

**Mediation of seed provisioning in the transmission of
environmental maternal effects in Maritime pine (*Pinus pinaster*
Aiton)**

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Abstract

While maternal environmental effects are increasingly recognized as an important source of phenotypic variation with relevant impacts in evolutionary processes, their relevance in long lived plants such as pine trees is largely unknown. Here, we used a powerful sample size and a strong quantitative genetic approach to analyze the sources of variation of early seedling performance and to indentify seed mass-dependent and -independent maternal environmental effects in Maritime pine. We measured seed mass of 8924 individual seeds collected from 10 genotypes clonally replicated in two environments of contrasting quality (favourable and stressful), and we measured seedling growth rate and biomass allocation to roots and shoots. Seed mass was extremely variable (up to 14-fold) and strongly determined by the maternal environment and the genotype of the mother tree. The favourable maternal environment led to larger cones, larger seeds and reduced seed mass variability. The maternal environment also determined the offspring phenotype, with seedlings coming from the favourable environment being 35% larger and with greater root:shoot ratio. Transgenerational plasticity appears, thus, to be a relevant source of phenotypic variation in the early performance of this pine species. Seed provisioning explained most of the effect of the maternal environment on seedling total biomass. Maternal environment effects on seedling biomass allocation were, however, determined through other seed mass-independent mechanisms, suggesting that other epigenetic regulation channels may be involved.

Key words: Conifer; Early seedling performance; Long-lived trees; Seed-mass variability; Seed mass; Seed weight; Transgenerational plasticity

1 INTRODUCTION

2 That mother plants can alter the phenotype of their offspring in response to the environmental
3 conditions where they grow has been reported in several species across a range of
4 environments (reviewed in Herman and Sultan, 2011). Increasing evidence is accumulating
5 that environmental maternal effects may be adaptive, enhancing the fitness of the offspring
6 when established under environmental conditions that resemble the maternal environment
7 (Galloway and Etterson, 2007; Herman and Sultan, 2011). This form of adaptive
8 transgenerational plasticity has been reported in response to several biotic and abiotic
9 environmental cues, including temperature (Yakovlev *et al.*, 2010), drought (Herman *et al.*,
10 2012), shade (Galloway and Etterson, 2007), nutrient availability (Kou *et al.*, 2011), salinity
11 (Boyko *et al.*, 2010), herbivory (Rasman *et al.*, 2012), or viral infection (Kathiria *et al.*,
12 2010). As a result of all these studies, environmental maternal effects are now recognized as a
13 relevant source of phenotypic variation that may play an essential role in local adaptations
14 (Herman and Sultan, 2011; Holeski *et al.*, 2012).

15 One of the most important transmission vehicles of maternal environmental effects is
16 via seed provisioning (Herman and Sultan, 2011). Environmental conditions and resource
17 availability may determine the amount of resources that the mother plants allocate to the
18 developing seeds, which, in turn, may affect seed mass thus shaping many traits in seedling
19 establishment and early growth, especially under stressful and competitive conditions (Castro
20 *et al.*, 2006; Metz *et al.*, 2010). Seed mass may be, thus, simply passively determined by the
21 available resources in the maternal environment, where impoverished conditions lead to
22 reduced seed mass, and thus, reduced seedling performance. However, maternal plants may
23 also actively respond to environmental stress by maintaining or even increasing seed mass, in
24 order to enhance the success rate of the inevitably reduced offspring number (Violle *et al.*,
25 2009). Additionally, responses of maternal plants to environmental conditions may affect not

1 only mean seed mass but also seed mass variation within individual plants (Halpern, 2005;
2 Violle *et al.*, 2009). Producing seeds of different size may be advantageous, especially in
3 heterogeneous or unpredictable environments, where the optimal seed size varies through
4 time and space (Charpentier *et al.*, 2012; Halpern, 2005).

5 Maternal environmental effects can also be transmitted by other mechanisms that are
6 not directly related to the amount of maternal resources allocated to seed provisioning, that is,
7 seed mass-independent mechanisms. Recent studies have shown that different environmental
8 factors may induce epigenetic changes in the mother trees that, transmitted through the seeds,
9 can alter gene activity in the offspring modifying its phenotype (Boyko and Kovalchuk, 2011;
10 Herman and Sultan, 2011). Transmission mechanisms of this seed mass-independent
11 transgenerational plasticity include DNA methylation, histone modification and changes in
12 small non-coding regulatory RNAs (Herman and Sultan, 2011). These epigenetic changes
13 may persist over the whole life cycle, and there is even evidence that they could be
14 transmitted across generations (Boyko *et al.*, 2010). In contrast, when maternal influence is
15 through the mediation of seed mass it tends to be more pronounced during the first stages of
16 seedling development, becoming less significant as the seedling ages (Elwell *et al.*, 2011).

17 Both seed mass-dependent and seed mass-independent maternal effects have interested
18 quantitative geneticists, evolutionary biologists and more recently genomic researchers, as
19 they are an uncontrolled source of phenotypic variation that could hamper the quantification
20 of true genetic variation (Bischoff and Mueller-Schaerer, 2010; Elwell *et al.*, 2011). Several
21 methods have been used to account for this potential bias in quantitative genetic studies.
22 Including seed mass as a covariate in the statistical analyses has been probably the most
23 frequent (Cendán *et al.*, 2012; Hereford and Moriuchi, 2005; Hovenden *et al.*, 2008) but this
24 method is generally insufficient to cover all maternal effects, as effects unrelated to seed
25 provisioning remain unaccounted for (Cendán *et al.*, 2012). Alternatively, maternal effects

1 may be controlled by growing parental genotypes in a common environment for at least one
2 generation prior to genetic testing (Bischoff and Mueller-Schaerer, 2010). However, this
3 would be inoperative for long-lived species with delayed reproduction, such as conifers.

4 The vast majority of previous studies about transgenerational plasticity in plants have
5 been focussed on annual plants, and very little information is available on the magnitude and
6 ecological relevance of this source of phenotypic variation in long-lived trees (but see Cendán
7 *et al.*, 2012; Yakovlev *et al.*, 2010). Conifers are, however, an interesting model for the study
8 of maternal effects (Yakovlev *et al.*, 2012). Conifer seeds contain a haploid endosperm of
9 maternal origin that directly transmits both resource reserves and epigenetic marks from the
10 mother to the offspring (Linkies *et al.*, 2010). The long-lasting period of seed maturation in
11 conifers (up to two years in the case of maritime pine) also broadens the window period
12 during which environmental cues can be imprinted into the seeds (Cendán *et al.*, 2012). On
13 the other hand, conifers have very large genome sizes with a large amount of non-coding
14 DNA that could play a relevant role in the gene regulation processes (Yakovlev *et al.*, 2012).
15 However, not all the particularities of conifer trees favour the expression of maternal effects.
16 Several particularities of their life history, such as the multiple reproductive cycles over their
17 lifetime, or the larger year to year environmental heterogeneity within their prolonged
18 lifespan, may largely alter the adaptive value and evolution of transgenerational plasticity in
19 conifers.

20 The aim of this study was to identify the sources of variation of seed mass and
21 offspring phenotype in a long-lived plant species, the Maritime pine (*Pinus pinaster* Aiton).
22 Our main goal was to determine whether the maternal environment influences the early
23 performance of the pine seedlings, and to disentangle at what extent these effects are mediated
24 by seed provisioning. We studied seed mass and early offspring phenotype of seeds collected
25 from ten genotypes of *P. pinaster* clonally replicated in two contrasting environments, one

1 with favourable conditions for the development of this pine species (in terms of growth and
2 reproduction rate), and the other one with less favourable conditions (see Supplementary
3 Material Table S1, SM-S1). Our experimental design allowed separating and quantifying the
4 influence of the maternal genotype, the maternal environment and their interaction on both
5 seed mass and early offspring phenotype. In particular, we were able to adequately separate
6 and compare seed mass-dependent and -independent maternal environment effects.
7 Preliminary studies with the same plant material showed significant maternal environment
8 effects on both the percentage and the timing of germination in this pine species (Cendán *et*
9 *al.*, 2012). Seed mass differences between maternal environments explained part but not all of
10 the observed germination patterns. Here we performed a more comprehensive study to
11 explore whether the maternal environment may also affect seedling performance, and the
12 extent to which these effects are mediated by seed mass differences.

14 MATERIALS AND METHODS

15 *Plant material*

16 Maritime pine is a widespread forest tree in Southwest Europe and North Africa. Due to its
17 rapid growth rate, this species is planted on a massive scale for timber production in
18 Mediterranean climates all over the world, with seeds commonly collected from clonal seed
19 orchards, i.e. plantations designed for seed production in which several clonal copies (ramets)
20 of a few selected genotypes are inter-crossed among each other. We took advantage of some
21 of these genetically controlled plantations and collected seeds in two clonal seed orchards
22 with exactly the same genetic material and spatial design but established in contrasting
23 environmental conditions. The two seed orchards were established within the Galician Tree
24 Breeding Program (Consellería do Medio Rural, Xunta de Galicia), and include 116 unrelated
25 *P. pinaster* genotypes selected for their superior growth, stem form and branching habit in

1 natural and planted stands within the Galician Coastal Population (NW Spain) (see Zas *et al.*,
2 2004 for details). Each selected genotype was clonally replicated by grafting scions on two-
3 year old seedlings. Within each seed orchard 10 ramets of each genotype were established
4 following a complete randomized design with 10 blocks and one ramet of each genotype per
5 block. Thus each seed orchard includes $116 \times 10 = 1160$ mother trees, and occupies around 3
6 ha. Environmental conditions strongly differ between the two seed orchards. The first
7 (Sergude 42° N, 8.45° W) is located in a site especially favourable for growing maritime pine,
8 with mild temperatures, adequate moisture all year round, and well drained, deep soils. The
9 second (Monfero, 43.52° N, 7.93°W) is located at the top of a hillside, with lower
10 temperatures, exposure to strong winds, and shallow soils (see SM Table 1 for more details).
11 Based on the large differences found in growth and reproduction rates between the two sites,
12 we will refer to Sergude as the *favourable* maternal environment, and to Monfero as the
13 *stressful* maternal environment.

14 ***Cone and seed sampling, processing and sowing***

15 For the present study we used 10 genotypes randomly selected among the 116 genotypes
16 included in the seed orchards. In January 2009 we collected 3-4 mature cones from 4-6 ramets
17 of each selected genotype within each maternal environment. Diameter at breast height was
18 measured in all selected mother trees (N = 103). Cones were randomly selected within the
19 crown of each sampled tree and collected using ladders and climbing tools. Damaged cones or
20 cones in damaged branches were avoided. A total of 373 cones were sampled. Cones were
21 opened by oven-drying at 35 °C, then weighed (± 0.001 g) and all seeds in each cone removed
22 and stored at 4 °C in labelled PET vials. The percentage of viable seeds in each cone was
23 determined by decantation in cold water, where empty seeds remained floating at the surface
24 (Cendán *et al.*, 2012). The number of filled and empty seeds and total seed mass per cone
25

1 were recorded after oven-drying the seeds a second time (7d, 35°C). A subsample of twenty-
2 four filled seeds per cone was randomly selected, individually weighted ($\pm 0.0001\text{g}$) and
3 stored at 4°C until sowing. In October 2009, 8924 seeds of known individual weight were
4 sown in vermiculite (0.5 – 2.0 mm coarse), using 96 wells sowing trays with 9 cm tall wells.
5 Trays were randomly disposed in a greenhouse with daily temperature fluctuating from 18 to
6 26°C. Germination and ontogenic development was followed every 2-3 days as in Lazcano *et*
7 *al.* (2010), and when the first set of true juvenile needles began to elongate, seedlings were
8 harvested and shoot (SDW) and root (RDW) dry weight (7d, 80°C) were determined. Total
9 dry weight (TDW) and root: shoot ratio (RSR) were derived from these values.

11 ***Statistical analyses***

12 Sources of seed mass variation were characterized by calculating the mean, minimum and
13 maximum, range of variation and coefficient of variation of seed mass at four levels: within
14 cones (N = 373), within individual mother trees (N = 103), within genotypes (N = 10), and
15 within maternal environments (N = 2).

16 For the analysis of cone weight we used a general linear mixed model with the PROC-
17 MIXED procedure of the SAS System (see results in SM Table S2 and Figure S1). We fitted a
18 hierarchical model similar to those used to solve a split-plot design with two levels of nested
19 experimental units (ramets and cones) in which measures from different cones within the
20 same ramet (3-4 sampled cones per ramet) were considered as repeated measures within the
21 same subject (Littell *et al.*, 2006). The effect of maternal environment (E) (the two twin seed
22 orchards) and the effect of block nested within each maternal environment (B(E)), which
23 account for the effect of spatial variation within each seed orchard, were the fixed effects of
24 the model. The maternal genotype (G) and the interaction between maternal environment and
25 maternal genotype ($G \times E$) were considered random factors. Micro-environmental variation at

scales lower than the block size and other phenotypic effects associated to individual ramets such as the effect of the rootstock, ontogeny or biotic interactions were accounted for by including the effect of individual ramets ($G \times B(E)$) as a random factor in the model. Diameter at breast height of each tree was also included as a fixed covariate in this model in order to account for possible allometric effects, but it was removed because it was not significant and did not improve the resolution of the model.

The effect of design factors on seed mass and seedling growth traits was also analyzed with a general mixed model, but in this case the hierarchical model fitted was similar to those used to solve a split-split design with three levels of nested experimental units (ramet, cones and seeds) (Littell *et al.*, 2006), in which values within each experimental level were considered repeated measures within the same subject. That is, measures from different seeds within a cone and measures from different cones within a ramet were assumed to be dependent measures within cones and ramets, respectively. Besides the factors included in the model described above, the mixed model also included the random effect of cones within each ramet (Cone ($G \times B \times E$)). Individual cone weight was included as a covariate in the analysis of seed mass, whereas covariation with germination time was considered in the analyses of seedling growth traits.

To discern the extent to which the effects of the maternal genotype and the maternal environment on seedling traits are mediated by seed provisioning effects we compare the results of the mixed models including and excluding the covariation of seed mass (SM) and its interaction with the maternal genotype ($SM \times G$) and the maternal environment ($SM \times E$). These last two terms account for the possible variation across genotypes and environments in the slopes of the covariation of seedling traits with seed mass.

We also analyzed whether the maternal environment affects variation in seed mass within individuals rather than mean seed mass. To this end we analysed the coefficient of

variation in seed mass within each maternal tree using a conventional general mixed model with the fixed effects E and B(E), and the random effects G and G×E. We used the coefficient of variation as the estimate of seed mass variation as it is less likely to be influenced as an artefact of variation in mean seed mass (Crean and Marshall, 2009).

The statistical significance of the variance components for each random factor in all statistical models was assessed using likelihood ratio tests, where the differences in two times the log-likelihood of the models including and excluding that random factor are distributed as one tailed χ^2 , with one degree of freedom (Littell *et al.* 2006). To estimate the percentage of total variation explained by each factor we repeated all analyses, considering all factors as random factors.

RESULTS

Sources of variation of seed mass

Maritime pine seed mass in the studied accessions was found to be extremely variable, ranging from 10 to 140 mg, with an overall mean of 66.7 mg and a coefficient of variation of 31 % (Table 1). Variability of seed mass was high both within individual trees and within cones, with some cones and some trees showing coefficients of variations higher than 20%, and more than 60 mg of differences between their lightest and heaviest seeds (Table 1). As well as the large within-individual variation, variation across individual trees was also very high, with mean seed mass spanning a sevenfold range of magnitude (from 15 to 116 mg, Table 1).

Seed mass significantly varied among genotypes and was highly influenced by the quality of the maternal environment (Table 2). Across genotypes, mean seed mass showed a twofold range of variation, ranging from 44.5 to 89.3 mg (Table 1). Across environments, seed mass was nearly 50% greater in the favourable environment than in the stressful

environment (Table 1). Seed mass significantly covaried with the weight of the cones which explained more than half of the variation of seed mass (Table 2). Cone weight was also highly variable among maternal environments and maternal genotypes, which together explained up to 70% of the total variation in cone weight (see Table S2 and Figure S1). Accordingly, the effect of both the maternal environment and the maternal genotype on the seed mass was much larger when the covariation with cone weight was not adjusted. The percentage of seed mass variation explained by these factors increased from 17.2 and 8.9% when accounting for cone weight covariation (Table 2) to 51 and 14% when excluding the covariable from the model, respectively. Accordingly, the reaction norms of the seed mass were clearer flatter and compressed when accounting for the covariation with cone weight (Figure 1). On the other hand, the lack of genotype by environment interaction (Table 2) indicates that the seed mass was significantly and similarly higher in the favourable maternal environment for all the 10 studied genotypes (Figure 1).

The maternal environment not only affected the mean seed mass, but also significantly modulated the within-individual variability in the seed mass ($F_{1,9} = 25.4$, $p < 0.001$; Table S4). Trees growing in the stressful maternal environment had a higher coefficient of variation in seed mass (14.5 ± 5.6 %) than trees growing in the favourable environment (10.8 ± 0.5 %). In contrast, the maternal genotype did not affect the variation in seed mass ($\chi^2_1 = 0.2$; $p = 0.327$), and neither there was interaction between the genotype and the maternal environments ($\chi^2_1 = 0.8$; $p = 0.186$; Table S4).

Sources of variation of offspring phenotype

Without accounting for seed mass covariation, both the maternal environment and the maternal genotype significantly affected the total dry mass and the root:shoot ratio of the resulting offspring (Table 3, left). A significant interaction was also observed in the case of

seedling total dry mass. Seedlings coming from the favourable maternal environment were 35% larger than those coming from the stressful maternal environment and showed a larger root:shoot ratio (Figure 2, triangles).

However, the variation among maternal genotypes became non-significant for both variables when the seed mass was considered as a covariate in the statistical models, whereas that of the maternal environment was strongly reduced for the total dry weight (Table 3, right; circles in Figure 2a). The covariation with seed mass was significant for the two seedling traits and especially strong in the case of the total dry weight. The significant SM×E and SM×G interactions (Table 3, right) also indicate that the slope of the covariation significantly differed among maternal environments and maternal genotypes. In the case of seedling total dry weight, the slope was significantly steeper for seeds coming from the stressful maternal environment (6.38 ± 0.30) than for seeds from the favourable maternal environment (5.29 ± 0.23) ($F_{1,1958} = 7.5$, $p = 0.006$; Figure 3). When the statistical model accounted for all these covariation terms, the significant effect found for the maternal environment (Table 3, right) indicates that the intercept of these relationships also significantly differed between maternal environments (Figure 3).

The covariation of the root:shoot ratio with the seed mass was weaker than for the total seedling dry weight and the slope of the covariation did not differ between maternal environments (no significant SM×E interaction) (Table 3). The effect of the maternal environment not only remained highly significant when accounting for seed mass covariation (Table 3, right) but it was also even higher (around 10% greater RSR in the favourable environment than in the stressful environment) than when seed mass covariation was not considered (5% greater RSR in the favourable environment, Figure 2b).

DISCUSSION

Seed mass variation

Because seed mass can considerably affect fitness (reviewed in Castro *et al.*, 2006), classical life history theory predicts that it should be under strong stabilizing selection and show little variation within populations (Silvertown, 1989). However, many empirical observations do not fit with this prediction, and the range of variation in seed mass within populations is usually very high and even greater than that across populations (Susko and Lovett-Doust, 2000). Our results are consistent with those findings and indicate an extremely high variation (up to 14-fold) in seed mass both across and within individual trees (Table 1). Several non-exclusive explanations have been proposed to explain the maintenance of intrapopulation variation in seed mass. First, through modulating the resources that the maternal plant can allocate to the seeds, the maternal environment can be an important source of seed mass variation (Donohue, 2009). Our results indicate a strong effect of the maternal environment on seed mass with seeds coming from the favourable environment being around 50% heavier than those coming from the stressful environment. This environmental effect was consistent for all the studied genotypes and explained more than half of the overall variation in seed mass. Our study also indicates considerable variation in seed mass across individuals of the same genotype within each environment, suggesting that microenvironmental variation and other phenotypic effects related to each single phenotype such as ontogenetic development or biotic interactions may also be important sources of seed mass variation. Additionally, allocation of resources to seeds can also vary among cones according to their position within the crown (Chanyenga *et al.*, 2011). The observed large variation in seed mass among cones and the strong relationship between mean seed mass and cone weight ($r^2 = 0.76$, $p < 0.001$, $N = 373$) may be reflecting these positional effects. However, environmental and genotypic effects on seed mass remained highly significant when accounting for covariation with cone weight, indicating that the maternal environment and the maternal genotype control the

variation in seed mass irrespective of the amount of resources allocated to the cone. Taking all these results together, we can conclude that seed mass in this pine species is strongly determined by the environmental conditions where the mother tree grows, and this may have important consequences for offspring development (see discussion below). Additionally, the observed genetic variation in seed mass may allow the evolution of this life history trait according to environmental conditions, providing that it affects offspring seedling performance, that the effect differs among maternal environments, and that a significant part of the genetic variation is heritable. Further research analyzing parent-offspring regression and/or family trails should determine the amount of additive variation within the observed genetic variation in seed mass in this pine species. Because seed mass plays a relevant role in many important life history processes, many studies have focussed on determining the sources of seed mass variation in different plant species, but the effects of the maternal environment and the maternal genotype have not always been adequately separated (Blodner *et al.*, 2007; Guo *et al.*, 2010), and when they have been (Galloway *et al.*, 2009; Halpern, 2005; House *et al.*, 2010) they have usually referred to annual plants (but see Stoehr *et al.*, 1998). Results are very variable. Some authors only found effects of the maternal environment (e.g. Galloway *et al.*, 2009), while others have found the opposite (e.g. Halpern, 2005). Most frequently, however, both factors have been found to significantly affect mean seed mass (e.g. Elwell *et al.*, 2011; Stoehr *et al.*, 1998). Our results agree with these studies, and clearly show a strong genetic component in seed mass as well as a large influence of the maternal environment (Table 2, Figure 1). Interestingly, the lack of a significant G×E interaction indicated that the effect of the maternal environment was similar for all the studied genotypes. However, the lack of genetic variation in plasticity should be interpreted with care as the studied genotypes were selected by the same criteria and shared a common phenotype. These genotypes do not represent, therefore, all the potential variation within the studied population.

Maternal environmental effects on seed mass variability

Our results indicate that the maternal environment not only affected the mean seed mass but also it significantly modulated the variability of seed mass within individual trees, with larger variation in trees growing in the stressful environment. These results are consistent with previous findings in which reduced resource availability has been associated with increased within-individual variability in seed mass (Halpern, 2005). Increased variability in seed mass may enhance the likelihood of establishing in a wider range of environmental conditions, and it has been proposed that this is an adaptive strategy that may increase mother fitness in heterogeneous or unpredictable environments (Crean and Marshall, 2009; Charpentier *et al.*, 2012). The opposite has also been reported, however. Under conditions of limited resources, mother plants may benefit from prioritizing a single optimum seed size that maximizes the success of the seedlings under the stressful conditions where they will establish (Violle *et al.*, 2009). In both cases variability in seed mass could be interpreted as a parental trait per se that may respond to selection according to the environmental conditions (Moles and Westoby, 2006). In this sense, the lack of genetic variation in seed mass variability observed here would imply a potential constraint on the evolution of this trait. Seed mass variability therefore seems to be a plastic trait controlled by environmental conditions alone, in accordance with results found by other studies in annual plants (Castellanos *et al.*, 2008).

Variability in seed mass may also arise due to other causes with no adaptive significance (Castellanos *et al.*, 2008; Halpern, 2005). Under stressful environmental conditions, developmental instability may limit the ability to equally provision all seeds due to constraints in resource allocation to seeds (Castellanos *et al.*, 2008). Our results do not indicate whether the observed differences in seed mass variability are merely a passive response to the environment or it is under other kind of control by the mother trees; to draw

conclusions about its adaptive value would require analysis of the fitness consequences of seed mass variability for the mother trees.

Maternal environment and seed mass effects on seedling performance

Our results indicated a strong effect of the maternal environment on offspring performance. Without accounting for seed mass covariation, seedlings derived from seeds from the favourable maternal environment showed both higher aboveground and belowground biomass. These results agree with other studies reporting larger offspring phenotypes of seeds coming from favourable maternal environments (Castro *et al.*, 2006; Leishman *et al.*, 2000). However, whether this maternal environmental effect is merely an indirect consequence of seed mass differences between environments or whether it may be an active transgenerational response mediated by other epigenetic mechanisms remains an unresolved question (Boyko and Kovalchuk, 2011; Cendán *et al.*, 2012; Hereford and Moriuchi, 2005). Our data shows that, when the covariation with seed mass is properly accounted for in the statistical model, the effect of the maternal environment on offspring biomass, although still significant, was strongly reduced (see Table 3). This result suggests that most of the transgenerational maternal effects on seedling size were mediated by effects related to seed provisioning. The observed differences in seedling total dry mass between maternal environments were, thus, derived principally from the large differences in seed mass between environments. However, our results also indicated that the influence of seed mass on seedling biomass differed between the seeds collected in the two maternal environments, with a strong and steeper seed-to-seedling biomass relationship for seeds from the stressful environment (Figure 3). That the benefits of heavier seeds are higher under stressful conditions has been demonstrated for different plant species (Metz *et al.*, 2010), but the key finding of our analyses is that the seed-to-seedling relationship was determined by the maternal environment rather than by the

environmental conditions where the seeds are grown, which, in our study, were similar for all seedlings. Two non exclusive mechanisms may explain these results. First, because of the large environmental differences between the two maternal sites, the quality of the resources allocated to the seeds may strongly differ between them (Violle *et al.*, 2009). Bigger seeds from the two environments may, thus, differ in their chemical reserve composition, and this seed quality may impact seedling performance. However, the fact that the relationship between seed mass and seedling biomass was steeper for the resources-deprived maternal environment reduces the likelihood of this argument. Alternatively, even though seed provisioning could determine most of quantitative phenotypic changes, epigenetic mechanisms regulated by the environmental conditions of the maternal plants and differentially expressed depending on the seed mass may also occur (that is, interactive effects between seed provisioning and epigenetic transgenerational programming). Those seed mass-dependent epigenetic mechanisms, i.e. a mixture of heritable and non-heritable maternal effects (sensu Boyko and Kovalchuk, 2011), are feasible whenever epigenetic regulation is resource-dependent. Other authors have also found a significant seed mass \times maternal environment interaction on seedling growth, but they did not study the responsible mechanisms (Hovenden *et al.*, 2008). Nevertheless, irrespective of the involved mechanisms, our results suggest that the maternal environment, rather than directly affecting the seedling total dry mass, modulates the extent to which the seedling phenotype depends on the seed mass, that is, a regulatory maternal effect.

The maternal environment not only affected total seedling dry mass, but also modulated the allocation of biomass to shoots and roots, with seedlings from the stressful maternal environment prioritizing relative biomass allocation to aerial parts. However, in contrast with what occurred with total seedling biomass, this effect was not related to differences in seed provisioning. In accordance to previous studies (Hovenden *et al.*, 2008),

1 when considering the whole data set we found that seed mass was negatively correlated with
2 seedling RSR, with the smaller seeds leading to seedlings with greater RSR. However, when
3 considering the effect of the maternal environment, we found that the stressful environment
4 strongly determined a lower seedling RSR, even when the seed mass in that environment was
5 the smaller. In consequence, the effect of the maternal environment on seedling biomass
6 allocation was even more pronounced when the statistical model properly accounted for the
7 seed mass covariation (Figure 2b). Thus, as reported for germination traits in this pine species
8 (Cendán *et al.*, 2012), the effect of the maternal environment on seedling biomass allocation
9 appeared to be mediated by seed mass-independent mechanisms.

10 Previous studies have also shown that the maternal abiotic environment can modulate
11 seedling biomass allocation (Hovenden *et al.*, 2008; Sultan *et al.*, 2009). Warmer maternal
12 environments have been found to produce offspring with greater root:shoot ratio in a grass
13 species (Hovenden *et al.*, 2008). The two maternal environments of the present study not only
14 differed, however, in the temperature regime but also had extremely different edaphic
15 properties (Cendán *et al.*, 2012), with the warmer environment also having the best quality
16 soils. According to this, one could expect that seedlings coming from the stressful
17 environment would benefit from prioritizing allocation to roots rather than to shoots in order
18 to favour the uptake of the limited edaphic resources of this environment (Moles and
19 Westoby, 2006; Sultan *et al.*, 2009), whereas those coming from the favourable environment
20 would benefit from prioritizing the growth of the aerial part in order to increase competitive
21 ability for light (Galloway and Etterson, 2007). The observed results, however, point in the
22 opposite direction. Different species, even closely related congeners, are known to show
23 different transgenerational plasticity in response to a particular maternal environment stress,
24 and these plastic responses do not always show an adaptive value (Sultan *et al.*, 2009).
25 Further studies, controlling the environmental factors responsible of the maternal stress and

using reciprocal cross transplanting experiments, are needed to explore the adaptive value of these transgenerational responses in this pine species.

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Figure Captions

Figure 1. Reaction norms for the mean seed mass of ten genotypes clonally replicated in two contrasting maternal environments. Best linear unbiased predictors (BLUPs) as obtained from the corresponding mixed model (a) without considering the cone weight as a covariate and (b) adjusting for the covariation with the cone weight.

Figure 2. Total dry weight (a) and root:shoot ratio (b) of the offspring of ten genotypes clonally replicated in two contrasted environments. Least square means (\pm s.e.) obtained from the mixed models including (circles) and excluding (triangles) the seed mass (SM) as a covariate are presented.

Figure 3. Linear relationship between the seed mass and the seedling total dry weight for the two maternal environments as obtained from a heterogenous slope covariation mixed model. The slopes significantly differ, with a steeper relationship for seeds coming from the stressful maternal environment, and the intercept also significantly differed between maternal environments.

Table 1. Basic statistics of seed mass variation (Overall variation) and range of variation (min-max) of seed mass basic statistics within cones, within ramets, within genotypes, and within each maternal environment (stressful and favourable).

	Overall seed mass	Within-cone range	Within-ramet range	Within-genotype range	Seed mass	
					Stressful	Favourable
N	8924	373	103	10	1	1
Mean (mg)	66.7	13.7 - 128.8	15.0 - 116.5	44.5 - 89.3	53.2	79.1
Minimum (mg)	10	10 -117	10 - 86	10 - 35	10	31
Maximum (mg)	140	17- 140	21 - 140	83 - 140	103	140
Range (mg)	130	7 - 61	11 - 65	67 - 105	93	109
Coefficient of variation (%)	31.4	4.4 - 22.9	6.8 - 20.2	20.2 - 38.0	29.1	22

4

Table 2. Sources of variation of mean seed mass for ten genotypes clonally replicated in two contrasting maternal environments. Degrees of freedom (DF) and *F*-ratios are shown for fixed effects; variance components (VarComp \pm s.e.) and associated χ^2 values showing their statistical significance are shown for random factors. Significant ($P < 0.05$) *P* values are typed in bold. The Variance component (%) column shows the percentage of total variation explained by each factor as obtained from a model in which all categorical factors are considered random¹.

Effect	Seed mass			
	DF or VarComp	<i>F</i> or χ^2	<i>P</i> value	Variance components (%)
Fixed factors				
Maternal environment [E]	1, 9	84.1	<0.001	17.2
Block(E) ²	15, 69	1.7	0.064	0.8
Cone weight	1, 8389	228.3	<0.001	58.1
Random factors				
Mother genotype [G]	57.2 \pm 29.3	13.0	<0.001	8.9
G \times E	1.0 \pm 4.0	0.1	0.376	0.0
Ramet [G \times B(E)]	28.5 \pm 6.0	107.3	<0.001	4.7
Cone(E \times G \times B) ²	19.6 \pm 1.9	1788.0	<0.001	3.2
Residual	43.7 \pm 0.7			7.1

¹ The percentage explained by the covariable (cone weight) was determined as the reduction of the total variance when including the covariate in the model.

² Block was nested within seed orchards, and cones were nested within ramets [G \times B(E)]

Table 3. Results of the general mixed models for the analysis of seedling total dry weight (a) and seedling root:shoot ratio (b) without accounting for seed mass (SM) covariation (left) and accounting for seed mass covariation (right). Degrees of freedom (DF) and *F*-ratios are shown for fixed effects; variance components (VarComp \pm s.e.) and associated χ^2 values showing their statistical significance are shown for random factors. Significant ($P < 0.05$) *P* values are typed in bold.

Variable Effect	Without accounting for SM covariation			Accounting for SM covariation		
	DF or VarComp	<i>F</i> or χ^2	<i>P</i> value	DF or VarComp	<i>F</i> or χ^2	<i>P</i> value
<i>a) Total seedling dry weight</i>						
Fixed factors						
Maternal environment [E]	1, 9	45.3	<0.001	1, 9	6.3	0.033
Block(E) ^a	15, 68	2.1	0.018	15, 68	1.5	0.140
Germination date	1, 1970	157.1	<0.001	1, 1958	246.1	<0.001
Seed mass (SM)				1, 1958	832.8	<0.001
SM \times E				1, 1958	7.5	0.006
SM \times G				9, 1958	3.2	<0.001
Random factors						
Genotype of the mother tree [G]	6225 \pm 3373	8.5	0.002	0	0.0	0.500
G \times E	1068 \pm 816	4.9	0.013	162 \pm 286	0.0	0.500
Ramet [G \times B(E)]	2586 \pm 563	89.5	<0.001	1245 \pm 280	80.3	<0.001
Cone(E \times G \times B)	1298 \pm 198	110.0	<0.001	590 \pm 120	47.2	<0.001
Residual	6005 \pm 191			4633 \pm 148		
<i>b) Seedling root shoot ratio</i>						
Fixed factors						
Maternal environment [E]	1, 9	5.5	0.044	1, 9	9.8	<0.001
Block(E) ^a	15, 68	1.3	0.202	15, 68	1.4	0.194
Germination date	1, 1970	19.9	<0.001	1, 1959	16.5	<0.001
Seed mass (SM)				1, 1959	4.4	0.037
SM \times E				- ¹	-	-
SM \times G				9, 1959	2.3	0.015
Random factors						
Genotype of the mother tree [G]	0.15 \pm 0.11	3.2	0.037	0	0.0	0.500
G \times E	0	0.0	0.500	0	0.0	0.500
Ramet [G \times B(E)]	0.21 \pm 0.15	2.4	0.061	0.26 \pm 0.16	3.6	0.029
Cone(E \times G \times B)	0.87 \pm 0.2	31.5	0.000	0.79 \pm 0.20	27.2	<0.001
Residual	8.40 \pm 0.27			8.41 \pm 0.27		

¹ The SM \times E term was excluded from the model as it was not significant ($F_{1,1958} = 0.0$, $p = 0.982$) and did not improve the resolution of the model.

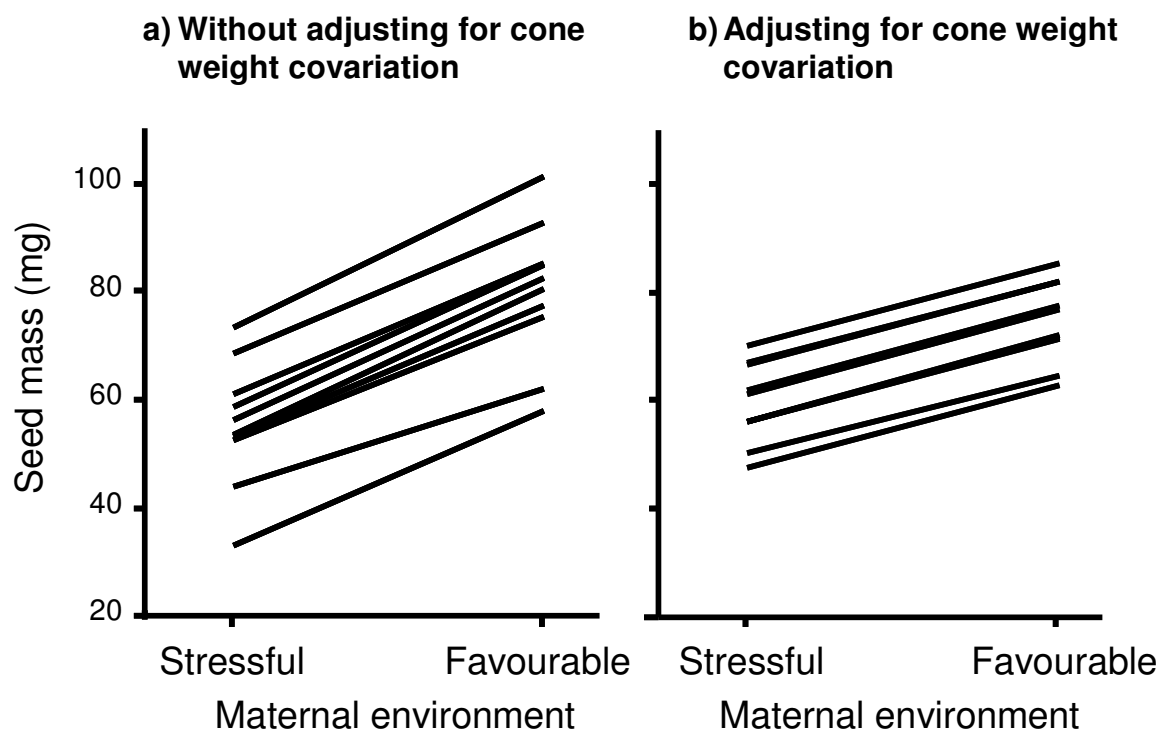


Figure 1

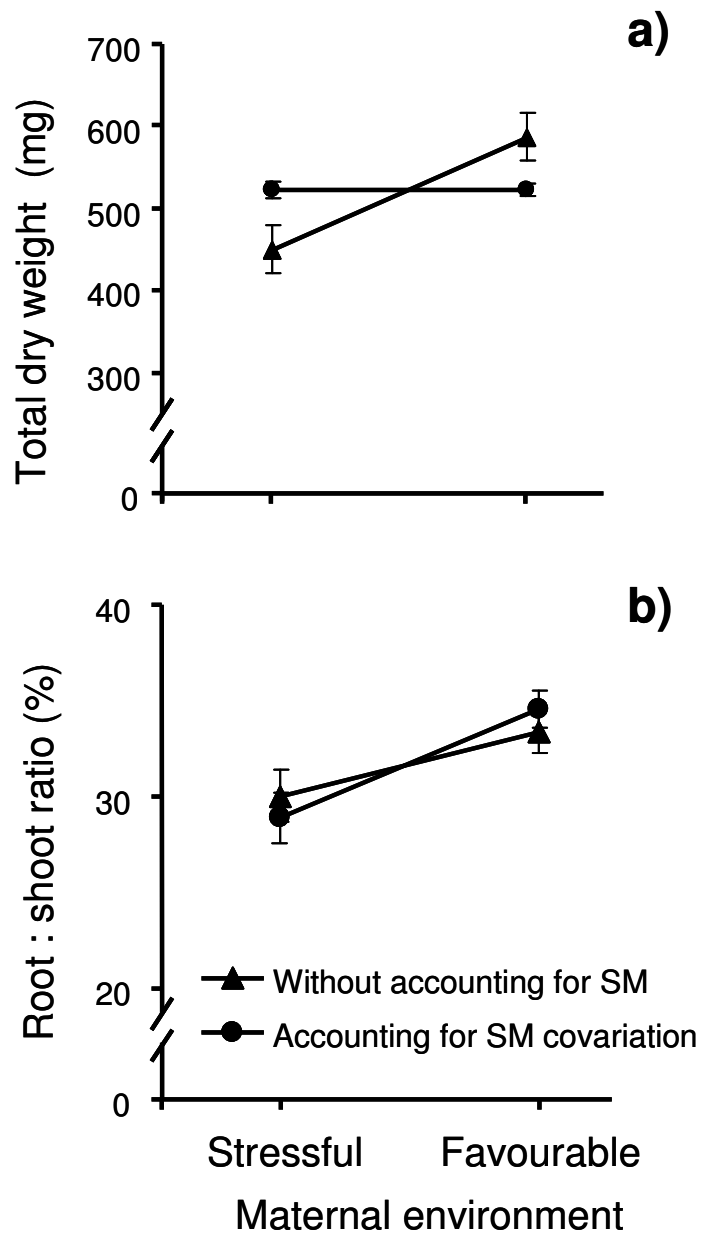


Figure 2

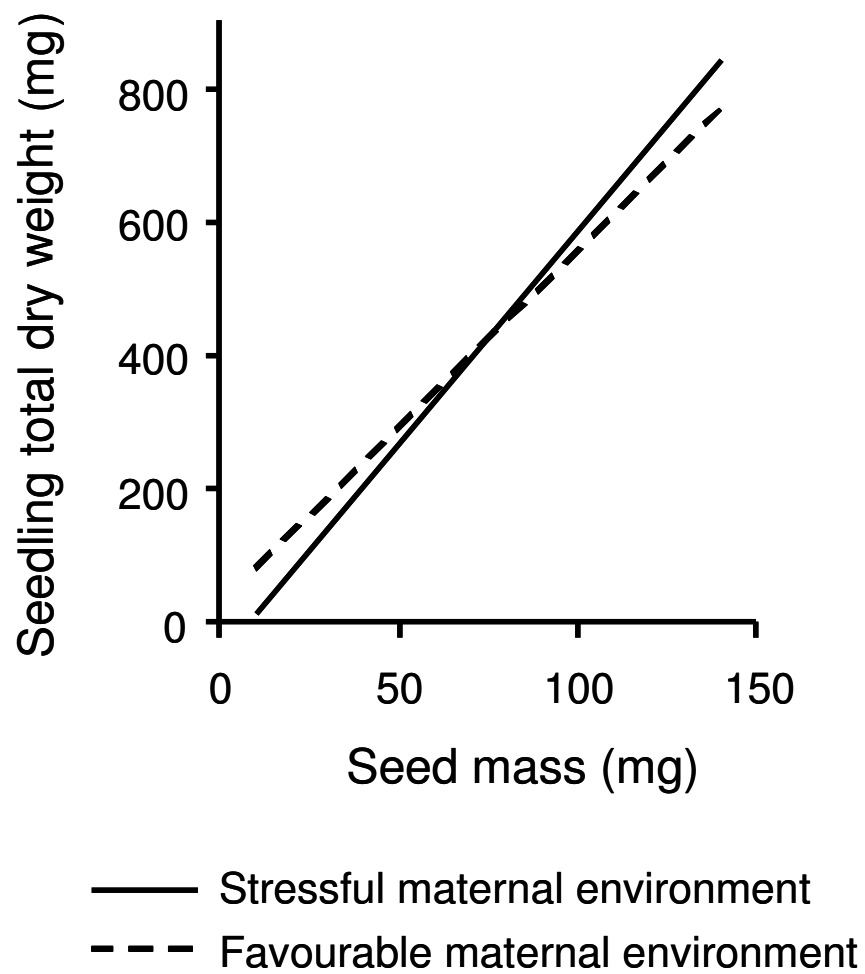


Figure 3

Supplementary Material

Mediation of seed provisioning in the transmission of environmental maternal effects in a pine tree.

Rafael Zas, César Cendán, Luis Sampedro

SM Table S1. Climatic, edaphic and dasometric characteristics of the two maternal environments of contrasting site quality, the high (Sergude), and the low quality clonal seed orchards (Monfero).

	Monfero	Sergude
Altitude (m)	615	258
Mean annual temperature (°C)	10.6	13.2
Maximum temperature (°C)	35.3	38.7
Minimum temperature (°C)	-8.0	-4.7
Mean temperature of the warmest month (°C)	15.5	19.2
Mean temperature of the coldest month (°C)	5.9	7.8
Number of frost-free months ^a	3	5
Annual precipitation (l m ⁻²)	1435	1445
Daily average wind speed (m s ⁻¹)	5.2	3.2
Number of windy days per year ^b	166	35
Soil pH (H ₂ O 1:2.5)	4.5	5.1
Soil depth (cm)	45.1 ± 3.2	> 120 cm
Tree age at sampling	20	27
Mean tree diameter at breast height (cm)	6.1 ± 0.3	20.9 ± 0.6
Annual individual tree growth in basal area (cm ² yr ⁻¹)	1.65 ± 0.15	13.6 ± 0.8
Number of cones per tree at age 9	3.8 ± 0.2	76.5 ± 11.0
Reproductive allocation (cones dm ⁻²) ^c	15.8 ± 2.8	47.8 ± 4.1

^a *sensu* Emberger et al. (1963) i.e. the period during which the average minimum temperature is over 7° C

^b average wind speed > 5 m s⁻¹

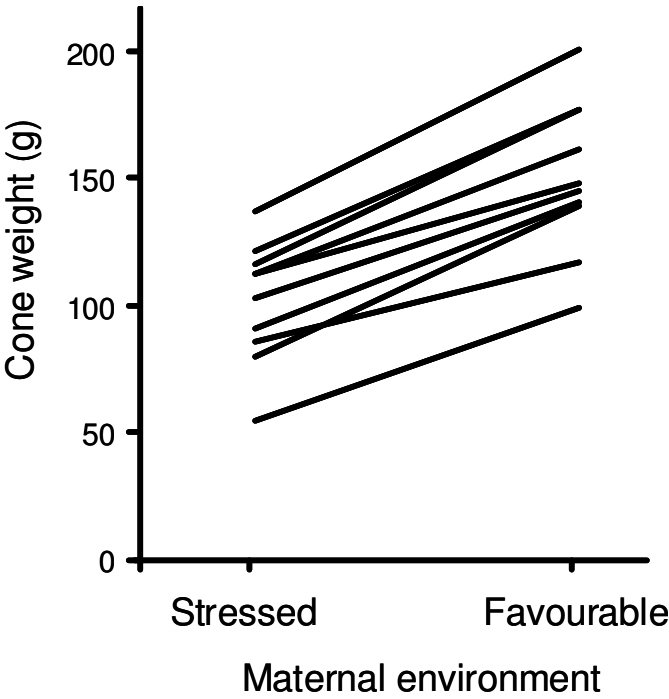
^c Number of cones per unit of basal area at breast height (modified from Climent et al., 2008)

SM Table S2. Sources of variation of mean cone weight for ten genotypes clonally replicated in two contrasting maternal environments. Degrees of freedom (DF) and *F*-ratios are shown for fixed effects; variance components (VarComp) and associated χ^2 values showing their statistical significance are shown for random factors. Significant ($P < 0.05$) *P* values are typed in bold. The Variance component (%) column shows the percentage of total variation explained by each factor as obtained from a model in which all factors are considered random.

Effect	Cone weight			
	DF or VarComp	<i>F</i> or χ^2	<i>P</i> value	Variance components (%)
Fixed factors				
Maternal environment [E]	1, 9	43.5	<0.001	46.9
Block(E) ¹	15, 69	1.9	0.032	0.9
Random factors				
Genotype of the mother tree [G]	692.0 ± 385.2	7.4	0.003	23.6
G × E	115.4 ± 109.8	2.3	0.065	1.4
Ramet [G × B(E)]	397.9 ± 95.2	62.5	0.000	14.7
Residual	405.0 ± 35.6			12.4

¹ Block was nested within seed orchards, and cones were nested within ramets [G × B(E)]

SM Figure S1. Reaction norms for the mean cone weight of ten genotypes clonally replicated in two contrasting maternal environments. Best linear unbiased predictors (BLUPs) as obtained from the corresponding mixed model (See Table SM2) are shown.



SM Table S4. Results of the general mixed model for the analysis of the coefficient of variation in seed mass within maternal individuals of ten genotypes clonally replicated in two contrasting maternal environments. Degrees of freedom (DF) and *F*-ratios are shown for fixed effects; variance components (VarComp \pm s.e.) and associated χ^2 values showing their statistical significance are shown for random factors. Significant ($P < 0.05$) *P* values are typed in bold.

Individual variation in seed mass			
Effect	DF or VarComp	<i>F</i> or χ^2	<i>P</i> value
Fixed factors			
Maternal environment [E]	1, 9	25.4	<0.001
Block(E) ^a	15, 69	0.9	0.614
Random factors			
Mother genotype [G]	0.34 \pm 0.82	0.2	0.327
G \times E	0.73 \pm 1.02	0.8	0.186
Residual	6.71 \pm 1.15		

SM References

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