Oecologia (2016) 180:91–102
DOI 10.1007/s00442-015-3446-8

POPULATION ECOLOGY - ORIGINAL RESEARCH

Pre-dispersal predation effect on seed packaging strategies and seed viability

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Received: 10 October 2014 / Accepted: 2 September 2015 / Published online: 23 September 2015

Abstract An increased understanding of intraspecific seed packaging (i.e. seed size/number strategy) variation across different environments may improve current knowledge of the ecological forces that drive seed evolution in plants. In particular, pre-dispersal seed predation may influence seed packaging strategies, triggering a reduction of the resources allocated to undamaged seeds within the preyed fruits. Assessing plant reactions to pre-dispersal seed predation is crucial to a better understanding of predation effects, but the response of plants to arthropod attacks remains unexplored. We have assessed the effect of cone predation on the size and viability of undamaged seeds in populations of *Juniperus thurifera* with contrasting seed packaging strategies, namely, North African populations with single-large-seeded cones and South European populations with multi-small-seeded cones. Our results show that the incidence of predation was lower on the single-large-seeded African cones than on the multi-small-seeded European ones. Seeds from non-preyed cones were also 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. It is possible that pre-dispersal seed predation has been a strong selective pressure in European populations with high cone predation rates, being a process which maintains 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 the African populations with single-large-seeded cones characterized by seed germination and filling rates higher than those in the 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. the allocation of total seed content into a single large seed or several small ones, constitutes one of the central trade-offs in plant ecology and evolution because, given finite resources, increased seed size comes at the expense of seediness or crop or brood size (Leishman 2001). In general, large-seeded species produce seedlings with higher establishment probabilities and growth rates, while small-seeded species produce more abundant offspring (Moles and Westoby 2004). Both the direct and accessory costs of producing a seed increase with seed

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Communicated by Anne Worley.

Electronic supplementary material The online version of this article (doi:10.1007/s00442-015-3446-8) contains supplementary material, which is available to authorized users.

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size (Lord and Westoby 2006). This is particularly true in fleshy fruits where the resources allocated to pulp, and consequently the parental cost of a seed, usually decrease with increasing seed number (Obeso 2004), leading to an optimal size/number combination. However, seed packaging is not constant within a species and demonstrates considerable intraspecific variability (Eriksson 1999; Mehlman 1993; Willson et al. 1990), which may represent an opportunity for selection.

Abiotic and biotic factors may act as selective agents that drive seed trait variability (Harper et al. 1970). Climatic conditions associated with geographical patterns influence physiological processes and may also partially explain the observed large-scale intraspecific variability in seed trait variability. In general, resources become less available with increasingly harsher climatic conditions, such as higher elevation, higher latitude and/or higher aridity, possibly resulting in a decrease in either seed viability or size (see, for example, 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, with a parallel adjustment for seed size (Alcantara and Rey 2003; Jordano 1995; Levey 1987), and lighter seeds tend to be retained longer in a bird's digestive tract, thereby increasing dispersal efficiency (Obeso et al. 2011). Post-dispersal predation may also exert a selective pressure on smaller seeds that can be buried easily, consequently avoiding disturbance and escaping predation (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 color, fruit and seed size and mast seeding (Kolb et al. 2007, and references therein). In particular, pre-dispersal predators may exert a selective pressure on fruits with smaller seeds or a low number of seeds as they preferentially consume larger fruits which contain either more seeds and thus have a higher seed-to-pulp ratio, which facilitates oviposition (Knight 1987), or larger seeds, which facilitates larger predator offspring (Moegenburg 1996). Consequently, pre-dispersal seed predation may influence the variability of seed packaging, and function as an effective selective pressure (Geritz 1998).

Ecological models assume that small seed sizes may reduce the resources lost per seed preyed upon (Sakai and Harada 2007). In addition, multi-seeded fruits can avoid the loss of the total seed content, thereby reducing the seed predation rate, i.e. the number of damaged seeds per total number of seeds in each fruit. Satiating predators either with a surplus of flowers and/or immature fruits or by producing seedless fruits can be two potential plant 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 early detection of the infection and selective abscission of the fruit, with a negative effect on the predator (Bonal and Muñoz 2008; Fernandes and Whitham 1989; Verdú and García-Fayos 1998). Selective seed abortion as a response to predation is likely to occur in multi-seeded fruits when there is a second intact seed in the fruit to prevent co-infestation (Meyer et al. 2014). Plants may also reduce the resources allocated to attacked fruits, leading to a reduction in the size 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 due to the elimination of a competing seed within a cone, suggesting a lack of active response from the plant. Understanding how plants react to predator attack is therefore fundamental to our understanding of plant ecology since the response of the plant 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. For instance, in multi-seeded fruits, seed predators may eat either all 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 different predator's feeding behavior could imply different costs for predated plants that might trigger different plant responses to the attack.

The dioecious conifer *Juniperus thurifera* (Spanish juniper) has female berry-like cones that vary in seed size and number of seeds per cone between the populations situated on either side of the Strait of Gibraltar. These populations have two different seed packaging strategies which consist of multi-small-seeded cones in the European populations and single-large-seeded cones in the African populations (Boratyński et al. 2013). *J. 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 two predators display different feeding behaviors (García 1998; Rouault et al. 2004; El Alaoui El Fels and Roques 2006; Llorente and Alonso 2006) that may exert different effects on seed packaging, with wasps parasitizing individual seeds per cone and mites using seeds as growth chambers and feeding on as many seeds as they can. Also, seed abortion and pre-dispersal predation in *J. thurifera* may not be homogeneously distributed across its European populations

(Montesinos et al. 2010). This 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.

We report here our assessment of how pre-dispersal seed predation influences seed traits on the Mediterranean endemic tree *J. thurifera* by (1) exploring differences in pre-dispersal seed predation (i.e. predation rates, predator identity and preferences) and plastic responses to predation in populations with contrasting seed packaging strategies, and (2) assessing whether seed viability of the co-occurring seeds within a preyed cone is reduced under conditions of 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 which grows up to a height of 20 m with a conical to round or irregular crown. It is a long-lived (up to 600 years) tree that is endemic to continental areas of the Western Mediterranean Basin at altitudes ranging from 200 to 3400 m a.s.l. (DeSoto et al. 2014; Gauquelin et al. 1999). It has been suggested that *J. thurifera* was a dominant species during the cold stages of the Pleistocene (Carrión et al. 2003), but it currently has a disjunct distribution throughout south-western Europe and North Africa. The most abundant populations can be found in Spain (200,000 ha) and in the region of 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 cones (length 3–4 mm) shed their pollen. Female cones are wind-pollinated, fertilized late in April (5–6 months after pollination) and ripen in about 20 months. The ripe female cones (hereafter referred to as cones) are dark purple and berry-like with a whitish waxy bloom and 7–11 mm in diameter; they usually contain one to five seeds, rarely more than six seeds but have been recorded to contain up to ten seeds (Amaral-Franco 1986; Boratyński et al. 2013). Seed packaging strategies differ between the populations of the different continents, with the African populations having single-large-seeded cones containing relatively fewer, larger and heavier seeds (average number of seeds 1.3; length 4.8 mm; weight 62 mg/seed) and the European populations containing multi-small-seeded cones with smaller and lighter seeds [average number of seeds 3.6; length 3.8 mm; weight 40 mg/seed; Boratyński et al. 2013; Electronic Supplementary Material (ESM) Fig. S1]. Based on the number of seeds per cone for the closest relatives of *J. thurifera* (Farjon 2005; Adams 2008), its ancestor is likely to have been a multi-seeded cone species (DeSoto et al., unpublished data). 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, namely, the females of a chalcid wasp, *Megastigmus* sp. (Hymenoptera, Torymidae), and the mite *Trisetacus* sp. (Acarina, Nallepellidae). The female wasp *Megastigmus* sp. oviposits usually one egg per seed inside 8-month-old immature cones in the summer following fertilization (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 the seed and cone. *Megastigmus* sp. usually attacks only one seed per cone (García 1998). The mite *Trisetacus* sp. can attack several times at different seed developmental stages, usually before fertilization, and grows, forming colonies and feeding on the seeds for about 18 months. It usually damages more than one seed within a cone, and seeds become light brown and stick out of the cone (El Alaoui El Fels 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

Our study populations comprised five populations of *J. thurifera*, of which three were in Spain [Luna: in the Cantabrian Range (42°55′N, 5°51′W, 1228 m a.s.l.); Soria (41°47′N, 2°48′W, 1150 m a.s.l.); Monegros Desert (hereafter referred to as Monegros; 41°40′N, 0°21′W, 534 m a.s.l.)] and two were in the Moroccan High Atlas [Azzaden Oussem (hereafter referred to as Oussem; 31°06′N, 7°57′W, 2396 m a.s.l.); Tizi n' Techt (hereafter referred to as Techt; 31°10′N, 7°58′W, 1994 m a.s.l.)]. Luna and Soria were the moistest and coldest sampling sites, and Monegros and the High Atlas were the warmest and driest, with a water deficit almost all year round (see DeSoto et al. 2014 for a detailed description of the climate).

These populations represent the variability of seed packaging strategies found in *J. thurifera*, with single-large-seeded cones produced by the African populations in Morocco and multi-small-seeded cones produced by the European populations in Spain (ESM Fig. S1). We randomly collected approximately 1500 mature cones (at least 150 cones from each of 10 randomly chosen female trees) of each population at various positions around the crowns to avoid orientation effects. The cones were collected in October 2010 in Morocco and in 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 El Fels and Roques 2006; Llorente and Alonso 2006).

Pre-dispersal seed predation

To measure the incidence of pre-dispersal seed predation, we dissected 5240 cones collected from the study populations (approx. 100 cones/tree) with the aim to evaluate the relationship between predation and seed packaging strategies (single- vs. multi-seeded cones). These cones were first embedded in 1 % sodium hypochlorite solution for 2 days to remove the waxes and resins (García-González et al. 2009), then opened and classified as non-preyed cones with non-preyed seeds or preyed cones with at least one preyed seed (ESM Fig. S1). The number of preyed, non-preyed and non-developed or aborted seeds were counted for each cone, and the total number was taken as the seed number for the cone (i.e. the total number of ovules found within a cone). We explored the occurrence of pre-dispersal seed predation in 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 each preyed cone. Second, we calculated the cone predation rate for each tree (percentage of preyed cones per tree), classifying the cones into *Megastimus*-preyed or *Trisetacus*-preyed cones. When possible, we completed the dataset by checking for predator attack in an additional 100 cones per tree (total number of cones 9320). Cones predated by *Megastimus* have a different morphology than those predated by *Trisetacus*, and these two cone morphologies are easily distinguishable.

We evaluated the potential effect of seed predator on the size and viability of the remaining non-preyed seeds within a preyed cone (hereafter referred to as co-occurring seeds), comparing seed traits between *Megastimus*-preyed, *Trisetacus*-preyed and non-preyed cones. First, we weighed the non-preyed seeds of 30 non-preyed and 30 preyed cones per tree. We then performed a germination test to estimate seed viability in relation to the effect of predation (see "Seed viability").

Seed viability

Seed viability was assessed using a germination test for seeds collected in Luna, Soria, Monegros and Oussem. To test seed viability, we selected seeds which we considered to be a priori viable seeds based on the absence of any morphological evidence of damage and the inability to float in water (García-González et al. 2009). We performed two germination experiments to test whether cone predation affected seed viability (with seed size controlled for) and to explore general trends in seed viability between populations and seed packaging strategies (where only the effect of cone predation was tested). In the first experiment, 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 1536 seeds were used in this experiment, 64 seeds per tree, from six different trees and four populations. For each tree, four petri dishes filled with wet sand were used, and 16 seeds were sown per petri dish; the seeds were chosen firstly by predation level (from preyed or non-preyed cones) and secondly by seed size (small and large, with the 8 largest or smallest seeds selected at each predation level). In the second, larger experiment, we tested the effect of seed predation on germination using the remaining seeds collected from the four populations. We sowed 180 seeds in each tray filled with wet sand, with two to eight trays for each population, depending on the total number of seeds available per population (total number of seeds 3907). The origin, both tree and cone, of each seed was recorded.

The method described of García-González et al. (2009) was used to induce seed germination: seed scarification with sandpaper, followed by water stratification with four moisture–drought cycles and then by a 2-month cold stratification period at 5 °C. Following this treatment, all petri dishes and trays were maintained under controlled conditions [temperature 15 °C (day)/10 °C (night); light photosynthesis active radiation 80 μmol m^{-2} s^{-1}; light regime 16 h (light)/8 h (dark); humidity 85 %] from May to October 2012 and rotated weekly to avoid position effects within the growth chamber (Fitoclima D1200 PLH; Aralab Inc., http://www.aralab.pt/Aralab-Your-own-climate, Rio de Mouro, Portugal).

Following the germination experiments, we dissected approximately 200 non-germinated seeds (100 from preyed cones and 100 from 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, which had not been included in the germination experiment. Seeds that did not contain an embryo were considered to be empty seeds (ESM Fig. S1). Neither larvae nor adults of *Megastigmus* sp. or other predators in prolonged diapause were found inside any of the opened seeds.

Statistical analysis

In order to determine whether population and predation exerted an effect on the seed and cone traits of the studied *J. thurifera* trees we used generalized linear mixed models (GLMMs) in two different tests. GLMMs provide a flexible procedure to model traits that do not satisfy the assumptions of a standard linear model, while allowing fixed and random factors to be distinguished in the model. First, we analyzed whether seed predation rate or cone predation rate varied among populations and also estimated the predator dominance among populations by 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, taking into account 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 analyzed cone predation rate, seed predation rate and cone predation rate for each seed predator, assuming a binomial error distribution with a logit link and including population as a fixed factor (McCullagh and Nelder 1989). We also 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. The number of seeds was analyzed using a Poisson error distribution with log link function, and seed mass was analyzed assuming a Gaussian error distribution with an identity link. Germination rate and empty seeds occurrence were analyzed considering a binary error distribution with a logit link. In all analyses, the tree was considered to be a random factor, and only in the seed mass analysis was the cone nested within the tree also included as a random factor. We did not include cones with only one seed in the analyses of predation effect on seed mass, germination and emptiness due to the lack of co-occurring seeds. Since multi-seeded cones in the African tree populations were less frequent and predator frequencies varied among populations, we conducted type III tests and used the 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 statistical package 9.2 (SAS Institute, Raleigh, NC).

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 %; Soria 56 %) than in the Monegros population 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 were found to have a lower proportion of preyed seeds in each cone, with the Luna population having the highest seed predation rate (Fig. 1b). We observed differences in the dominance of seed predators among populations. The cone predation rate by the wasp *Megastigmus* was higher in the Monegros and African populations, while that by the mite *Trisetacus* was higher in the Luna and Soria populations (Fig. 1c).

Predator preferences and differential effects of predator type on uneaten seeds

Predator preferences for seed-packaging traits were found to be predator type-dependent (Table 1). *Megastigmus* showed a preference for cones with more seeds (i.e. higher total number of ovules within a cone) in the 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 shown in Fig. 1c). Our data shows that there was a significant interaction between predation and population, with differential effects of predation observed on seed traits according to the 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 varied between the European and African populations (Fig. 2b). European populations, which had higher predation levels of *Trisetacus* in general (Fig. 1c), had lighter seeds after predation by *Trisetacus*; this phenomenon was not found in African populations, with co-occurring uneaten seeds of cones preyed by *Trisetacus* being heavier than seeds from non-preyed cones at Techt (Fig. 2b).

Seed viability

Regarding seed viability, in the first experiment, in which we controlled seed size and predation, germination success was very low (overall average 5 %), with only 73 of 1536 seeds germinating. There were distinct inter-population differences, with the majority of the germinated seeds originating from Oussem, the African *J. thurifera* population. Within this population, seed mass exerted a positive and significant effect on germination ($\chi^2 = 22.52$, $P < 0.001$, $n = 60$; ESM Fig. S2). In the second experiment, 190 of 3907 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 and found that the mean germination rate per tree differed between populations, being 16.5 % in Oussem (Africa) and 5.4 % in Monegros, 1 % Soria and 0.6 % in Luna (Europe; Fig. 3a). Predation affected germination in the European populations, resulting in a greater than 75 % reduction in the viability of the co-occurring seeds within a preyed cone; in contrast, we found a non-significant

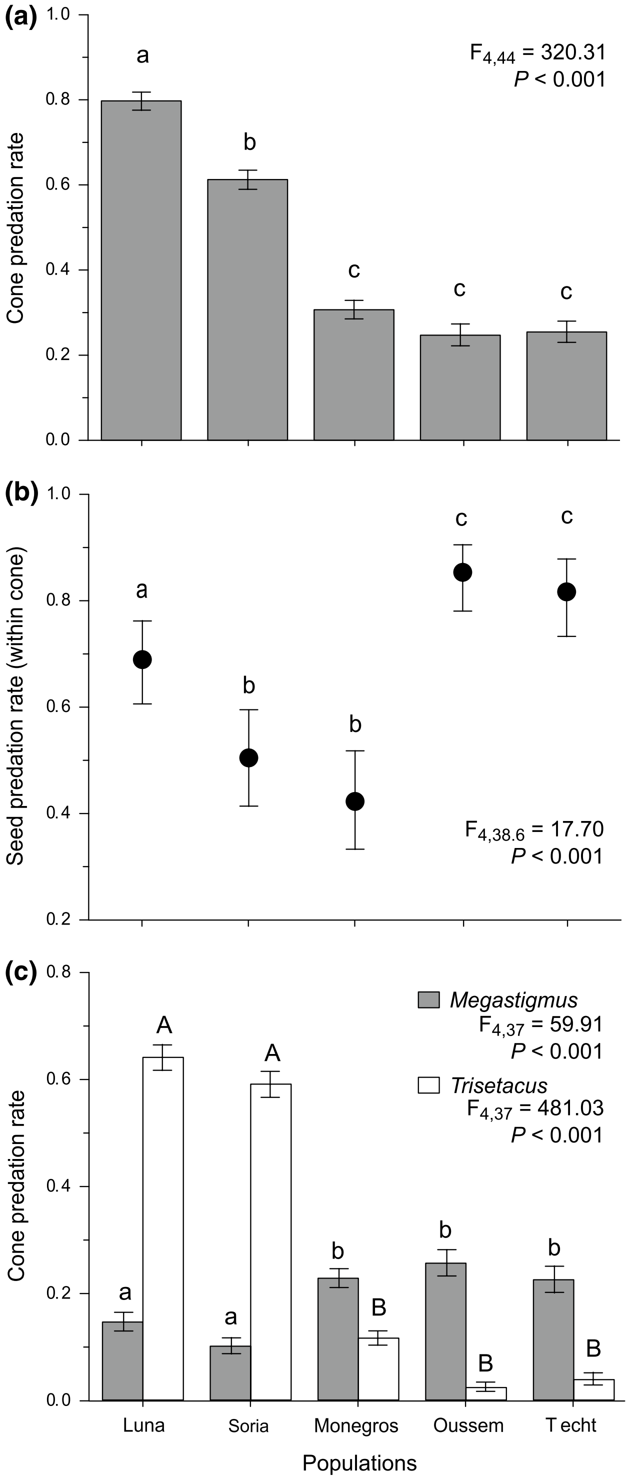

Fig. 1 Population differences in predation on cone predation rate per tree in each population of *Juniperus thurifera* (**a**; 9320 cones, 49 trees), seed predation rate within a cone in each population of *J. thurifera* (**b**; 2435 preyed cones, 49 trees) and cone predation rate per tree in each population of *J. thurifera* for each seed predator, *Megastigmus* and *Trisetacus* (**c**). *Bars*, *filled circles* Model-adjusted back-transformed least-square means, *whiskers* 95 % confident intervals (CI). *Different letters* (*lowercase* and *uppercase* for *Megastigmus* and *Trisetacus,* respectively) indicate significant differences ($P < 0.05$) in least-square means among populations

effect of predation on Oussem *J. thurifera* population (Table 2; Fig. 3a).

The main reason for the very low level of germination rate observed was that most of seeds were empty. Empty seeds could not be detected before the experiment by either the visual inspection or the floating test mainly due to the very thick coat of *J. thurifera* seeds (ESM Fig. S1). The occurrence of empty seeds significantly differed among populations, being lower in Oussem (76 %), Techt (83 %) and Monegros (87 %) *J. thurifera* populations than in those of Soria (95 %) and Luna (97 %; Fig. 3b). In general, the proportion of empty seeds was not affected by predation and was the only significant difference between preyed and non-preyed cones was in the *J. thurifera* population at Soria (Table 2; Fig. 3b).

Discussion

Our findings support a plastic response of *J. thurifera* trees to pre-dispersal seed predation that is probably caused by changing resource allocation to preyed cones. Preyed cones contained smaller seeds in the European *J. thurifera* populations but larger seeds in the African *J. thurifera* populations, particularly in those preyed by *Trisetacus*. In addition, the germination success of seeds from non-preyed cones in the European populations was threefold higher than that of intact seeds from preyed cones. The occurrence of empty seeds was higher in the European populations, indicating that the 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 were more likely to suffer from pre-dispersal seed predation in the two African populations and in the Monegros population, 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 may affect the development of co-occurring undamaged seeds in preyed fruits as a result of the plant's reaction to the attack (Bonal et al. 2007). One possibility is that plants allocate fewer resources to preyed fruits in order to reduce food availability to predator larvae and, consequently, co-occurring seeds may suffer from 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 the European *J. thurifera* populations, preyed cones contained smaller

Table 1 Results of the generalized mixed model (type III) to test the effect of the fixed factors predation, population and their interaction on the number of seeds and seed mass ($n = 9$–12 trees per *Juniperus thurifera* population)

	Number of seeds				Seed mass^{a}			
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			

Fixed factor predation in the generalized linear mixed models included three levels: seeds from *Megastigmus*-preyed, *Trisetacus*-preyed and non-preyed cones

[a] One-seeded cones were not included in the analysis

seeds than non-preyed cones, which reduced their viability, measured as germination success. In the African *J. thurifera* populations the effect appeared to be the reverse, with co-occurring seeds from the Techt *J. thurifera* population displaying a larger size, likely due to the elimination of a competing seed within the preyed cones, while no effect on co-occurring seeds was found in preyed cones from the Oussem *J. thurifera* population.

Our results support the notion that differences in seed size between preyed and non-preyed cones are better explained by the plants' response to the attack than by the preferential attack of predators. A reduction in seed size in preyed cones was a response to *Trisetacus* mites in the European *J. thurifera* populations, whereas this response was not found in the African populations. A smaller seed size in preyed fruits could be a plant's response to predation if predators were to prefer filled and larger seeds within fruits (Moegenburg 1996; Nalepa and Grisselli 1993; Rouault et al. 2004). Since the cones of the European *J. thurifera* populations are not yet fully developed during the period 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* may be able to select for filled seeds that contain a young embryo because they oviposit after plant fertilization in Cupressaceae trees (Rouault et al. 2004), which could result in an increased 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* did not differ in size from seeds collected from non-preyed cones in both the African and European populations. Moreover, the incidence of seed emptiness was not higher in preyed cones in those populations where *Megastigmus* was the main predator. It is unknown whether *Megastigmus* females can differentiate between fertilized and unfertilized ovules in Cupressaceae (Rouault et al. 2004). In Pinaceae, *Megastigmus* larvae can complete their development even in empty seeds, thereby eliminating the necessity of finding a filled seed (von Aderkas et al. 2005).

We hypothesize that divergent plant responses between the European and African *J. thurifera* populations could be due to geographic variations in predator distribution and abundance that can result in different selective scenarios, i.e. mosaic selection (Thompson 2005). We observed differences in the dominance of seed predators among populations, which is an interesting observation given their different predation strategies in terms of mobility and feeding behavior (Mezquida and Olano 2013). The feeding behavior of *Trisetacus* may result in a higher loss of plant resources, as several seeds are eaten during an attack on a cone, whereas *Megastigmus* individuals frequently parasite a single seed per cone (García 1998; El Alaoui El Fels and Roques 2006; Llorente and Alonso 2006; Rouault et al. 2004). Under high *Trisetacus* occurrence, an active reallocation of resources from preyed cones to non-preyed cones could be a beneficial plant strategy. This sort of response could not have evolved in populations where *Trisetacus* attacks are not very likely, such as the African *J. thurifera* populations.

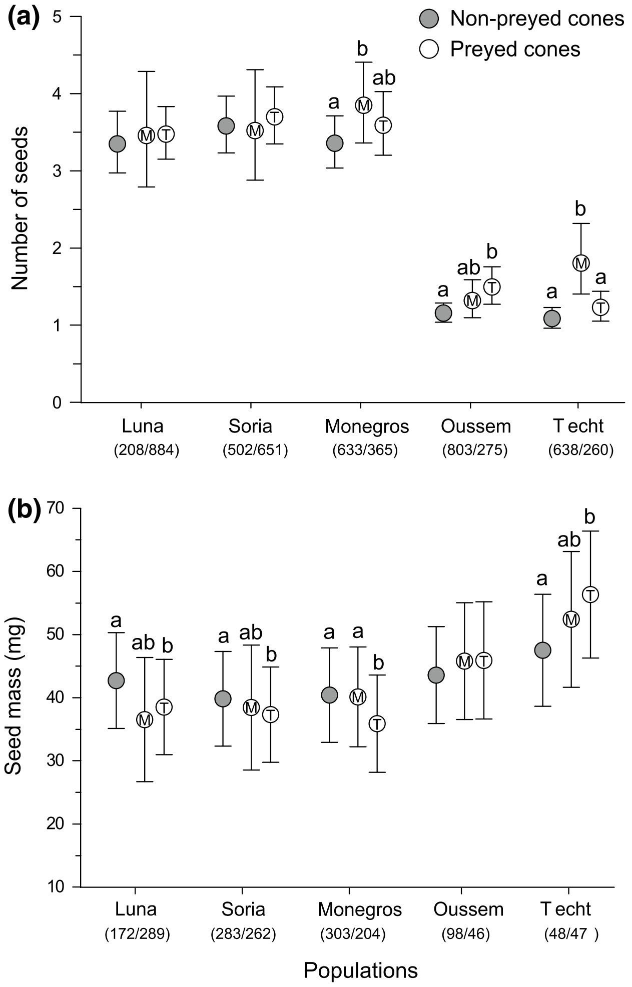

Fig. 2 Species-specific predation effect on the number of seeds per cone (**a**) and seed mass (**b**) in *Megastigmus* (*M*)- and *Trisetacus* (*T*)-preyed cones and non-preyed cones (*shaded circles*) in each *J. thurifera* population. The data are presented as model-adjusted back-transformed least-square means (*circles*) and 95 % CI (*whiskers*) based on the generalized mixed models in Table 1. *Different lowercase letters* indicate significant differences ($P < 0.05$) between least-square means among populations. *Numbers in parenthesis below figures* Sample sizes for non-preyed/preyed cones

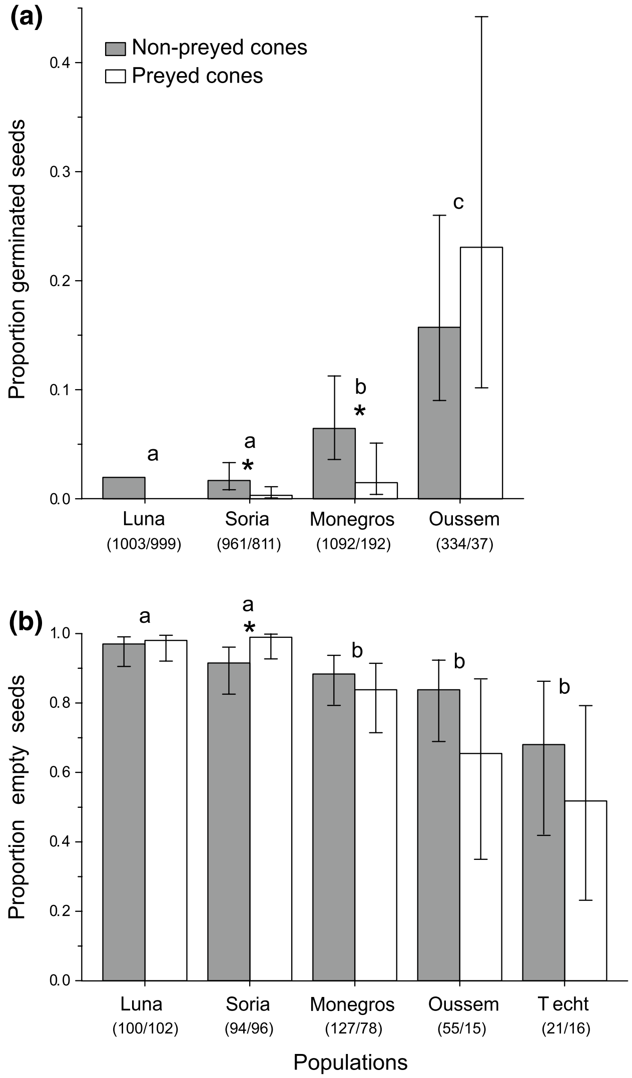

Fig. 3 Predation effect on germination (**a**) and emptiness (**b**) rates from preyed and non-preyed cones in each *J. thurifera* population. The data are presented as model-adjusted back-transformed least-square means (*bars*) and 95 % CI (*whiskers*)—based on the generalized mixed models in Table 2. *Asterisks* indicate significant differences ($P < 0.05$) between least-square means within populations, *different lowercase letters* indicate significant differences ($P < 0.05$) among populations. *Numbers in parenthesis below figures* Sample sizes for non-preyed/preyed cones

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

Empty seeds as a mechanism to reduce predation

The presence of empty seeds was common in the cones of the studied *J. thurifera* populations (>75 %) and is also a common occurrence in the cones of other *Juniperus* species (e.g. Adams et al. 2014; Fuentes and Schupp 1998;

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 *J. thurifera* population)

	Germinated seeds[ab]				Empty seeds[a]			
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			

[a] One-seeded cones were not included in the analysis

[b] The Luna population was not considered for the germination analysis since none of the seeds germinated from preyed *J. thurifera* cones

García et al. 2000), suggesting that it 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 reduce the impact of pre-dispersal seed predators, with the aborted seeds acting as predator sinks that allow the viable seeds to escape damage (Ghazoul and Satake 2009; Traveset 1993; Verdú and García-Fayos 1998). Nevertheless, seed emptiness might not necessarily be an evolutionary response to seed predation, and the production of cones with empty seeds may be triggered by specific conditions experienced by a plant during the reproductive stages. For example, empty seeds can be produced by deficient pollination (in *J. oxycedrus*, Ortiz et al. 1998) or by fertilization failure caused by asynchronous development of male or female gametophytes (in *J. communis*, Gruwez et al. 2013). However, male and female trees of *J. thurifera* have more or less synchronously masting flowering years and are usually not pollen-limited (Montesinos et al. 2012, 2010). A limited availability of resources could also restrict the number of viable seeds, resulting in an increased number of empty seeds. Nevertheless, the results from a resource supplementation experiment in this species do not support this hypothesis (Montesinos et al. 2010). In that experiment, plants with an ample supply of resources produced more ripe cones, but the proportion of empty seeds remained invariable and independent of resource availability.

The production of empty seeds could be maintained if the reproductive cost of allocating resources to cones with empty seeds were to be counterbalanced by the final establishment gain. First, the waste of resources is reduced when seeds are aborted early, ultimately resulting in 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 terms of seed predation avoidance, acting as decoys for predators (Traveset 1993). Thirdly, deceptive fruits could also attract a higher number of seed dispersers that will also disperse some of the viable seeds (Jordano 1989). *J. thurifera* may therefore 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

The studied *J. thurifera* populations showed different predation levels and either different seed packaging strategies or different seed viability. Our results are in partial agreement with the predictions of the Sakai and Harada's (2007) model on the effect of predation on the evolution of seed size/number. This 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). Consistent with this model, predators may have exerted selective pressure on seed packaging in *J. thurifera* towards multi-seeded cones in the European populations, where a high level of cone pre-dispersal predation was observed. This selection would not occur in the Moroccan *J. thurifera* populations where predation is lower and the larger seeds would 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 in the proportion of eaten seeds as a result of the size/number trade-off of the whole plant. These authors based their prediction on the assumption that the cost of predation to the plant increases with an increasing number of seeds. However, their model does 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 plant, and finite resources within a fruit may also lead to a size/number trade-off, resulting in a constraint in the increase in seed size because it comes at the expense of producing fewer seeds (Leishman 2001). Therefore, Sakai and Harada's (2007) model may only partially predict the effect of pre-dispersal predation 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 the Monegros *J. thurifera* population despite the low predation pressure observed. Under low predation pressure, large-seeded cones could be favored 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). An unfavorable climate could thus act as a selective regime towards the single-large seed strategy in the African *J. thurifera* populations, which are mainly located in the High Atlas Mountains at above 2000 m a.s.l. and have to cope with a drier and warmer climate and a longer summer drought than do the European *J. thurifera* populations (DeSoto et al. 2014). Of the European *J. thurifera* sites studied, Luna and Soria are located on the Spanish Plateau at up to 1300 m a.s.l., and their *J. thurifera* populations are able to withstand a colder and more humid climate than those at the other sites; in comparison, Monegros has a climate more similar to that found at the African sites (DeSoto et al. 2014). Therefore, climate on its own does not explain why seed packaging in the Monegros *J. thurifera* population is similar to that in the Luna and Soria *J. thurifera* populations and different from that in the African populations.

The theoretical adaptation of seed packaging to low predation pressure in the Monegros *J. thurifera* population may have been constrained by other selective pressures that maintain multi-seeded cones. For example, the multi-small-seed strategy of the European *J. thurifera* populations yields a benefit since seed number can be directly translated into fitness and is negatively related to seed cost (Leishman 2001). Second, small seeds tend to be regurgitated less often and retained longer in the digestive tract of birds; therefore, 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 the latter are less likely to be found and consumed by predators (Gómez 2004). In addition, seeds of small-seeded species can also be buried more easily and escape from disturbances (Peco et al. 2003). Finally, a high inter-population gene flow (Teixeira et al. 2014) might prevent a divergence of seed packaging traits among the European *J. thurifera* population maintaining multi-small-seeded cones in Monegros. All of these hypotheses are not mutually exclusive and thus deserve further research.

Conclusions

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

Acknowledgments We are especially grateful to MD García-González for her valuable advice on nursery procedure and to R Heleno for the early review of the manuscript. We also thank D Caimel, M Esteve, L Lope and P Lorenzo for helping in the laboratory, and M Alifriqui for his help in the field work. The comments and suggestions of A Worley, KL Gross and three anonymous referees improved the manuscript.

Author contribution statement LDS and RT originally formulated the idea. LDS, SRE and CN conceived and designed the experiments. LDS and DT performed the experiments. LDS analyzed the data. All the authors wrote the manuscript.

Compliance with ethical standards

Funding This work was supported by Fundação para a Ciência e a Tecnologia (FCT) with the project MEDIATIC (PTDC/AAC-CLI/103361/2008). LDS and RT were supported by a postdoctoral fellowship from FCT (SFRH/BPD/70632/2010) and Spanish Ministry of Education (BVA 2010-0375) respectively.

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval statement This article does not contain any studies with human participants or animals performed by any of the authors.

Legal statement The experiments comply with the current laws of Portugal in which the experiments were performed.

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