

**1Heritability of seed weight in Maritime pine, a relevant trait in the transmission  
2of environmental maternal effects**

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## 23Abstract

24Quantitative seed provisioning is an important life-history trait with strong effects on  
25offspring phenotype and fitness. As for any other trait, heritability estimates are vital for  
26understanding its evolutionary dynamics. However, being a trait in between two generations,  
27estimating additive genetic variation of seed provisioning requires complex quantitative  
28genetic approaches for distinguishing between true genetic and environmental maternal  
29effects. Here, using Maritime pine as a long-lived plant model, we quantified additive genetic  
30variation of cone and seed weight (SW) mean and SW within-individual variation. We used a  
31powerful approach combining both half-sib analysis and parent-offspring regression using  
32several common-garden tests established in contrasting environments to separate G, E and  
33G×E effects. Both cone weight and SW mean showed significant genetic variation **but were**  
34**also influenced** by the maternal environment. Most of the large variation in SW mean was  
35attributable to additive genetic effects ( $h^2 = 0.55 - 0.74$ ). SW showed no apparent G × E  
36interaction, particularly when accounting for cone weight covariation, suggesting that the  
37maternal genotypes actively control the SW mean irrespective of the amount of resources  
38allocated to cones. Within-individual variation in SW was low (12%) **relative** to between-  
39individual variation (88%), and showed no genetic variation but was largely affected by the  
40maternal **environment**, with greater variation in the less favourable sites for pine growth. In  
41summary, results were very consistent between the parental and the offspring common  
42garden tests, and clearly indicated heritable genetic variation for SW mean but not for within-  
43individual variation in SW.

44

45**Keywords:** Additive genetic variation, Conifer, Transgenerational plasticity, Long-lived  
46plants, Phenotypic plasticity, *Pinus pinaster*, Quantitative genetics, Seed weight variability,  
47Seed provisioning, Seed mass

## 48Introduction

49Seed weight (SW) is an important life-history trait with strong effects on offspring phenotype  
50and ultimately on fitness. The influence of SW on germination (Norden *et al.*, 2009), early  
51growth (Hanley *et al.*, 2007), survival (Metz *et al.*, 2010), abiotic stress tolerance (Gomaa  
52and Xavier Pico, 2011), and biotic resistance (Solla *et al.*, 2011) of the offspring is well  
53documented for many different plant species, including conifer trees (Sorensen and  
54Campbell, 1993; Surlis *et al.*, 1993; Castro, 1999; Wennstrom *et al.*, 2002; Parker *et al.*,  
552006; Blade and Vallejo, 2008). Given the relevance of SW for offspring fitness, it could be  
56expected that SW would show little variation within populations as a result of stabilizing  
57selection forces (Silvertown, 1989). The scientific literature is, however, replete of empirical  
58studies reporting large **intraspecific** variation in SW (reviewed in Castro *et al.*, 2006). Both  
59maternal environmental effects (Roach and Wulff, 1987) and the size-number trade-off  
60(Sadras, 2007) have been repeatedly identified as relevant factors maintaining intraspecific  
61variation in SW. However, many other factors can all also contribute, including parental-  
62offspring (de Jong *et al.*, 2011) or paternal-maternal (Willi, 2013) conflicts in the optimum  
63seed size, sibling competition within plants or fruits (Banuelos and Obeso, 2003), temporal  
64and spatial instability of the optimum size (Charpentier *et al.*, 2012), limited resources  
65constraining the ability of plants to control individual seed size (Vaughton and Ramsey,  
661998), and conflicting selective pressures acting on SW depending on the phase of  
67recruitment (Gomez, 2004; Lazaro and Traveset, 2009). This unpredicted intraspecific  
68variation has driven an interesting field of research in recent decades.

69       The theoretical prediction of reduced additive variance in SW within populations  
70arises because natural selection is expected to eliminate genotypes with SW departing from  
71the optimum for each environment. But in order to evolve in such a way, phenotypic  
72variation in SW must have a genetic basis, and this genetic variation must be heritable

73(Silvertown, 1989; Sadras, 2007). Separating the environmental and genetic effects in seed  
74traits is, however, a complicated task (Zas *et al.*, 2013). Seed weight is a complex trait that  
75bridges the transition between plant generations. Seeds include tissues of both maternal and  
76embryonic origin, with triploid (or maternal-haploid in gymnosperms) and diploid genetic  
77material. Because of their close connection with the mother plant, seeds are influenced not  
78only by their own genotype (House *et al.*, 2010), but also by the maternal genotype  
79(Schwaegerle and Levin, 1990; Platenkamp and Shaw, 1993; Wolfe, 1995), and by the  
80environmental conditions where the mother plant has grown (Roach and Wulff, 1987). The  
81maternal environmental effect has often been confounded by the effect of the maternal  
82genotype (Vaughton and Ramsey, 1998; Susko and Lovett-Doust, 2000; Voeller *et al.*, 2012;  
83Sober and Ramula, 2013), which, in turn involves both nuclear and extranuclear effects  
84(Lipow and Wyatt, 1999) and can differentially affect the different seed tissues because of  
85their different genetic contributions (Lacey *et al.*, 1997). Accurate quantification of additive  
86genetic variation -essential for understanding the evolution of SW- will thus require complex  
87quantitative genetic approaches that allow the relative genetic and environmental maternal  
88effects to be distinguished (Mazer, 1987). Such experimental approaches are difficult to  
89implement, especially in long-lived plants such as conifer trees (Zas *et al.*, 2013), and this  
90may explain why there are no previous studies reporting narrow-sense heritability for seed  
91traits in long-lived trees, in which these confounding factors are properly accounted for.  
92Previous studies with conifer trees have reported total genetic variation for seed traits  
93(without distinguishing additive and non-additive variance) but failed to differentiate the  
94effect of the maternal genotype from that of the maternal environment (Matziris, 1998;  
95Castro, 1999; Roy *et al.*, 2004; Bilir *et al.*, 2008; Carles *et al.*, 2009). Taking advantage of a  
96long-term experimental device of common garden tests established within a tree breeding

97programme, in the present paper we were able to properly differentiate these factors and  
98accurately estimate narrow-sense heritability of seed traits of Maritime pine.

99       The strong plasticity and the low heritability estimates of SW observed in wild  
100populations of several species have motivated some authors to question the idea of stabilizing  
101selection on SW (Silvertown, 1989). However, a more recent [meta-analysis](#) found  
102considerable heritability estimates for SW in many herbaceous crops, despite the fact that  
103domestication and breeding are supposed to have led to a reduction in the available genetic  
104variation (Sadras, 2007). This apparent contradiction between wild populations and crops  
105may arise from the difficulties in accurately estimating the heritability of wild populations in  
106which many confounding factors (e.g. maternal effects, G×E interaction) can operate.  
107Additionally most of the studies have focused on the [heritability of SW mean](#), and neglected  
108the within-individual variation in SW which in many cases can be as high as or even higher  
109than between-individual variation (Holland *et al.*, 2009). Within-individual variation in SW  
110has, in fact, been considered a fitness-correlated trait, as SW variability may provide fitness  
111benefits in heterogeneous or unpredictable environments, providing a buffer against  
112environmental variability and constituting a form of bet-hedging (Charpentier *et al.*, 2012).  
113When the temporal or spatial environmental heterogeneity impedes to settle an optimum seed  
114size, producing variable seed sizes could be an advantageous strategy that maximizes the  
115fitness of the mother plant. Again, heritable variation in this trait should exist in order to  
116allow evolutionary responses to environmental selective pressures. However, very few  
117previous studies have quantified the heritability of within-individual variation in SW  
118(Halpern, 2005; Castellanos *et al.*, 2008), and none in long-lived woody plants.

119       Recently, analyzing two clonal seed orchards established in contrasting environmental  
120conditions, we have found the SW of Maritime pine to be extremely variable, and highly  
121influenced by both the maternal environment and the maternal genotype (Zas *et al.*, 2013).

122Seed weight also influenced offspring performance mediating the transmission of both  
123genetic and environmental maternal effects (Zas *et al.*, 2013). In this study we went a step  
124further by aiming (i) to determine the extent to which the observed genetic variation in SW is  
125heritable, and (ii) whether there is a genetic correlation between SW and adult tree growth  
126potential. Here, we analyzed mean SW and individual seed-weight variation of the open-  
127pollinated progenies of the genotypes included in those clonal seed orchards analyzed in the  
128previous paper (Zas *et al.*, 2013). This allowed us to estimate narrow-sense heritability using  
129both half-sib analysis and parent-offspring regression, using in both cases several common  
130garden tests established in contrasting environmental conditions to disentangle G, E and G×E  
131effects. Results will help to understand the adaptive value and evolutionary dynamics of these  
132interesting traits.-

133

#### 134Material and Methods

##### 135Experimental approach

136To address our objectives we took advantage of different genetic trials established within the  
137frame of the breeding programme of Maritime pine in Galicia (NW Spain, Consellería de  
138Medio Rural, Xunta de Galicia). Within that programme, unrelated superior genotypes ('*plus*  
139trees' with outstanding phenotypes in terms of growth, stem form and branching habit for  
140timber production) were selected in the early 80's from an extensive field survey in natural  
141and man-made plantations within the Spanish North West Coastal provenance of Maritime  
142pine. Open-pollinated seeds and scions collected from those plus trees were used for the  
143establishment of a series of open-pollinated progeny trials and two twin clonal seed orchards,  
144respectively (see Figure 1).

145 Several open-pollinated progeny trials were established in 1994-95 across NW Spain  
146following a randomized block design, with ten blocks and 5-tree row plots (Zas *et al.*, 2004).

147In the present study we analysed cones and seeds from two of those progeny trials: Lalin  
148(42.64° N, 7.99° W) and Cortegada (42.21° N, 8.11° W), which were the worst and the best  
149sites respectively in terms of growth (Zas *et al.*, 2004).

150 In the clonal seed orchards, the scions were grafted on two year-old seedlings by  
151substituting the terminal bud to obtain several clonal replicates of each genotype. Ten copies  
152(*ramets*) of each plus-tree genotype were established in each of two clonal seed orchards  
153(“Sergude” and “Monfero”) following a randomized block design with 10 blocks and one  
154ramet of each genotype per block. Environmental conditions drastically differed between the  
155two seed orchards, one of them (Sergude, 42.82° N, 8.45° W) with favourable conditions for  
156pine growth and reproduction, and the other (Monfero, 43.52° N, 7.93° W) with stressful  
157edaphoclimatic conditions (environmental *stresses* with low winter temperatures, exposed to  
158continuous and intense winds, and shallow and impoverished soils) (Table 1).

159

#### 160*Sampling and assessments*

161In this study we analyzed cone and seed traits in 10 genotypes randomly selected among the  
162116 genotypes included in each seed orchard, and in 10 open-pollinated families established  
163in the two progeny trials, nine of which corresponded to the maternal genotypes studied in  
164the seed orchards.

165 In each progeny trial, and for each selected open-pollinated family, up to 3 mature  
166cones were collected from 3 out of the 5 trees per block, in 5 randomly selected blocks (i.e.,  
16715 individual half-sib trees per family). Because of tree mortality and lack of available cones  
168for sampling in some trees, we sampled 133 and 143 trees in Cortegada and Lalin,  
169respectively, resulting in a total of 773 sampled cones. Sampling was performed in March  
1702009 when trees were 14 years-old.

171 In the two seed orchards 3-4 mature cones were collected from 4-6 ramets of each  
172selected genotype in each of the two clonal seed orchards. A total of 373 cones were sampled  
173from 103 different individual trees. The analysis of the sources of variation of seed weight in  
174this collection of cones and its influence on offspring performance has been published  
175elsewhere (Zas *et al.*, 2013). Sampling was performed in January 2009, when trees were 20  
176years old in Monfero and 27 years old in Sergude.

177 Cones were randomly selected within the crown of each tree, collected using ladders  
178and climbing tools and individually labelled. Damaged cones or cones in damaged branches  
179were avoided. Cones were opened in the oven at 35 °C, weighed, and all seeds collected and  
180stored at 4 °C in labelled PET vials. Empty seeds were separated from filled seeds by  
181decantation in cold water. A subsample of filled seeds ( $n = 24$  in the seed orchards and  $n = 16$   
182in the progeny trials) was randomly sampled from each cone and seeds were individually  
183weighed ( $\pm 0.0001$  g). A total of 8924 seeds from the seed orchards and 12464 seeds from the  
184progeny trials were considered in the analyses. Individual cone weight mean, SW mean and  
185within-individual variation in SW were computed for each sampled tree. To estimate the  
186variation of SW within individual trees we used the coefficient of variation as it is the  
187parameter least influenced by the variation in the mean (Crean and Marshall, 2009). Diameter  
188at breast height was measured for all sampled trees.

189

#### 190 *Statistical analyses*

191 In the present study we estimated additive genetic variance and narrow sense heritability of  
192cone and seed traits by analyzing (i) the relation between family and within-family variances  
193as estimated by mixed model analyses in the progeny trials, and (ii) parent-offspring  
194regression between the genetic values estimated in the clonal seed orchard and the progeny  
195trials (Figure 1).

196 Tree diameter, mean cone weight and the mean and within-individual tree variation in  
197SW in the progeny trials were analyzed by fitting mixed models with site as a fixed factor,  
198and family and family  $\times$  site interaction as random factors. Blocks within sites and the  
199interaction of blocks and families (representing the variation among multi-tree plots) were  
200also included in the models as random factors. Variance components were estimated using  
201the REML method as implemented in the MIXED procedure of SAS (Littell *et al.*, 2006). To  
202explore and interpret the genotype  $\times$  environment (G $\times$ E) interaction, we first fitted a *full*  
203*mixed model* in which we assumed heterogeneity of residual variance and an unstructured  
204family (co)variance structure. Then, by fitting different *reduced models* constraining different  
205elements of the family and residual covariance structures we tested for different hypotheses  
206on the causes of the G $\times$ E interaction (see details in de la Mata and Zas, 2010). Specifically  
207we tested whether there was heterogeneity of residual and family variances across sites, and  
208whether the genetic correlation between sites was significantly greater than zero (zero being  
209complete independence) and significantly lower than one (one indicating perfect correlation  
210or parallel reaction norms). Hypothesis testing regarding the constraints imposed on the  
211residual and family covariance structures was done by comparing the restricted log-  
212likelihoods (RLL) of the reduced model and the full model, where the differences in two  
213times the log-likelihood of these two nested models are distributed as one-tailed  $\chi^2$  with  
214degrees of freedom given by the difference in the number of covariance parameters between  
215both models (Fry, 2004). The statistical significance of each specific variance component was  
216also assessed using likelihood ratio tests by fitting reduced models in which we fixed the  
217given (co)variance parameter to 0. Finally, we used the most parsimonious model (i.e., that  
218with the lowest number of parameters that fitted as well as the full model) to estimate the best  
219linear unbiased predictors (BLUPs) for each family (i.e. the offspring genetic values).

220 In order to compute parent-offspring regressions, we used the estimates of the  
221 parental values for seed and cone weight in the two clonal seed orchards obtained by *Zas et*  
222*al. (2013)*. Analyses of the cone weight mean and the mean and variation in SW were  
223 performed by fitting mixed models with the sites as a fixed factor and the blocks within sites,  
224 the genotypes and the site  $\times$  genotype interaction as random factors (*Zas et al., 2013*). Best  
225 linear unbiased predictors (BLUPs) as obtained from those mixed models were used to  
226 characterize the parental genetic values for each trait.

227 For the SW mean we implemented two types of analyses for both the progeny trials  
228 and the seed orchards, including and excluding the cone weight as a covariate in the  
229 statistical models. The two approaches led to different covariance parameter estimates and  
230 BLUPs and thus to different heritability estimates.

231 Narrow-sense heritability was estimated both by means of parent-offspring regression  
232 and by analyzing the similarities between half siblings in the progeny trials. In the first case  
233 we regressed the BLUPs of the open-pollinated families on the BLUPs of their respective  
234 maternal parents as estimated from the mixed models described above. As the original  
235 maternal trees were located far away from each other, they are assumed to be unrelated and  
236 thus the heritability was estimated as two times the slope of the parent-offspring linear  
237 relationship (Roff, 1997). Similarly, the standard error of the heritability was twice the  
238 standard error of the regression slope. On the other hand, heritability was also calculated as  
239 the ratio of additive genetic variance to total phenotypic variance, with variance estimates  
240 derived from the analyses of the open-pollinated progeny trials. Pine families were assumed  
241 to be true *half-sibs*, and thus the additive genetic variance was estimated as four times the  
242 family variance ( $\sigma^2_A = 4 \cdot \sigma^2_F$ , where  $\sigma^2_F$  is the family variance). *Phenotypic variance was*  
243 *estimated as the sum of family, among-plot and within-plot (residual) variances.* In this case,  
244 s.e. of heritability were estimated by the Delta method upon the asymptotic estimates of the

245variances and covariances of the variance components of the mixed model (Lynch and  
246Walsh, 1997). Pines are wind-pollinated and predominantly outcrossers, and self pollination  
247and correlated paternity have been shown to be extremely low in Maritime pine (de-Lucas *et*  
248*al.*, 2008). However, we can not discard some level of full-sibling within our studied  
249families.

250 To estimate genetic correlations between different traits we carried out mixed  
251bivariate repeated measures analyses (Holland, 2006) on the data obtained in the progeny  
252trials. For each pair of traits, we assumed that the values of the two traits on a given tree are  
253repeated measures on the same subject.

254

## 255**Results**

256In the progeny trials we found significant variation among families for cone weight mean and  
257SW mean but not for tree diameter and within-individual variation in SW (Table 2). All these  
258four traits were significantly influenced by the environment (Table 2). Trees were smaller in  
259Lalín than in Cortegada, but cones and seeds were significantly greater in the former than in  
260the latter site (Table 3). Individual variation of SW was, however, larger in Cortegada, i.e.,  
261the site with lighter seeds (Table 3). The effect of the environment on the different traits was  
262apparently similar for all the ten studied families, as revealed by the lack of any significant  
263family  $\times$  site interaction (Table 2). However, the likelihood-based analyses of the G $\times$ E  
264interaction revealed different interpretations of the interaction depending on the trait being  
265considered. No trait showed a correlation estimate that significantly differed from one (i.e.  
266perfect correlation, Table 4), but this was due to the high standard errors for the estimate of  
267the genetic correlation between sites. In fact, the estimate of genetic correlation was  
268significantly different from zero only in the case of the mean SW (Table 4), suggesting that  
269only for this trait **can we** actually assume a high correlation between sites. Consequently,

270reaction norms for SW were parallel but those for cone weight showed large ranking changes  
271across environments (Figure 2). Family variances were similar in the two sites for all the four  
272traits but residual variances significantly differed between sites in the case of tree diameter  
273and individual-variation in seed weight (Table 4).

274 Heritability estimates were low for cone weight but moderate to high for SW mean  
275(Table 5, Figure 3). The two estimation methods (parent-offspring regression, and half-  
276sibling design) led to fairly similar heritability estimates, with no clear trend *differentiating*  
277*the methods* (Table 5, Figure 3). Using the half-sibling design, heritability estimate of SW  
278was higher when the analysis did not account for cone weight covariation, but the inclusion  
279of the covariate did not affect the heritability when estimated *using* parent-offspring  
280regression (Table 5).

281 Cone weight and the SW mean *were just moderately correlated at the phenotypic*  
282*level ( $r = 0.628$ ,  $N = 773$ ,  $p < 0.001$ ), but* showed a strong positive genetic correlation (Table  
2836). However, we found no significant genetic correlation among the other studied traits.  
284Genetic correlations with tree diameter were inestimable because of the lack of additive  
285genetic variation for this trait (Table 6).

286

## 287**Discussion**

### 288*Heritability of mean seed weight*

289Despite being a plastic trait subjected to environmental variation, SW mean appeared to be a  
290genetically variable and highly heritable trait in this pine species, with most of the large  
291observed genetic variation being attributable to additive genetic effects. Results also indicate  
292that the genetic variation was consistent across sites, with parallel reaction norms despite the  
293large differences in environmental conditions. Interestingly, results from the parental and  
294offspring common gardens were consistently similar, with strong differences in SW between

295sites, large total or additive genetic variances and lack of genotype  $\times$  environment interaction  
296in both cases (see also *Zas et al.*, 2013). Because in this study parental trees proceed from a  
297single artificial phenotypic selection event, the chances are that only a small range of the  
298actual genetic variation of the original population is explored. It is therefore likely that the  
299additive genetic variance in SW mean within the whole population could actually be even  
300larger than that reported here. On the other hand, heritability was estimated assuming that  
301families are true half sibs, but we can not completely **rule out** that some of the seedlings from  
302the same maternal tree are, in fact, full siblings. Although not very likely because of the  
303**mating** system and gene flow in Maritime pine (*de-Lucas et al.*, 2008), this could have caused  
304a slight heritability overestimation.

305       The vast majority of the many studies exploring the sources of variation of SW (most  
306of them performed in herbaceous or annual plants) have identified the variation among  
307mother plants as the most important source of variation (*Castro*, 1999; *Halpern*, 2005; *Lazaro*  
308and *Traveset*, 2009; *Sober and Ramula*, 2013). However, most of these studies failed to  
309distinguish whether this effect has an additive genetic component or whether it is related to  
310phenotypic plasticity (i.e. maternal environmental effects) or genetic maternal effects (e.g.  
311*Voeller et al.*, 2012). When these effects have been successfully isolated, SW mean has not  
312always shown a strong genetic control; indeed, several studies on wild species have either  
313failed to detect within-population genetic variation in SW mean (*Schwaegerle and Levin*,  
3141990; *Wolfe*, 1995; *Castellanos et al.*, 2008), or have reported very low heritability estimates  
315(*Mazer*, 1987; *Biere*, 1991; *Platenkamp and Shaw*, 1993). These reports of low heritability of  
316SW have prompted some authors to challenge the idea of stabilizing selection for a particular  
317seed size (*Silvertown*, 1989). However, there is now ample evidence that SW is a variable  
318and heritable trait that can respond to the selection pressures imposed by the environmental  
319conditions. Empirical evidence for the evolution of SW arises, for example, from studies

320showing strong genetic differentiation in SW among plant populations growing in contrasting  
321environmental conditions, with the SW of each population matching the optimal predicted  
322SW (Lazaro and Traveset, 2009). Accordingly, climatic or geographic gradients in SW have  
323been described in many different plant species (Murray *et al.*, 2004), including conifers (Liu  
324*et al.*, 2013), although in most cases it is not clear whether the observed variation is due to  
325genetic differentiation or phenotypic plasticity.

326       Importantly, the controversy around the heritability of seed traits may be related to the  
327difficulties associated with the determination of the genetic architecture of a trait that is in  
328between two generations. Many studies have considered all seed properties to be offspring  
329traits (e.g. Byers *et al.*, 1997), and accordingly have interpreted the seeds of each mother  
330plant as half-sibs (e.g. Carles *et al.*, 2009). In most of these cases, including almost all the  
331studies reporting heritability for seed mass in conifer trees (Matziris, 1998; Bilir *et al.*, 2008;  
332Sivacioglu and Ayan, 2008; Carles *et al.*, 2009), the reported heritability estimates were, in  
333fact, clonal repeatability estimates. The statistical analyses used have not accounted,  
334however, for the fact that seeds of a given mother plant are, indeed, non-independent  
335repeated measures within the same subject (Carles *et al.*, 2009). In other cases, seed traits  
336have been assumed to be a property of the mother plant, and separation of the effects of the  
337maternal environment and the maternal genotype is difficult unless the maternal plants are  
338replicated in contrasting environments (Mazer and Gorchov, 1996). Separating genetic and  
339environmental effects is, however, essential for estimating the narrow-sense heritability of  
340seed weight accurately (Mazer and Gorchov, 1996). This has been typically approached by  
341means of diallel or related breeding designs, in which the differences among paternal families  
342are assumed to be due to additive genetic effects, whereas differences among half-sib  
343maternal families include not only the additive genetic variance but also maternal  
344environment and maternal genetic effects (Schwaegerle and Levin, 1990; Wolfe, 1995;

345Mazer and Gorchoy, 1996; Lipow and Wyatt, 1999). Here, we used an alternative approach  
346based on parental-offspring regression and maternal [half-sibs](#) resemblance. Both approaches  
347have been recognized as problematic, as the resemblance of maternal half-sibs may be due to  
348causes other than additive Mendelian genetic variation (Mazer and Gorchoy, 1996). For  
349example, the use of mother-offspring regression for estimating heritability of SW mean has  
350been criticized because the maternal environmental effect –usually one of the most important  
351sources of variation of SW (Zas *et al.*, 2013)– can be transmitted through several generations,  
352so that the offspring from mother plants with large seeds may be more vigorous, and, in turn,  
353may also bear large seeds independent of the genotype (Mazer, 1987). Our experimental  
354approach resists this criticism for three reasons. Firstly, although it is well known that big  
355pine seeds lead to bigger seedlings (Castro, 1999), the effect of SW on the offspring is  
356usually limited to early stages and tends to diminish or even disappear at older ages (Vivas *et*  
357*al.*, 2013). Secondly, and more relevant, both parent and offspring genetic values were  
358estimated in two common garden tests sited in contrasting environmental conditions, thereby  
359increasing the reliability of our results. On the one hand, most environmental maternal effects  
360were successfully accounted for by clonally replicating the parental genotypes in the two  
361contrasting environments (Zas *et al.*, 2013). On the other hand, by replicating each maternal  
362genotype within each macro-environment into several individual copies (ramets) established  
363following a block design, we also accounted for microenvironmental variation within each  
364site, diminishing at the same time the possible interference of the rootstock variation on seed  
365traits (Zas *et al.*, 2013). Thirdly, the seeds used for the establishment of the progeny trials  
366were collected from the original selected trees in which scions were also sampled for  
367vegetative propagation and establishment of the clonal seed orchards. Thus, the parental  
368values of SW were estimated using individual trees different from those in which seeds were  
369collected for the establishment of the progeny trials. Across-generation maternal effects are

370thus not likely to have influenced our results, although it is acknowledged that they cannot be  
371completely ruled out.

372         Although we cannot completely discard the idea that observed differences between  
373the maternal half-sibs could be partially due to non-genetic factors such as environmental or  
374genetic maternal effects, the close resemblance between maternal and offspring SW suggests  
375that SW is, in any case, transmitted through generations and thus, the resulting phenotypic  
376variation could respond to selection pressures. There is now increasing evidence that the  
377biological information transmitted from one generation to the next is not limited to the DNA  
378sequence (Jablonka and Raz, 2009). Non-genetic information, including parental effects, can  
379also be inherited across generations, contributing to the resemblance between individuals,  
380and thus to the evolutionary dynamics of populations (Rapp and Wendel, 2005; Bossdorf *et*  
381*al.*, 2008; Danchin *et al.*, 2011). This has been recently formalized into the term *inclusive*  
382*heritability*, which represents the percentage of phenotypic variation that is transmitted across  
383generations irrespective of the mechanisms of transmission, i.e., including both genetic and  
384non-genetic inheritance (Danchin *et al.*, 2011). The heritability estimate of SW reported here  
385could therefore to some extent be reflecting an estimation of the inclusive heritability of SW,  
386including both direct additive genetic effects and the transgenerational transmission of other  
387information related to the maternal genotype and the maternal environment. Although our  
388experimental approach suggests that non-genetic effects are likely to be of less relevance than  
389additive genetic effects (see discussion above), the relative contribution of both sources of  
390inheritance should be formally tested as they affect evolutionary dynamics in different ways  
391(Danchin *et al.*, 2011). For example, evolutionary changes due to additive genetic effects are  
392commonly irreversible whereas those due to non-genetic inheritance are not, and ancestral  
393phenotypes could be recovered when the environmental conditions revert (Rapp and Wendel,  
3942005). The lack of consistence in the reported heritability estimates of SW for crops and wild

395populations (Silvertown, 1989; Sadras, 2007) may be also related to the degree to which  
396genetic and non-genetic inheritance are experimentally differentiated.

397 Another interesting result regarding the quantitative genetics of the mean SW in this  
398pine species is the lack of any genotype  $\times$  environment interaction in both the parental and  
399the offspring series of common garden tests (see also *Zas et al.*, 2013). Despite the large  
400environmental effect, the genetic variation in mean SW remained almost the same in the  
401different environments, with nearly parallel reaction norms across environments. This was  
402especially the case when accounting for cone weight covariation, suggesting that the maternal  
403genotypes actively control the mean SW irrespective of the amount of resources allocated to  
404the cones (*Zas et al.*, 2013). The lack of G $\times$ E interactions agrees with other studies in  
405perennial plants that found a high correspondence across years in the relative variation in  
406mean SW among maternal individuals (Castro, 1999; Koenig *et al.*, 2009). These  
407observations have been interpreted as further evidence of a high maternal genetic control of  
408SW (Castro, 1999). It should be noted, however, that the lack of G $\times$ E interaction in the  
409present study may be simply due to the selection process of the parental genotypes, that is  
410likely to have **reduced** the available genetic variation within the studied population (*Zas et*  
411*al.*, 2013). Additionally, the low number of families analyzed may have also favoured a  
412reduced G $\times$ E interaction. We can not discard that increasing the number of families and  
413extending the range of the explored within-population variation, the G $\times$ E interaction could  
414become larger.

415

#### 416*Within-individual seed weight variability*

417Contrary to what was observed for mean SW, within-individual variation in SW showed no  
418genetic variation associated with the maternal lineage. The results were, again, very  
419consistent between the parental and the offspring common garden tests (see also *Zas et al.*,

4202013). While both the maternal genotypes and the half-sibs did not statistically differ in the  
421variation of SW within individual trees, the environmental conditions appeared to have an  
422important effect, with greater variation in the sites least favourable for pine growth, i.e. in  
423those sites with the lowest SW mean. Producing seeds of different sizes might be an adaptive  
424strategy to heterogeneous or unpredictable environments in which the optimum seed size is  
425not clearly defined (Castellanos *et al.*, 2008; Charpentier *et al.*, 2012) or, alternatively, may  
426simply be an indirect consequence of the physiological constraints that limit equal provision  
427among all seeds when resources are scarce (Vaughton and Ramsey, 1998).

428       Variation in SW within individual plants is often among the most substantial sources  
429of intraspecific variation in SW (Vaughton and Ramsey, 1998; Susko and Lovett-Doust,  
4302000). Our results do not show, however, a high variation in SW within individual trees.  
431From the analyses of the parental common garden tests, within-individual variation in SW  
432(including among and within cone variation) explained around 12% of the total observed  
433phenotypic variation, whereas variation between-individuals (including genetic maternal  
434effects and macro and microenvironmental maternal effects) was 88% (Zas *et al.*, 2013).  
435These results suggest that individual trees of this pine species effectively control the mean  
436size of the seeds they produce, and agree with the strong genetic control of mean SW  
437discussed above. On the other hand, both the lower within-individual variation in SW and the  
438lack of genetic differences among half-sibs or among parental genotypes in the variation of  
439seed size within individual trees suggest that within-individual variation in SW has little  
440potential to evolve as an adaptive trait per se.

441

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452

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592

### Figure captions

593

594Figure 1. Schematic representation of the experimental approach. Heritability of cone and  
595seed weight was estimated both through (1) a half-sibling analysis of cone and seed traits  
596assessed in two open-pollinated progeny trials, and (2) parent-offspring regression between  
597the parent and offspring genetic values (BLUPs) estimated across the two clonal seed  
598orchards and the two open-pollinated progeny trials, respectively.

599

600

601Figure 2. Reaction norms for within-individual cone weight (a) and seed weight mean (b) of  
602ten half-sib families of *P. pinaster* across two progeny trials (Site 1 = Cortegada; Site 2 =  
603Lalín). Best linear unbiased predictors (BLUPs) as obtained from the corresponding mixed  
604models are shown.

605

606

607Figure 3. Parent – offspring regression for cone weight and seed weight mean of *P. pinaster*.  
608Each point represents the best linear unbiased predictor (BLUP) of the parental value and the  
609offspring value as obtained from the analysis of two clonal seed orchards (parents) and two  
610half-sib progeny trials (offspring). N = 9 parental genotypes and 9 half-sib families. Error  
611bars denote the standard error of the corresponding BLUPs.

612

613Table 1. Climatic, edaphic and dasometric characteristics of the two maternal seed orchards  
614(Sergude and Monfero) and the two open-pollinated family trials (Cortegada and Lalin).

	Seed orchards		Progeny trials	
	Monfero	Sergude	Cortegada	Lalín
Latitude (° N)	43.52	42.82	42.21	42.64
Longitude (° W)	7.93	8.45	8.11	7.99
Altitude (m)	615	258	530	700
Mean annual temperature (°C)	10.6	13.2	12.6	11.3
Annual precipitation (mm)	1435	1445	1106	1202
Soil pH in H <sub>2</sub> O (soil:water, 1:2.5)	4.5	5.1	4.6	4.6
Soil depth (cm)	45.1	> 120 cm	60.9	59.5
Tree age at sampling	20	27	14	14
Spacing	5 x 5	5 x 5	3 x 3	3 x 3
Mean tree diameter at breast height (cm)	6.1 ± 0.3	20.9 ± 0.6	10.0 ± 0.1	7.7 ± 0.1
Mean tree height (cm)	-	-	558.1 ± 3.1	440.9 ± 2.1

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620 Table 2. Summary of the mixed model for the analysis of cone weight, seed weight mean and  
 621 variation of seed weight within individual trees in the two progeny trials. Environment was  
 622 treated as a fixed factor, and family, blocks (not shown) and the interactions involving these  
 623 terms were considered random factors. Degrees of freedom (DF), F ratios and associated  
 624 probability values are shown for fixed effects. REML estimates of variance components ( $\pm$   
 625 s.e.) and the likelihood ratio test ( $\chi^2$ ) testing whether they are significantly greater than zero  
 626 are shown for random factors. A heterogeneous residual mixed model was fitted for variation  
 627 in seed mass.

628

	Cone weight			Seed Weight Mean			Seed Weight Variation		
	DF	F	p > F	DF	F	p > F	DF	F	p > F
<i>Fixed factors</i>									
Environment (E)	1,16	5.6	<b>0.031</b>	1,16	8.3	<b>0.011</b>	1,16	13.7	<b>0.002</b>
<i>Random factors</i>	Var Comp	$\chi^2$	p > $\chi^2$	Var Comp	$\chi^2$	p > $\chi^2$	Var Comp	$\chi^2$	p > $\chi^2$
Family (F)	40.6 $\pm$ 55.8	9.0	<b>0.001</b>	24 $\pm$ 13.8	19.3	<b>0.000</b>	0.91 $\pm$ 1.37	2.2	0.069
F x E	57.1 $\pm$ 59.1	1.9	0.084	0 $\pm$ 0	0.0	0.500	0.4 $\pm$ 1.5	0.1	0.376
Residual	(Cortegada) 709.9 $\pm$ 73.5			85.3 $\pm$ 9.0			69.7 $\pm$ 8.8		
	(Lalín)						22.2 $\pm$ 2.7		

629

630 Table 3. Least square means ( $\pm$  s.e.) for tree diameter, cone weight, seed weight mean and  
 631 within-individual variation of seed weight in *Pinus pinaster* based on the analysis of two  
 632 clonal seed orchards (Monfero and Sergude) and two open-pollinated progeny trials  
 633 (Cortegada and Lalín) as estimated from the corresponding mixed models.

634

Variable	Clonal seed orchards		Progeny trials	
	Monfero	Sergude	Cortegada	Lalín
Tree diameter (cm)	6.1 $\pm$ 0.3	20.9 $\pm$ 0.6	18.6 $\pm$ 0.7	16.2 $\pm$ 0.7
Cone weight Mean (g)	101.5 $\pm$ 10.1	150.6 $\pm$ 9.6	113.2 $\pm$ 4.1	125.3 $\pm$ 4.1
Seed Weight Mean (mg)	53.2 $\pm$ 0.2	79.1 $\pm$ 0.3	60.9 $\pm$ 1.8	65.1 $\pm$ 1.9
Seed Weight Coefficient of Variation (%)	14.4 $\pm$ 0.6	10.8 $\pm$ 0.5	19.4 $\pm$ 0.8	15.9 $\pm$ 0.6

635

636 Table 4. Results of different likelihood ratio tests for testing different hypothesis on the  
 637 relevance and interpretation of the family  $\times$  environment interaction across the two progeny  
 638 trials.

639

<i>Hypothesis testing</i>	Diameter		Cone weight		Mean SW		Variation SW	
	$\chi^2$	$P > \chi$	$\chi^2$	$P > \chi$	$\chi^2$	$P > \chi$	$\chi^2$	$P > \chi$
Homogeneity of family variances	0.0	0.500	0.1	0.376	0.1	0.376	0.2	0.327
Homogeneity of residual variances	16.9	<b>0.000</b>	0.3	0.292	2.4	0.061	38.2	<b>0.000</b>
Genetic correlation not different from zero	0.0	0.500	0.6	0.219	10.9	<b>0.000</b>	0.4	0.264
Genetic correlation not different from one	0.0	0.500	1.7	0.096	0.0	0.500	0.1	0.376

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644

645 Table 5. Narrow sense heritability estimates ( $h^2 \pm \text{s.e.}$ ) for cone weight and seed weight mean  
 646 of *Pinus pinaster* derived from the analysis of similarities between half-sibs in progeny trials,  
 647 and from parental-offspring relationships. Heritability of seed weight is shown with and  
 648 without accounting for the covariation with cone weight in the corresponding mixed models.  
 649

	Cone weight	Seed weight	
		Not accounting for cone weight covariation	Accounting for cone weight covariation
Half-sibs relationship	0.17 $\pm$ 0.08	0.74 $\pm$ 0.27	0.56 $\pm$ 0.12
Parent-offspring regression	0.25 $\pm$ 0.09	0.58 $\pm$ 0.17	0.55 $\pm$ 0.19

650

651 Table 6. Restricted maximum likelihood estimates of genetic correlation ( $\pm$  s.e.) between tree  
 652 diameter, cone weight (CW), seed weight mean (SW) and seed weight variation within  
 653 individual trees, estimated as coefficient of variation (CV-SW). Significant correlation  
 654 estimates ( $p < 0.05$ ) are given in boldface. N = 10 open-pollinated families.

655

	Cone Weight	Seed Weight	CV-SW
Diameter (D)	$1.52 \pm 1.87$ <sup>ns</sup>	$1.47 \pm 1.87$ <sup>ns</sup>	$0.00 \pm 1.07$ <sup>ns</sup>
Cone weight mean (CW)	-	<b><math>0.99 \pm 0.06</math></b> <sup>***</sup>	$0.54 \pm 0.69$ <sup>ns</sup>
Seed weight mean (SW)		-	$0.19 \pm 0.53$ <sup>ns</sup>
Variation in seed weight (CV-SW)			-

656 ns = not significant; \*\*\* =  $p < 0.001$

657