Neighbouring Scots pine populations from contrasting climatic regions show substantial variability but consistent response to warming

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ABSTRACT

Natural tree populations consist of individuals that exhibit intraspecific adaptive variation at a range of geographic scales, as a result of the balance between gene flow and selection. The spatial distribution and magnitude of such variation will influence the capacity of populations to adapt to forthcoming changing environmental conditions. The native Scots pine populations in Scotland represent what remains of the iconic Caledonian forest. Despite being distributed within a relatively narrow geographic area these populations occur across a steep East-West environmental gradient of increasing rainfall and temperature. We hypothesised that western populations compared to those from the east may be better adapted to warmer conditions and as a consequence, may respond differently to the increased temperature predicted during climate change. We conducted an experiment lasting 22 weeks in controlled environment chambers using a nested hierarchical design based on material from different regions (west or east), populations and families (half sibs). We examined the effects of two temperature scenarios, current temperature and warmer predicted temperature on percentage germination as well as growth and morphology of above and below-ground traits. Most of the variation occurred at the family level. Nevertheless, significant regional and population differences were detected, where eastern populations invested more in roots, exhibited fewer stomatal rows per needle and produced thinner roots. In addition, warmer temperatures had strong effects on early growth that were consistent in material from both regions and resulted in earlier germination, greater growth and biomass, but these effects were not accompanied by shifts in biomass partitioning. Although the strong effect of warming suggested temperature limitation for early growth in the study areas under current conditions our results did not support the idea that low seedling recruitment resulting from poor emergence and early growth would lead to a decline in Scottish populations of Scots pine under a warmer climate. Our results are informative regarding the adaptive potential in the populations and will contribute to the development of appropriate forest conservation strategies.

1. Introduction

Intraspecific genetic variation determines adaptive potential and is a key part of plant functional ecology (Hoffmann and Sgrò, 2011; Savolainen et al., 2013). Consequently, this variation has been the subject of increasing research interest, particularly in the context of climate change, given its crucial role in determining the response of a species to new environmental conditions (Alberto et al., 2013; Anderson, 2016). The amount of intraspecific genetic variation is a consequence of the balance between selective pressures and gene flow, together with mutation and drift (Loveless and Hamrick, 1984). The intensity and direction of selective pressures differ across the distribution range of a species, leading to a shift in optimal conditions maximising fitness depending on the local environment (Savolainen et al., 2007), and

resulting in higher frequencies of genotypes tuned for local optimum conditions at different sites. However, gene flow acts in opposition to natural selection (Kawecki and Ebert, 2004) and, as a consequence, populations show variation around the optimum, with a magnitude determined by the strength of selection. Intraspecific genetic variation will be key for maintaining the resilience of forest ecosystems against environmental changes. Therefore, in tree species, which are predicted to be highly vulnerable to rapid climate change due to their long life cycles (Dyderski et al., 2018), an understanding of intraspecific adaptive variation is crucial to plan strategies aimed at optimising their resilience to the expected changes.

During the last century global mean temperature has increased by a mean of 1.7ºC, and even if greenhouse gas emissions were to cease immediately, temperature is predicted to continue rising through the 21st Century (IPCC, 2021). Temperature plays an important role in several components of individual performance such as seed germination, growth and survival (Arft et al., 1999; Chidumayo, 2007; Walck et al., 2011), an effect that is particularly acute at species' altitudinal limits (Harsch et al., 2009; Greenwood et al., 2015; Matías et al., 2017) or in cold areas where temperature-induced drought is not a limiting factor (Wilmking et al., 2004). Increases in global temperature are therefore likely to have important consequences for species persistence and for forest population dynamics at a range of scales (Peñuelas and Boada, 2003; van Mantgem et al., 2009; Matías and Jump, 2015; Matías et al., 2016). Many predictions often make the assumption that species will respond homogeneously to warmer temperatures (Germain and Lutz, 2020; Koide et al., 2016) but this omits to consider differences in the extent of local intraspecific adaptive potential, which may result in very distinct responses among populations. Studies at continental scales that have taken intraspecific variation into consideration have found that this aspect of diversity may be key to buffer the negative impacts of climate change on the distribution of species (Benito Garzón et al., 2011, 2019; Valladares et al., 2014; Oney et al., 2013; Reich and Oleksyn, 2008; Matías and Jump, 2014; Matías et al., 2017). However, adaptive responses to warmer temperatures at smaller spatial scales have received substantially less attention (but see Wilmking et al., 2004; Benito Garzón et al., 2011).

Adaptive divergence has been found among tree populations at relatively small spatial scales, most probably due to heterogeneity in local selection pressure (Salmela et al., 2013; Donnelly et al., 2016). However, much genetic diversity is of low adaptive significance in a consistent home environment or involves variation that may only become selectively important under novel environments and is therefore 'cryptic' until exposed to new conditions. Therefore, this cryptic variation has the potential to gain adaptive significance under a climate change scenario (Gibson and Dworkin, 2004) and can strongly influence the ability of tree natural populations to adapt to new local conditions (Paaby et al., 2014). Cryptic genetic variation should be revealed by direct experimental manipulation of conditions (Paaby et al., 2014) and therefore be detected by increases in within-population phenotypic variation when populations are experimentally transplanted into novel environments (Ledon-Rettig et al., 2014; Donnelly et al., 2018). In addition, phenotypic plasticity, defined as the range of phenotypes a single genotype can express in response to cues from its environment, may also operate when novel conditions are present (Nicotra et al., 2010). Consequently, there is a need to understand both the extent of population adaptive potential and how variation in adaptive traits might interact with climate in order to improve our ability to predict the response of particular tree populations under global warming conditions.

In plants, the seedling life stage represents the time at which the action of natural selection is at its strongest and is when the greatest proportion of a generational cohort is lost (Green et al., 2014). Their size and fragility makes seedlings particularly susceptible to disturbances (Vizcaíno-Palomar et al., 2014), and they usually respond faster to environmental changes than adult trees (Lloret et al., 2009).

Consequently, consideration of germination and establishment, which are key fitness components, will be critical to understand the response of trees to novel environments and to identify the abiotic drivers to which populations are locally adapted (Alberto et al., 2013). An assessment of the differential contribution of above and below ground functional traits can provide a useful way to characterize plant growth and resource use strategies, and has been shown to vary considerably at different organisational levels (Hajek et al., 2013; Donnelly et al., 2016; Violle et al., 2007).

Scots pine (*Pinus sylvestris* L.) is one of the most widely distributed tree species in the world and, at a continental scale, shows strong patterns of intraspecific adaptive variation (Andersson and Fedorkov, 2004; Matías and Jump, 2014; Matías et al., 2014). The north-western limit of Scots pine species distribution occurs in Scotland and, as a globally important peripheral population, Scottish populations of Scots pine may contain unique genetic variation in terms of adaptive potential. Scottish populations have maintained high levels of neutral (Sinclair et al., 1998; Wachowiak et al., 2011) and adaptive genetic diversity (Salmela et al., 2011; Salmela et al., 2013; Perks and Mckay, 1997; Perry et al., 2016; Donnelly et al., 2016) despite several millennia of isolation with respect to the core continental populations. Regarding neutral variation, previous studies found that low levels of population differentiation persist in Scottish populations, suggesting that effective population size, together with extensive gene flow, has been high sufficient to limit the effects of genetic drift. (González-Díaz et al., 2018). Scots pine forests in Scotland experience a temperate and oceanic climate, but the remaining native populations are scattered over a highly heterogeneous landscape which varies in aspect and altitude. Consequently, there is an increase in mean temperature and rainfall patterns from East to West, with markedly different climatic conditions (in terms of temperature, precipitation and growing season length) at the longitudinal extremes (Salmela et al., 2010). The contrasting landscape and climatic conditions across such a narrow spatial scale makes Scottish populations a unique model system to study underlying processes of intraspecific adaptive variation and their responses to the predicted changes in a future warmer climate.

Populations from colder sites are more likely to be experiencing the limiting effects of cold temperatures and are more likely to reveal cryptic genetic variation when exposed to warmer conditions, as compared to populations from warmer sites. We test these ideas by comparing Scots pine populations from an East to West climatic gradient across Scotland. Studies under controlled conditions which mimic natural environment and/or the conditions predicted under climate change constitute an excellent way to evaluate intraspecific variation in key functional traits, and help to disentangle the underlying processes (environmental vs. genetic) involved in the responses to novel pressures (e.g. warmer temperature). The main objective of this study was to characterize the intraspecific genetic variation in Scots pine early growth traits (seed mass, seedling emergence, biomass accumulation and key above & belowground functional traits) among and within populations, to relate this variation to home site environment, and to estimate the likely response of those populations to the projected changes in climate for the area. Specifically, we sought to address the following questions: (1) do early growth traits in plants growth under current temperature regimes differ in populations from western compared to eastern Scotland? and (2) does the extent of variation in response to warmer conditions differ in populations from the west compared to those in the east of Scotland? Studies that aim to address these questions may reveal whether adaptive genetic divergence is evident and whether the level of cryptic genetic variation varies among populations. We hypothesized that (1) early growth traits will differ in populations from the west compared to those in the east as a consequence of the steep longitudinal climatic gradient under current conditions, and (2) the response of early growth traits to warmer temperatures will be stronger in populations from the eastern range. By addressing these questions, we aim to improve our understanding of the scale of variation under current conditions and the capacity of Scots pine to tolerate predicted changes in conditions at the

seedling stage, and consequently better predict its population dynamics.

2. Material and methods

2.1. Study populations and seed collection

Cones were collected in the winter of 2015 from Scots pine (*Pinus sylvestris* L.) mother trees growing in four natural populations representing two of the most westerly (BE(W): Benn Eighe and SH(W): Shieldaig) and two of the most easterly (GT(E): Glen Tanar and GD(E): Glen Derry) populations in Scotland (Table 1). With the objective of capturing maximum variation within each population, mature cones were collected from mother trees located along transects at three altitudinal levels, covering the whole altitudinal range of each population (Table 1). Four half sib families were sampled in each altitudinal level (12 families per population in total). Care was taken to ensure that sampled mother trees grew at least 40 m apart, as family clusters of Scots pine can occur at distances of less than this in Scottish pinewoods (González-Díaz et al., 2017). Altitude was subsequently found not to explain significant variation in the studied traits and is therefore not considered in any subsequent analysis. To summarize, the sampling design was: 2 regions (East and West) x 2 populations per region x 12 families per population, resulting in 4 populations and 48 families sampled in total.

Cones were oven dried at 45 °C for 3–4 days which seeds were extracted. Cone size was classified as large, medium or small and seeds were pooled within a maternal progeny. Number of viable and non-viable seeds was visually evaluated and recorded, using full seeds as a proxy of viability in contrast to empty seeds. Viability was defined as the proportion of viable seeds present in the total set of seeds (viable plus non-viable). Viable seeds were weighed to estimate mean seed biomass per family within each population.

2.2. Experimental design

Ten replicates of each of the 48 families were separated in individual pots on June 2015 at the Controlled Environmental Facilities of the University of Stirling (UK). However, three seeds were initially surface sown in each pot to ensure a sample per replicate, thinning to one when multiple seedlings emerged, and retaining the first one to emerge. Tubular pots (6.5 cm diameter, 45 cm height), were filled with a 2:1 mixture of peat and river sand. A bottom layer of gravel was used to improve drainage. Before sowing, each pot was irrigated with 200 ml of soil microbial inoculum resulting from the maceration of roots and soil beneath adult Scots pine trees growing at the University of Stirling, in central Scotland, to favour formation of mycorrhizas (Matías and Jump, 2014). In total 480 pots were randomized in blocks inside four Snijders Scientific MC1750E (Tilburg, Netherlands) controlled environment chambers (inner space 1.8 m length × 0.75 m wide × 1.2 m high), resulting in 120 pots in each chamber.

The experimental design applied a nested structure to examine families within populations. Two temperature treatments were applied throughout the experiment from sowing to harvest: half of the samples (48 families belonging to the four populations with 5 individual replicates) were grown in current temperature (CT) and the other half in predicted warmer temperature (WT). Two chambers were used for each

of the temperature treatments, each treatment contained 5 replicates of each of the 48 families (240 pots in each treatment, 120 pots per chamber). Temperature for the CT treatment was established by calculating the mean day and night values for each month of the growing season using data from a weather station located nearest to each of the sampled populations for the period 1981–2010: Kinlochewe (57.613 N, −5.308 W, 25 m.a.s.l.) and Aultbea (57.859 N, −5.636 W, 11 m.a.s.l.) nearest to the western populations, and Aboyne (57.077 N, −2.836 W, 140 m.a.s.l.) and Braemar (57.006 N, −3.397 W, 339 m.a.s.l.) nearest to the eastern populations, available at http://metoffice.gov.uk. The temperature regime CT represented the mean across these four weather stations, ensuring all plants under CT received the same temperature regime. The WT treatment was set up with an increase of 5 °C over that of CT, at both day and night temperatures (Table 2), which is the predicted increase in mean temperature under the UKCP09 high emission scenario by 2080 at the 50% probability level (http://ukclimateprojections.metoffice.gov.uk/). This high emission scenario is based on the SRES A1F1 emission scenario (IPCC, 2014). Day and night temperatures were set up with a constant duration of 16 and 8 h respectively (experimental temperature values are summarised at Table 2). Light intensity was fixed with a photosynthetic photon flux density of 210 mol m^2 s^{-1}, rising progressively at dawn and decreasing at dusk for 1 h, which is the value representative for forest understory (Valladares et al., 2004). The appropriate watering regime was calculated on the basis of the mean rainfall value for each weather station during the growing season (May to September), for the period 1981–2010, resulting in monthly precipitation levels of 86.2 l/m^2. Watering was applied twice per week, assuming therefore that water availability was not a limitation in our study. Air relative humidity and CO_2 concentration were kept constant at 70% and 891 mg m^{-3} (460 ppm) respectively. To minimise any possible chamber effect, all pots were rotated between the different chambers, spending at least one month in each chamber after programming for the appropriate treatment conditions, whilst also randomising block position within chambers. However, block composition was kept constant throughout the duration of the experiment. Soil moisture was measured every ten days during the experiment in all pots over the surface 5 cm by the time-domain reflectometry method (SM300; Delta-T devices, Cambridge, UK), and values were recorded two days after irrigation. Monitoring of emergence was carried out every two days until the last seedling emerged.

On 26th November 2015, after 22 weeks in the controlled

Table 2
Temperature (day/night) during the experiment for the two treatments: Current Temperature (CT) and Warmer Temperature (WT).

Week	Equivalent	CT		WT	
		Day Temp. (16 h.)	Night Temp. (8 h.)	Day Temp. (16 h.)	Night Temp. (8 h.)
1-4	May	14 °C	6 °C	19 °C	11 °C
5-8	June	16 °C	8 °C	21 °C	13 °C
9-13	July	18 °C	10 °C	23 °C	15 °C
14-17	August	17 °C	9 °C	22 °C	14 °C
18-22	September	15 °C	7 °C	20 °C	12 °C

Table 1
Location of study sites and altitudinal ranges where the mother trees were sampled, as well as mean temperature and precipitation in each location.

Population	Code	Latitude	Longitude	Altitudinal Range (m a.s.l.)	Altitude range of transect per site (m a.s.l.)			Mean annual Temp. (°C)	Annual Rainfall (mm)
					Low	Medium	High		
Glen Tanar	GT(E)	57.02	2.86	276-418	276-278	323-350	404-418	7.40	780
Glen Derry	GD(E)	57.03	3.58	438-525	438-464	466-493	508-525	6.80	932
Benn Eighe	BE(W)	57.63	5.40	9-222	9-20	41-140	196-222	8.53	2282
Shieldaig	SH(W)	57.50	5.63	6-242	6-49	111-144	230-242	8.40	1467

environment chambers simulating a complete growing season under natural conditions, all seedlings were harvested and cut at the root collar to divide each plant into above and belowground parts. Roots were washed gently but thoroughly taking care to keep loss of root tissue to a minimum, and measurements of maximum root and shoot length and fresh biomass were taken. Five needles were collected from each seedling, two from the upper, two from the lower and one from the middle part of the plant. The number of stomatal rows at midway along the needle on its adaxial (upper) surface was counted with the aid of a stereo Leica light microscope (x10 objective). Stomatal rows were used as a proxy of stomatal densities. After assessment, needles were scanned using a scanner at 300 d.p.i. and returned to material from their respective seedling. Assessment of scanned needle dimensions was done using ImageJ software, v.1.36b (Abramoff et al., 2004). In addition, clean fresh roots were placed flat on a sheet of paper and scanned. The obtained images were edited manually with Adobe Photoshop® in order to remove any shadows that would cause spurious measurements to be recorded. The structure and morphology of scanned roots was analysed using WinRhizo (Regent Instruments Inc., Quebec, Canada, 2000), obtaining the following morphological parameters (considering all main and fine roots): total root area, total root length, root diameter, root volume, number of root tips and forks. Dry biomass (g) was recorded after both roots and shoots were dried in the oven for a minimum of 3 days at 45 °C.

In total, nineteen early growth traits that are likely to be experiencing the effects of natural selection were considered (Supplementary Table S1), of which two refer to the seed stage, mean viable seed weight (SEW) and seed viability (SEV), considering full seeds as a proxy of viable seeds. Date of seedling emergence (EME) was recorded as soon as the seeds were observed at the substrate surface within pots, and survival (SUR) was checked every two days and recorded over the duration of the experiment. All the remaining traits were recorded at the end of the experiment: Total dry biomass (TDB), which is the sum of shoot dry and root dry biomass; plant height (SHL); maximum root length (ROL); root mass fraction (RMF) which is the ratio of root dry biomass to total dry biomass. Other below-ground traits were total root length (TRL), which represents the sum of the lengths of all the fine roots; average root diameter (AVD); root volume (ROV); number of tips (TIP); number of forks (FOR); branching intensity (BRI), which is the ratio of the number of tips to the total root length; specific root area (SRA), which is the ratio of surface area to root dry biomass and the specific root length (SRL), which is the ratio of total root length to the root dry biomass. Finally, above-ground traits that were measured were needle length (NEL), needle width at its midpoint (NEW) and the mean number of stomatal rows (STO) were measured in five needles per individual. Mean values are given ± Standard deviation.

2.3. Data analysis

2.3.1. Seeds and seedling emergence

A nested analysis of variance (ANOVA) was used to test differences in seed biomass (SEW) and viability (SEV) among and within populations. To analyse differences in emergence (EME), only the date of emergence of the first of the three seeds sown per pot was considered, and the cumulative emergence ratio per treatment per region on each experimental day (e.g. days after sowing) was calculated, which indicates the cumulative proportion of seedlings emerged. A cumulative binary logistic regression was used to evaluate the effect of temperature, region and day of emergence in the emergence ratio with the glm function, using a binomial error distribution. Experimental day (i.e. days after sowing), temperature, region and the interaction between the three factors were considered as the predictor variables. Survival (SUR) was estimated as the proportion of emerged seedlings that survived to the end of the experiment.

2.3.2. Intraspecific variation of early growth traits and response to temperature

Mixed effect models were used to analyse the genetic variance components of the response of early growth traits and to test the effect of temperature with the statistical package "lme4″. To identify the best-supported model we constructed all possible combinations of alternative models, from the maximal model considering both the main effects and the pair-wise interactions between the fixed effects. Models were fitted by *Maximum Likelihood* (ml) and model selection was performed using backward stepwise selection to minimize the *corrected Akaike Information Criterion* (AICc). The selected model included temperature and region as fixed factors (and the interaction of both for a few traits) and population, family and block as a random factor, and it was fitted with a *Restricted Maximum Likelihood* (REML) algorithm to obtain parameter estimates.

$$\text{Trait} = \mu + \text{Temperature} + \text{Region} + \text{Population} + \text{Family} + \text{Block} + \varepsilon \quad (1)$$

This model (1) was applied to two types of data-sets: (i) all data containing the source of variation "temperature", and (ii) data separately by temperature treatment, removing the source of variation "temperature" from the Eq. (1). Residuals were tested for normality and homoscedasticity, and log transformation of the variables was applied when necessary.

The model containing all data, which included the temperature treatment, was analysed in order to quantify the variance explained for each of the factors. To estimate the variance of the trait of interest associated with a specific fixed effect, the *k*-th, we used the Eq. 2.

$$\sigma^2_{fk} = \frac{\text{var}(\beta_k x_{ik})}{\sum_j \text{var}(\beta_k x_{ij})} \sigma^2_f \quad (2)$$

Where x_{ij} is the explanatory variable data for variables $j = 1, ..., p$, and β_k is the estimated fixed effect.

The models for the individual temperature treatments were fitted in order to calculate the variance explained for each of the factors and to estimate additive genetic variation, heritability and evolvability of the trait of interest in each treatment. Narrow-sense heritability (h^2), which corresponds to the proportion of total phenotypic variation attributed to additive genetic variation, proportional to the rate of short-term responses to selection (Bolnick et al., 2011), were estimated as indicated in Eq. 3.

$$h^2 = \frac{V_A}{V_P} = \frac{4 \, V_{fam}}{V_{fam} + V_{block} + V_{res}} \quad (3)$$

Where V_A is the additive genetic variance and V_P is the phenotypic variance; V_{fam}, V_{block}, V_{res}.

Standard errors (*SE*) for heritabilities (h^2) were estimated as suggested by (Visscher, 1998), indicated in Eq. 4.

$$\text{SE}_{h^2} = 4 \sqrt{\frac{2(1 - \frac{h^2}{4})^2 \, [1 + (s-1)\frac{h^2}{4}]^2}{s(s-1)(f-1)}} \quad (4)$$

Where *s* is the number of individuals per family, and *f* is the number of families.

The genetic coefficient of variation CV_A was estimated according to (Houle, 1992) following Eq. 5, and is a standardised measure of variation normalised by the trait mean. It provides a measure of the evolvability of a trait.

$$CV_A = \frac{\sqrt{V_A}}{\mu_{\text{Trait}}} \times 100 \quad (5)$$

Where μ_{Trait} is the mean of the trait of interest.

Differences among regions for each temperature treatment were evaluated by comparing pairs of models by AIC_C for each of the studied traits. The model with the same formula as Eq. (1) without the

temperature factor, with region as a fixed effect, was compared to a null model which did not include the region effect.

To detect differences in soil moisture among the different treatments and regions (or populations), repeated measures ANOVA was used. As expected under different temperature regimes soil moisture availability varied among treatments (F=101.14, $d.f.$=1, $p < 0.0001$), being significantly lower in the WT compared to the CT temperature treatment. However, no significant differences in soil moisture availability were observed between pots containing plants from different regions or populations within treatments. All statistical analyses were carried out in the R software (v. 3.5.2, 2018, The R Foundation for Statistical Computing) in order to assess the level of intraspecific variability at different levels (region or population and family) and the effect of the predicted increase of temperature.

3. Results

3.1. Seeds and seedling emergence

Viability of seeds differed significantly among regions (F= 1.56, $d.f.$=1, $p = 0.04$) and populations (F= 4.00, $d.f.$=3, $p = 0.01$), which ranged from the lowest viability at BE(W) (61.73% $\pm$ 18.79) and SH(W) (62.08% $\pm$ 11.55), followed by GD(E) (65.35% $\pm$ 5.36), and the highest seed viability was at GT(E) (70.62% $\pm$ 9.47). These differences were not accompanied by differences in seed biomass among regions nor populations, whose mean values were 5.69 mg $\pm$ 1.9. In addition, and as expected, seed biomass differed among cone sizes (F=5.98, $d.f.$=2, $p = 0.006$), with larger cones producing heavier seeds.

A total of 1428 seedlings emerged during the experiment (99.2%), but only the first seedling to emerge was kept from the initial three per pot, with the earliest emergence occurring nine days after sowing. Temperature had a strong effect on the emergence ratio ($z = -16.37$, $p<0.0001$), with the warmer temperature advancing the average date of emergence by 10 days (mean time to emergence, CT= 26.23 days $\pm$ 8.39, WT=15.32 days $\pm$ 4.08) (Table 3, Fig. 1). Region had a smaller but significant effect on the emergence ratio ($z = 4.57$, $p<0.0001$), with emergence occurring faster in the populations from east grown under the CT regime. Day of emergence had a significant effect on the emergence ratio of a seedling in a region ($z = 33.61$, $p<0.0001$), indicating that the rank order of emergence for regions changed over time. Day of

Table 3
Parameters of the Logistic Regression of cumulative emergence ratio over time of Scots pine. Experimental day (DAY) refers to the day of emergence; TreatmentW refers to the warmer treatment, PopulationGT(E) refers to GT(E); PopulationBE(W) refers to BE(W) and PopulationSH(W) refers to SH(W).

Variables	Estimate	SE	z value	Pr (>|z|)
(Intercept)	-3.92	0.14	-27.11	< 0.0001 * **
DAY	0.16	0.00	30.14	< 0.0001 * **
TreatmentW	-0.91	0.25	-3.59	< 0.001 * *
PopulationGT(E)	-3.72	0.36	-10.21	< 0.0001 * **
PopulationBE(W)	-2.10	0.26	-7.95	< 0.0001 * **
PopulationSH(W)	-2.03	0.26	-7.60	< 0.0001 * **
DAY:Treatment(W)	0.12	0.01	10.24	< 0.0001 * **
DAY:PopulationGT(E)	0.16	0.01	11.43	< 0.0001 * **
DAY:PopulationBE(W)	0.07	0.01	7.44	< 0.0001 * **
DAY:PopulationSH(W)	0.07	0.01	7.21	< 0.0001 * **
Treatment(W):Population GT(E)	-0.98	0.70	-1.38	ns
Treatment(W):PopulationBE(W)	0.96	0.46	2.1	0.0357 *
Treatment(W):PopulationSH(W)	0.49	0.46	1.07	ns
DAY:Treatment(W):Population GT (E)	0.22	0.04	5.07	< 0.0001 * **
DAY:Treatment(W):PopulationBE (W)	0.06	0.02	2.55	0.0108 *
DAY:Treatment(W):PopulationSH (W)	0.07	0.02	2.71	< 0.001 * *

Significant P-values are indicated as *P < 0.05; * *P < 0.01; * **P < 0.001; ns, non significant
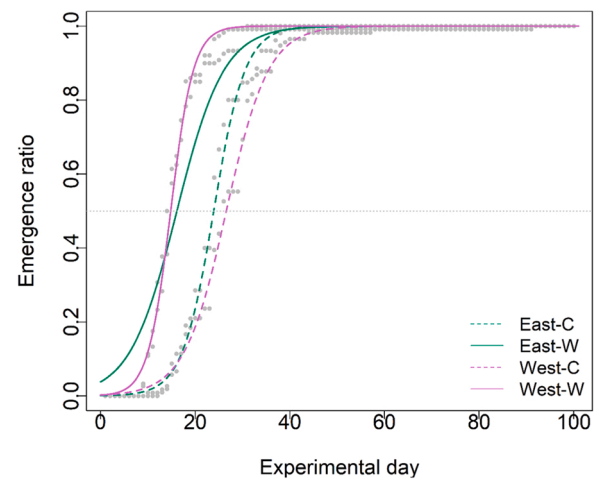

Fig. 1. Predicted cumulative emergence ratio over time (experimental day) across temperature treatments (Current Temperature, C and Warmer Temperature, W) and region (East and West) using a binomial error distribution. Grey dots represent observed data.

emergence had a significant interaction with region, indicating that the change over time of the emergence ratio differed among regions. Furthermore, triple interactions between region, day and treatment were also observed (Table 3, Fig. 1). Survival was 100% on both treatments during the experiment.

3.1.1. Intraspecific variation of early growth traits

All traits showed substantial intraspecific adaptive variation, and the largest proportion of genetic variation was partitioned within the populations (among families) rather than among regions or populations for most traits. Family variance was relatively large for most traits (V_{FAM}, CT: 0–17.43%) (Table S2), and those values were reflected in the high estimates of narrow-sense heritability (h^2, CT: 0–0.77). In addition, the estimates of evolvability were relatively large (CV_A, CT: 0–48.31). Although the variance attributable to region was smaller than that for family in the majority of cases, it was nevertheless significant for different traits (V_{REG}, CT: 1.61–6.55%), as was indicated by the adequate support for the inclusion of region as a fixed effect for those traits in CT (TableS2). Furthermore, differences among populations were also present in most of the traits (V_{POP}, CT: 0.61–9.01%) (but see RMF, BRI, SRA, SRL).

Regional differences were evident for some early growth traits. Although total biomass (TDB) did not differed among regions (Fig. 2a), western populations displayed smaller biomass allocated to roots (RMF) compared to the eastern ones in CT (Fig. 2b). Remarkable differences were evident among regions in other root traits, with western populations having shorter specific root length (SRL) (Fig. 2c), thicker roots (AVD) (Fig. 2d) and lower specific root area (SRA). Branching intensity (BRI) was greater in western compared with eastern populations (Fig. 2e). Regarding above ground traits, western populations had a significantly greater number of stomatal rows (STO) (Fig. 2f) and thicker needles under CT, and the number of stomatal rows was highly correlated with the total biomass (R^2=0.37, $p < 0.001$) and the needle width (R^2=0.34, $p < 0.001$).

3.1.2. Response to temperature of early growth traits

Overall, temperature $\times$ region interactions were found for only a minority of the traits assessed in the study, which means that all populations responded consistently under current and warmer temperature (but see TRL and FOR in Table 4). Temperature had a strong effect on most of the measured traits, and explained up to 49.63% of the variance (Table 4). The temperature treatment (WT) resulted in significantly greater total biomass (Fig. 2a), taller shoots and deeper roots (we did
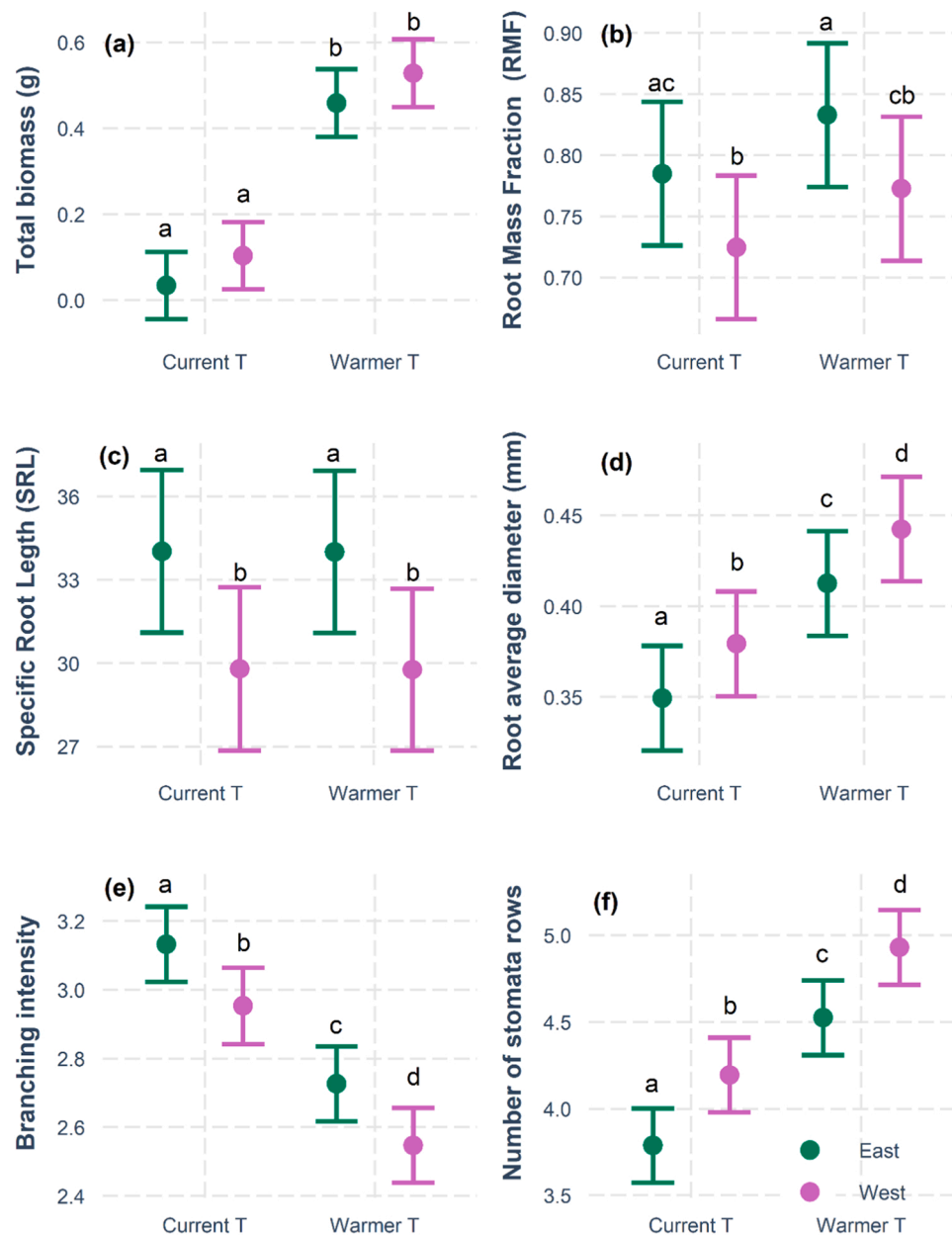
Fig. 2. Model predictions for (a) total dry biomass TDB, (b) root mass fraction RMF, (c) Specific Root Length SRL, (d) root average diameter AVD, (e) branching intensity BI and (f) number of stomata rows STO of Scots pine seedlings from the two regions (East vs. West) across the temperature treatment (Current T, Warmer T). Error bars represent ± SE. The letters on top of the bars show the results of the post hoc Tukey multiple comparison test.

not observe root growth limitation due to pot height). However, there was no or little difference between the plants subjected to the CT and WT regimes in terms of biomass allocation (Fig. 2b). In addition, most of the below ground traits were highly influenced by the warmer temperature treatment, which resulted in longer (i.e. considering the total length), thicker (Fig. 2d), and more voluminous roots, with more forks and more root tips. However, the warmer temperature reduced the branching intensity of roots (Fig. 2e), and had little or no effect on SRA and SRL (Fig. 2c) respectively. Warmer temperature also had a strong effect on the above ground traits, resulting in greater numbers of stomatal rows per needle (Fig. 2f) and longer and thicker needles (see Table 5 for mean values of traits). Narrow sense heritability did not show consistency across traits in the direction in which it shifted in response to the WT treatment (Table S2). However, for most of the traits not related to root structure, C_{VA} was greater under WT (Fig. 3) for both eastern and western populations (Table 5).

4. Discussion

Our study aimed to evaluate intra-specific genetic variation in early growth traits (i.e. germination and growth of above- and belowground traits) of Scots pine seedlings under current and predicted elevated temperatures. We examined populations from the north-western limit of the distribution of the species, and compared populations located at the extremes of a steep East-West environmental gradient in this highly heterogeneous geographic location. Despite the fact that our sampled populations were located within a relatively small geographic area, we detected a substantial amount of intra-specific genetic variation within and among regions and populations. Therefore, in agreement with our first hypothesis, early growth traits significantly differ in populations from western compared to eastern Scotland. Although warmer conditions had a strong effect on early growth traits by advancing seedling emergence date and enhancing above and below-ground biomass, there

Table 4
Summary of early growth traits analyses by treatment and region. Model subsets were generated and compared by AICc to assess the importance of Treatment, Region, and their interaction as fixed effects. The best models were determined by AICc, and the presence (+) or absence (-) of fixed effects are indicated for each of the traits listed. ΔAICc values represent the difference in AICc between the null model and the best. The most parsimonious model was determined using AIC (Akaike Information Criterion) as an indicator of both parsimony and likelihood, where difference lower than 1 indicated no support for the most complex model. Variances are indicated by the source of variation as follow: V_{TREAT}, treatment variance; V_{REG}, region variance; V_{POP}, population variance; V_{FAM}, family variance; V_{BLOCK}, block variance and V_{RES}, residual variance.

Trait	Trait code	Mean CT	Mean WT	Treatment	Region	Treatment X Region	df	ΔAIC	V_{TREAT} (%)	V_{REG} (%)	$V_{TREAT\ X\ REG}$ (%)	V_{POP} (%)	V_{FAM} (%)	V_{BLOCK} (%)	V_{RES} (%)
Total Dry Biomass	TDB	0.05	0.38	+	-	-	6	36.47	49.33	-	-	2.01	7.42	3.98	37.26
Shoot Height	SHL	17.16	64.15	+	-	-	6	33.00	44.02	-	-	1.42	3.94	3.87	46.75
Root Depth	ROL	300.26	435.57	+	-	-	6	28.70	37.45	-	-	2.83	4.22	1.86	68.04
Root Mass Fraction	RMF	0.42	0.43	-	+	-	6	2.24	-	1.78	-	0.78	0.00	3.67	93.81
Total Root Length	TRL	86.63	608.36	+	+	+	8	38.58	22.74	0.10	27.83	2.29	6.14	3.69	37.21
Average Diameter	AVD	0.36	0.43	+	+	-	7	13.37	18.32	4.11	-	3.73	1.47	12.49	59.88
Root Volume	ROV	0.1	0.99	+	-	-	6	36.29	49.63	-	-	2.40	5.23	3.94	38.79
Tips	TIP	267.24	1449.04	+	-	-	6	36.41	48.27	-	-	1.43	6.71	3.76	39.83
Forks	FOR	370.54	2976.11	+	+	+	8	29.73	21.35	0.15	26.81	2.15	6.19	8.68	34.67
Branching intensity	BRI	3.04	2.63	+	+	-	7	23.61	9.97	1.93	-	0	0.84	1.01	86.24
Specific Root Area	SRA	0.04	0.04	+	+	-	8	5.83	5.43	0.90	-	0.93	0.80	7.28	84.65
Specific Root Length	SRL	31.79	31.89	-	+	-	7	3.83	-	4.73	-	1.40	2.67	6.85	84.35
Needle Length	NEL	17.9	33.37	+	+	-	7	46.64	45.12	-	-	1.84	7.46	1.81	43.76
Needle Width	NEW	0.81	1.001	+	-	-	8	29.18	19.12	1.41	-		7.92	2.45	69.10
Stomata Rows	STO	3.99	4.73	+	+	-	7	26.64	13.66	3.88	-	0.03	16.14	1.07	65.20

Table 5
Genetic coefficient of variation (CV_A) for each trait by temperature treatment (Current vs. Warmer) and region (E, East and W, West) and the magnitude and direction of the CV_A change from the current to the warmer temperature treatment for each region. ↑ indicates an increase of CV_A from current to warmer temperatures, ↓ indicates a decrease and no symbol indicates no change.

Trait	Trait code	CV_A Current temperature		CV_A Warmer temperature		Magnitude and direction of CV_A change	
		E	W	E	W	E	W
Total Dry Biomass	TDB	11.3060	18.9024	57.6070	78.8302	↑46.3010	↑59.9278
Shoot Height	SHL	12.5192	0.0015	12.6662	4.8936	↑0.1471	↑4.8920
Root Depth	ROL	2.8300	3.2451	2.1296	0	↓0.7004	↓3.2451
Root Mass Fraction	RMF	0	0	8.5542	10.4474	↑8.5542	↑10.4474
Total Root Length	TRL	7.3761	9.0427	12.6011	11.4528	↑5.2250	↑2.4101
Average Diameter	AVD	0	12.9926	0	2.6140	0	↓10.3785
Root Volume	ROV	0.9306	23.0905	88.8901	227.0365	↑87.9595	↑203.9460
Tips	TIP	6.2698	7.3476	8.9181	10.0585	↑2.6482	↑2.7108
Forks	FOR	7.9048	6.4938	10.5733	10.1586	↑2.6685	↑3.6648
Branching intensity	BRI	8.3719	15.2692	8.4357	0	↑0.0637	↓15.2692
Specific Root Area	SRA	24.6132	15.5426	0	0	↓24.6132	↓15,5426
Specific Root Length	SRL	17.5692	20.0465	0.0028	0	↓17.5664	↓20.0465
Needle Length	NEL	5.4616	5.4355	9.9503	9.7177	↑4.4888	↑4.2822
Needle Width	NEW	42.9255	53.6570	395.5220	1471.6380	↑352.5965	↑1417.9810
Stomatal Rows	STO	0	16.5650	0	0	0	↓16.5650

was a consistent response to warmer temperature in populations from both western and eastern Scotland, which did not support our second hypothesis, in which we hypothesised that early growth traits response to warmer temperatures would be stronger in eastern populations. This result indicates that the amount of cryptic genetic variation associated with temperature response does not differ among regions or populations. In summary, this study provides experimental evidence of the genetic variation but similar effect of warmer temperatures on seedling performance of the north-western limit Scots pine populations that inhabit at the extremes of an East-West environmental gradient.

4.1. Substantial intra-specific genetic variation at the species' north-western limit

We observed notable intra-specific genetic variation in early growth traits of Scots pine seedlings derived from seed collected from individuals growing at the north-west distributional limit of the species, with some traits showing a particularly large variation resulting either from local adaptation or from a high plasticity. At the regional level, the eastern populations in our sample showed greater seed viability, faster germination, more investment in roots, thinner roots and fewer needle stomatal rows in comparison to those from the west. Although we
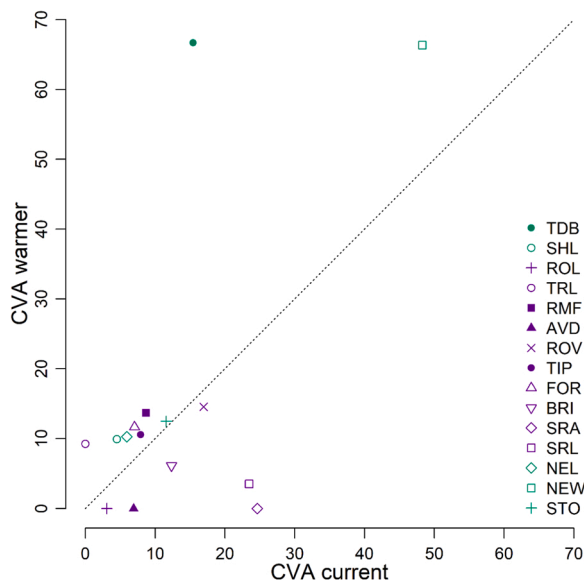
Fig. 3. Plasticity to change of the coefficient of genetic variation (CV_A) of Scots pine seedlings across the temperature treatment (current and warmer). Refer to table S1 for trait code key. Green symbols represent above ground traits, purple symbols represent below ground traits. Symbols above the dashed 1:1 line indicate greater CV_A under future conditions.

cannot discern the specific factors driving these differences (i.e. temperature, precipitation, etc.), selective forces at the home environment might have imposed adaptive genetic divergence related to these traits.

Local climate, particularly temperature and water availability, generally influences plant development in all different stages (Savolainen et al., 2007; Hatfield and Prueger, 2015). Seed viability is the primary factor determining the success of recruitment in tree populations, and is usually controlled and positively correlated with the temperature at the home environment (Reyes and Casal, 2002; Kullman, 2007). However, despite the lack of differences in seed mass across populations found in our study, populations originating from colder eastern areas of Scotland had greater values of seed viability, and the reason for this difference remains unclear. This result is accompanied with the greater biomass investment into roots in seedlings from eastern populations and a root structure characterised by greater specific root length based on thinner and more highly branched roots, which would likely satisfy the need to explore deeper into the soil fraction. Under natural conditions, cold and aridity usually promote greater biomass investment into roots, thereby enhancing the ability to uptake water and nutrients, more limited in such environments (Luo et al., 2012). Although a global meta-analysis attributes allocation shifts only to temperature (Reich et al., 2014), common garden trials indicate that plants that invest a greater proportion of biomass in their roots might have a selective advantage in arid conditions (Matías et al., 2014). In fact, in Mediterranean pines allocation, water availability is the most relevant factor driving allocation to roots. As eastern Scottish populations experience both colder and drier conditions compared to those in the west, our results suggest that individuals from colder and drier conditions may achieve a selective advantage by being genetically predisposed to allocate more biomass to roots. Similar results were reported by Comas and Eissenstat (2004) in species needing stronger soil exploration and root defences. In addition, wind has been shown to act as a selective force in Scotland (González-Díaz et al., 2018) and in the western populations, trees are more exposed to strong west-south-westerly winds (Dore et al., 2006) coming directly from the Atlantic Ocean. In our trial, western populations had thicker roots than populations from the east, a trait that provides superior anchorage for the plant (Alvarez-Uria and Körner, 2007). Western populations could therefore be under selective pressure

to produce individuals with a stronger anchorage system whilst the need to penetrate soil to find water is lower than experienced by the populations from the east. It is worth mentioning that we did not explore soil properties at the home sites, such information may have helped to explain the results found.

We also found consistent differences in the number of stomatal rows, which was greater towards the West, a result supported by a previous study (Donnelly et al., 2016). The density of stomata on leaf surfaces in situ has been interpreted as a response to differences in moisture availability (Hogan et al., 1994; Brewer and Nuñez, 2007), decreasing with reduced rainfall as an adaptation to conserve water in drier areas, and also suggested to decrease with increasing altitude (Hultine and Marshall, 2000), presumably also assumed to be a water availability adaptation. Although we did not measure the stomatal density, when the number of stomatal rows was standardized the by the width of the leave it was significantly greater for western populations. We therefore suggest that the differences observed in our common garden trial might indicate that the exposure of populations from the East to drier and higher altitude conditions in their local sites may have driven genetic adaptation towards the development of lower stomata rows. Maternal effects might have also significant control on seed and at first-year seedling performance in Scots pine (Castro, 1999). This is because the interaction of environment and maternal genotype influences the fitness and plasticity of the progeny irrespective of the genotype of the latter (Bose et al., 2020). However, it should be noted that our study was based on early seedlings, and the effect of both the climatic environment of the seedling and the maternal adaptation to its environment may be reduced in older stages, so genetic differences might be reduced in older ontogeny stages. This represents an important limitation in clarifying the role that adaptive variation may play under warmer scenarios.

In long-lived outcrossing trees, a higher proportion of the species' genetic diversity is usually harbored within populations than among them (Hamrick et al., 1992). Accordingly, we found a larger extent of within-population variation (ranging between 4% and 18% of the variance under current conditions, with few exceptions; Table S2) than that attributable to among-region (1.6 to 6.5%) and among-population (0.6 to 9%) variation. In our study, the large amount of variation among families was reflected in substantial trait heritability values, which indicate the component of the phenotypic variation of the population that is under genetic control and is inherited from parental trees (Wray and Visscher, 2008). We observed moderate to high values of heritability ($h^2 = 0.0.4-0.49$ for CT), that were particularly large for total biomass and number of stomatal rows (over 0.7). Furthermore, heritability estimates indicate that Scots pine has the genetic architecture required for trait evolution driven by natural (or human directed) selection (Ahrens et al., 2018). Large estimates of heritability are usually associated with fluctuating natural selection (Bell, 2010; Donnelly et al., 2016). Therefore, temporal or spatial variation (e.g. seasonal climatic variation or spatial heterogeneities) might have caused fluctuation in the selective forces acting on the studied Scottish populations, especially for stomatal rows and total biomass which exhibit particularly high heritabilities. However, we cannot disregard the considerable standard errors attached to these estimates - since high accuracy can only be achieved with much larger sample sizes (Donnelly et al., 2016). Furthermore, some maternal effects might also be present in our estimates (denoted, among others, by the positive, albeit weak, correlation between seed mass at the outset and seedling mass at the end of the experiment). However, it seems that on balance the evidence indicates that growth traits are likely to retain substantial heritable adaptive capacity.

4.2. Response to a warmer temperature

Temperature is an important factor influencing plant development (Hatfield and Prueger, 2015). We detected a strong effect of warmer temperature in substantially advancing seedling emergence by 10 days

and dramatically enhancing biomass and some above- and belowground traits by up to 10 fold. However, the extent of variation in response to warmer temperature was consistent in both west and east populations for most traits, indicating that the level of cryptic genetic variation did not vary among regions or populations and that similar capacities for phenotypic plasticity were present. The strong effect of the warmer condition suggests temperature limitation for early growth in material from both regions under current conditions. Although the lowest temperature simulated in our experiment remained above the minimum temperature required for root growth (4–6 ℃) (Alvarez-Uria and Körner, 2007), it is likely that the current temperature (CT) regime is sufficiently low to slow the growth of both below- and aboveground traits.

Seedling emergence is a key stage that sets the context for subsequent development and natural selection (Verdú and Traveset, 2005; Donohue et al., 2010). Therefore, earlier emergence in response to the warmer temperature may result in a longer first growing season providing seedlings with more time to develop and establish before the onset of adverse autumn conditions (Jones et al., 1997; Castro, 2006; Richter et al., 2012a; Matías and Jump, 2014). In addition, if warming is accompanied with adequate precipitation and does not cause resource limitation, it could increase tree metabolic processes in the subsequent seedling stages that, in turn, would lead to higher biomass accumulation (McMahon et al., 2010). Earlier germination and increased growth under warmer conditions are common for species inhabiting areas which are temperature-limited, as they are usually restricted by cold (Pulkkinen et al., 2013; Milbau et al., 2009; Overdieck et al., 2007). Hence, our results, together with the climate predictions for Scotland, where the increase in temperature is not expected to exceed the optimal and water availability is not predicted to decrease, suggest that warmer temperature and its consequent advancement of emergence might have a consistent positive effect in early recruitment across the Scottish distribution. As a counterpoint, this positive effect does not remove the risk of late spring frost at higher altitudes, so seedlings emerging earlier in the growing season may be vulnerable to cold damage.

Although the warmer treatment had an effect on seedling emergence and most of the other assessed traits, it did not cause shifts in carbon allocation (RMF) in any of the studied regions. A lack of consistency in response is evidenced in previous studies in which warmer temperatures have resulted in a greater carbon allocation to shoots (Cerasoli et al., 2014), roots (Pumpanen et al., 2012; Delucia et al., 1994; Matías et al., 2016; Domisch et al., 2001) or no change (Overdieck et al., 2007). Furthermore, for Scots pine, when the increase in temperature was combined with low water availability, a greater investment in roots has usually been found (Matías et al., 2016; Richter et al., 2012b). Therefore, the lack of shift in biomass allocation for most populations in our study could be related to the absence of water limitation. Interestingly, warmer temperatures had a strong effect on the structure of the belowground system, with an increase in the length of roots, greater number of tips and forks as well as an increase in the root diameter. The growth of fine roots, which are primary organs for water and nutrient acquisition and are responsible for transferring resources between belowground and aboveground parts, is usually positively correlated with temperature (as long as the optimal limit is not surpassed) and also with nutrient uptake, root respiration and root mortality (Pregitzer et al., 2000). Hence, an increase in the root length, diameter and number of tips and forks is a common response to warmer conditions. Interestingly, we found that branching intensity, which governs exploration through the soil matrix and thus may also affect nutrient acquisition, was reduced under warmer conditions although with some regional differences. Some authors have previously related higher root branching (Alvarez-Uria and Körner, 2007) and higher root growth (Reich et al., 2014) with greater tolerance to cold environments. Thus, the simulated warmer conditions (WT) might have favoured the reduction of root branching, which supports the idea that the lower investment into roots and the reduced branching observed in the western compared to eastern sites.

In terms of aboveground traits, an increase of stomatal rows was observed under warmer conditions, and this was positively correlated with the increase in needle width. In contrast, some previous results found that warmer temperature resulted in a reduction in stomatal density in recently collected individuals compared with those collected many years before and preserved as herbarium specimens (Beerling and Chaloner, 1993), although this was also usually associated with an increase of CO_2 over recent decades. Number of stomata should be governed by a trade-off between CO_2 uptake and water loss: higher CO_2 availability would imply less need for apertures so stomata density can be reduced. As CO_2 concentration was kept constant in our experiment, warmer temperature but no changes in CO_2 might explain the increase in the number of stomatal rows. Nevertheless, it seems plausible that confounding effects of temperature and CO_2 might be expected under more realistic warming conditions.

Finally, our results showed that the genetic coefficient of variation (CVA) shifted under warmer conditions, however, traits did not respond homogenously to novel environments (i.e. WT in our experiment). In particular, CVA estimates were greater in the majority of the traits for most of the traits not related to root structure under the warmer conditions, suggesting that more variation might be released under novel conditions, but it declined for some root traits. Exposure to stress or novel conditions has the potential to release cryptic genetic variation (Hoffmann and Merilä, 1999; Gibson and Dworkin, 2004; Donnelly et al., 2016, 2018), which might be the case for most traits in our study. However, the opposite response was found for some root traits (e.g. SRA or SRL) and the reason for such trends remains unclear. The presence of cryptic genetic variation in natural populations, which is only revealed under novel conditions, may have an important role in facilitating adaptation to rapid environmental change. However, western and eastern populations responded consistently to experimental warmer conditions (no interaction between temperature and region for most traits, but see emergence ratio; and similar direction and magnitude in the CVA change for most traits, but see ROV and NEW), which indicates that the level of cryptic genetic variation did not significantly vary among regions or populations and similar capacities for phenotypic plasticity were present. Thus, in a context of climate change, Scots pine populations from the east and the western regions might perform similarly.

5. Conclusions

Substantial levels of adaptive genetic variation can be present at small spatial scales for Scots pine, with adaptive genetic variation usually larger within than among population, resulting in most traits being highly heritable, which might allow populations to genetically adapt to warmer conditions. Although we did not find evidence for differences in cryptic genetic variation nor in plasticity across populations located in a small climatic gradient in response to warmer conditions, our results suggest that more variation (i.e. cryptic or plasticity) might be released under novel conditions. Overall, the results reported in this study suggest that a decline of the Scottish populations of Scots pine under a warmer climate due to poor seedling recruitment, decreased seedling emergence and worst seedling performance- seems unlikely. Although our results are informative regarding the extent of phenotypic plasticity and adaptive potential in the populations, it is not possible to draw conclusions at the community level. Along with Scots pine, other species might benefit from warmer conditions, and the overall increase in success of Scots pine may not be realised if other species in the community also thrive. Furthermore, common garden trials and experimental set ups might prevent some natural disturbances, such us wind, spring frost, humidity diurnal variation or herbivore and pathogen attack, which could imply some additional trade-offs not detected here (e.g. bigger early growth might be more vulnerable to wind and herbivores). Therefore, our results must be taken with caution, and more research is needed to improve our understanding of how adaptive responses in

Scots pine might be modulated by interactions with other species and environmental factors not tested here. Nevertheless, the findings of this study can contribute to take management decisions on the future of Scots pine. Thus, the intra-specific differences observed in this study at small spatial scales together with the amount of variation released under novel conditions might improve the possibilities for the species to genetically adapt to warmer conditions. Such results highlight the importance of preserving local forest genetic resources to cope with the expected changes in climate.

CRediT authorship contribution statement

P. G. D., S. C., L. M. and A. S. J. designed the research. P. G.D. and S. C. conducted field and P. G.D. conducted experimental-based work. P. G. D. conducted data analysis. A. S. J. and S.C. supervised the research project. P. G.D., S. C., L. M. and A. S. J. wrote the manuscript with the contribution of R. A. E. and J. E. C.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envexpbot.2023.105603.

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