Costs of female reproduction in a conifer tree: a whole-tree level assessment

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Summary

1. Costs of reproduction lie at the core of basic ecological and evolutionary theories, and their existence is commonly invoked to explain adaptive processes. Despite their sheer importance, empirical evidence for the existence and quantification of costs of reproduction in tree species comes mostly from correlational studies, while more comprehensive approaches remain missing. Manipulative experiments are a preferred approach to study cost of reproduction, as they allow controlling for otherwise inherent confounding factors like size or genetic background.

2. Here, we conducted a manipulative experiment in a Pinus halepensis common garden, removing developing cones from a group of trees and comparing growth and reproduction after treatment with a control group. We also estimated phenotypic and genetic correlations between reproductive and vegetative traits.

3. Manipulated trees grew slightly more than control trees just after treatment, with just a transient, marginally non-significant difference. By contrast, larger differences were observed for the number of female cones initiated 1 year after treatment, with an increase of 70% more cones in the manipulated group. Phenotypic and genetic correlations showed that smaller trees invested a higher proportion of their resources in reproduction, compared with larger trees, which could be interpreted as an indirect evidence for costs of reproduction.

4. Synthesis. This research showed a high impact of current reproduction on reproductive potential, even when not significant on vegetative growth. This has strong implications for how we understand adaptive strategies in forest trees and should encourage further interest on their still poorly known reproductive life-history traits.

Key-words: costs of reproduction, manipulative experiment, plant development and life-history traits, reproduction, trade-off, vegetative growth

Introduction

Life-history theory assumes that trade-offs exist between functions linked to fitness, expressed as costs in terms of survival, growth and reproduction (Roff 1992). The study of the costs of reproduction has a long tradition in evolution and ecology, although the approaches used by both disciplines usually differ (Reznick 1992). Four approaches have been used to illustrate the costs of reproduction (Reznick 1985): phenotypic correlations, experimental manipulations of reproduction, genetic correlations and selection experiments. When considering evolutionary change, it is only genetically based costs (i.e. genetic correlations and selection experiments) that are relevant to future adaptation and also to test the assumptions of life-history theory, as only genetic information will be passed on to future generations (Reznick 1985). On the other hand, phenotypic correlations and, preferably, experimental manipulations illustrate physiological costs of reproduction provided that confounding factors such as environmental and genetic variability are controlled (Obeso 2002). The study of costs of reproduction in trees is more complex than in short-lived taxa, as investment in growth, maintenance and reproduction might vary among life stages (Thomas 2011). In fact, investment in maintenance is a key difference between long- and short-lived plants (Bonser & Ladd 2011), which is likely to have a deep influence on the expression of costs of reproduction (Sala et al. 2012). Despite more challenging, the study of costs of reproduction in trees deserves particular attention as trees are foundation species upon which forest ecosystems depend (Petit & Hampe 2006). Also, trees are long-lived organisms and, as such, they must withstand changing environmental conditions much more than short-lived
plants (Willson 1983). Therefore, previous results derived mainly from short-lived species might not hold for tree species (Barringer, Koenig & Knops 2013; Santos-del-Blanco et al. 2013).

Available evidence for tree species highlights a physiological trade-off between reproduction and growth, with several recent papers (Alla et al. 2011; Sánchez-Humanes, Sork & Espelta 2011; Sala et al. 2012; Barringer, Koenig & Knops 2013; Zywiec & Zielonka 2013) and a review (Thomas 2011) helping to define mechanistic links between both functions. Thus, reproduction has commonly been related to reduced growth in terms of stem diameter and shoot elongation. But other traits like leaf size and chemistry and photosynthesis, as evidenced by a correlation between reduced leaf nitrogen and chlorophyll content (Obeso 1998; Leal & Thomas 2003), have been also used to illustrate costs of reproduction in trees. Negative correlations between current and future reproduction, that is demographic costs of reproduction, reflect a more important trade-off than between reproduction and growth. They are thought to be important for the occurrence of masting (Iwasa & Satake 2004; Barringer, Koenig & Knops 2013), as it is assumed that individuals only reproduce above a certain threshold of resources, and resources are depleted by reproductive events (Isagi et al. 1997). Demographic costs of reproduction have, however, been rarely explored in non-masting species with some exceptions in cold climate tall shrubs (Elmqvist et al. 1991; Fox & Stevens 1991; Cipollini & Whigham 1994; Houle 2001).

Studies reporting on physiological costs of reproduction in trees are mainly based on correlational studies (Thomas 2011). The most basic approach consists on relating a variable of interest, usually growth, with naturally occurring variable levels of reproduction (or vice versa) at different hierarchical levels, from shoots to whole the tree (Sánchez-Humanes, Sork & Espelta 2011) and in one or several populations or species (Barringer, Koenig & Knops 2013) along one or several growth seasons (Pulido et al. 2014). Some studies have used manipulation experiments, helping to generate more variability than naturally available for water or nutrient resources (Sánchez-Humanes & Espelta 2011; Pulido et al. 2014). Moreover, direct manipulation of reproduction is widely considered a more straightforward method for highlighting costs of reproduction (Reznick 1992; Obeso 2002). This approach has been used with some cold climate tall shrubs (Elmqvist et al. 1991; Fox & Stevens 1991; Cipollini & Whigham 1994; Fox 1995; Houle 2001) as well as with a rain forest palm (Cunningham 1997). Overall, results from these studies indicate that plants with artificially reduced reproductive effort tend to reproduce more, but not grow more, than non-manipulated plants. These results have been interpreted as demographic costs of reproduction (Elmqvist et al. 1991; Fox & Stevens 1991; Cipollini & Whigham 1994; Houle 2001). Nonetheless, it seems that the study of costs of reproduction in woody plants has been prematurely abandoned, as derived from the limited number of species and growth types assayed so far and the lack of recent publications. Likely, this is because manipulation of reproduction in trees poses great practical challenges in such large organisms.

Genetically based costs of reproduction in trees are even more scarcely reported in the plant ecology literature given that genetic trials must be planted and cared after at least until trees reach reproductive maturity, and then there is the issue of defining the variables that best represent investment in growth and in reproduction. There are, however, some examples in the forestry literature reporting on negative genetic correlations between reproductive and vegetative traits (Schmidtling 1981; El-Kassaby & Barclay 1992; Santos-del-Blanco et al. 2010). Non-significant or positive correlations between current reproduction and growth or future reproduction often come from studies where environmental confounding factors like tree size are not controlled, as large trees often also have access to a larger amount of resources (Almqvist, Jansson & Sonesson 2001; Hamner & Aitken 2001; Bilir et al. 2002). Moreover, the use of genotypes selected for enhanced growth in breeding programmes can incorporate a severe bias that limits a broad interpretation of results. Specifically, because the focus is on a particularly narrow set of genotypes, suffering likely modifications of reproductive allometry and thus the expression of costs of reproduction (El-Kassaby & Barclay 1992). Data from tree breeding programmes could nonetheless be used to illustrate genetic costs of reproduction by comparing reproductive traits in progenies from trees submitted and not submitted to phenotypic selection. This, however, has not yet been performed, and there is only indirect evidence for genetic costs of reproduction derived from artificial selection (Varghese et al. 2009; Santos del Blanco et al., unpubl. data).

In this study, we address the joint study of physiological and genetic costs of production on a precocious Mediterranean pine whose reproductive ecology is becoming reasonably well understood, the Aleppo pine (Pinus halepensis Ait.) (Ne’veman, Goubite & Nathan 2004; Climent et al. 2008; Ne’veman et al. 2011; Santos-del-Blanco et al. 2013). This combination of approaches is quite unique and brings together approaches from ecology and evolutionary biology. We do so by taking advantage of the opportunities provided by a large common garden experiment, which combines the maximum genetic diversity available for the species in its western range and uniform environmental conditions. We specifically (1) test the existence of a physiological trade-off between current reproduction and subsequent growth and reproduction at the whole-tree level by experimentally manipulating reproduction in young P. halepensis trees. We therefore expect cone removal to increase vegetative growth and eventually, subsequent reproduction. Also, if costs of reproduction are higher for small trees, as they have a proportionally higher reproductive investment (Santos-del-Blanco et al. 2010), we would expect larger differences in growth and reproduction among this size class. And (2) provide estimates of phenotypic and genetic correlations between vegetative and reproductive traits from common garden experiments, indicative of costs of reproduction.

Material and methods

STUDY SITE

Our experiment was carried out in a provenance-progeny common garden planted in 1995 with 1-year-old seedlings. Size and female reproduction were periodically registered for all trees in the common garden since establishment. Details can be accessed in (Santos-del-Blanco et al. 2010) www.genforees.es. All mother trees from which seeds were collected were chosen at random in natural Spanish populations, therefore without any phenotypic selection. Moreover, besides providing uniform conditions for all trees, the common garden approach provided a wide genetic diversity over which to test our hypothesis.

STUDY SPECIES

Pinus halepensis has several features that make it a suitable species for the study of costs of (female) reproduction. First, as it is a monocious species, that is, separate female and male cones appear on each individual tree, individual study of male and female sexual functions is more convenient than in hermaphroditic species. Secondly, reproduction in this species is precocious, especially regarding female reproduction, as it produces female cones as early as 3 years old. Thirdly, it has a relatively small size at maturity, which at early ages enables manipulation without need of special equipment like scaffolds or elevator platforms. Fourthly, it has a high reproductive allocation and produces cones regularly throughout its life, which facilitates the detection of costs of reproduction (Grivet et al. 2013). And fifth, it is a widespread species, with populations putatively adapted to varying ecological conditions that might reveal compromises between reproduction and other life-history traits (Climent et al. 2008).

Pinus halepensis has a 2-year female cone developmental cycle; thus at any time, there are potentially two cohorts of female cones developing simultaneously on the same tree (Pardos et al. 2003) (Fig. 1). Female floral induction of vegetative buds takes place at the end of the summer and female cones emerge the following spring (Enescu 1987), generally from vigorous branches of the upper half of the crown (Shimida et al. 2000). Pollination then takes place in spring, but ovule fecundation and seed and cone development are arrested until the following year. First-year female cones then fully grow along the season completing their development 2 years after pollination. Developed cones are attached to their bearing branches through a very thick peduncle, so they rarely drop after final development (Ne’eman, Goubitz & Nathan 2004). Female cones in P. halepensis trees typically precede male cones in one or several years at the onset of reproductive life. Therefore, female reproduction is clearly more relevant during early stages in this species (Ne’eman et al. 2011), unlike other related pine species like P. pinea (Santos-del-Blanco et al. 2012) or Pinus pinea (Shimida et al. 2000).

MANIPULATIVE EXPERIMENT

In May 2010, when trees were 16 years old, a manipulative experiment was carried out. At that time, still about half of the trees in the common garden remained vegetative or had produced a low number of cones (less than five). We imposed a minimum threshold of five cones in the previous 2 years for the trees to be eligible for the experiment. The aim was to increase power in detecting significant differences, as removing a low number of cones might not be as a strong enough stimulus given the size of the trees. Among those with at least five cones, we selected trees evenly in the range of 5–61 cones per tree by subdividing them into three categories, low (5–10 cones), medium (11–18 cones) and high (19–61 cones) reproduction. In total, 110 trees from 23 of 28 populations were selected, randomly assigning half of them to the treatment group and leaving the other half as a control group (CTR group hereafter). We checked that there were no statistical differences in size or female reproduction between the control and treatment groups previous to treatment, and later on we controlled statistically for random population effects.

All developing female cones, both of current year (strobili) and previous year (conelets), were counted and removed from trees in the treatment group (FCR group hereafter). Special care was taken when removing the cones as not to damage the branches. Current and previous year female cones were also counted in trees from the control group.

We preferred a whole-tree assessment of the treatment effects because compensation between plant parts could mask processes at the branch level (Karlsson & Mendez 2005), as suggested by a previous correlational study in P. halepensis (Ne’eman et al. 2011). Also, it is at the whole-organism level where the study of costs of reproduction is most relevant in ecological and evolutionary terms (Salguero-Gómez, Shefferson & Hutchings 2013). We did not assess costs of male reproduction for two reasons. First, because of the above-mentioned prevalence of female gender during early reproductive stages (Ne’eman et al. 2011) and, second, because a similar removal of male cones in pines is not possible without severely damaging vegetative structures of the affected shoot.

Fig. 1. Developmental stages of female strobili in Pinus halepensis. (a) Female strobili in spring, soon after budburst. (b) Female conelets in spring, 1 year after budburst. (c) Fully developed and ripe 2-year-old female cones.

At the end of the 2011 autumn growth season, thus 2 years after treatment a radial wood microcore was extracted at breast height from each tree. Wood microcores were mounted on a frame, sanded and radial growth for years 2009, 2010 and 2011 measured with a tree-ring measurement station. Basal area for each tree and year was calculated from diameter at breast height in late 2011 and yearly radial growth increments. Current year female cones were also counted in 2011. Basal area and female reproduction provided information to assess costs of reproduction from a whole-tree approach, focusing on their phenotypic outcome, but not on their proximate causes.

**DATA ANALYSIS**

Differences in vegetative growth one and 2 years after treatment were evaluated with linear mixed models which included treatment as a fixed factor and basal area previous to treatment as a covariate. Model term significance was evaluated by means of likelihood ratio tests. The interaction between treatment and covariate was non-significant, and therefore, it was removed from final models. The provenance (population of origin) of each individual was included as a random factor in the model to avoid pseudoreplication.

Count data for female cones was analysed with Poisson generalized linear mixed models. We first tested for differences between treatment and control groups in absolute number of cones produced after the treatment (counted in late 2011). As before, treatment was defined as fixed, but we used no covariate. Population was included as a random factor as well as an observation-level random factor to account for overdispersion in our data (Elston et al. 2001). Finally, to check for differences in reproductive allometry between treatments, we included the logarithm of basal area in 2011 as a covariate in the model.

Complementarily, phenotypic and genetic correlations between reproduction and size were calculated with measurements at 16 years from the entire trial (28 populations 144 families, 1305 trees). We calculated correlations between biomass in 2009 (as data were available for all individuals) and absolute (reproductive investment) or relative (reproductive allocation) reproduction until 2009. Reproductive allocation for each individual tree was calculated dividing the number of developing cones, corresponding to the two last cohorts (Climent et al. 2008), by total biomass, derived from diameter at breast height and allometric models (Karlsson & Mendoza 2005; Ruiz-Feinado, del Rio & Montero 2011). Finally, we also calculated correlations between biomass growth between 2005 and 2009 and number of cones produced during that period. Here, each variable reflects investment either in vegetative growth or in reproduction, thus providing a more conventional estimate of costs of reproduction. Phenotypic correlations were estimated as Pearson’s correlation coefficients between phenotypic values after correcting for population effects. As a proxy for genetic correlations, we calculated Pearson’s correlation coefficients between family corrected means. All models and tests were performed with R packages lme4 v. 0.9999999-0 and lmerTest v.1.2-0.

**Results**

Manipulated trees, that is, those with removed current and first-year female cones, FCR group, grew slightly more than control trees after treatment, but differences only approached significance in 2010 and disappeared the following year (Table 1 and Fig. 2).

By contrast, the female cone removal treatment showed a greater effect in subsequent reproduction. FCR trees produced, on average about 70% more female cones than control trees (Table 1 and Fig. 3). We found only additive effects of treatment on reproductive allometry, that is, interaction between reproduction and size was non-significant, indicating a similar effect of experimental treatment across tree sizes, and no evidence for increased costs of reproduction among the smallest trees. Also, manipulated trees produced many more cones that removed corresponding to the previous year (n = 55, t_{df} = -2.51, P = 0.015). However, it was difficult to classify this result as an overcompensatory response, as it is not possible to isolate the effect of treatment from that of ontogeny (trees were larger one season after treatment) and weather (weather might have been more benign one season after treatment). In fact, control trees also produced more cones one season after treatment than those that had been removed, although differences were less evident than for FCR trees (n = 55, t_{df} = -2.51, P = 0.044).

The phenotypic correlation between size and reproduction, either absolute or relative, was positive and significant, that is, bigger trees, or those that grew more, yielded more female cones (Table 2). However, the correlation between size and number of cones per unit size (RA) was negative. Among family genetic correlations between size and reproductive investment were positive and significant but lower than phenotypic correlations, while those with reproductive allocation were negative and significant (Table 2).

**Discussion**

Experimental removal of developing female cones in young *P. halepensis* resulted in enhanced production of female cones 1 year later, even when treatment had only a minor effect on vegetative growth. To our knowledge, this is the first account on somatic costs of reproduction in a forest tree assessed by a manipulative experiment.

Induction of vegetative meristems into meristems giving rise to female cones takes place after summer (Enescu 1987), and induction depends on nutrient content on branches, such that vigorous branches with higher nitrogen content are more likely to bear female cones (Ne’eman et al. 2011). Then, fruit development takes place partially at the expenses of stored N and P, that in the case of the masting species *P. albicaulis* can be even depleted (Sala et al. 2012) but with no apparent effect on vegetative growth. By removing developing female cones, trees in our experiment were probably left with a surplus of readily available resources that could be allocated to other functions or stored. Similar results have been reported.
Fig. 2. Scatterplots showing the relationship between basal area of *Pinus halepensis* trees before and after a treatment consisting in the removal of developing female cones. (a) Relationship 1 year after treatment (2010) and (b) 2 years after treatment (2011). Solid dots, control group; open dots, female cone removal treatment.

Fig. 3. Boxplots comparing control *Pinus halepensis* individuals (Control) with others subjected to experimental removal of developing female cones (Cone removal); (a) basal area in 2010, one growth season after experimental manipulation and (b) number of female cones in 2011, one reproductive season after experimental manipulation.

Table 2. Phenotypic and genetic (family) correlations between vegetative and reproductive size in *Pinus halepensis*. 95% confidence intervals are given in brackets. RI correlation between absolute vegetative and reproductive sizes. RA correlation between absolute vegetative size and reproductive allocation. R-GI correlation between growth in the period 2005–2009 and the number of cones produced.

<table>
<thead>
<tr>
<th>Phenotypic</th>
<th>RI (0.42–0.50)</th>
<th>P-value</th>
<th>Family</th>
<th>R-GI (0.27–0.36)</th>
<th>P-value</th>
</tr>
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<tbody>
<tr>
<td>n = 1305</td>
<td>&lt;0.001</td>
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<td>RI</td>
<td>0.38 (0.23–0.51)</td>
<td>&lt;0.001</td>
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<tr>
<td>RA</td>
<td>–0.40 (–0.44)</td>
<td>&lt;0.001</td>
<td>RA</td>
<td>–0.43 (–0.56)</td>
<td>&lt;0.001</td>
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<td>to –0.35</td>
<td>&lt;0.001</td>
<td></td>
<td>R-GI</td>
<td>0.21 (0.05–0.36)</td>
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in other woody species like *Salix* spp. and *Alnus viridis* (Elmqvist et al. 1991; Fox & Stevens 1991; Houle 2001). Thus, reproductive meristem induction in the following year could be influenced in two ways. First, indirectly by the investment of those resources into vegetative growth that could help to acquire and store more resources. And secondly, directly by readily stored extra resources the previous year. Measurement of nutrient (Ne’eman et al. 2011; Sala et al. 2012) and carbohydrates (Ichie et al. 2013) at the branch and tree level could help elucidate between those alternatives. Our results suggest that manipulation of reproduction can have greater carry-over effects on reproduction compared with immediate effects on vegetative growth.

Our experiment was carried out with relatively young *P. halepensis* trees. While 16 years can be close to the life expectancy for the species under some environmental conditions, that is, short fire return interval (Tessler et al. 2014), normal life expectancy is typically longer (Grivet et al. 2013). If older trees were also subjected to experimental manipulation, the expression of costs of reproduction might differ from that in our experiment. For example, large trees have higher maintenance costs, and their growth is less vigorous (Ne’eman et al. 2011). Regarding future reproduction, female reproductive allocation is lower in adult trees compared with young trees, while male reproductive allocation becomes higher (Ne’eman et al. 2011). Thus, experimental removal of female reproductive structures might have a stronger indirect effect on the male reproductive function.

Experimental manipulation of reproduction proved critical in the detection of costs of reproduction in our study. Indeed, manipulative experiments have been recommended for the study of costs of reproduction in plants, while controlling for confounding factors like size and genetic background (Obeso
2002). For example, large trees invest more in both vegetative and reproductive growth, leading to positive correlations (de Jong & KlKinhamer 2005) which indeed are the base of allometric relationships between size and reproduction (Niklas & Enquist 2003). Such an example is also provided by our data at the phenotypic level. Therefore, only rarely a negative correlation between absolute size and reproduction is described but see (Hansen, García & Ehlers 2013). Besides size effects, other environmental factors can blur the detection of costs of reproduction, but also produce false positives (Knops, Koening & Cameron 2007). Experimental manipulations are therefore recommended whenever possible.

Despite the high number of trees and contrasted origin of the families included in our study, we only found mixed support for phenotypic and genetic costs of reproduction in this species, indicative of evolutionary constraint. A negative relationship of growth and reproduction was only evident at the individual and family level when comparing absolute size and reproductive allocation, also reported in (Santos-del-Blanco et al. 2010). Such comparisons have raised concern about spurious correlations (Klinkhamer & de Jong 1990), not least because they use an absolute measure of size instead of growth. However, they also describe a biological reality, that is, trees actually invest a fraction of their resources in reproduction. Thus, the problem might not be the use of such correlations but their straightforward interpretation as evidence for costs of reproduction (see below).

In our common garden study, smaller trees invested a higher proportion of their resources in reproduction, compared with larger trees (Table 2), a pattern also described in (Climent et al. 2008). This could be caused by the cost of reproduction burdening the growth of those trees that started to reproduce at smaller sizes (Climent et al. 2008) or just by specific allometric patterns that might determine a more intense relative reproduction at young ages and/or smaller size. To the extent that the allometry of reproduction is genetically determined for a species, one could interpret the difference between the theoretical reproductive allocation and the actual reproductive allocation as driven by costs of reproduction, that is, the predicted reproductive allocation, or that of a control group, can provide a reference by which decide whether the relationship between size and the actual reproductive allocation can or cannot be influenced by costs of reproduction. Also here, manipulative experiments would be a preferred tool.

Even if present, as suggested by our manipulation experiment, several factors could have affected our ability to detect such physiological and genetic costs of reproduction by means of phenotypic and genetic correlations. First, family estimates had high errors as the number of trees per family was relatively low. Secondly, neutral genetic variability in Spanish populations of Aleppo pine is low, following recent range expansion (Grivet et al. 2011). Thus, although neutral and adaptive genetic variability need not be correlated, and despite our comprehensive sampling effort, our sample might lack enough genetic variation. Nonetheless, this would challenge recent reports on *P. halepensis* describing significant variation in reproductive life-history traits (Santos-del-Blanco et al. 2013), which we expected to trade-off against reproductive traits.

And thirdly, costs of reproduction might exist, as implied by allocation theory and suggested by our manipulative experiment, but not expressed either phenotypically or genetically under natural, that is, unmanipulated, conditions. At the phenotypic level, for example, environmental heterogeneity causing differences in resource availability among trees will blur the expression of costs (Friedman & Barrett 2011). In turn, at the genetic level, genes causing among-tree differences in resource acquisition, like for example below- or above-ground competition, will have pleiotropic effects and cause a positive genetic correlation between growth and reproduction. Other variables that might influence the expression of costs of reproduction are environmental plastic effects (Santos-del-Blanco et al. 2013) and other morphological traits like serotiny (Ne’e’mann, Goubitz & Nathan 2004). In particular, cone serotiny shows genetic variation both within and between populations (A. Hernández-Serrano, M. Verdú, L. Santos del-Blanco, Climent, S. C. González-Martínez, J. Pausas, unpubl. data), and there are evidences indicating that serotiny can imply additional reproductive costs in Aleppo pine (Salvatore et al. 2009; Espelta, Arnan & Rodrigo 2011). Also, our controlled manipulative experiment showed that the direct impact of reproduction on vegetative growth is scarce, at least in the short term, and phenotypic and genetic correlations could just be reflecting this effect.

Reproduction in trees has been an overlooked trait in studies addressing adaptation, while the relationship between fitness and reproduction is much more direct than that with vegetative traits. A deeper knowledge on tree reproductive ecology is thus still needed to understand adaptive processes in forest stands, and the study of costs of reproduction is certainly important in this regard. Our experiment has shown that as long as reliable approaches and adequate model organisms are chosen, simple methods can be useful to illustrate costs of reproduction in trees. Specifically, demographic costs of reproduction seem to be particularly important and now further effort is needed to deepen into their causes and consequences.

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**Data Accessibility**

Data used in this research can be accessed through the Spanish Network of Genetic Trials (GENFORED) website: www.genfored.es.
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