Title: Pre-dispersal predation effect on seed packaging strategies and seed viability.

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Abstract

Understanding intraspecific seed packaging (i.e. seed size/number strategy) variation across different environments may allow a better knowledge of the ecological forces that drive seed evolution in plants. Particularly, pre-dispersal seed predation may influence seed packaging strategies triggering the reduction of resources allocated to undamaged seeds within preyed fruits. Assessing plant reaction to pre-dispersal seed predation is crucial for a better understanding of predation effects; however, this sort of plant response to arthropod attacks remains unexplored. We assessed the effect of cone predation on the size and viability of undamaged seeds on populations of *Juniperus thurifera* with contrasting seed packaging strategies, single-large-seeded cones in North African populations and multi-small-seeded cones in Southern Europe. Single-large-seeded African cones suffered from lower cone predation incidence compared to multi-small-seeded European cones. Seeds from non-preyed cones were larger and had a higher germination success than uneaten seeds from preyed cones, but only in populations with multi-seeded cones and in cones attacked by *Trisetacus* sp., suggesting a differential plastic response to predation. Pre-dispersal seed predation might be a strong selective pressure in European populations with high cone predation rates. This process might maintain multi-small-seeded cones and empty seeds as a strategy to save some seeds from predation. Conversely, pre-dispersal predation might not have a strong effect in African populations, which displayed single-large-seeded cones with seed germination and filling rates higher than in European populations. Our results indicate that differences in pre-dispersal seed predators and predation levels may affect both selection on and intraspecific variation in seed packaging.

Keywords: *Juniperus thurifera*; seed number; seed size; deceptive fruits

Introduction

Seed packaging, i.e. allocation of total seed content into a single large seed or several small ones, constitutes one of the central trade-offs of plant ecology and evolution because, given finite resources, an increase in seed size comes at expense of producing fewer seeds (Leishman 2001). In general, large-seeded species produce seedlings with higher establishment probabilities and growth rates, while small-seeded species produce a more abundant offspring (Moles and Westoby 2004). Both, direct and accessory costs of producing a seed increase with seed size (Lord and Westoby 2006). Particularly, in fleshy fruits, the resources allocated to pulp, and consequently the parental cost of a seed, usually decrease as seed number increases (Obeso 2004), leading to an optimal size/number combination. However, seed packaging presents considerable intraspecific variability instead of being constant within a species (Eriksson 1999; Mehlman 1993; Willson et al. 1990), and this may represent an opportunity for selection.

Abiotic and biotic factors might act as selective agents driving seed trait variability (Harper et al. 1970). Climatic conditions associated with geographical patterns influence physiological processes and may explain the intraspecific variability found at large scale. In general, a reduction in resources availability in harsher climatic conditions, such as high-elevation, high-latitude or high aridity, may lead to a decrease in either seed viability or size (e.g. García et al., 2000; Guo et al., 2010; Moles et al., 2004; Murray et al., 2004). Dispersers can also act as selective agents; fruit size can be constrained by gape width, adjusting also seed size (Alcantara and Rey 2003; Jordano 1995; Levey 1987). Lighter seeds tend to be longer retained in bird guts and, thus, dispersal efficiency increases (Obeso et al. 2011). Post-dispersal predation may also exert a selective pressure towards smaller seeds that can be buried easily and escape from disturbances and predators (Gómez 2004; Peco et al. 2003). Finally, pre-dispersal predation may be a crucial selective pressure since it occurs when seeds are developing (Janzen 1971). Pre-dispersal predation can influence plant reproductive traits, such as crop size, morphology and size of reproductive structures, flowering phenology, fruit colour, fruit and seed size and mast seeding (Kolb et al. 2007, and references therein). Particularly, pre-dispersal predators may exert selective pressure towards fruits with smaller seeds or low number of seeds. They preferentially attack larger fruits that contain either more seeds with a higher seed-to-pulp ratio which facilitates the oviposition (Knight 1987) or larger seeds which produce larger predator off-spring (Moegenburg 1996). Therefore pre-dispersal seed predation might influence the variability of seed packaging and function as an effective selective pressure (Geritz 1998).

Models assume that small seed sizes may reduce the resources lost per seed preyed (Sakai and Harada 2007). In addition, multi-seeded fruits could avoid the loss of the total seed content reducing seed predation rate, i.e. the number of damaged seeds per total number of seeds in each fruit. Satiating predators with a surplus of flowers, or immature fruits, or producing seedless fruits can be strategies to reduce the negative consequences of pre-dispersal predation (Stephenson 1981; Stowe et al. 2000; Traveset 1993). Likewise plants may maintain non-viable inbred seeds making them available to predators to mitigate the impact on viable outcrossing seeds (Ghazoul and Satake 2009).

Plants may plastically respond to pre-dispersal seed predators through detection of the infection and selective abscission of the fruit causing a negative effect on the insect (Bonal and Muñoz 2008; Fernandes and Whitham 1989; Verdú and García-Fayos 1998). In multi-seeded fruits, seed abortion is likely to occur when there is a second intact seed in the fruit to prevent co-infestation (Meyer et al. 2014). Plants might also reduce the resources allocated to attacked fruits leading to a size decrease of the remaining non-preyed co-occurring seeds, which could have a detrimental effect on seed viability (Fernandes and Whitham 1989; Verdú and García-Fayos 1998). However, if plants do not respond to the attack by resource reduction, non-preyed co-occurring seeds would be larger by the elimination of a competing seed within a cone, suggesting a lack of active response from the plant. Understanding how plants react to insect attack is fundamental since it will change the final cost of predation and the expected optimum strategy of seed packaging (Sakai and Harada 2007), although the latter has been poorly explored. In addition, seed predators may differ in their feeding behaviour imposing different costs to plants. In multi-seeded fruits, seed predators may eat either most of the seeds they can (e.g. Herrera 1984) or only one seed within a fruit leaving some uneaten viable seeds per preyed fruit (e.g. Bradford and Smith 1977). This could imply different costs for predated plants that might trigger different plant responses to the attack.

The dioecious conifer *Juniperus thurifera* (Spanish juniper) displays female berry-like cones that vary in seed size and number per cone between both sides of the Strait of Gibraltar, presenting two different seed packaging strategies: European multi-small-seeded cones and African single-large-seeded cones (Boratyński et al. 2013). *Juniperus thurifera* cones are commonly preyed by pre-dispersal seed predators, mainly wasps and mites, which oviposit before seed ripening and consume the resources allocated to seeds (Llorente and Alonso 2006; Mezquida and Olano 2013). These predators seem to display different feeding behaviour (García 1998; Rouault et al. 2004; El Alaoui and Roques 2006; Llorente and Alonso 2006) that may exert different effects on seed packaging. Wasps parasitize individual seeds per cone, whereas mites use seeds as growth chamber feeding on as many seeds as they can. Also, *J. thurifera* seed abortion and pre-dispersal predators may not be homogeneously distributed across its European populations (Montesinos et al. 2010). The combination of different seed packaging strategies and different pre-dispersal predation pressures makes this species an exceptional model to investigate the plastic response of plant populations to seed predation. In this paper we assessed how pre-dispersal seed predation influences seed traits on the Mediterranean endemic tree *J. thurifera* by (i) exploring pre-dispersal seed predation differences (i.e. predation rates, predator identity and preferences) and plastic responses to predation in populations with contrasting seed packaging strategies, and (ii) assessing whether seed viability of the co-occurring seeds within a preyed cone is reduced under high predation. The evolutionary consequences of pre-dispersal predation on seed-packaging are also discussed.

Materials and Methods

Study species

Juniperus thurifera L. (Cupressaceae) is a dioecious conifer tree up to 20 m height with a conical to round or irregular crown. It is a long-lived (up to 600 years) tree endemic to continental areas of the western Mediterranean Basin at altitudes ranging from 200 to 3400 m (DeSoto et al. 2014; Gauquelin et al. 1999). It has been suggested as a dominant species during the cold stages of the Pleistocene (Carrión et al. 2003) and currently, its range is over a disjunct distribution in South Western Europe and North Africa. The most abundant populations are in Spain (200,000 ha) and the Medium and High Atlas Mountains in Morocco (30,000 ha; Gauquelin et al. 1999, and references therein).

Reproduction starts in early winter when male flower cones (3-4 mm long) shed their pollen. Female flower cones are wind-pollinated, late-fertilised in April (5-6 months after pollination) and ripen in about 20 months. The ripe female cones (hereafter cones) are dark purple berry-like with a whitish waxy bloom, 7-11 mm in diameter and usually contain 1-5 seeds rarely more than 6 and up to 10 (Amaral-Franco et al. 1986; Boratyński et al. 2013). Seed packaging strategies differ between both continents: (i) single-large-seeded cones from African populations with fewer, larger and heavier seeds (averages of 1.3 seeds, 4.8 mm length and 62 mg/seed) and (ii) multi-small-seeded cones from European populations with smaller and lighter seeds (averages of 3.6 seeds, 3.8 mm length and 40 mg/seed; Boratyński et al. 2013; Online Resource, Fig. S1). Based on the number of seeds per cone for the closest relatives of *J. thurifera* (Farjon 2005; Adams 2008), the ancestor was likely a multi-seeded cone species (DeSoto et al. unpublished). Cone dispersers are birds, mainly thrushes (*Turdus* spp.), and mammals, mainly small carnivores and sheep (Escribano-Avila et al. 2012). Two types of pre-dispersal predators frequently eat *J. thurifera* seeds. Females of a chalcid wasp, *Megastigmus* sp. (Hymenoptera, Torymidae), oviposit usually one egg per seed inside eight-month immature cones in summer after fertilisation (Rouault et al. 2004). The resulting larva develops within the seed, pupates the next summer, and emerges as an adult through an exit hole in both seed and cone. *Megastigmus* sp. usually attacks only one seed per cone (García 1998). The other predator, the mite *Trisetacus* sp. (Acarina, Nallepellidae), can attack several times at different seed developmental stages, usually before fertilisation, and grows forming colonies and feeding on the seeds during 18 months. Usually it damages more than one seed within a cone and seeds become light brown and stick out of the cone (El Alaoui and Roques 2006; Llorente and Alonso 2006; Montesinos et al. 2010). It is very rare to find both predators in the same cone (Mezquida and Olano 2013).

Study sites and sampling procedure

We selected five populations, three in Spain: Luna (in the Cantabrian Range; 42°55'N, 5°51'W; 1,228m), Soria (41°47'N, 2°48'W; 1,150m) and Monegros Desert (here after Monegros; 41°40'N, 0°21'W; 534m) and two in Moroccan High Atlas: Azzaden Oussem (hereafter Oussem; 31°06'N, 7°57'W; 2,396m) and Tizi n' Techt (hereafter Techt; 31°10'N, 7°58'W; 1,994m). Luna and Soria were the moistest and coldest sampling sites, whereas Monegros and the High Atlas were the warmest and driest with water deficit almost all year round (see DeSoto et al. 2014 for a detailed description of the climate).

These populations represented the variability of seed packaging strategies of the species, African single-large-seeded cones in Morocco and European multi-small-seeded cones in Spain (Online Resource, Fig. S1). In each population ca. 1,500 mature cones (at least 150 cones from 10 randomly chosen female trees) were haphazardly collected all around the crowns to avoid orientation effects. Sampling was done in October 2010 in Morocco and May-June 2011 in Spain since seed predators usually emerge from the seeds (and cones) in late spring in Spain and in late summer in Morocco (El Alaoui and Roques 2006; Llorente and Alonso 2006).

Pre-dispersal seed predation

To measure the incidence of pre-dispersal seed predation, a total of 5,240 cones from the study populations (ca. 100 cones/tree) were dissected to evaluate the relationship between predation and seed packaging strategies (single vs. multi-seeded cones). Cones were embedded in sodium hypochlorite solution 1% for two days to clean the waxes and resins (García-González et al. 2009). Then, we opened the cones and classified them as non-preyed cones with non-preyed seeds or preyed cones with at least one preyed seed (Online Resource, Fig. S1). We counted preyed, non-preyed and non-developed or aborted seeds for each cone, and referred that as seed number (i.e. the total number of ovules found within a cone). We explored the pre-dispersal seed predation occurrence on each population using two different proxies. First, we estimated the seed predation rate as the number of preyed seeds in relation to the total number of seeds in those preyed cones. Second, we calculated the cone predation rate for each tree (percentage of preyed cones per tree) and classified them into *Megastimus*-preyed or by *Trisetacus*-preyed cones. When possible, we completed the dataset by checking predator attack in 100 cones more per tree (9,320 cones). Cones predated by *Megastimus* and by *Trisetacus* present different morphology and were easily distinguished during the process.

We evaluated the potential effect of seed predator on the size and viability of the remaining non-preyed seeds within a preyed cone (hereafter co-occurring seeds) comparing seed traits between *Megastimus*-preyed, *Trisetacus*-preyed and non-preyed cones. To do that we weighed the non-preyed seeds of 30 non-preyed and 30 preyed cones per tree. Then, we performed a germination test to estimate seed viability dependant to predation influence (see below).

Seed viability

Seed viability was assessed using a germination test for seeds collected in Luna, Soria, Monegros and Oussem. We selected seeds that did not have any morphological evidence of damage and did not float in water, therefore considered *a priori* viable seeds (García-González et al. 2009). We performed two germination experiments to test whether cone predation affected seed viability and to explore general trends in seed viability between populations and seed packaging strategies. In a first experiment, seed size was controlled, while in the second experiment we only tested the effect of cone predation. (1) In order to study the effect of seed size and seed predation on the germination of co-occurring seeds we designed a factorial experiment with three factors: population, seed size and cone predation. A total of 1,536 seeds were used in this experiment, 64 seeds per tree, from six different trees and four populations. For each tree four Petri dishes were filled with wet sand and 16 seeds which were chosen firstly by predation level (from preyed or non-preyed cones), and secondly by seed size (small and large) selecting the eight largest or smallest seeds for each predation level. (2) A larger experiment was set up to test the effect of seed predation on germination using the remaining seeds collected in the four populations. 180 seeds were sown in each tray filled with wet sand and 2-8 trays were used for each population, depending on the total number of seeds available (3,907 seeds in total). The origin, both tree and cone, of each seed was also recorded.

We followed the method described by García-González et al. (2009) to induce seed germination: seed scarification with sandpaper followed by water stratification with four cycles of moisture-drought, and a two-month cold stratification at 5ºC. Afterwards all Petri dishes and trays were maintained in controlled conditions (15°C day / 10ºC night temperature, light PAR 80μmol $m^{-2}s^{-1}$, 16h photoperiod, 85% humidity) from May to October 2012 and rotated weekly to avoid position effects within the growth chamber (Fitoclima D1200 PLH, Aralab Inc.).

After the germination experiments, we dissected ca. 200 non-germinated seeds (100 from preyed cones and from 100 non-preyed cones) per population of the first experiment to check for the occurrence of an embryo. We also dissected 220 seeds of six trees from the Techt population, not included in the germination experiment. Seeds that did not contain an embryo were considered as empty seeds (Online Resource, Fig. S1). Neither larvae nor adults of *Megastigmus* sp. or other predators in prolonged diapause were found inside any seed opened.

Statistical analysis

In order to determine whether population and predation exerted an effect on seed and cone traits we carried out generalized linear mixed models (GLMMs). GLMMs provide a flexible way to model traits that do not satisfy the assumptions of a standard linear modelling, allowing at the same time the distinction between fixed and random factors in the model. We performed two sorts of tests. First, we analysed whether seed predation rate or cone predation rate varied among populations. We also estimated the predator dominance among populations computing cone predation rates for each seed predator. Second, we evaluated whether seed packaging varied between preyed or non-preyed cones among populations and predators. Seed packaging evaluation considered both cone traits (number of seeds, i.e. total number of ovules produced within a cone) and seed traits (seed size and seed viability).

We analysed cone predation rate, seed predation rate and cone predation rate for each seed predator considering a binomial error distribution with a logit link and including population as a fixed factor (McCullagh and Nelder 1989). We explored the variation in seed and cone traits including both the population and predation type (*Megastimus*-preyed, *Trisetacus*-preyed and non-preyed cones) and their interaction as fixed factors. We analysed the number of seeds considering a Poisson error distribution with log link function. To analyse seed mass we assumed a Gaussian error distribution with an identity link. Germination rate and empty seeds occurrence were analysed considering a binary error distribution with a logit link. In all analyses, the tree was considered as a random factor, and only in seed mass analysis the cone nested within tree was included also as random factor. We did not included cones with only one seed in the analyses of predation effect on seed mass, germination and viability because the lack of co-occurring seeds. Since multi-seeded cones in African populations were less frequent and predator frequencies varied among populations, we conducted Type III tests and used Satterthwaite approximation to calculate the denominator degrees of freedom to overcome the problems due to unbalanced factorial designs (Quinn and Keough 2002). Differences between least-squares means were tested pairwise through multiple comparisons. We fitted GLMMs using the GLIMMIX procedure of SAS (SAS Statistical package 9.2).

Results

Pre-dispersal predation pressure and seed packaging

Cone predation rate (i.e. the proportion of preyed cones per tree) was significantly higher in the two European multi-seeded cone populations, Luna (78 %) and Soria (56 %), than in Monegros and the populations from Africa (26 %; Fig. 1a). However, a higher seed predation rate (i.e. proportion of preyed seeds in each cone) was detected in the African populations since they were mainly single-seeded cones. Multi-seeded European populations suffered a lower proportion of preyed seeds in each cone, Luna being the population with the highest seed predation rate among them (Fig. 1b). We observed differences in the dominance of seed predators among populations. Cone predation rate by the wasp *Megastigmus* was higher in Monegros and African populations, while predation rate by the mite *Trisetacus* was higher in Luna and Soria populations (Fig. 1c).

Predator preferences and differential effects of predator type on uneaten seeds

Predator preferences on seed-packaging traits depended on the type of predator (Table 1). *Megastigmus* showed preference for cones with more seeds (i.e. higher total number of ovules within a cone) in Monegros and Techt populations where this predator was more frequent, while *Trisetacus* showed no preference for cones with a particular seed number (Fig 2a; except in Oussem, which had the lowest predation rate by this species as it is shown in Fig. 1c). Differential effects of predation on seed traits depending on the population were observed since there was a significant interaction between predation and population (Table 1). *Megastigmus* did not lead to changes on the size of co-occurring uneaten seeds in preyed cones (Fig. 2b). By contrast, *Trisetacus* triggered changes in the size of co-occurring seeds in preyed cones, although this effect was divergent between European and African populations (Fig. 2b). European populations, which had higher predation levels of *Trisetacus* in general

(Fig. 1c), showed lighter seeds after *Trisetacus* predation. However, this was not found in African populations where co-occurring uneaten seeds of cones preyed by *Trisetacus* were heavier than seeds from non-preyed cones in Techt (Fig. 2b).

Seed viability

Seeds in the first experiment, controlling seed size and predation, showed a low germination success (overall average 5 %) because only 73 out of 1,536 seeds germinated. There were sharp differences between populations, with the majority of the germinated seeds belonging to the African population Oussem. Within this population, seed mass exerted a positive significant effect on germination ($\chi^2_1 = 22.52$, $P < 0.001$, n = 60; Online Resource, Fig. S2). In the second experiment, 190 out of 3,907 seeds (5 %) germinated. Due to the low germination rate of the European populations, we combined the whole dataset of the two germination experiments for the analysis. Mean germination rate per tree differed between populations, 16.5 % in Oussem (Africa), and 5.4 % in Monegros, 1 % Soria and 0.6 % in Luna (Europe, Fig. 3a). Predation did affect germination in the European populations reducing more than 75 % the viability of the co-occurring seeds within a preyed cone, whereas a non-significant effect of predation was found in Oussem (Table 2, Fig. 3a).

The main reason for the very low level of germination rate observed was that most of seeds were empty. Neither the visual inspection nor the floating test allowed detecting empty seeds before the experiment mainly due to the very thick coat of *J. thurifera* seeds (Online Resource, Fig. S1). The occurrence of empty seeds significantly differed among populations being lower in Oussem (76 %), Techt (83 %) and Monegros (87 %) than in Soria (95 %) and Luna (97 %, Fig 3b). In general, the proportion of empty seeds was not affected by predation being only significantly different between preyed and non-preyed cones in Soria (Table 2, Fig. 3b).

Discussion

Our findings support a plastic response of *J. thurifera* trees to pre-dispersal seed predation probably caused by changing resource allocation to preyed cones. Preyed cones contained smaller seeds in the European populations but larger seeds in the African populations. In addition, in the European populations seeds from non-preyed cones had a germination success three times higher than intact seeds from preyed cones. Empty seeds occurrence was higher in the European populations, indicating that production of a high proportion of empty seeds by this species could be a strategy to reduce seed loss due to pre-dispersal predation. We also observed that cones with more seeds are more likely to suffer from pre-dispersal seed predation in Africa and Monegros, suggesting predation preference on multi-seeded cones especially by *Megastigmus* wasps. Our results indicate that differences in pre-dispersal seed predators and predation levels may affect both selection on and intraspecific variation in seed packaging.

Plastic response to the effect of predation on seed packaging and viability

Predation might affect the development of the co-occurring undamaged seeds in preyed fruits as a result of the plant reaction to the attack (Bonal et al. 2007). It has been suggested that plants could allocate fewer resources to preyed fruits to reduce food availability for larvae and, consequently, co-occurring seeds may experience both a growth rate constraint and high abortion probability (Fernandes and Whitham 1989; Verdú and García-Fayos 1998). In support of these predictions, we found that in European populations, preyed cones contained smaller seeds compared to non-preyed cones, reducing their viability, measured as germination success. Conversely, in the African populations the effect seems the opposite. In Techt, co-occurring seeds displayed a larger size likely due to the elimination of a competing seed within preyed cones and in Oussem a reduction on seed germination was not found when a cone was preyed.

Our results support that differences in seed size between preyed and non-preyed cones are better explained by the plant response to the attack rather than by the preferential attack of predators. Seed size reduction on preyed cones was a response to *Trisetacus* mites in European populations, whereas this was not found in African populations. A smaller seed size in preyed fruits could be produced if predators can choose filled and larger seeds within fruit (Moegenburg 1996; Nalepa and Grisselli 1993, Rouault et al. 2004). Since cones are not developed when *Trisetacus* attacks, this mite may not be able to select for cone and seed traits suggesting an active plastic response of European plants. Conversely, it has been suggested that *Megastigmus* insect might be able to select filled seeds, containing a young embryo, because they oviposit after plant fertilisation in Cupressaceae trees (Rouault et al. 2004) which could result on an increase of the proportion of empty seeds in preyed cones. However, our results do not support the hypothesis of female preference because uneaten seeds of cones preyed by *Megastigmus* showed no differences in size compared to seeds from non-preyed cones in African and European populations. Moreover, seed emptiness was not higher in preyed cones in the populations where *Megastigmus* was the main predator. It is still unknown whether *Megastigmus* females can differentiate between fertilised and unfertilised ovules in Cupressaceae (Rouault et al. 2004). As it happens in Pinaceae, the development of their larvae might be completed even in empty seeds avoiding the necessity of finding a filled seed (von Aderkas et al. 2005).

We hypothesize that divergent plant response between Europe and Africa could have been produced by a geographic variation on predator distribution and abundance that can result in different selective scenarios, i.e. mosaic selection (Thomson 2005). We observed differences in the dominance of seed predators among populations, which is interesting given their different predation strategies that vary according to their mobility and feeding behaviour (Mezquida and Olano 2013). *Trisetacus* feeding behaviour could result in a higher resource lost when they attack a cone, eating several seeds, whereas *Megastigmus* individuals frequently parasite a single seed per cone (García 1998; Rouault et al. 2004; El Alaoui and Roques 2006; Llorente and Alonso 2006). Under an environment with high probability of *Trisetacus* attack, an active reallocation of resources from preyed cones to non-preyed cones could be advantageous. This sort of response could not have evolved in populations where *Trisetacus* attacks are not very likely, such as those studied in Africa.

Megastigmus, a flying insect, might exert selection on cone traits choosing larger cones. Besides being easily detected, large fruits with a higher seed-to-pulp ratio seem to facilitate the oviposition of the insect (Herrera 1984; Knight 1987). In Monegros and African populations, where *Megastigmus* was more abundant, predation by this insect was higher in cones with more seeds. Therefore, this predator might actively select large cone sizes (Turgeon et al. 1994), usually having more and smaller seeds but displaying a larger variation in seed size (DeSoto et al. unpublished), and consequently might affect seed packaging. Further research is needed to address whether the different predators act as effective selective pressure and whether the predation levels observed in these populations are maintained in time and, consequently, whether predation has been exerting disruptive selection on seed packaging.

Empty seeds as mechanism to reduce predation

The common presence of empty seeds in cones of *J. thurifera* (higher than 75% in the studied populations) and in other *Juniperus* species (e.g. Adams et al. 2014; Fuentes and Schupp 1998; García et al. 2000) may be an adaptive mechanism to reduce the impact of high levels of pre-dispersal predation. Many tree species abort seeds or fruits as a mechanism to dilute the impact of pre-dispersal seed predators because the aborted seeds act as predator sinks allowing the viable seeds to escape damage (Ghazoul and Satake 2009; Traveset 1993; Verdú and García-Fayos 1998). Nevertheless, seed emptiness might not be necessarily an evolutionary response to seed predation and the production of cones with empty seeds may be triggered by particular conditions that a plant experiences during reproductive stages. For instance, empty seeds could be produced by deficient pollination (in *J. oxycedrus*, Ortiz et al. 1998) or by fertilisation failure caused by asynchronous development of male or female gametophytes (in *J. communis*, Gruwez et al. 2013). However, *J. thurifera* male and female trees present more or less synchronously masting flowering years and are usually not pollen limited (Montesinos et al. 2012; Montesinos et al. 2010). A limited availability of resources could also restrict the number of viable seeds increasing the presence of empty seeds. Nevertheless, results from a resource supplementation experiment in this species do not support this hypothesis (Montesinos et al. 2010). In that experiment, plants with resource supply produced more ripe cones but the proportion of empty seeds remained invariable and independent of resource availability.

The phenomenon of producing empty seeds could be maintained if the reproductive cost of allocating resources to cones with empty seeds is counterbalanced by the final establishment gain. Firstly, the waste of resources is reduced when having early-aborted and ultimately empty seeds. Seed emptiness might evolve when the number of uneaten seeds exceeds the optimum number of seeds that one plant can develop (Sakai and Harada 2007). Secondly, empty-seeded cones of gymnosperms could have the same adaptive role as parthenocarpic fruits of angiosperms in seed predation avoidance, acting as a decoy for predators (Traveset 1993). Thirdly, deceptive fruits could also attract a higher number of seed dispersers that will also disperse some viable seeds (Jordano 1989). Then, *J. thurifera* may benefit from producing cones with a higher number of empty seeds or even with no offspring when seed predation pressure is high in order to reduce the global incidence of predation within the population by preserving undamaged viable seeds and maintaining the attraction of dispersers.

Evolutionary consequences of pre-dispersal predation on seed-packaging

Different predation levels and either seed packaging strategies or seed viability were found in the studied populations. Our results partially match the predictions forecasted by the Sakai and Harada's model (2007) on the effect of predation on seed size-number evolution. The model forecasts that if predation is high or unpredictable, plants will increase the number of ovules produced to ensure a minimum offspring number (Sakai and Harada 2007). Consistently, predators may have exerted selection on seed packaging in *J. thurifera* towards multi-seeded cones in European populations, where high level of cone pre-dispersal predation was observed. This selection would not happen in Moroccan populations where predation is lower and larger seeds could increase germination rates and seedling establishment (Moles and Westoby 2004; Seltmann et al. 2006). The model developed by Sakai and Harada (2007) also predicts an increase in seed size under an increment of the proportion of eaten seeds as a result of the size/number trade-off of the whole plant. The authors based their prediction on the assumption that the cost of predation for the plant increases with an increasing number of seeds. However, this model did not take into account the hierarchical resource investment in several seeds within a fruit. The size/number trade-off may not be restricted to the individual, and finite resources within a fruit may also lead to a size/number trade-off constraining the increase in seed size because it comes at expense of producing fewer seeds (Leishman 2001). Therefore, Sakai and Harada's model (2007) might only partially predict pre-dispersal predation effect on multi-seeded species.

Although the evolutionary response of seed packaging to predation seems to be consistent with the prediction of increasing seed number, a multi-small-seeded cone strategy was maintained in Monegros despite the low predation pressure observed. Under low predation pressure, large-seeded cones could be favoured in stressful environments because larger seeds benefit from size-related advantages to survive the hazards of establishment, such as drought, shade, defoliation, burial depth, competition, and nutrient shortage (Moles and Westoby 2004). Unfavourable climate could, thus, act as selective regime towards the single-large seed strategy in African *J. thurifera* populations. These populations are mainly located in the High Atlas Mountains above 2,000 m a.s.l., and have to cope with a drier and warmer climate with a longer summer drought (DeSoto et al. 2014). Within European populations, Luna and Soria are located in the Spanish Plateau up to 1,300 m a.s.l. and withstand colder and more humid climate, while Monegros has a climate more similar to that found in the African populations (DeSoto et al. 2014). Therefore climate by its own does not explain why seed packaging in Monegros is similar to Luna and Soria and different from the African populations.

The theoretical adaptation of seed packaging to low predation pressure in Monegros may have been constrained by other selective pressures that maintain multi-seeded cones. For instance, European multi-small-seed strategy yields a benefit since seed number can be directly translated into fitness and is negatively related to seed cost (Leishman 2001). Second, seeds tend to be less regurgitated, longer retained in bird guts and, thus, dispersal efficiency increases in small-seeded species (Jordano 1995; Obeso et al. 2011). Third, post-dispersal predation may also exert a positive selective pressure towards smaller seeds, since they are less likely to be found and consumed, but also seeds of small-seeded species can be more easily buried and escape from disturbances (Peco et al. 2003; Gómez 2004). Finally, a high inter-population gene flow (Teixeira et al. 2014) might prevent divergence of seed packaging traits among European populations maintaining multi-small-seeded cones in Monegros. All these hypotheses are not mutually excluding, and, thus, deserve further research.

Conclusions

Seed predators have an impact on the size of the non-preyed co-occurring seeds within a cone. In European populations, these co-occurring seeds may be deprived of resources because trees may identify seed attack, particularly by the predominant *Trisetacus* predator, and therefore try to reduce the resources allocated to preyed cones compromising seed viability. In African populations, trees may not react to the attack and non-preyed seeds would be larger by the elimination of a competing seed within a cone. The two seed packaging strategies, via the seed size/number trade-off, of *J. thurifera* could be subjected to selection by pre-dispersal predation leading to different consequences on population dynamics. African one-larger-seeded cones could be a better strategy than European multi-smaller-seeded cones under low predation rates since seed germination success was higher in African populations. In European populations a considerable fraction of reproductive investment is lost through deceptive cones with empty seeds probably to avoid predation. High incidence of seed emptiness could represent a problem in understanding the patterns of reproductive allocation and in the conservation of the species.

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TABLES

Table 1. Results of generalized mixed model (Type III) to test the effect of the fixed factors predation (seeds from *Megastigmus*-preyed, *Trisetacus*-preyed and non-preyed cones), population and their interaction on the number of seeds and seed mass (n = 9-12 trees per population)

	Number of seeds				Seed mass*			
Fixed effects	df num	df den	F	*P*	df num	df den	F	*P*
Predation	2	5161	12.28	< 0.001	2	2078	0.00	0.996
Population	4	5161	81.72	< 0.001	4	47.93	2.07	0.099
Population × predation	8	5161	2.40	0.014	8	1885	2.58	0.008
Random effects			Z	p			Z	p
Tree			3.94	< 0.001			4.31	< 0.001
Cone (tree)							17.38	< 0.001
No. of cones	5222				1752			
No. of seeds					4605			

* one-seeded cones were not included in the analysis

Table 2. Results of generalized mixed model (Type III) to test the effect of the fixed factors predation (seeds from preyed vs. non-preyed cones), population and their interaction on seed germination and emptiness (n = 6 trees per population)

	Germinated seeds*†				Empty seeds*			
Fixed effects	df num	df den	F	*P*	df num	df den	F	*P*
Predation	1	3404	7.73	0.005	1	672	0.09	0.762
Population	2	3404	20.75	< 0.001	4	672	8.21	< 0.001
Population × predation	2	3404	5.65	0.004	4	672	1.83	0.122
Random effect			Z	*P*			Z	*P*
Tree			1.81	0.035			1.12	0.131
No. of seeds	173 germinated out of 3427				633 empty out of 704			

* one-seeded cones were not included in the analysis.

† Luna population was not considered for the germination analysis since none seeds germinated from preyed cones

Figure legends

Fig. 1 Population difference in predation on a) cone predation rate per tree in each population (9,320 cones; 49 trees), b) seed predation rate within a cone in each population of *Juniperus thurifera* (2,435 preyed cones; 49 trees), and c) cone predation rate per tree in each population for each seed predator: *Megastigmus* and *Trisetacus*. Bars are model-adjusted back-transformed least-square means and 95% confident intervals. Significant differences of least-square means among populations are indicated with different letters, lowercase and uppercase for *Megastigmus* and *Trisetacus* respectively ($P < 0.05$)

Fig. 2 Species-specific predation effect on a) the number of seeds per cone and b) seed mass in preyed M, *Megastigmus*, and T, *Trisetacus*, and non-preyed cones in each population. The figures are model-adjusted back-transformed least-square means and 95% confident intervals based on the generalized mixed models in Table 1. Significant differences between least-square means among populations are indicated with different letters ($P < 0.05$; sample sizes for non-preyed/preyed cones are indicated in parenthesis)

Fig. 3 Predation effect on a) germination and b) emptiness rates from preyed and non-preyed cones in each population. Bars are model-adjusted back-transformed least-square means and 95% confident intervals based on the generalized mixed models in Table 2. Significant differences between least-square means within populations are indicated with asterisks ($P < 0.05$) and among populations with different letters (sample sizes for non-preyed/preyed cones are indicated in parenthesis).

Figure 1

Click here to download Figure: Fig1_PredationR4.eps

Figure 1

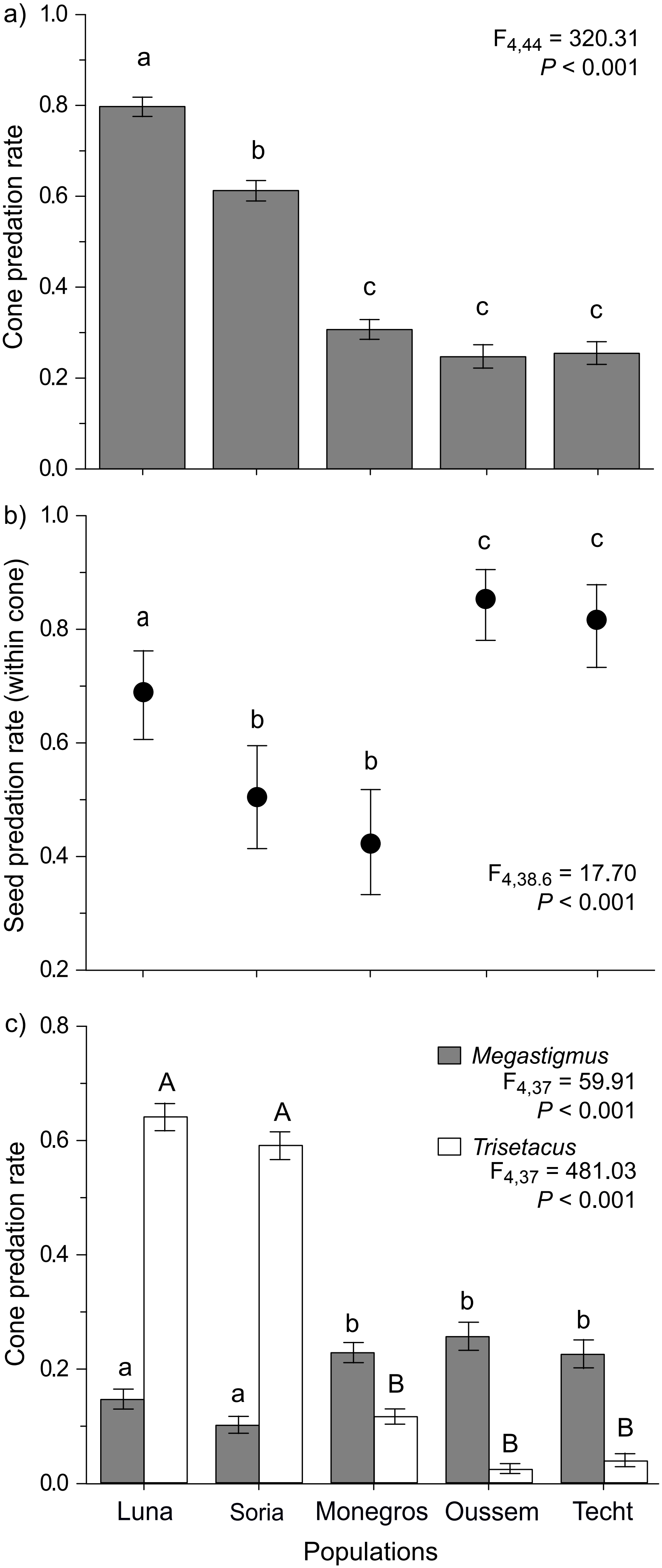


Figure 2

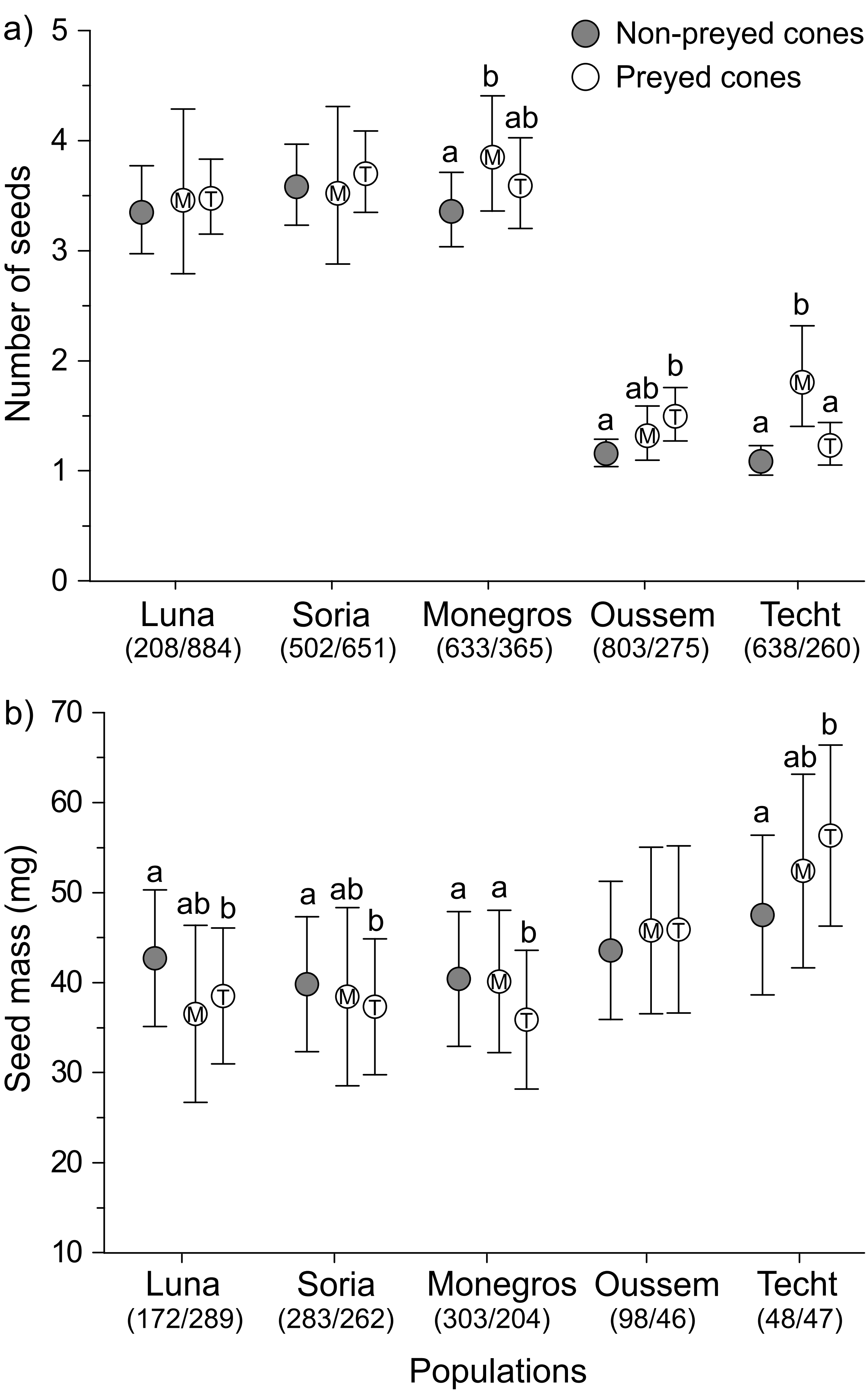
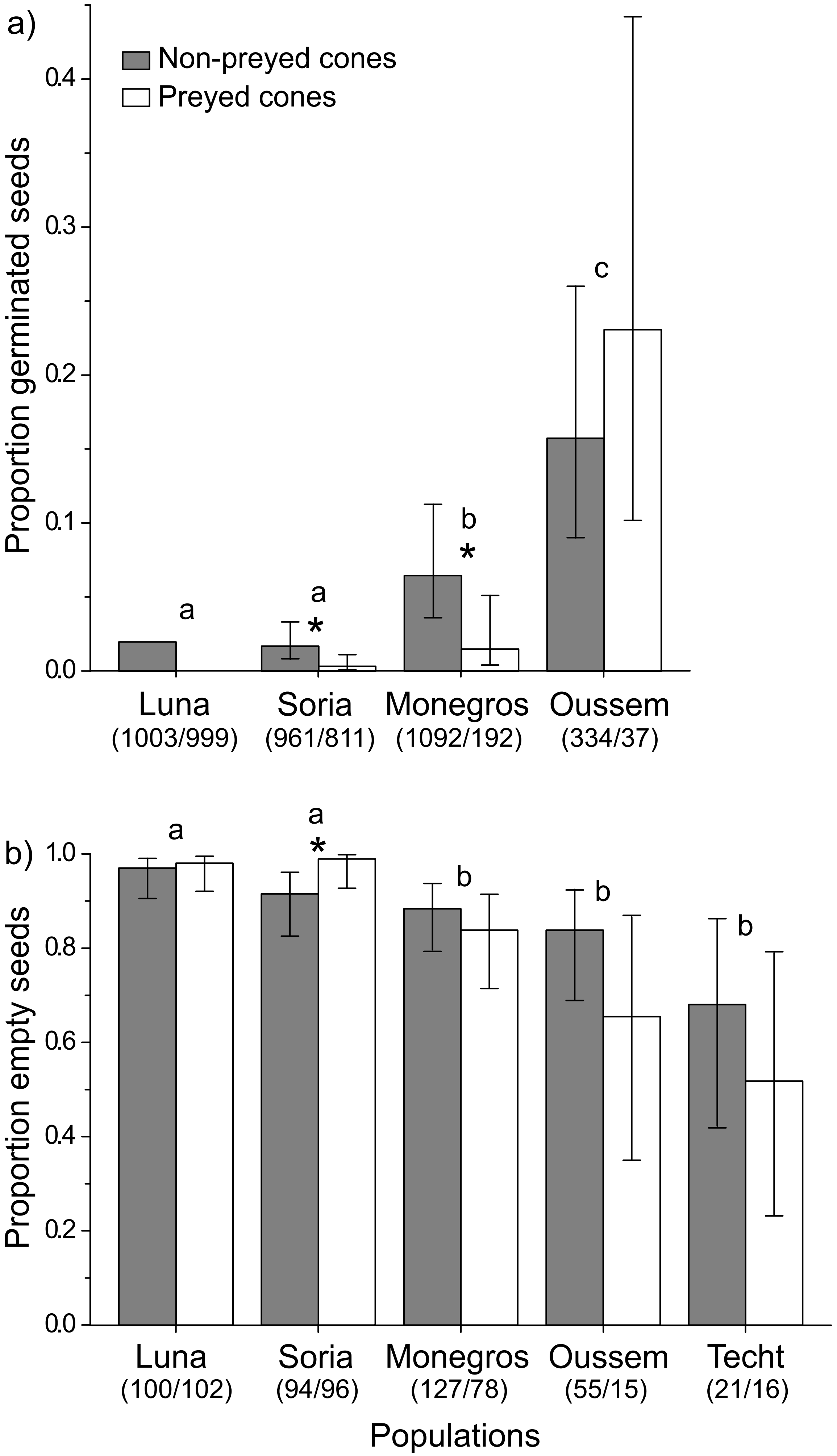

a)
Number of seeds
0
1
2
3
4
5
Non-preyed cones
Preyed cones
M
T
a
b
ab
Luna
(208/884)
Soria
(502/651)
Monegros
(633/365)
Oussem
(803/275)
Techt
(638/260)
b)
Seed mass (mg)
10
20
30
40
50
60
70
Luna
(172/289)
Soria
(283/262)
Monegros
(303/204)
Oussem
(98/46)
Techt
(48/47)
Populations

Figure 3


a)
Non-preyed cones
Preyed cones
Proportion germinated seeds
0.0
0.1
0.2
0.3
0.4
a
a
*
b
*
c
Luna
(1003/999)
Soria
(961/811)
Monegros
(1092/192)
Oussem
(334/37)
b)
Proportion empty seeds
0.0
0.2
0.4
0.6
0.8
1.0
a
a
*
b
b
b
Luna
(100/102)
Soria
(94/96)
Monegros
(127/78)
Oussem
(55/15)
Techt
(21/16)
Populations