8, 14–16, and 34–36 d. The number of leaves was Box-Cox transformed to yield a continuous response variable and meet assumptions of homoscedasticity and normality of residuals.

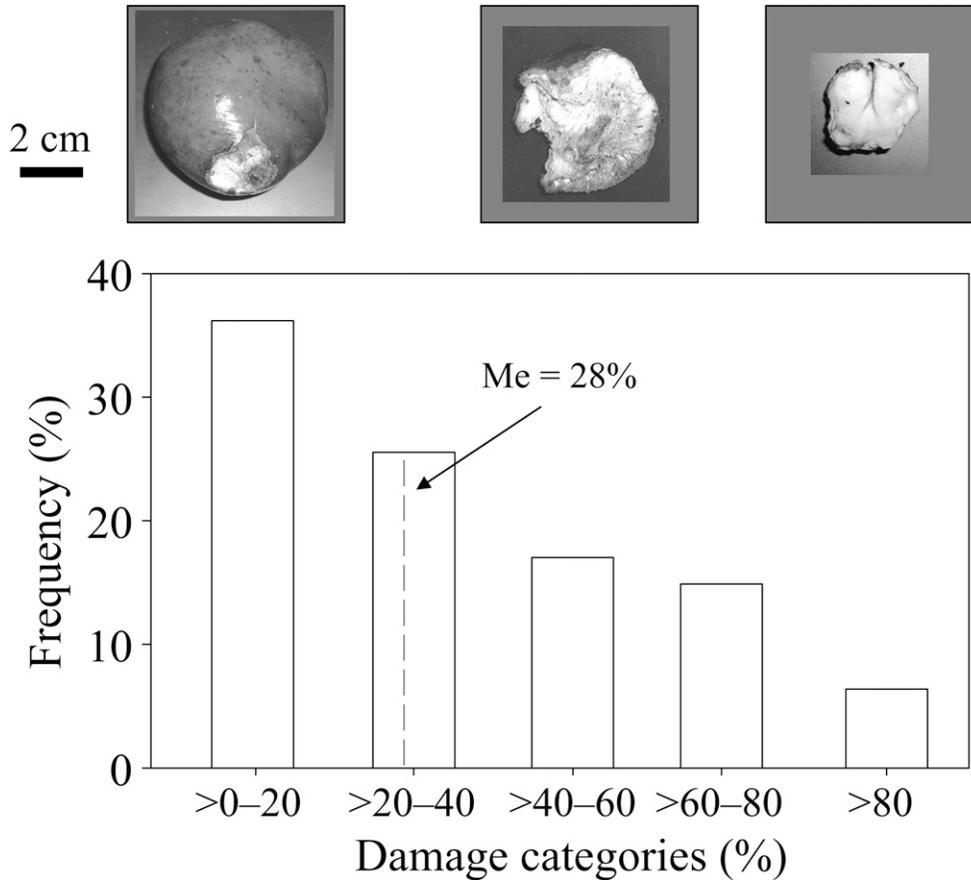


Fig. 1. Damage to attacked seeds of *Aesculus californica* collected at the Jasper Ridge Preserve, California. Top, left to right, photographs of damage categories: >0-20, >40-60, and >80%. Bottom, frequency of seeds in categories of damage; broken line shows median (Me) damage.

RESULTS

Seed characterization—Mean mass (± 1 SE) of intact seeds ($N = 137$) was 58.1 ± 1.9 g (Fig. 1), while seeds with evidence of vertebrate attack ($N = 48$) had a mean mass of 45.7 ± 3.2 g. Although the typical estimated damage (mass loss) among attacked seeds was relatively low (median = 28%), the range was considerable, extending from 3% to a striking 90% (Fig. 1).

Patterns of germination—Seed predation had a positive impact on the speed of germination. The median time for germination of attacked seeds was almost one-third the median time for germination of intact seeds ($U_s = 1644.5$, $P < 0.001$; Fig. 2A). In contrast, the germination potential of attacked seeds was reduced nearly by half: only 25 of 47 (53.2%) germinated, while among intact seeds, 79 of 81 (97.5%) did so (Fig. 2A). This contrast in germination success was highly significant ($G = 39.18$, $df = 1$, $P < 0.001$). Moreover, while 91% of the intact seeds that germinated were able to sprout, only 72% of the attacked and germinated seeds sprouted ($G = 17.57$, $df = 1$, $P < 0.001$). In contrast, there was no difference in the median time to sprout between attacked and intact seeds that germinated (13 and 12 d, respectively; $U_s = 787.5$, $t = 1.52$, $P = 0.13$; data not shown). Seeds that did not germinate had rotted by the end of the study.

Although there was considerable variation in damage levels among seeds that germinated, as well as among seeds that were

unable to germinate (Fig. 2B), there was a significant relationship between percentage of seed mass loss and germination probability. The logistic regression shows that germination probability gradually decreased as the percentage of mass consumed increased ($P = 0.0045$, $N = 51$; Fig. 2B).

Seedling growth—We found that the rates of growth, measured as height increment, were higher for seedlings that emerged from intact seeds (median = 1.21 cm/day) than for seedlings emerged from attacked seeds (median = 0.87 cm/day; $U_s = 853.5$, $P = 0.012$) (Fig. 3A). Likewise, we found a significant effect of seed condition (attacked or intact, $P = 0.027$), time ($P < 0.001$), and their interaction ($P < 0.001$) on seedling growth (leaf production). This result reflects that, with time, the number of leaves per plant increasingly differed between seedlings emerging from the two types of seed (Fig. 3B). At the end of the experiment, seedlings that emerged from intact seeds had four more leaves, on average, than seedlings from attacked seeds.

Regression analyses to examine the relationship between seed mass of intact seeds and size (number of leaves) of seedlings at three different times after germination (Fig. 3C) showed that size of seedlings was not affected by seed mass at day 7 of growth, because the slope of the relationship was statistically indistinguishable from zero ($R^2 = 0.007$, $P = 0.4967$, $N = 69$, Fig. 3C). In contrast, we found an increasingly significant positive relationship between seed mass and seedling size with time

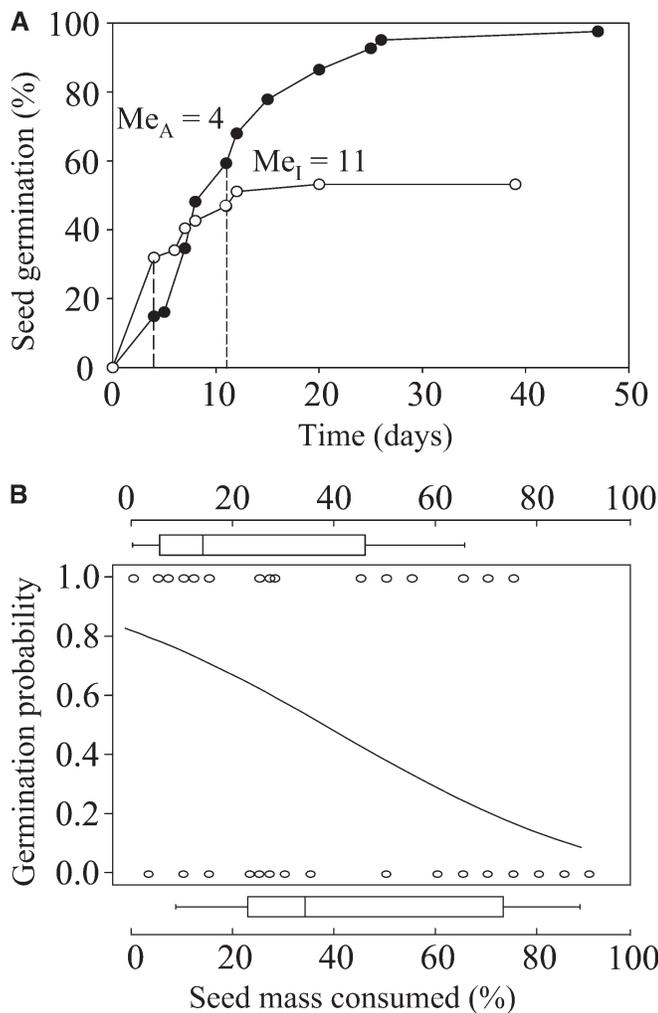


Fig. 2. Impact of damage to seeds of *Aesculus californica* on germination. (A) Germination of intact (black dots) and attacked (white dots) seeds over time in the greenhouse. Me_I = median number of days to germination for intact seeds; Me_A = median number of days to germination for attacked seeds. (B) Relationship between seed damage and the probability of germination. Central panel shows the relationship as described by logistic regression analysis. Upper and lower box-plots show the distribution of damage for seeds that germinated and of those that failed to germinate, respectively; lines of boxes indicate from left to right, 25th percentile, median, and 75th percentile; whiskers indicate 90th and 10th percentiles.

($R^2 = 0.117$, $P = 0.0132$, $N = 52$, at day 15; and $R^2 = 0.120$, $P = 0.0110$, $N = 53$, at day 35).

DISCUSSION

Effects of rodent attack on germination and sprouting patterns—Attack by rodents had a clear impact on the performance of seeds and seedlings of *A. californica*. On the one hand, attack reduced germination success, sprouting, and growth of seedlings. On the other hand, attack by rodents significantly reduced the time to germination. Among intact seeds, germination success (>97%), as well as subsequent sprouting (91%), were markedly high. Intact seeds of the closely related species *A. octandra* are also reported to have very high levels of germination success in

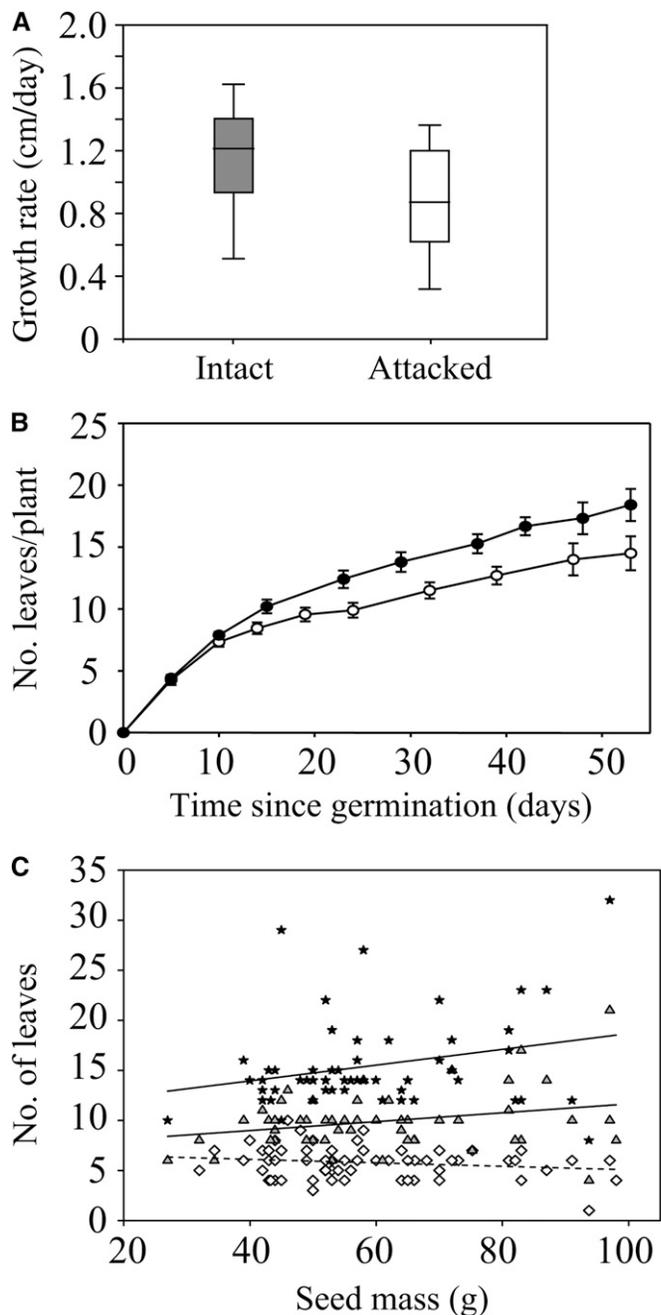


Fig. 3. Relationship between damage and seedling growth. (A) Growth rates of seedlings that emerged from intact and attacked seeds, measured from sprouting to approximately day 40 of development. Bottom, central, and upper lines in boxes correspond respectively to the 25th percentile, median, and 75th percentile. Whiskers indicate 10th and 90th percentiles. (B) Number of leaves per plant, over time, of seedlings that emerged from intact (black dots) and attacked (white dots) seeds. Each data point corresponds to the mean of between 18 and 34 intact seeds and between 8 and 14 attacked seeds. Error bars represent ± 1 SE. (C) Relationship between the weight of intact seeds and the size (number of leaves) of their corresponding seedlings at three different times during the experiment. Diamonds correspond to day 7 ($R^2 = 0.007$, $P = 0.49$), triangles to day 15 ($R^2 = 0.117$, $P = 0.01$), and stars to day 35 ($R^2 = 0.120$, $P = 0.01$).

the field (Levy, 1984). In comparison, only 53% of the attacked seeds germinated, and 72% of these germinated seeds sprouted. Therefore, in spite of the negative effects of attack on germination and sprouting, a relatively large proportion (38%) of the initial number of attacked seeds produced seedlings, compatible with the hypothesis that seeds of *A. californica* are able to tolerate attack by vertebrate predators probably because of their large mass. However, there was a large amount of variation in the percentage of mass loss among attacked seeds that successfully germinated and also among those that failed to germinate. These results suggest that, in contrast to what happens in some species of oak, in which vertebrate consumption of the endosperm containing the embryo is discouraged by the presence of tannins (Steele et al., 1993), in the case of *A. californica*, rodents may on occasion kill seeds (i.e., impede their germination).

Germination success of partially attacked seeds is usually reduced compared to the germination of intact seeds (Janzen, 1976; Cipollini and Stiles, 1991; Koptur, 1998; Fukumoto and Kajimura, 2000; Branco et al., 2002; Leiva and Fernández-Alés, 2005; Vallejo-Marín et al., 2006; Xiao et al., 2007), but damage has also been shown not to have a significant effect on (Dalling et al., 1997; Vallejo-Marín et al., 2006) or to even increase (Karban and Lowenberg, 1992; Steele et al., 1993) germination. This variation in the response of seeds of different species to damage seems to be a function of the level of attack they receive but also of their level of tolerance to damage (Janzen, 1976; Koptur, 1998; Branco et al., 2002). For example, in some species seed damage affecting just 10% of endosperm mass leads to a complete failure to germinate (Vallejo-Marín et al., 2006), while other species such as *Prioria copaifera* are able to fully compensate, because germination of seeds in which mass losses are as high as 60% is similar to that of intact seeds (Dalling et al., 1997). Results of this study indicate that *A. californica* falls in the category of species with a great capacity to tolerate seed damage because seeds with up to 75% of their mass consumed are able to germinate.

Seeds of *A. californica* attacked by rodents germinated faster than intact seeds, a response similar to that observed in acorns of *Quercus suber* and *Q. mongolica*, seeds of the shrubs *Sesbania drummondii* and *Gossypium* spp. infested by insects, and some tropical rain forest species in which damage was artificially inflicted (Karban and Lowenberg, 1992; Branco et al., 2002; Ceballos et al., 2002; Vallejo-Marín et al., 2006; Yi and Zhang, 2008). Observed reductions in the time to germination as a consequence of insect infestation range from 17% in *Q. mongolica* to 42% (high seed damage) in *Q. suber* (Branco et al., 2002; Yi and Zhang, 2008). Therefore, it seems that within a certain range of variation, increased seed mass loss caused by animal attack may lead to faster germination. Mechanisms that may be involved in this effect include a release in the physical constraint that the endosperm imposes on radical elongation (Branco et al., 2002), an increase in the activity of enzymes such as amylase that promote germination (Yi and Zhang, 2008), and scarification of the seed coat (Karban and Lowenberg, 1992; Koptur, 1998). Yet, regardless of the specific underlying mechanism, the question remains as to what consequences this change in germination time has on seedling establishment. Increased speed of germination in attacked seeds might be adaptive by allowing early seedling emergence before intraspecific competition intensifies, thus favoring chances of establishment (Karban and Lowenberg, 1992). However, the consequences of increased speed of germination due to predation are context-dependent (see Karban and Lowenberg 1992).

In the case of *A. californica*, we posit that the consequences of predation would depend on the timing of attack. For example, because seed release of *A. californica* usually starts late in the dry season, early attack leading to early germination might expose seeds to desiccation. On the contrary, if attack occurs after the onset of the rainy season, early germination might allow seedlings emerged from attacked seeds to have an earlier start to take full advantage of the rainy season to grow. Elucidation of the net effect of accelerated germination for the performance of *A. californica* seedlings warrants further work.

The possible incidence of multiple events of attack on a single seed might reduce the effectiveness of tolerance as a way to deal with the effects of predation. However, observations from an ongoing experiment in the field suggest that the incidence of repeated events of attack by vertebrates is low (E. Mendoza, unpublished data).

Because predators displace seeds upon attack (E. Mendoza, unpublished data), the possibility arises of predation also operating as a means of secondary dispersal. An important role of secondary dispersal by rodents for seedling establishment has been suggested in a closely related species, *A. turbinata* (Hoshizaki et al., 1997). It is known from other temperate, large-seeded species that rodents such as squirrels scatter-hoard seeds for later consumption and, in doing so, protect seeds from attack by other vertebrate and invertebrate predators (Vander Wall, 1990). Moreover, when cached or scatter-hoarded seeds are not recovered, germination and seedling establishment may be favored. Likewise, it has been proposed that during the time seeds remain cached and during germination, the concentration of chemical compounds decreases, making seeds more accessible to consumers (Smallwood et al., 2001). Therefore, the influence seed mass might have in plant performance does not conclude at the germination and early seedling growth stages, but includes the potential for seedlings to resprout or compensate for foliage loss caused by folivores (Dalling and Harms, 1999). Large seeds may thus be an adaptation to enable plants to resprout once dispersed by rodents, and not only an adaptation to grow faster. How patterns of seed dispersal and attack relate to seed mass variation and seedling establishment is an important next step to examine for *A. californica* at our study site.

Effects of rodent attack on seedling performance—Attack affected not only germination but also seedling growth. Seedlings that emerged from attacked seeds were shorter and had fewer leaves than did seedlings that were the same age but emerged from intact seeds. These results indicate that seedlings that emerged from attacked seeds were able to only partially compensate for the effect of seed damage.

The contrast in size between seedlings that emerged from intact and attacked seeds and the fact that a relationship between seed mass and seedling size became increasingly evident with time suggest that seed reserves were used not only to subsidize the initial growth of seedlings but also to support their longer-term growth. These findings are consistent with the fact that seeds attached to the fully developed seedlings still had a noticeable amount of endosperm, which was gradually exhausted as seedlings grew, and with the report that seedling survival and growth are affected by experimental cotyledon excision weeks after germination in large-seeded species such as *Q. rugosa* (Bonfil, 1998) and *Gustavia superba* (Dalling and Harms, 1999; Kitajima, 2003).

Larger size can help seedlings escape from the many hazards they face during their growth, including herbivory, competition,

or physical damage. In addition, seed size correlates with the capacity to repetitively resprout after severe shoot damage (Dalling et al., 1997; Dalling and Harms, 1999; Green and Juniper, 2004). Therefore, while the large reserves in the seeds of *A. californica* seem to be a proximate trait providing tolerance to the damage caused by rodent attack, it remains to be tested what the impact of seed attack is on seedling growth and plant establishment in the field, as compared to the performance of seedlings emerged from intact seeds. Moreover, the demographic consequences of predation leading to seed or seedling death depend on the critical number of plants that can be recruited into the population (Louda, 1982). If tolerance to damage allows the buckeye tree to escape partial damage by predators, resulting in later plant recruitment, large seed mass could be seen as an adaptive trait for the species.

The seeds of *A. californica* seem to combine traits related to resistance (presence of chemical compounds) as well as tolerance to predation. This combination of antipredation traits can be a profitable strategy in ecological settings where mortality hazards vary in time and space and seeds are exposed to a variety of predators (Xiao et al., 2007). Moreover, because contacts (probability of encountering) between seeds and predators are more likely to result in plant death than contacts between established plants and herbivores, the presence of a combination of defense mechanisms might be particularly favored in seeds as a strategy to reduce the impact of a coterie of omnipresent predators (in our case, potentially, deer, mice, squirrels, and invertebrates) and increase plant regeneration and population growth.

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