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ARTICLE

Using among-year variation to assess maternal effects in *Pinus aristata* and *Pinus flexilis*

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Abstract: Maternal effects, the effect of the maternal environment during development on offspring growth, can complicate the interpretation of common garden studies. Growing one or more generations in a common environment can help minimize maternal effects, but is often not practical with long-lived species. In *Pinus aristata* Engelm. and *Pinus flexilis* James, we assessed maternal effects by growing offspring sourced over multiple years from the same mother trees, comparing growth traits between source years. Additionally, we explored the effect of maternal environment on seed characteristics by collecting five twig clippings from each mother tree and measuring characteristics indicative of the relative vigor of the tree during each seed source year. The effect of year was significant for twig growth characteristics, seed size, and seedling performance. For both species, there were significant relationships between the relative inter-annual (RIA) variation in seed mass and the RIA variation in numerous seedling traits including cotyledon length, seedling total dry mass, and needle length. Variation in seed mass was not predicted by yearly variation in the maternal plant's phenotypic traits. These results support the hypothesis that maternal effects translate into variation in early seedling growth and suggest possibilities to statistically account for them in common garden studies involving long-lived species.

Key words: greenhouse, local adaptation, maternal environment, *Pinus aristata*, *Pinus flexilis*.

Résumé : L'effet maternel, c'est-à-dire l'effet de l'environnement maternel durant le développement sur la croissance de la progéniture, peut compliquer l'interprétation d'études de transplantation. Faire croître une ou plusieurs générations dans un environnement transplanté peut contribuer à minimiser l'effet maternel, mais cette alternative n'est souvent pas pratique pour les espèces longévives. Les auteurs ont évalué l'effet maternel chez *Pinus aristata* Engelm. et *Pinus flexilis* James en cultivant la progéniture provenant des mêmes arbres-mères à chaque année pendant plusieurs années, comparant leurs traits de croissance entre les années d'origine. De plus, elles ont exploré l'effet de l'environnement maternel sur les caractéristiques des graines en récoltant cinq petites branches de chaque arbre-mère et en mesurant les caractéristiques indicatives de la vigueur relative de l'arbre à chaque année d'origine. L'effet de l'année était significatif sur le plan des caractéristiques de croissance des petites branches, la taille des graines et la performance des pousses. Chez les deux espèces, des relations significatives étaient observées entre la variation interannuelle relative (IAR) de la masse des graines et la variation IAR de plusieurs traits des pousses incluant la longueur du cotylédon, la masse sèche totale des pousses et la longueur des aiguilles. La variation de la masse des graines ne pouvait être prédite par la variation annuelle des traits phénotypiques maternels du plant. Ces résultats appuient l'hypothèse que l'effet maternel se traduit dans la variation de la croissance annuelle des pousses et suggèrent la possibilité d'en tenir compte sur un plan statistique lors d'études de transplantation impliquant des espèces longévives. [Traduit par la Rédaction]

Mots-clés : serre, adaptation locale, environnement maternel, *Pinus aristata*, *Pinus flexilis*.

Introduction

As climate shifts away from historical norms and invasive species continue to spread, research is being conducted to understand methods for conserving threatened plant species. Conservation actions such as targeted restoration or assisted migration must take into account the source population to ensure successful establishment and continued growth (McKay et al. 2005). Common garden studies are often used to test for genetic differentiation, local adaptation, and trait plasticity among populations that could lead to maladaptation in conservation actions. However, maternal effects, or the influence of the environment during seed development on offspring performance, can influence early seedling growth, complicating the interpretation of common garden studies (Roach and Wulff 1987; Bischoff and Müller-Schärer 2010).

Maternal effects can impact offspring directly through seed provisioning or indirectly by influencing gene expression (Roach and Wulff 1987; Galloway 2005). In gymnosperms, both the seed coat and the megagametophyte, which provide resources for the germinating seedling, are of maternal origin (Kramer and Kozlowski 1979). Large seeds are generally associated with large seedlings because there are increased reserves available for the seedling to use early in its life (Reich et al. 1994; Westoby et al. 1996). Provisioning due to maternal effects has been found to impact seed size, germination, and early growth of many plant species (Roach and Wulff 1987), including trees (Reich et al. 1994; Sultan 1996; Castro 1999; Castro et al. 2008, Oliver and Borja 2010; González-Rodríguez et al. 2012). Mother tree age and stand density have also been found to influence germination and early seedling growth in *Pinus thunbergii* (Mao et al. 2014).

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The emerging field of epigenetics explores how the environment during seed development influences gene expression in offspring, unrelated to changes in DNA sequence (Wolffe and Matzke 1999). Epigenetics can be thought of as a "memory" of environmental conditions during seed development (Johnsen et al. 2005). For example, Johnsen et al. (2005) found that temperature during seed development affected timing of bud set and cold hardiness in Norway spruce progeny. In another case, maternal environment was found to influence tolerance in *Pinus pinaster* to the pathogen *Fusarium circinatum* (Vivas et al. 2013), possibly mediated by plant carbohydrates (Vivas et al. 2014).

Common garden studies rely on the assumption that variation among seed collection locations is due to genetic differentiation; however, some of the phenotypic variation observed in these studies has been attributed to environmental maternal effects (Bischoff and Müller-Schärer 2010). Without additional measures, researchers using field-collected seeds cannot be sure whether variation in offspring growth is due to genetic or environmental factors. It is generally accepted that maternal effects are strongest in the earliest stages of a plant's life and then diminish over time, often lasting at least one growing season (Roach and Wulff 1987; Reich et al. 1994). Growing one or more generations from random crosses in one common environment can minimize variation due to maternal environment (Bischoff and Müller-Schärer 2010), though this approach is often not feasible for long-lived species that do not reach reproductive maturity for decades.

Alternatively, for long-lived plants, we suggest estimating maternal effects by comparing the growth of seedlings raised from seed sampled from multiple years from the same maternal plant (Carles et al. 2009). The modular growth patterns of pines permit the use of historic growth data from the mother tree twigs to assess phenotypic response to environmental variation. A limited number of studies have used this method to examine maternal effects (Castro 1999; Castro et al. 2008; Oliver and Borja 2010), few have highlighted the effect of the maternal environment on offspring growth (Cendán et al. 2013; Zas et al. 2013), and none have used mother tree twig traits as indicators of among-year environmental variation.

Pinus aristata Engelm. (Rocky Mountain bristlecone pine) and *Pinus flexilis* James (limber pine) are of conservation interest in the Rocky Mountains because of their ecological importance to high elevation forest systems and they are currently threatened by the invasive pathogen (*Cronartium ribicola*) that causes white pine blister rust (Schoettle and Sniezko 2007). Planting seedlings with genetic resistance to white pine blister rust is a key management option to sustain populations of these species (Burns et al. 2008). Improved understanding of the importance of maternal effects in these species will help with plant material development and increase the success of conservation efforts by clarifying the interpretation of common garden studies that explore genetic differentiation among populations.

The overall goal of this study was to determine whether environmental variation among years affects tree growth, seed provisioning, and maternal environmental effects on seedling traits. Specifically, we tested: (1) whether year-to-year differences in environment during seed maturation, as detected by mother tree twig traits, affect offspring seed characteristics; and (2) whether those differences in seed traits between source years correspond to differences in early seedling growth. We predicted that increased variation in twig growth between source years would correspond to increased variation in seed mass, which would relate to enhanced variation in seedling growth. A strong relationship between traits would indicate the presence of maternal effects such that a covariate could be used for year-to-year variation in common garden studies of long-lived plant species.

Materials and methods

Seed source

Seed sources originated across northern Colorado (Fig. 1) from multiple open-pollinated mother trees per population. To minimize both relatedness among progeny and the possibility of self-pollination, mother trees were spaced at least 60 m apart and open-pollinated cones were collected from the upper canopy of each tree. Both *P. aristata* and *P. flexilis* seeds had been previously collected in 2008 and 2009 and stored at –20 °C by Rocky Mountain Research Station (Ft. Collins, Colorado, USA).

Experimental design

We used *P. aristata* seeds from 12 mother trees and three populations sourced from 2008 and 2009 and *P. flexilis* seeds from 14 mother trees and five populations sourced from 2008 and 2009 (Table 1). Trees were selected based on overall health and vigor and had a minimum diameter of 15 cm at 1.4 m height. During the summer of 2012 we collected five twig clippings from the upper canopy of each mother tree to detect environmental effects on mother tree growth during seed development in each seed-producing year. Straight twigs with no branching or cone development were collected from the south side of each tree in full sun to minimize variation due to position within the tree. Each twig was cut and separated at the bud scale scar to isolate twig growth from each of the last 10 years. Using the methods of Schoettle (1994), the following measurements were taken on each year's twig growth, focusing on growth completed in 2008 and 2009, the years during seed maturation: increment length (twig extension), mean needle length, number of fascicles, and specific leaf area (SLA). SLA was calculated from the projected leaf area of that year's needle cohort (Delta-T Area Meter System; Delta-T Devices, Cambridge, England) divided by the dried mass of the needle cohort (65 °C, 48 h). The purpose of including the twigs was to characterize mother tree growth during seed provisioning, so tree twig data from the year of seed collection was used in analyses for all traits except number of fascicles, which is determined in the bud the year prior to cone maturity. Pollination in these species occurs in the summer of year 1; however, fertilization does not take place until the following spring (year 2), after which the seeds mature rapidly and are shed in the fall (year 2) (U.S. Department of Agriculture, Forest Service 1974). By focusing on environmental conditions during the year of seed collection, we concentrated on the time frame in which seed provisioning takes place. Traits measured for twigs, seeds, and seedlings are summarized in Table 2 (for raw data see Supplementary Tables S1–S3).[1]

To test for a relationship between twig growth and local annual weather conditions, we used precipitation and temperature data collected daily by a USDA Natural Resources Conservation Service Snowpack Telemetry (SNOTEL) station. Accumulated precipitation (Sensotec 100" Transducer; Honeywell, Columbus, Ohio, USA) and mean temperature (Extended Range; Omega Engineering, Inc., Stamford, Connecticut, USA) data were gathered from the Bear Lake SNOTEL site 322, which is central to the study sites.

X-radiography (model XPERT 80; Kubtec, Milford, Conneticut, USA) was used to ensure the presence of a full embryo (Willan 1987) before 30 seeds·family^{-1}·year^{-1} were randomly selected for inclusion in the study. Each ellipsoidal seed was weighed and maximum length and width were estimated. *Pinus flexilis* seeds were hydrated in a 1% solution of H_2O_2 before undergoing a 1–2 °C

[1]Raw data describing twig, seed, and seedling characteristics measured in this study are reported in Tables S1, S2, and S3, respectively, which are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/cjb-2014-0085.

Fig. 1. Seed source locations in northern Colorado for *Pinus aristata* (white) and *P. flexilis* (black). Enlarged inset in upper right corner shows seed sources in Rocky Mountain National Park. See Table 1 for abbreviation definitions.

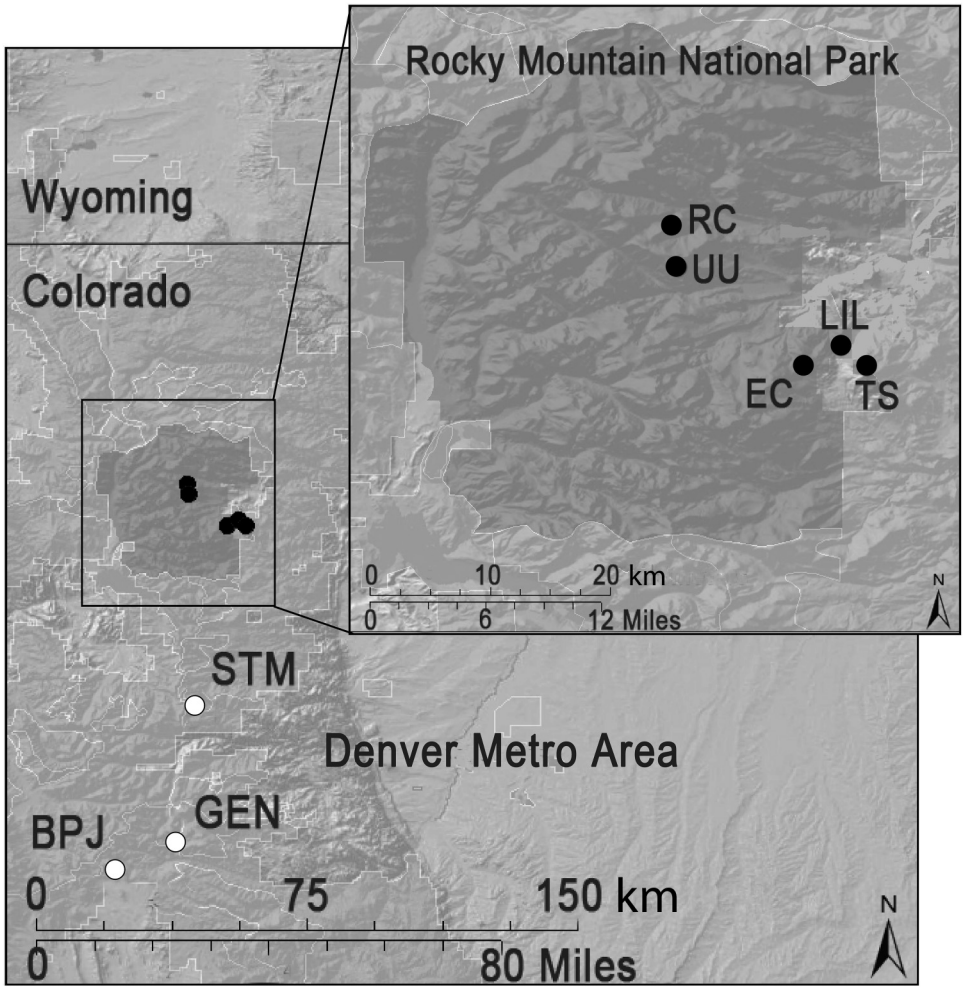


Table 1. Seed sources included in this study; open-pollinated seed was sampled from each mother tree in both 2008 and 2009.

Site	Site abbreviation	Latitude (°N), Longitude (°W)	No. of mother trees
Pinus aristata			
St. Mary's	STM	39.82946, –105.65435	7
Geneva	GEN	39.51573, –105.71334	2
Jefferson Beaver Ponds	BPJ	39.43129, –105.84575	3
Pinus flexilis			
Rainbow Curve	RC	40.39890, –105.66900	5
Upper Ute	UU	40.37140, –105.66500	2
Estes Cone	EC	40.29507, –105.56979	3
Lily Lake	LIL	40.30910, –105.54200	3
Twin Sisters	TS	40.29560, –105.52300	1

moist stratification period of 6 weeks in a growth chamber (Precision Scientific LT-105; Perry, Iowa, USA). Seeds were kept separate in labeled mesh bags, strung over a sealed plastic tub filled with approximately 2.5 cm of water, rinsed weekly, and checked for mold. Moldy seeds were rinsed with a 1% solution of H_2O_2. After stratification, seeds were sown in a planting medium, a 1:1 ratio of 4P mix (Conrad Fafard Inc., Agawam, Massachusetts, USA) and sand to increase drainage, in 656 mL deepots (D40h; Stuewe and Sons, Inc., Tangent, Oregon, USA). *Pinus aristata* seeds needed no treatment prior to sowing; seeds were sown directly into pots with the same soil mixture. Days to germination was not evaluated in this study.

Thirty seeds from each maternal tree and each source year were randomly assigned to 10 replicate blocks in a temperature-controlled greenhouse room at Colorado State University to monitor differences in early seedling growth between source years. Once sown, seeds were watered daily until germination was complete, then watered 2–3 times per week and fertilized weekly with Jack's Professional 20–8–20 Forestry (JR Peters, Inc., Allentown, Pennsylvania, USA) at 100 ppm. Over 87% of *P. flexilis* seedlings and 92% of

Table 2. Traits measured from twigs of mother trees, seeds, and seedlings.

Mother tree twig traits	Seed traits	Seedling traits
Increment length	Seed mass	Stem height
Twig needle length	Seed length	Cotyledon length
Specific leaf area	Seed width	Primary needle length
Number of fascicles		Relative growth rate
		Stem diameter
		Total dry biomass
		Root-to-shoot ratio

P. aristata seedlings emerged within a 9 day period, making them relatively the same age when measurements were taken; this variation in age is reasonable given the slow growth rate of both species. The greenhouse was kept at temperatures between 17 and 22 °C during the duration of the study with a 16 h (light) – 8 h (dark) photoperiod.

Seedling performance was assessed by measuring stem height, cotyledon length, primary needle length, relative growth rate (RGR), stem diameter, and dry biomass (Table 2). Stem and cotyledon length were measured once 40 days after sowing as these did not change over time, while measurements of RGR, primary needle length, and mortality were made at 80, 140, and 210 days after sowing. RGR was calculated as follows:

$$(1) \qquad RGR = \frac{\ln(s_2 - s_1)}{(t_2 - t_1)}$$

where s is equal to the length of needles in millimetres and t is equal to time in days. After 8 months, all seedlings were harvested for stem diameter measurements and dried at 65 °C for 7 days to determine root and shoot biomass. Stem diameter measurements were made with calipers directly below the cotyledons to ensure consistency among samples.

Data analysis

Analyzing each species separately, we tested: (1) whether twig characteristics, seed size, and seedling performance varied between years within each family; (2) whether differences in twig characteristics between years were related to the difference in seed size within each family; and (3) whether the difference in seed size between years was related to differences in seedling performance within each family. To determine whether there was inter-annual variation in each response variable for twig growth, seed size, and seedling performance, we used generalized linear mixed models with a random effect of site and fixed effects of year, family nested within site, and the interaction between year and family. We treated family as fixed to avoid testing interaction terms between variables of different classes. For models of twig characteristics, we also included twig as a repeated measures subject with compound symmetric error structure because the same twigs were measured for both years. For models of seedling performance, we also included a random effect of block within the greenhouse common garden. In cases where there were significant interactions between year and family, suggesting genetic variation among families in response to inter-annual variation, we interpreted year effects for each family individually.

The effect of variation in twig growth traits between years on variation in seed size between years was analyzed through linear regressions. Similarly, linear regressions were used to examine the effect of variation in seed size on variation in seedling performance. To assure independence of data points for each regression, we took the mean value of the twig, seed, and seedling traits for each maternal tree and each source year. To accurately compare mean traits between years, independent of differences in mean traits among families, we calculated the relative inter-annual (RIA) variation in each trait according to the following equation

$$(2) \qquad RIA = 1 - \left(\frac{Trait_{y2} - Trait_{y1}}{Trait_{y1}}\right)$$

where $Trait_{y2}$ is the mean trait value in seed source year 2 and $Trait_{y1}$ is the mean trait value in seed source year 1 for a given family. The RIA variation takes into account variation among families for twig growth, seed size, and seedling growth, allowing for an accurate comparison of inter-annual variation independent of differences in mean traits among families. The regression model used to determine the effect of twig growth differences during seed provisioning on seed mass differences was

$$(3) \qquad RIA_{seed} = RIA_{twig}$$

where RIA_{seed} is the RIA difference in seed mass between years and RIA_{twig} is the RIA difference in a twig trait between years. The regression model used to determine the effect of seed size differences on seedling growth differences observed in the greenhouse was

$$(4) \qquad RIA_{seedling} = RIA_{seed}$$

where $RIA_{seedling}$ is the RIA difference in a seedling trait. Transformations were needed to obtain a normal distribution for some traits. A square root transformation was used for RGR measured between 140 and 210 days (*P. aristata*) and RGR between 20 and 60 days (*P. flexilis*). RGR measured between 80 and 140 days was transformed as x^2 (*P. aristata*). An inverse transformation was used for cotyledon length, stem height, and needle length at 180 days (*P. flexilis*). Normality was monitored through the Shapiro–Wilk test and the distribution of residuals in quantile–quantile plots and histograms. Data for seed length, width, and mass were used in a principal component analysis; the resulting factor PC1 had seed mass accounting for 91% of the total variance in *P. aristata* and 82% in *P. flexilis*, so seed mass was used in future analyses for ease of interpretation. All statistical analyses were conducted with SAS 9.3 (SAS Institute, Cary, North Carolina, USA).

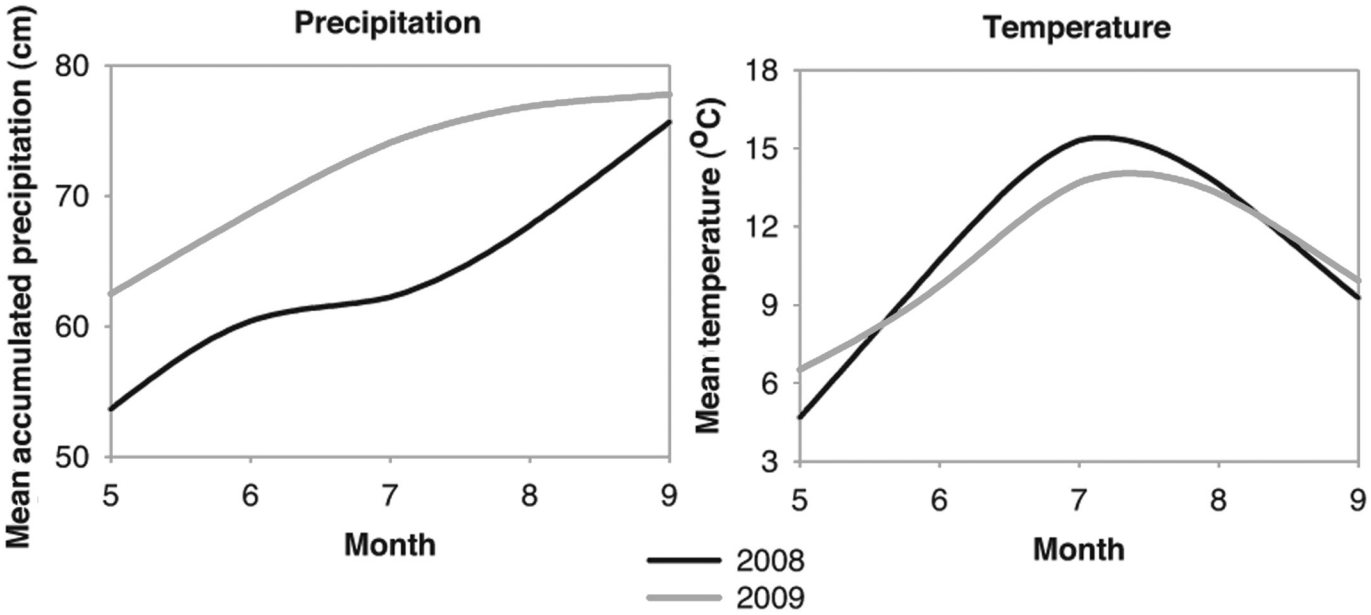
Results

Weather data revealed that 2008 was warmer, with temperatures averaging over 1.6 °C greater in July, and drier, with 80 mm less accumulated precipitation during the summer months, than in 2009 (Fig. 2). These weather patterns were reflected in other SNOTEL sites throughout the area and are within the normal range of weather over the last 15 years.

Trait variation among years

Significant interactions were observed between year and family for some twig, seed, and seedling traits (Table 3). For the mother tree twig traits, the year × family interaction was significant for needle length and specific leaf area in *P. aristata* and increment length, needle length, and number of fascicles in *P. flexilis*. Although families differed in the magnitude of response to year for some traits, in general most families had longer needles (both species), longer increment length (*P. flexilis*), and lower specific leaf area (*P. aristata*) in 2008, the dry year (for raw data see Supplementary Tables S1–S3).[1] Yearly variation was not evident in increment length or number of fascicles of *P. aristata* or specific leaf area of *P. flexilis*, but variation among mother trees was evident for these traits. For both species, seed mass varied among mother trees in response to year (Table 3). However, as with twig traits, the year × family interaction resulted primarily from differences

Fig. 2. Mean accumulated precipitation (left) and temperature (right) during the growing season for seed collection years 2008 (black) and 2009 (gray). Data from USDA Snowpack Telemetry station 322.



among families for the magnitude of the year effect; seeds collected in 2008 were, on average, larger than those from 2009 for all but one family of each species. For seedling performance, the year × family interaction was significant for stem, cotyledon, and needle length at most time intervals (both species), total dry mass (*P. aristata*), and relative growth rate between 20 and 60 days (*P. flexilis*) (Table 3). Values for all of these variables were generally greater in 2008 than 2009, although the magnitude varied for families. Main effects of year were detected for relative growth rates (both species), stem diameter, total dry mass, and root-to-shoot ratio (*P. flexilis*), and needle length at 80 days (*P. aristata*) (Table 3). With the exception of root-to-shoot ratio and relative growth rates, these variables had greater values in 2008 compared with 2009. Variation among mother trees that did not depend on year was evident for stem diameter (both species), early relative growth rate (*P. aristata*), and root-to-shoot ratio (*P. flexilis*) (Table 3).

Linear regression among traits

Maternal tree twig growth, seed provisioning, and progeny performance were greater in 2008 than 2009, however, inter-annual variation in mother tree twig traits did not correlate linearly with inter-annual variation in seed mass (Table 4). RIA variation in seed mass did significantly influence inter-annual variation in seedling performance for some traits. For *P. aristata*, $RIA_{seed\ mass}$ was positively related to $RIA_{seedling\ biomass}$ at 8 months but negatively to $RIA_{seedling\ needle\ length}$ at 7 months ($p < 0.05$) (Fig. 3, Table 4). This negative relationship was driven by a single outlier; once removed, no significant relationship existed. For *P. flexilis*, $RIA_{seed\ mass}$ positively influenced $RIA_{cotyledon\ length}$, $RIA_{stem\ diameter}$ at 8 months, $RIA_{stem\ length}$, and $RIA_{seedling\ needle\ length}$ at 20 days, 120 days, and 180 days ($p < 0.05$) (Fig. 4, Table 4).

Discussion

The overall goal of this study was to determine whether maternal environment during seed development affects traits of the resultant seedlings by assessing the effect of environmental variation among years on mother tree growth, seed provisioning, and seedling performance. Specifically, we tested: (1) whether year-to-year differences in environment during seed maturation, as detected by mother tree twig traits, affected offspring seed characteristics; and (2) whether those differences in seed traits between source years corresponded to differences in early seedling growth. There were significant between-year differences in mother tree growth traits, yet they were not significantly related to seed and seedling traits in linear regressions. However, we clearly found that variation in seed mass between years correlated with early seedling growth, especially in *P. flexilis*, and that seed mass was highly correlated with seedling cotyledon length.

Differences in maternal tree growth between seed source years were evident in twig clippings, with increased growth occurring in 2008, the warm dry year. Although the year with most mother tree growth corresponded to the year with largest seeds, this correlation was not statistically significant (Table 4). RIA differences in seed size between seed source years revealed significant relationships with seedling traits. In *P. aristata*, increased variation in seed mass between years was associated with increased variation in total dry mass of seedlings, explaining 27% of the variation in seedling traits. In *P. flexilis*, RIA variation in seed mass was positively associated with variation in cotyledon length, stem height, stem diameter, and needle length at 20, 120, and 180 days. These positive relationships indicate that, on average, large seeds produced large seedlings in the same year. In the strongest relationship, seed mass variation explained 66% of variation in cotyledon length, suggesting cotyledon length is a good indicator of maternal effects.

This study focused on maternal environment as a source of variation, though variation in paternal genotype might have also played a role in offspring variation. The mother trees were open-pollinated, meaning the pollen cloud responsible for fertilization could have come from a variety of pollen sources and likely differed year-to-year. Offspring trait variation could therefore have been influenced by the environment during seed maturation on the mother tree as well as during pollen development and the fact that different pollen sources (and thus paternal genotypes) may have fertilized the maternal plant in different years. Though paternal genotype may have led to offspring variation in this study, others have found that the maternal plant influences progeny traits to a much higher degree. Burgess and Husband (2004) found that variation in fitness of red and white mulberry hybrids was influenced mainly by maternal, rather than paternal, plant genotype. *Eucalyptus globulus* seed germination traits have also been found to be most heavily influenced by maternal plant (Rix et al.

Table 3. Results of the generalized mixed model using a random effect of site and fixed effects of year, family nested within site, and the interaction between year and family.

Response	Effect	df		F	p
		Numerator	Denominator		
Pinus aristata					
Twig increment length	Year	1	59	0.00	0.95
	Family(site)	11	48	2.71	0.01
	Year × family(site)	11	48	0.72	0.71
Twig needle length	Year	1	48	17.26	0.00
	Family(site)	11	48	2.03	0.05
	Year × family(site)	11	48	3.61	0.00
Number of fascicles	Year	1	59	1.43	0.23
	Family(site)	11	48	2.52	0.01
	Year × family(site)	11	48	1.12	0.37
Specific leaf area	Year	1	47	11.21	0.00
	Family(site)	11	64	5.53	<0.0001
	Year × family(site)	11	47	2.71	0.01
Seed mass	Year	1	588	184.17	<0.0001
	Family(site)	11	108	160.92	<0.0001
	Year × family(site)	11	588	13.37	<0.0001
Stem length	Year	1	519	108.62	<0.0001
	Family(site)	11	107	19.50	<0.0001
	Year ×family(site)	11	517	3.95	<0.0001
Cotyledon length	Year	1	512	121.02	<0.0001
	Family(site)	6	1	2.25	0.47
	Year × family(site)	11	510	4.95	<0.0001
Needle length at 20 days	Year	1	515	132.41	<0.0001
	Family(site)	11	115	17.54	<0.0001
	Year × family(site)	11	513	3.01	0.00
Needle length at 80 days	Year	1	523	8.37	0.00
	Family(site)	11	147	1.64	0.09
	Year × family(site)	11	514	1.06	0.39
Needle length at 140 days	Year	1	529	0.72	0.40
	Family(site)	11	154	4.06	<0.0001
	Year × family(site)	11	520	1.79	0.05
Stem diameter	Year	1	521	2.72	0.05
	Family(site)	11	99	4.06	0.04
	Year × family(site)	11	513	0.80	0.64
Total dry mass	Year	1	517	10.86	0.00
	Family(site)	8	1	2.93	0.42
	Year × family(site)	11	515	1.95	0.03
Root-to-shoot ratio	Year	1	525	0.18	0.60
	Family(site)	11	109	5.37	0.63
	Year × family(site)	11	515	0.96	0.48
Relative growth rate 3 between 20 and 60 days	Year	1	524	22.92	<0.0001
	Family(site)	11	164	2.01	0.04
	Year × family(site)	11	515	0.91	0.53
Relative growth rate 5 between 80 and 140 days	Year	1	528	10.22	0.00
	Family(site)	11	1	2.99	0.45
	Year × family(site)	11	519	1.41	0.16
Pinus flexilis					
Twig increment length	Year	1	44.64	26.19	<0.0001
	Family(site)	13	57.03	7.14	<0.0001
	Year × family(site)	10	44.59	2.26	0.03
Twig needle length	Year	1	46.68	2.96	0.09
	Family(site)	13	57.79	8.05	<0.0001
	Year × family(site)	10	46.61	2.30	0.03
Number of fascicles	Year	1	45.8	0.00	0.95
	Family(site)	13	56.82	7.48	<0.0001
	Year × family(site)	10	45.8	2.10	0.04
Specific leaf area	Year	1	54.33	0.55	0.49
	Family(site)	13	56.34	15.78	<0.0001
	Year × family(site)	10	44.5	1.33	0.25
Seed mass	Year	1	685	393.71	<0.0001
	Family(site)	13	126	70.43	<0.0001
	Year × family(site)	13	685	9.62	<0.0001
Stem length	Year	1	450	19.71	<0.0001
	Family(site)	9	1	4.59	0.35
	Year × family(site)	10	422	2.31	0.01

Table 3 (*concluded*).

Response	Effect	df		*F*	*p*
		Numerator	Denominator		
Cotyledon length	Year	1	452	47.06	<0.0001
	Family(site)	13	139	40.81	<0.0001
	Year × family(site)	10	423	3.08	0.00
Needle length at 20 days	Year	1	435	48.18	<0.0001
	Family(site)	13	167	15.59	<0.0001
	Year × family(site)	10	407	2.92	0.00
Needle length at 120 days	Year	1	439	9.13	0.00
	Family(site)	13	163	11.90	<0.0001
	Year × family(site)	10	415	2.50	0.01
Needle length at 190 days	Year	1	426	19.00	<0.0001
	Family(site)	13	147	13.07	<0.0001
	Year × family(site)	10	397	3.01	0.00
Stem diameter	Year	1	380	9.98	0.00
	Family(site)	13	120	11.46	<0.0001
	Year × family(site)	10	401	1.29	0.23
Total dry mass	Year	1	371	10.96	0.00
	Family(site)	13	113	18.12	<0.0001
	Year × family(site)	10	393	1.04	0.41
Root-to-shoot ratio	Year	1	368	4.18	0.06
	Family(site)	13	110	3.19	0.00
	Year × family(site)	10	392	0.65	0.77
Relative growth rate between 20 and 60 days	Year	1	412	9.16	0.00
	Family(site)	13	129	3.74	<0.0001
	Year × family(site)	9	373	3.41	0.00
Relative growth rate between 120 and 190 days	Year	1	295	1.99	0.30
	Family(site)	13	157	1.36	0.34
	Year × family(site)	9	290	0.44	0.91

Note: A significant year × family(site) interaction indicates trait variation among families in response to year.

2012). Similar patterns of high maternal influence in offspring trait variation have also been found with annual plants (Antonovics and Schmitt 1986; Byers et al. 1997).

The seed source years included in this study were determined by what seed was available and the years were not extreme for weather. The study duration was also relatively short in relation to the species' lifespans, lasting less than 1 year; however, this time period captured the stage when maternal effects were expected to be greatest. Time and resources permitting, it would be interesting to test whether the difference in magnitude of maternal effects is greater when comparing seeds that developed in more diverse environmental conditions than those in this study and observing seedling growth trends over a longer period of time. Possibly, because these species exhibit masting reproductive patterns, displaying variable and synchronized cone production, seeds mature only in response to certain climatic cues. This has been found in other tree species (Pérez-Ramos et al. 2010; Mooney et al. 2011; Smaill et al. 2011) and may make it difficult to repeat this study using seed that developed during very different environmental conditions because they may not mature in those years, thereby reducing maternal effects over time.

Factors other than environmental conditions can also contribute to seed size variation, such as the cone size or location of seed within the cone (McGinley et al. 1990). However, Zas et al. (2013) found that *Pinus pinaster* seed size depended on maternal genotype and maternal environment even when accounting for the amount of resources allocated to seed development. There can also be a trade-off in offspring size and number, where few, large seeds are produced (Smith and Fretwell 1974), which could also contribute to seed mass variation. It is possible that this type of heterogeneity within mother trees and among years obscured significant relationships between twig traits and seed size.

Maternal effects due to seed provisioning as studied here are more easily explained than some other causes of maternal effects, such as epigenetics. In study systems with prolonged maternal effects, such as this one where maternal effects lasted throughout the 8 months of the study, the influence of the maternal environment on seedling performance should be assessed prior to interpreting studies to test for genetic differentiation among populations. Studies to determine the presence of genetic differentiation in seedling experiments should first determine the influence of maternal effects in the species and then account for the difference in seedling size statistically. Bischoff and Müller-Schärer (2010) found in their study with angiosperms that using seed mass as a covariate was not effective in reducing maternal effects and tended to underestimate genetic differentiation in common gardens. They suggested using initial plant size to best account for maternal provisioning. We found in this study that cotyledon length in *P. flexilis* best represented the inter-annual variation in the maternal plant seed provisioning; the years with large seeds were associated with large cotyledons, a finding mirrored in Reich et al. (1994). This trend is consistent with biological mechanisms of provisioning. Growth of cotyledons is related to the size of the megagametophyte reserve in the germinating seedling (Sasaki and Kozlowski 1970), so large seeds are able to produce seedlings with long cotyledons. Measuring cotyledon length on a young seedling is easier than measuring the mass of each individual seed and tracking the resultant seedling. Therefore, we propose using cotyledon length as a covariate to account for any residual maternal effects in greenhouse common garden studies with *P. flexilis* sourced over multiple years or from geographically or climatically varied populations. Maternal effects were least influential in *P. aristata* (as shown by decreased variation in seed mass due to the year effect and diminished relationships between RIA variation in seedling growth traits and RIA variation in seed mass), and cotyledon length was not highly correlated with seed size, questioning the need for any statistical correction in that species.

In this study, we predicted that increased variation in twig growth would correspond to increased variation in seed mass, which would relate in turn to large variation in seedling growth.

Table 4. Results of the linear regression analyses determining (A) whether the relative inter-annual (RIA) variation in mother tree twig traits predicted the RIA variation in seed mass and (B) whether the RIA variation in seed mass predicted the RIA variation in seedling traits, including the species, variable measured from either the twig or the seedling, slope, and r^2 and p values for each regression equation (n = 12 for *Pinus aristata* and n = 11 for *P. flexilis*).

		Slope	r^2	p
(A) Mother tree twig trait (explanatory variable)				
Pinus aristata	RIA twig increment length	0.04	0.00	0.89
	RIA twig needle length	0.16	0.06	0.46
	RIA number of fascicles	0.06	0.01	0.73
	RIA specific leaf area	0.49	0.19	0.16
Pinus flexilis	RIA twig increment length	−0.13	0.06	0.46
	RIA twig needle length	0.36	0.08	0.40
	RIA number of fascicles	0.11	0.03	0.29
	RIA specific leaf area	−0.49	0.04	0.57
(B) Seedling growth trait (response variable)				
Pinus aristata	RIA cotyledon length	0.22	0.26	0.09
	RIA stem length	0.31	0.20	0.15
	RIA seedling needle length at 20 days	0.18	0.15	0.22
	RIA seedling needle length at 50 days	−0.03	0.01	0.82
	RIA seedling needle length at 80 days	−0.04	0.01	0.75
	RIA seedling needle length at 140 days	−0.25	0.23	0.11
	RIA seedling needle length at 210 days	−0.25	0.33	0.05
	RIA stem diameter	0.12	0.16	0.20
	RIA seedling dry biomass	1.13	0.34	0.05
	RIA root-to-shoot ratio	0.04	0.01	0.78
	RIA relative growth rate (RGR) between 20 and 50 days	−1.06	0.30	0.07
	RIA RGR between 50 and 80 days	−0.20	0.02	0.67
	RIA RGR between 80 and 140 days*	−0.97	0.10	0.31
	RIA RGR between 140 and 210 days*	0.47	0.01	0.74
Pinus flexilis	RIA cotyledon length*	1.21	0.65	0.00
	RIA stem length	1.49	0.41	0.03
	RIA seedling needle length at 20 days	2.05	0.54	0.01
	RIA seedling needle length at 60 days	1.05	0.26	0.09
	RIA seedling needle length at 120 days	1.83	0.41	0.03
	RIA seedling needle length at 180 days*	0.80	0.47	0.02
	RIA stem diameter*	1.62	0.37	0.05
	RIA seedling dry biomass	0.09	0.00	0.93
	RIA root-to-shoot ratio	0.22	0.05	0.51
	RIA RGR between 20 and 60 days*	1.99	0.23	0.27
	RIA RGR between 60 and 120 days	−0.25	0.03	0.63
	RIA RGR between 120 and 190 days	1.62	0.12	0.40

*Transformation needed to achieve normality; slopes are back-transformed to be comparable.

Fig. 3. Relationships between the relative inter-annual (RIA) variation in seed mass and the RIA variation in (*a*) seedling dry mass at 8 months and (*b*) seedling needle length at 7 months for *Pinus aristata* ($p < 0.05$). Trend lines represent the best fit from the linear regression analysis. The filled circle in graph *b* is an outlier that drives the negative relationship; when removed from the analysis, no significant trend is present. RIA values >1 indicate 2008 had larger seed or seedling traits while RIA values <1 indicate 2008 had smaller traits than observed in 2009. Non-significant relationships between RIA variation and seed mass and RIA variation in cotyledon length, stem height, needle length at 20, 50, 80, and 140 days, stem diameter, root-to-shoot-ratio, and relative growth rate are not shown.

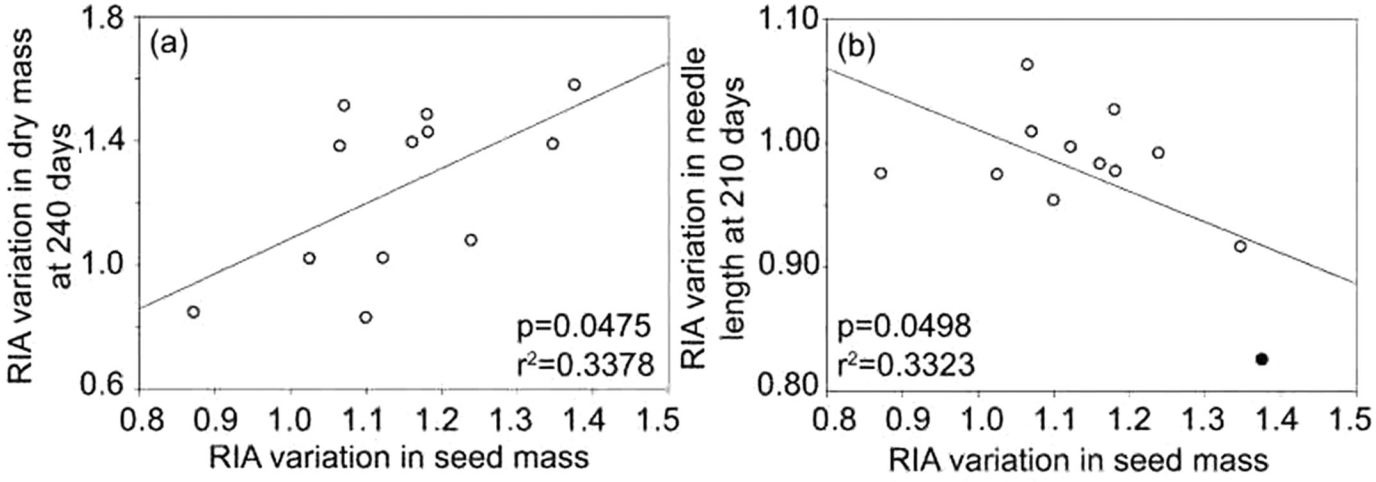
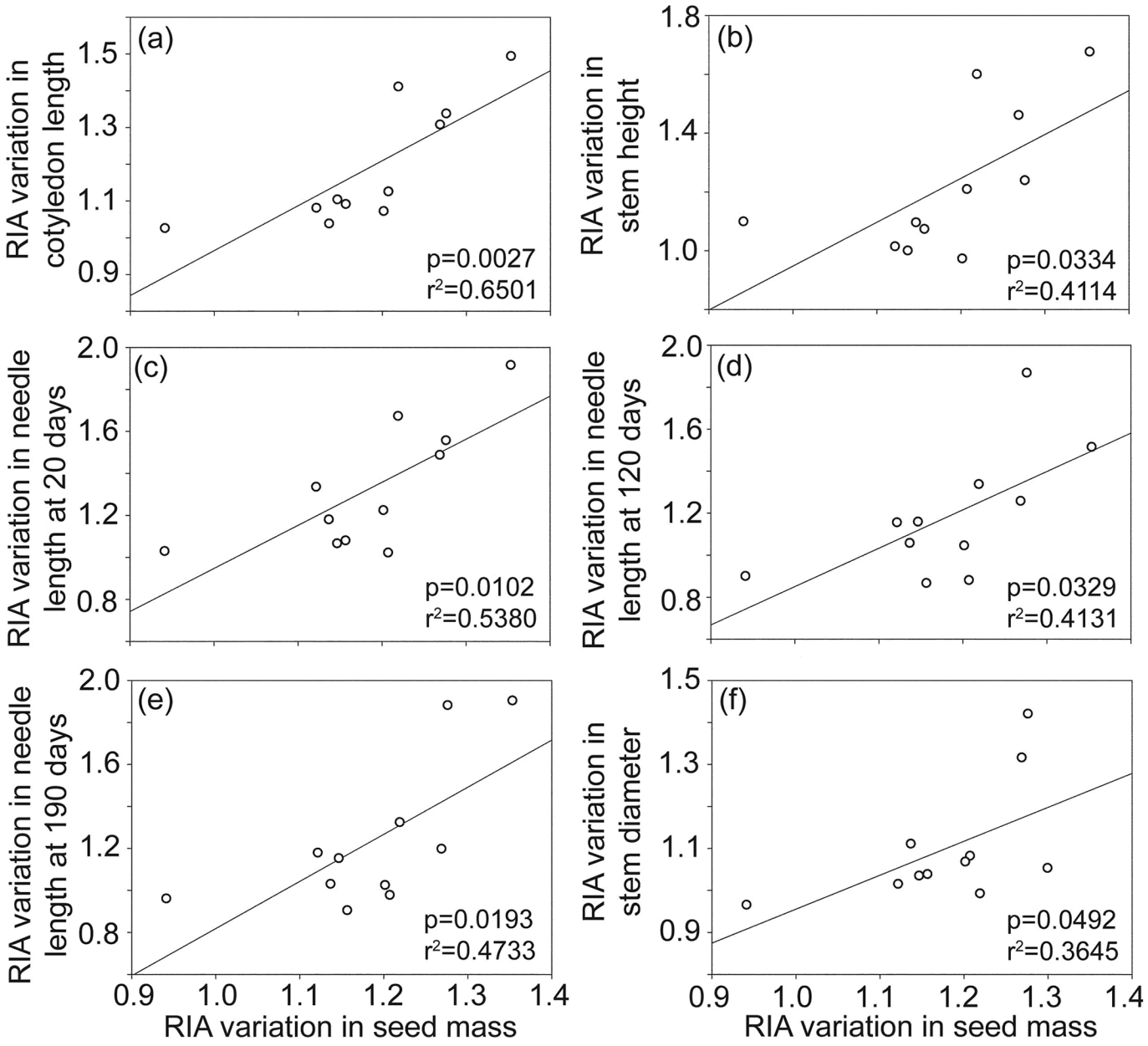


Fig. 4. Relationships between the relative inter-annual (RIA) variation in seed mass and the RIA variation in the following seedling traits for *Pinus flexilis*: (*a*) cotyledon length, (*b*) stem height, needle length at (*c*) 20 days, (*d*) 120 days, and (*e*) 190 days, and (*f*) stem diameter ($p < 0.05$). Trend lines represent the best fit from linear regression analyses. The positive relationship in each case signifies that years with large seeds produced large seedlings. RIA values >1 indicate 2008 had larger seed or seedling traits while RIA values <1 indicate 2008 had smaller traits than observed in 2009. Non-significant relationships between RIA variation and seed mass and RIA variation in stem height, needle length at 60 days, dry biomass, root-to-shoot ratio, and relative growth rate are not shown.



Though inter-annual variability was found in twig growth, seed size, and seedling growth, differences in twig growth were not significantly related to differences in seed mass. For many traits, however, RIA seed mass variation was related to variation in seedling performance, indicating that maternal effects influence early seedling growth in *P. aristata* and *P. flexilis*. Overall, examining differences in twig growth between years using our sampling intensity was not effective in predicting seed size. However, examining seed collected over 2 years from the same maternal plant was effective in determining the influence of maternal effects on early growth traits of offspring. The use of this technique to study the impact of maternal effects in other long-lived species will help to clarify the interpretation of common garden studies, preventing the pitfall of mistaking environmental for genetic differences in progeny performance.

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