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Drought tolerance and plasticity in the invasive knapweed *Centaurea stoebe* s.l. (Asteraceae): effect of populations stronger than those of cytotype and range

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- **Background and Aims** Spotted knapweed (*Centaurea stoebe* s.l., Asteraceae) is native to Europe, where it occurs as a diploid (2*x*EU) and tetraploid cytotype (4*x*EU), but so far only the tetraploid has been reported in the introduced range in North America (4*x*NA). In previous studies, significant range shifts have been found towards drier climates in 4*x*EU compared with 2*x*EU, and in 4*x*NA when compared with the native range. In addition, 4*x* plants showed thicker leaves and reduced specific leaf area compared with 2*x* plants, suggesting higher drought tolerance in 4*x* plants. It is thus hypothesized that the 4*x* cytotype might be better pre-adapted to drought than the 2*x*, and the 4*x*NA better adapted than the 4*x*EU due to post-introduction selection.
- **Methods** Plants of the three geocytotypes (2*x*EU, 4*x*EU and 4*x*NA), each represented by six populations, were subjected to three water treatments over 6 weeks in a greenhouse experiment. Plasticity and reaction norms of above- and below-ground biomasses and their ratio, survival rate, stomatal conductance and carbon isotope discrimination were analysed using linear and generalized linear mixed effect models.
- **Key Results and Conclusions** Above-ground and total biomasses of European tetraploids were slightly less affected by drought than those of European diploids, and 4*x*EU plants maintained higher levels of stomatal conductance under moderate drought than 4*x*NA plants, thus supporting the pre-adaptation but not the post-introduction evolution hypothesis. Plasticity indexes for most of the traits were generally higher in 2*x*EU and 4*x*NA than in 4*x*EU plants, but these differences were not or were only marginally significant. Interestingly, the effect of population origin and its interaction with treatment was more important than the effects of geocytotype and range. Population means for the control treatment showed several significant associations either with latitude or some aspect of climatic data, suggesting evolution of local adaptations, especially within the 2*x*EU and 4*x*EU geocytotypes.

Key words: Biological invasions, biomass partitioning, carbon isotope discrimination, *Centaurea stoebe*, drought tolerance, local adaptations, plasticity, polyploidy, spotted knapweed, water use efficiency.

INTRODUCTION

As a consequence of the frequent incidence of polyploidization (whole genome duplication), many vascular plant species exhibit variation in ploidy. Most detailed studies focusing on the distributions of cytotypes in their native ranges have shown spatial segregation at the meso- and macrospatial scales (e.g. Mosquin and Small, 1971; Soltis, 1984; Mandáková and Münzbergová, 2006; Mráz *et al.*, 2008) with sympatry only rarely detected (e.g. Duchoslav *et al.*, 2010; Šingliarová *et al.*, 2011). Allo- or parapatric distribution of cytotypes could be explained by two main non-exclusive processes: (1) the cytotypes might have different adaptive potential as polyploidization may have triggered important structural and physiological changes, which in turn may have influenced the position and breadth of their ecological niches (Levin, 1983); (2) a non-adaptive scenario suggests that historic factors such as different centres of origin/refugia or colonization routes have shaped the ranges of cytotypes (e.g. Mandáková and Münzbergová, 2006; Mráz *et al.*, 2008).

Interestingly, a shift in cytotype distributions has frequently been recorded also in diploid–polyploid complexes of invasive species (te Beest *et al.*, 2012 and references therein). Introduced populations are often cytotypically depleted and/or show altered frequencies when compared with native populations (te Beest *et al.*, 2012). Because plant introductions are usually stochastic events, strong founder events may cause the loss of cytotype diversity in a new range. This may especially happen when cytotypes are strongly spatially segregated in their native range, which increases the chance that only a subset of the cytotypic diversity is introduced (Lafuma *et al.*, 2003; Kubátová *et al.*, 2008). Indeed, strong founder events in invasive populations are rather the rule than the exception (Dlugosch and Parker, 2008). Alternatively, cytotype shifts in the introduced ranges may be explained by genetically based differences in eco-physiological tolerance to various environmental factors. In a more tolerant cytotype, stress will have a lower effect on performance (e.g. above-ground biomass, seed set) than in a more sensitive cytotype. However, as stress always has an effect, even in stress-tolerant plants, it could be manifested in so-called underlying traits (Alpert and Simms, 2002), such as above-ground:below-ground biomass ratio), which may react more plastically in order to maintain an overall fitness stability. Therefore, performance and underlying traits may often show opposite reactions to stressful factors. This 'trade-off' model (Chapin, 1980; Fernández and Reynolds, 2000; Lambers and Poorter, 2004) may, however, be trait-dependent for underlying traits (Couso and Fernández, 2012). Because of higher gene copy numbers in polyploids (Levin, 2002), one might expect broader ecological tolerance and/or an increased level of plasticity in polyploids when compared with diploids. However, experimental studies testing these expectations for increased tolerance (Bretagnolle and Thompson, 1996; Li *et al.*, 1996; Pustovoitova *et al.*, 1996; Sugiyama, 1998; Xiong *et al.*, 2006; Ntuli and Zobolo, 2008; Saleh *et al.*, 2008; Li *et al.*, 2009; Maherali *et al.*, 2009; Ci *et al.*, 2010; van Laere *et al.*, 2011) or plasticity (Meerts, 1992; Bretagnolle and Thompson, 1996, 2001; Petit and Thompson, 1997; Münzbergová, 2007; Hahn *et al.*, 2012; Kolář *et al.*, 2014; Fialová and Duchoslav, 2014) do not provide general support for these hypotheses, probably because of taxon-dependent responses to treatments.

Based on the results of recent cytogeographical and ecological studies and spatial distribution modelling of spotted knapweed (*Centaurea stoebe* s.l.), one of the most aggressive plant invaders in North America (Sheley *et al.*, 1998), it has been suggested that the tetraploid cytotype ($2n = 4x = 36$) may be better pre-adapted to drier climatic conditions than the diploid one ($2n = 2x = 18$) and that this pre-adaptation might contribute to its invasion success and the absence of the diploid cytotype in North America (Broennimann *et al.*, 2007; Treier *et al.*, 2009; Henery *et al.*, 2010; Mráz *et al.*, 2011; Hahn *et al.*, 2012; Collins *et al.*, 2013; Broennimann *et al.*, 2014). Indeed, despite largely sympatric occurrences of diploid and tetraploid populations in their native European range (Treier *et al.*, 2009) and evidence of multiple introductions (Mars *et al.*, 2008), only the latter ones have been convincingly reported in the introduced range (Mráz *et al.*, 2011). Broennimann *et al.* (2007) and Treier *et al.* (2009) further showed that the climatic niche of the native European tetraploid has shifted towards a drier and warmer climate compared with native European diploids. Interestingly, this shift was even more pronounced in introduced tetraploid American populations, making tetraploid *C. stoebe* an exceptional case among holarctic plant invaders, among which in general niche conservancy has been found (Petitpierre *et al.*, 2012). In agreement with the pre-adaptation hypothesis on increased drought tolerance in the tetraploid cytotype, tetraploid plants produce longer roots than diploids (Collins *et al.*, 2011, 2013) and show more reduced specific leaf area and inversely increased leaf dry matter content than diploid plants (Henery *et al.*, 2010; Mráz *et al.*, 2011; but see Hahn *et al.*, 2012), which is a pattern typical of plants adapted to more xeric habitats (Wright *et al.*, 2001; Knight and Ackerly, 2003). Our previous experimental studies, furthermore, showed that North American tetraploids grew faster (Henery *et al.*, 2010) and flowered earlier (Mráz *et al.*, 2011; Hahn and Müller-Schärer, 2013) than native European tetraploids. This suggests post-introduction evolution in introduced populations, which has been explained (e.g. by Henery *et al.*, 2010) as a trade-off between growth and defence traits [the hypothesis of evolution of increased competitive ability (EICA); Blossey and Nötzold, 1995] or by directional selection on both defence and growth traits (Ridenour *et al.*, 2008). However, it remains unknown whether such post-introduction evolution in introduced populations has also been manifested in traits involved in drought tolerance.

In our study we aimed to experimentally test (1) the pre-adaptation hypothesis [higher tolerance to drought in native European tetraploids (4*x*EU) compared with diploids (2*x*EU)] and (2) post-introduction adaptive evolution (increased drought tolerance in 4*x*NA compared with 4*x*EU). To assess the level of drought tolerance, we cultivated plants of the three geocytotypes (continent by cytotype combinations) under three different levels of soil moisture for 6 weeks in the greenhouse and measured different traits related to performance and fitness (total and above-ground biomass, survival) and underlying ecophysiological traits (root:shoot ratio, stomatal conductance, carbon isotope discrimination). We expected that the performance trait of more tolerant geocytotype(s) would be less affected by water stress than that of the less tolerant geocytotype(s), while underlying traits responsible for adjustment of the water regime might react more plastically in more tolerant geocytotype(s). Moreover, Mráz *et al.* (2011) and Hahn and Müller-Schärer (2013) recently found an indication for strong between-population phenotypic differentiation within the 2*x*EU geocytotype, but not in the 4*x*EU and 4*x*NA geocytotypes. Such a pattern has been tentatively explained by an older distributional range in the 2*x* cytotype, populations of which might have had a longer time to adapt to local conditions, in contrast to both 4*x*EU and mainly 4*x*NA, which underwent recent range expansion. In agreement with putatively stronger local differentiation in 2*x*EU compared with the other geocytotypes, we expected more significant associations between tested traits and geographical/environmental variables in the 2*x*EU geocytotype.

MATERIALS AND METHODS

Study species

Centaurea stoebe (Asteraceae) is a herbaceous species distributed from western-most Asia to Western Europe (Ochsmann, 2000). It is represented by the two cytotypes, diploid ($2n = 2x = 18$) and tetraploid ($2n = 4x = 36$), the latter being an interspecific hybrid between the diploid cytotype and a yet unknown taxon (Mráz *et al.*, 2012*a*). The cytotypes differ in morphology (Mráz *et al.*, 2011) and life cycle, diploids being predominantly biannual monocarpic while tetraploids are short-lived perennial polycarpics, often already flowering in the first year (Boggs and Story, 1987; Müller, 1989; Ochsmann, 2000; Müller-Schärer *et al.*, 2004; Henery *et al.*, 2010; Mráz *et al.*, 2011).

Plant material

Seeds of *C. stoebe* were collected from 18 populations in 2005–2008. Each geocytotype (2*x*EU, 4*x*EU, 4*x*NA) was represented by six populations covering a large area of the respective ranges (Supplementary Data Table S1). Seeds were dried and stored at room temperature until sowing. Twenty seeds from five different mother plants per population were sown at the beginning of October 2009 in 2 × 2 × 4 cm bedding cells with a mixture of nutrient-poor TKS1 soil (Floragard, Oldenburg, Germany; three parts) and fine-grained sand (one part). Dates of each germination event were noted at intervals of 2 d. After 3 weeks, randomly selected healthy seedlings of similar size were transferred into 2-L slightly cone-shaped pots (with a larger diameter at the top), with the bottom filled with a 2-cm thick layer of river gravel and the remaining volume filled with the same soil–sand mixture as that used for seedlings. Since one 4*x*EU population of *C. stoebe* (DE4) did not germinate, it was replaced by one of the well-germinated populations (BG6) and named BG6-bis. We used plant material directly grown from field-collected seeds as a previous study showed little maternal effect in *C. stoebe* s.l. compared with genetic and environmental effects (Weiner *et al.*, 1997).

Experimental design

The pots were arranged into five blocks (five parallel greenhouse benches) in a completely randomized design within each block. In total, we used 270 plants of *C. stoebe*, representing three geocytotypes with six populations of each, and, if possible, five different seed plants (families) of each population. Each seed family was represented by three plants and to each of them we applied one of the three different water treatments (see below). To summarize, the experimental design was as follows: three geocytotypes × six populations × five seed families × three treatments. The experiment was performed in a greenhouse under controlled light (16 h day/8 h night; EYE Clean Arc^{TM} 400 W lamps) and temperature (23 °C/15 °C, day/night) from 9 November 2009 to 15 December 2009. To account for differences in seedling size at the beginning of the experiment, we measured the number of rosette leaves (NRL) and the length of the longest leaf (LL), the product (LL × NRL) being a good proxy for initial biomass (Henery *et al.*, 2010; $r = 0{\cdot}53$, $P < 0{\cdot}001$ in our study). Initial biomass was used as a covariate for subsequent analyses.

Three different water treatments (T1, T2 and T3) using a 50 ml beaker were applied to individual plants (in passages of 50 ml per plant) to allow the water to permeate into the soil slowly and thus to minimize gravimetric water loss. The T1 plants (control) were watered more or less every third day with 150 ml of tap water (the cumulative amount of water per pot at the end of experiment was 1800 ml); T2 (moderate water stress) plants were watered only every 6th day, firstly with 150 ml, later with 50 ml (the cumulative amount of water was 600 ml); and T3 (severe water stress) plants were watered every 9th day with 50 ml (the cumulative amount of water at the end of experiment was 300 ml).

Measurements

Soil humidity (SH, %) was measured using a Theta Probe soil moisture sensor (Delta T Devices, UK) equipped with rods 6 cm long. Soil moisture was measured at the beginning of the experiment (SH1) on a subset of 90 randomly selected *C. stoebe* plants (two plants per geocytotype/treatment and block) to assess possible initial variation among geocytotypes and treatments. During the experiment, soil moisture was assessed three times (SH2–SH4, in the 1st, 2nd and 6th weeks of treatment) just before stomatal conductance measurements and watering. Water loss (WL, %) was derived from soil humidity measures using the formula $WL = 1 - (SH2/SH1)$, indicating the reduction in soil humidity between two treatments.

Stomatal conductance (SC, mol m^{-2} s^{-1}) is an important trait involved in water regimes and depends on the state of opening/closing of the stomatal complex. This reacts to changing conditions, such as light intensity, humidity and carbon dioxide concentration, and allows the plant to regulate gas exchange, and thus transpiration and photosynthesis (Hsiao, 1973). Stomatal conductance was measured on 45 plants of *C. stoebe* from the 90 selected for soil moisture measurements using a porometer (AP4; Delta T Devices, UK), which was calibrated before each measurement. We measured stomatal conductance on three well-developed leaves per plant and the averaged value was used for statistical analyses. Measurements were taken between 0900 and 1100 h to avoid daytime variation and repeated four times during the experiment (SC1–SC4), with the first measurement (SC1) taken at the beginning of the experiment before the initiation of the treatments. As we could not get stabilized values in plants from the T2 and T3 treatments in the later phase of the experiment (SC3 and SC4), only the SC2 measurement was used to test for effects of treatment and treatment × geocytotype interactions. In addition, we tested whether the plants of the three geocytotypes from the control treatment (T1) differed in stomatal conductance during the experiment (SC1–SC4).

Carbon isotope discrimination (δ^{13}C, ‰) reflects the stomatal limitation of photosynthesis and thus gas exchange and transpiration rate in plants, and it is strongly correlated with water-use efficiency (ratio of water used in plant metabolism to water lost by the plant through transpiration; Farquhar *et al.*, 1989). Gas exchange and transpiration decrease in parallel with decreasing soil humidity due to the closing of stomata, which is mirrored in lower values of δ^{13}C and increased water-use efficiency (and drought tolerance). However, under well-watered conditions δ^{13}C increases and water-use efficiency decreases (Farquhar *et al.*, 1989). In contrast to stomatal conductance, δ^{13}C provides a cumulative value of the transpiration history of the leaf during its whole lifespan. We assessed δ^{13}C and element levels (N, C, %) using the same set of plants as for the stomatal conductance measurements. At the end of experiment, we harvested three leaves of each plant, excluding the central veins and petioles, which were, however, taken into account for biomass measurements. The leaves were subsequently dried at 60 °C for 3 d and ground for isotope and element analyses, performed in the Isolab at the Institut für Pflanzenwissenschaften ETH, Zürich. Carbon isotope discrimination in the leaves was calculated using the formula proposed by Farquhar *et al.* (1989).

Survival of each plant was recorded at the end of the experiment.

Above- and below-ground biomasses of all plants were assessed at final harvest. Plants were firstly separated into above- and below-ground parts, and the roots were then washed with water and meticulously cleaned and left to dry. Shoot and dry masses were determined after drying at 60 °C for 3 d.

To compare plasticity in four biomass traits among geocytotypes, we calculated a mean relative plasticity index for each seed family. Firstly, we averaged the values for each treatment and seed family and then subtracted the lower averaged value measured in a stressful environment (T2 or T3) from the higher averaged value obtained in the control treatment (T1), with the exception of root:shoot biomass ratio, where the calculation was performed in the opposite direction. This value was then divided by the mean of the two values (T1 and one of T2 or T3, respectively) to obtain the relative plasticity index (Hahn *et al.*, 2012).

Statistical analyses

We used linear and generalized linear mixed effect models [LMMs and GLMMs, the lmer function in the lme4 package

(Bates and Maechler, 2009) with the identity link function for normally distributed data and the logit link function for binomial data] and the likelihood ratio test (LRT, comparing a fully fitted model to the model from which the tested term was removed) to assess the effects of treatment, plant origin (geocytotype) and their interactions on the parameters measured. Difference in tolerance to drought between 2*x*EU and 4*x* EU (pre-adaptation hypothesis) and between 4*x*EU and 4*x*NA (post-introduction evolution hypothesis), were tested using two separate data sets. Block was considered in all models as a random factor and initial biomass as a covariate. For those variables with a balanced design with respect to treatment, block and population, the population nested within geocytotype was considered as an additional random factor. As the tests using geocytotype comparisons revealed that population effects were much stronger that geocytotype effects, we performed additional LMM and GLMM tests within each geocytotype separately. Population, treatment and their interactions were treated as fixed factors, initial biomass as a covariate and block as a random factor. If necessary the response variables were transformed to improve the normality of distribution of residuals. Specifically, biomass and carbon isotope discrimination values were square root-transformed, and leaf nitrogen amount and stomatal conductance values were log-transformed.

Differences among geocytotypes (2*x*EU versus 4*x*EU and 4*x*EU versus 4*x*NA) in plastic responses in four biomass traits to water treatment (T2 versus T1 and T3 versus T1, separately) were assessed using LMMs, with the geocytotype as the fixed factor and population nested within geocytotype as a random factor.

In addition, we tested for a potential relationship between selected population means (T1 values for seedling emergence, initial biomass, total, above- and below-ground biomasses) and 19 macroclimatic variables of the sampling sites derived from the WorldClim model (Hijmans *et al*., 2005) to explore putative local differentiation and adaptation. Putative associations were assessed within each geocytotype separately (the BG6-bis population was removed from these tests as the collection site was the same as for BG6) using linear regression models (LMs).

All analyses and plotting were performed in the R environment (R Development Core Team (2009).

RESULTS

Initial measurements before treatment application

Significant differences among the geocytotypes were found in the initial biomasses (LL × NRL; product of length of the longest leaf and number of rosette leaves). While European diploids did not differ in this trait from the European tetraploids (mean ± s.d.; 78.3 ± 25·9 for 2*x*EU), North American tetraploids accumulated significantly greater initial biomass than European tetraploids (4*x*NA, 94·4 ± 24·2; 4*x*EU, 74·37 ± 22·4; Table 1). Moreover, significant population effects on initial biomass were found in both 2*x*EU versus 4*x*EU and 4*x*EU versus 4*x*NA comparisons (Table 1) and among-population differences in this trait were more pronounced within the 2*x*EU and 4*x*EU geocytotypes than within the 4*x*NA geocytotype (Table 2, Fig. 1). There was no significant correlation between initial biomass and date of seedling emergence per population (Spearman correlation test: $\rho = -0{\cdot}193$, $P = 0{\cdot}6$). Similarly, there was no significant difference among geocytotypes in mean seedling emergence (LMM, $P = 1$, 2*x*EU, 10·4 ± 1·7 d; 4*x*EU, 10·1 ± 1·9; 4*x*NA, 10·2 ± 2·0). The geocytotypes did not differ in initial soil humidity (SH1, mean ± s.d.; 2*x*EU, 32·7 ± 5·3 %; 4*x*EU, 31·5 ± 4·2 %; 4*x*NA, 30·8 ± 4·3 %), or in stomatal conductance (SC1, mean ± s.d.; 2*x*EU, 0·68 ± 0·33 mol m^{-2} s^{-1}; 4*x*EU, 0·63 ± 0·31 mol m^{-2} s^{-1}; 4*x*NA, 0·73 ± 0·27 mol m^{-2} s^{-1}; LMMs, $P > 0{\cdot}05$ for both parameters).

Treatment effects and differences among geocytotypes and populations

The effect of drought on soil moisture in the pots was highly significant (GLMMs, $P < 0{\cdot}001$ in SH2, SH3 and SH4 measurements; Supplementary Data Table S2), but there was no significant effect of geocytotype or its interaction with treatment during the experiment (GLMMs, results not shown). Stomatal conductance strongly depended on soil humidity (linear regression on the whole data set for SH2/SC2 measurements, $r = +0{\cdot}66$, $P < 0{\cdot}001$; Supplementary Data Fig. S1).

Water stress had a very strong and generally negative effect on growth, survival and physiological parameters except the amount of leaf nitrogen in the 2*x*EU versus 4*x*EU comparison (Table 1, Fig. 2). As expected, significantly more plants ($N = 32$, 11·8 %) were dead at the end of the experiment in the most severe treatment (T3) than in the two remaining treatments ($N = 2$, 0·7 %). More 2*x*EU ($N = 15$, 16·7 %) than 4*x*EU ($N = 10$, 11·1 %) and 4*x*NA plants ($N = 9$, 10 %) were dead at the end of the experiment, but neither these differences nor the geocytotype × treatment interactions were significant (Table 2). Since the effect of drought was stronger on above- than on below-ground biomass, the root–shoot ratio also increased (Table 1). The 2*x*EU geocytotype produced significantly more above-ground biomass than the 4*x*EU geocytotype (2*x*EU, 1·27 ± 0·69 g; 4*x*EU, 1·06 ± 0·53 g) and also had a significantly greater proportion of leaf carbon in the dry leaf biomass (2*x*EU, 42·6 ± 1·5 %; 4*x*EU, 41·4 ± 1·2 %; Table 1).

Different responses of geocytotypes to drought treatment (significant geocytotype × treatment interactions) were recorded in above-ground and total biomass production in the 2*x*EU versus 4*x*EU comparison and in stomatal conductance in the 4*x*EU versus 4*x*NA comparison (Table 1, Fig. 2). In the 4*x*EU versus 4*x*NA comparison, the European tetraploids achieved greater values of stomatal conductance (SC2) than the North American plants (4*x*EU, 0·38 ± 0·23 mol m^{-2} s^{-1}; 4*x*NA, 0·32 ± 0·23 mol m^{-2} s^{-1}; Table 1), which was caused by higher values obtained in the T2 treatment in 4*x*EU plants (Fig. 2). When comparing repeated stomatal conductance measurements of the T1 plants (control) across the duration of the experiment (SC1–SC4), we found a consistent, though statistically non-significant, trend towards greater stomatal conductance in 4*x* plants (especially in 4*x*NA plants) as compared to the 2*x* cytotype (Supplementary Data Fig. S2).

Initial biomass treated as a covariate significantly explained variation in many parameters measured at the level of geocytotypes and within each geocytotype (Table 1, Table 2). It is noteworthy that block (greenhouse benches) taken as a random factor contributed significantly to the overall variation in some models (Table 1). Plants placed in marginal blocks (benches) lost less water than plants situated on the middle benches (Supplementary Data Fig. S3), most likely because of lower temperature in the greenhouse margins.

TABLE 1. *Results of linear mixed effect models, showing maximum log likelihood ratio values and degrees of freedom (subscript numbers) testing the effect of three water treatments,* Centaurea stoebe *geocytotype and their interactions on different fitness and physiological variables. For variables for which we had a balanced design, block and population nested within geocytotype were considered as random factors; for others only block was included as a random factor*

Fixed and random effects/ response variables	Initial biomass	Above-ground biomass	Below-ground biomass	Total biomass	Below-/ above-ground biomass	Survival	Water loss in soil	Carbon isotope discrimination	Stomatal conductance	Leaf carbon	Leaf nitrogen
2*x*EU versus 4*x*EU											
Treatment	NI	$249{\cdot}0_4$*** ↓	$161{\cdot}0_4$*** ↓	$262{\cdot}2_4$*** ↓	$10{\cdot}0_4$* ↑	$48{\cdot}6_4$*** ↓	$85{\cdot}4_4$*** ↓	$62{\cdot}5_4$*** ↓	13·6** ↓	$10{\cdot}5_4$* ↓	$3{\cdot}6_4$
Geocytotype	$0{\cdot}148_1$	$10{\cdot}7_3$* ↑	$4{\cdot}3_3$	$10{\cdot}2_3$* ↑	$4{\cdot}8_3$	$3{\cdot}2_3$	$3{\cdot}9_3$	$2{\cdot}4_3$	$1{\cdot}9_2$	$15{\cdot}0_3$*** ↑	$2{\cdot}8_3$
Geocytotype × treatment	NI	$8{\cdot}635_2$*	$2{\cdot}8_2$	$8{\cdot}8_2$ *	$0{\cdot}9_2$	$1{\cdot}7_2$	$3{\cdot}9_2$	$0{\cdot}7_2$	$1{\cdot}5_2$	$4{\cdot}4_2$	$0{\cdot}8_2$
Initial biomass	NI	$23{\cdot}803_1$***	$25{\cdot}6_1$***	$33{\cdot}1_1$***	$1{\cdot}9_1$	$3{\cdot}3_1$	$10{\cdot}4_1$**	$5{\cdot}3_1$*	$3{\cdot}1_1$	$0{\cdot}1_1$	$3{\cdot}5_1$
Population	$76{\cdot}709_1$***	$33{\cdot}367_1$***	$22{\cdot}6_1$***	$25{\cdot}2_1$***	$38{\cdot}9_1$***	0_1	NI	NI	NI	NI	NI
Block	NI	$4{\cdot}374_1$*	$7{\cdot}4_1$**	$6{\cdot}6_1$*	0_1	0_1	NT	NT	NT	NT	NT
4*x*EU versus 4*x*NA											
Treatment	NI	$222{\cdot}9_4$*** ↓	$147{\cdot}4_4$*** ↓	$232{\cdot}6_4$*** ↓	$15{\cdot}3_4$** ↑	$40{\cdot}1_4$*** ↓	$98{\cdot}9_4$*** ↓	$80{\cdot}6_4$*** ↓	43·4*** ↓	$2{\cdot}1_4$	14·9** ↑
Geocytotype	$5{\cdot}4_1$* ↓	$5{\cdot}9_3$	$1{\cdot}7_3$	$5{\cdot}5_3$	$1{\cdot}8_3$	$3{\cdot}2_3$	$1{\cdot}9_3$	$2{\cdot}8_3$	$10{\cdot}7_3$* ↓	$2{\cdot}9_3$	$1{\cdot}4_3$
Geocytotype × treatment	NI	$3{\cdot}0_2$	$1{\cdot}6_2$	$3{\cdot}4_2$	$0{\cdot}7_2$	$2{\cdot}8_2$	$1{\cdot}6_2$	$2{\cdot}8_2$	10·2**	$1{\cdot}579_2$	$1{\cdot}4_2$
Initial biomass	NI	$26{\cdot}2_1$***	$11{\cdot}7_1$***	$27{\cdot}1_1$***	$1{\cdot}2_1$	$3{\cdot}9_1$	$30{\cdot}3_1$***	$13{\cdot}4_1$***	17·0***	$0{\cdot}548_1$	5·614*
Population	$38{\cdot}0_1$***	$7{\cdot}1_1$**	$10{\cdot}3_1$**	$38{\cdot}9_1$***	$9{\cdot}8_1$**	0_1	NI	NI	NI	NI	NI
Block	NI	$1{\cdot}8_1$	$3{\cdot}5_1$	$6{\cdot}6_1$*	$3{\cdot}5_1$	0_1	NT	NT	NT	NT	NT

NI, not included; NT, not tested.

Arrows in the treatment row indicate the direction of response of variables to increasing water stress [from T1 (control treatment) to T3 (severe drought stress)] and the directions of arrows in the geocytotype row show whether the tested variable reached a higher (↑) or lower (↓) value in the first geocytotype compared with the second.

$*P < 0{\cdot}05$, $**P < 0{\cdot}01$; $***P < 0{\cdot}001$.

TABLE 2. *Results of linear mixed effect models, showing maximum likelihood ratio values and degrees of freedom (subscript numbers) testing the effect of population within each of the* Centaurea stoebe *geocytotypes and three water treatments on above- and below-ground biomass and their ratio. Block was considered as a random factor*

Fixed effects/response variables	Initial biomass	Above-ground biomass	Below-ground biomass	Total biomass	Below-/above-ground biomass	Survival
2*x*EU geocytotype						
Treatment	NI	$182\cdot2_{12}$***	$98\cdot5_{12}$***	$179\cdot2_{12}$***	$9\cdot8_{12}$	$39\cdot7_{12}$***
Population	$61\cdot6_{5}$***	$85\cdot6_{15}$***	$27\cdot6_{15}$*	$64\cdot8_{15}$***	$59\cdot6_{15}$***	$8\cdot8_{15}$
Population × treatment	NI	$25\cdot3_{10}$**	$5\cdot2_{10}$	$20\cdot2_{10}$**	$6\cdot2_{10}$	$4\cdot7_{10}$
Initial biomass	NI	$12\cdot8_{1}$***	$10\cdot5_{1}$**	$16\cdot6_{1}$***	$0\cdot9_{1}$	$5\cdot2_{1}$*
4*x*EU geocytotype						
Treatment	NI	$112\cdot1_{2}$***	$98\cdot4_{12}$***	$121\cdot5_{2}$***	$43\cdot0_{12}$*	$25\cdot1_{12}$*
Population	$58\cdot4_{5}$***	$18\cdot4_{15}$	$47\cdot1_{15}$***	$21\cdot9_{15}$	$43\cdot0_{15}$***	$3\cdot5_{15}$
Population × treatment	NI	$6\cdot4_{10}$	$18\cdot5_{10}$*	$7\cdot2_{10}$	$17\cdot1_{9}$*	0_{10}
Initial biomass	NI	$9\cdot3_{1}$**	$10\cdot5_{1}$**	$12\cdot2_{1}$**	$0\cdot7_{1}$	$2\cdot7_{1}$
4*x*NA geocytotype						
Treatment	NI	$165\cdot6_{2}$***	$105\cdot3_{12}$***	$133\cdot7_{2}$***	$20\cdot1_{12}$	$24\cdot2_{12}$*
Population	$16\cdot2_{5}$**	$49\cdot0_{15}$***	$37\cdot0_{15}$**	$57\cdot3_{15}$***	$9\cdot9_{15}$	$12\cdot2_{15}$
Population × treatment	NI	$33\cdot1_{10}$***	$25\cdot6_{10}$**	$39\cdot6_{10}$***	$8\cdot4_{10}$	$7\cdot6_{10}$
Initial biomass	NI	$11\cdot5_{1}$***	$1\cdot7_{1}$	$10\cdot6_{1}$**	$2\cdot5_{1}$	$3\cdot1_{1}$

NI, not included.
*$P < 0{\cdot}05$; **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$.

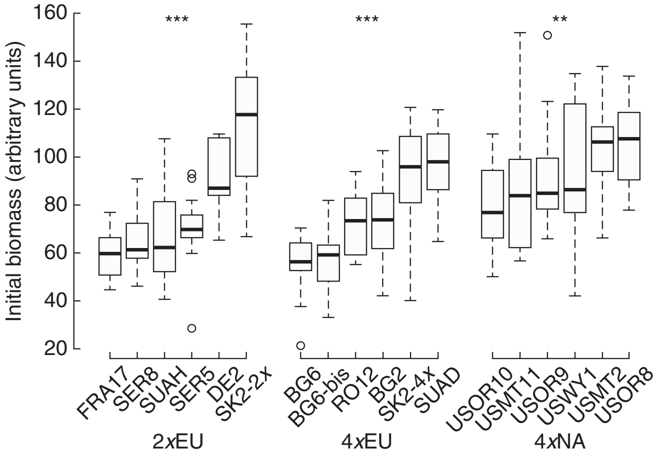

FIG. 1. Boxplots showing among-population variation in initial biomass (product of the length of the longest leaf and number of rosette leaves) within each of three studied geocytotypes of *Centaurea stoebe*. Statistical significances of population effect (**$P < 0{\cdot}01$; ***$P < 0{\cdot}001$) were assessed using linear mixed effect models (Table 2).

Significant population × water treatment interactions were found in the 2*x*EU and 4*x*NA populations for above-ground and total biomasses and in the 4*x*EU and 4*x*NA populations for below-ground biomass (Table 2, Fig. 3).

Differences in plasticity among geocytotypes

European diploids and North American tetraploids showed higher values for relative plasticity indices than European tetraploids for most of the biomass traits (Fig. 4, Supplementary Data Table S3), but these differences were only marginally significant in the 2*x*EU versus 4*x*EU comparison ($0{\cdot}05 < P < 0{\cdot}1$) for root:shoot ratio (higher in 4*x*EU) and below-ground biomass (higher in 2*x*EU), and non-significant in the 4*x*EU versus 4*x*NA comparison ($P > 0{\cdot}1$ for all traits).

Associations between geographical locations of populations, corresponding macroclimatic parameters and plant fitness variates

Linear regression models applied within each geocytotype separately revealed some significant associations.

In the 2*x*EU populations, total and above-ground biomasses were positively associated and root:shoot biomass was negatively associated with latitude (all $P < 0{\cdot}05$). Furthermore, a significant, negative correlation was found between below-ground biomass and the amount of precipitation (four different climatic variables; Table 3). In addition, initial and total biomasses were positively associated with precipitation seasonality.

In the 4*x*EU populations, significant, positive latitudinal trends were found in initial biomass. While above-ground, below-ground and total biomasses were positively correlated with precipitation seasonality, the opposite association was found with amount of precipitation in the driest quarter (Table 3). A negative association was also found between initial biomass and the amount of precipitation in the coldest quarter (Table 3).

In the 4*x*NA populations, less pronounced associations between biomass traits and geographical location or climatic variables were found despite the fact that the climatic niche of American populations was broader than the niches of European diploid and tetraploid populations (Supplementary Data Fig. S4). Our analyses further revealed negative correlations between maximum temperature of the warmest month and total- and below-ground biomasses and root:shoot biomass ratio, as well as a positive correlation between longitude and the day of seedling emergence (Table 3).

DISCUSSION

Treatment effects and differences among geocytotypes

As expected, increased drought strongly affected all traits, causing on the one hand significant decreases in above-ground, below-ground and total biomasses, survival rate, stomatal

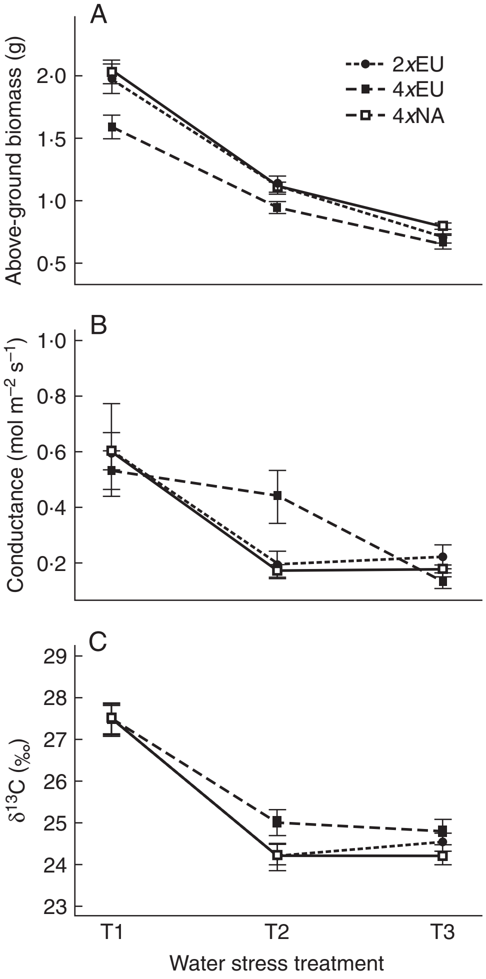

FIG. 2. Reaction norms of (A) above-ground biomass, (B) stomatal conductance and (C) carbon isotope discrimination (as a proxy of water-use efficiency) to three water treatments (cumulative amount of water: T1, 1600 ml; T2, 600 ml; T3, 300 ml) in three *Centaurea stoebe* geocytotypes. Values are mean ± s.e. based on individual plants.

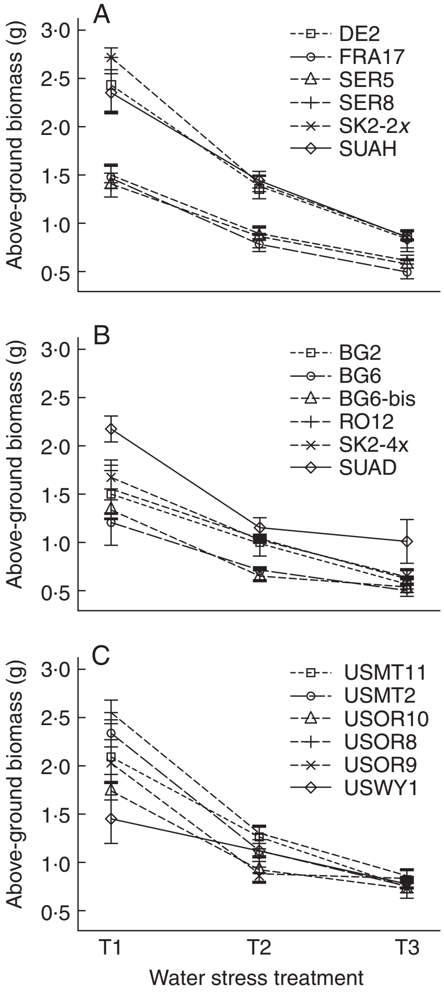

FIG. 3. Reaction norms of above-ground biomass to three water treatments (cumulative amount of water: T1, 1600 ml; T2, 600 ml, T3, 300 ml) in (A) European diploid (B), European tetraploid and (C) North American tetraploid populations of *Centaurea stoebe* s.l. Values are mean ± s.e. based on individual plants.

conductance and leaf carbon content, and on the other hand significantly increased water-use efficiency (decreased carbon isotope discrimination), root:shoot biomass and amount of leaf nitrogen (the last trait in the 4*x*EU versus 4*x*NA comparison only).

Initial biomass, which was used as a covariate in the models, explained a significant part of the variation in most plant performance parameters and therefore this trait should be taken into consideration in similarly conducted studies. Because there was no significant relationship between initial biomass and the day of seedling emergence, we suggest that differences in initial biomass found among geocytotypes resulted from differences in biomass accumulation during early growth. Our data thus confirm the results of previous common garden and greenhouse experiments, which demonstrated that North American populations are able to allocate resources faster during early growth (Henery *et al.*, 2010) and start to flower earlier than European tetraploids (Mráz *et al.*, 2011; Hahn and Müller-Schärer 2013). Accelerated growth in invasive populations of *C. stoebe* s.l. could be interpreted as a trade-off between growth and defence (the EICA hypothesis; Blossey and Nötzold 1995), as indeed Broz *et al.* (2009) found lower defence-related gene expression in the North American geocytotype. However, Ridenour *et al.* (2008) found no trade-off in the re-allocation of resources to growth and defence, as invasive populations showed similar or even higher resistance against specialists and generalist herbivores compared with native European populations, which indicates directional selection on both defence and competitive traits in invasive populations (Ridenour *et al.*, 2008).

The 2*x*EU plants produced significantly more above-ground and total biomass and accumulated more carbon in the leaf lamina than the 4*x*EU plants during the 1·5 months of our experiment. Earlier studies showed that the leaf lamina of 2*x* plants was more dissected than that of 4*x* plants (Henery *et al.*, 2010; Mráz *et al.*, 2011), which is expected to decrease leaf carbon content due to a greater abundance of supporting tissues (veins, sclerenchyma) (Niinemets *et al.*, 2007). Therefore, the greater carbon

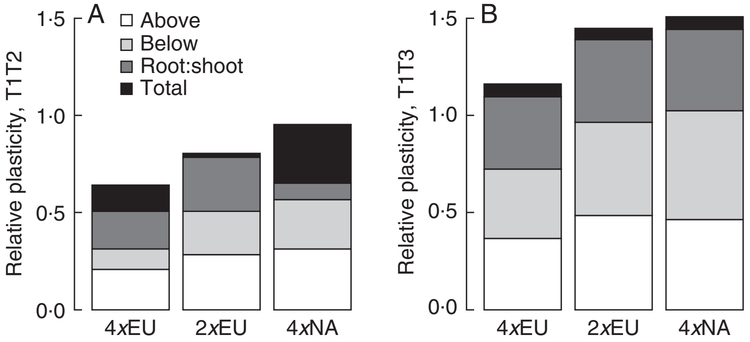

FIG. 4. Relative plasticity indexes for four traits of three *Centaurea stoebe* s.l. geocytotypes. (A) T1–T2 comparison (control versus moderate water stress). (B) T1–T3 comparison (control versus severe water stress).

TABLE 3. *Results of regression analyses, showing* F *values testing for putative associations between climatic variables derived from WorldClim model, latitude, longitude and altitude, and average population values of fitness traits [all but INBIOM and EMER were taken from the control treatment (T1)]*

Climatic and geographical variables/plant traits	INBIOM	TBIOM	ABIOM	BBIOM	BBIOM:ABIOM	EMER
2*x*EU geocytotype ($N = 6$)						
Latitude	1·3	**10·5*** ↑	**15·7*** ↑	0·2	**14·4*** ↓	2·4
Longitude	0·2	0·1	0	2·0	0·3	0·4
Altitude	2·4	6·7 ($P = 0·06$) ↓	5·3 ($P = 0·083$) ↓	0·9	0·5	0·9
BIO5	1·2	1·8	1·4	1·2	0·04	0·9
BIO12	0·3	0·7	0·4	**15·9*** ↓	0·5	0·002
BIO14	1·0	1·8	1·1	**15·5*** ↓	0·2	0·04
BIO15	**14·8*** ↑	**11·5*** ↑	7·6 ($P = 0·05$) ↑	1·9	0·4	0·4
BIO17	1·4	2·3	1·4	**13·5*** ↓	0·1	0·02
BIO19	2·1	2·8	1·7	**11·9*** ↓	0·05	0·07
4*x*EU geocytotype ($N = 5$)						
Latitude	**14·6*** ↑	5·7 ($P = 0·096$) ↑	7·7 ($P = 0·069$) ↑	1·6	0·3	0
Longitude	0·1	0·3	0·09	1·5	4·7	0·2
Altitude	4·7	1·8	2·5	0·4	1·0	0
BIO5	1	0·2	0·4	0	1·5	0
BIO12	0·01	0	0·01	0·2	1·1	0·6
BIO14	0·9	2·4	2·1	2·5	0·1	0·5
BIO15	5·9 ($P = 0·091$) ↑	**56·4**** ↑	**30*** ↑	**16·4*** ↑	0	0
BIO17	7·3 ($P = 0·073$) ↓	**10·6*** ↓	**13·5 *** ↓	3·0	0·1	0·4
BIO19	**15·5*** ↓	5·8 ($P = 0·094$) ↓	9·5 ($P = 0·054$) ↓	1·2	0·8	0·2
4*x*NA geocytotype ($N = 6$)						
Latitude	0·4	1·5	1·7	1·1	0·1	1·4
Longitude	0·2	1	0·9	1·5	4·8 ($P = 0·094$) ↑	**8·1*** ↑
Altitude	7·2 ($P = 0·062$) ↓	2·7	2·9	2·3	1·3	0
BIO5	1·1	**8·1*** ↓	6·0 ($P = 0·057$) ↓	**13·8*** ↓	**21·9**** ↓	1·8
BIO12	1·1	0	0	0·1	2·4	0·1
BIO14	0·9	0·9	1	0·8	0·2	1·4
BIO15	0·8	1	1·1	0·5	0·1	3·5
BIO17	0·1	0·8	0·8	0·6	0·2	1·6
BIO19	0·5	0·2	0·2	0·5	6·0 ($P = 0·071$) ↑	4·9 ($P = 0·09$) ↓

The directions of statistically significant associations (in bold) between fitness variables and geographical position of populations and corresponding climatic variables are indicated with arrows.

$^*P < 0·05$; $^{**}P < 0·01$; for marginally significant differences ($P < 0·1$), exact P values are given in parentheses.

N, number of populations; INBIOM, initial biomass; TBIOM, total biomass; ABIOM, above-ground biomass; BBIOM, below-ground biomass; BBIOM:ABIOM, below-:above-ground biomass ratio; EMER, speed of seedling emergence; BIO5, maximum temperature in warmest month; BIO12, annual precipitation; BIO14, precipitation of the driest month; BIO15, precipitation seasonality (coefficient of variation); BIO17, precipitation of driest quarter; BIO19, precipitation of coldest quarter.

content in the leaf lamina of diploid plants might be due to between-cytotype differences in their leaf structure. In general, cells of diploid plants are smaller and denser than those of polyploids (Levin, 2002) and thus contain more biomass in the cell walls, which are built principally from structural carbohydrates.

We found some significant geocytotype × treatment interactions in 2*x*EU versus 4*x*EU and 4*x*EU versus 4*x*NA comparisons. In the first comparison, after controlling for initial biomass, the above-ground and total biomasses of 2*x*EU plants were more responsive to water stress than those of 4*x*EU plants, suggesting better tolerance of drought in European tetraploids than in European diploids. In the same line of evidence, higher, but not significantly higher, mortality recorded in 2*x*EU compared with 4*x*EU plants suggests that 4*x*EU plants perform better under water shortage than 2*x*EU plants. One possible explanation for slightly better drought tolerance in European tetraploids could be a different root architecture and a higher root:shoot ratio between the geocytotypes. Although we did not find a statistically significant increase in below-ground biomass and root:shoot ratio in 4*x*EU compared with 2*x*EU plants (Table 1), Collins *et al.* (2013) reported that 2-week seedlings of European tetraploids had significantly larger total root length, tap root length, surface area, root volume and below-ground biomass and root:shoot ratio than European diploids. These root traits, which are considered to be an important mechanism of adaptation to water stress (Chapin *et al.*, 1993), might allow 4*x*EU plants to perform better under drought conditions compared with 2*x*EU plants. Such a slight increase in pre-adaptation to drier habitats could thus contribute at least partly to the macro- and microspatial distributional patterns of tetraploids in Europe (see Introduction and Ochsmann, 2000; Mráz *et al.*, 2012*b*; Fig. 1 in Broennimann *et al.*, 2014), but these patterns were probably mainly triggered by historical processes (Mráz *et al.*, 2012*a*, *b*). A recent molecular study by Mráz *et al.* (2012*a*) showed that tetraploid *C. stoebe* s.l. is a younger allopolyploid derivate that probably originated in south-eastern Europe and has recently spread to other parts of Europe, primarily along main transport corridors. The younger age, geographical origin and recent spread might thus explain the prevalence of tetraploid populations in south-eastern Europe and their lower frequencies in Central and Western Europe (Mráz *et al.*, 2012*a*).

In the 4*x*EU versus 4*x*NA comparison, European tetraploids retained a significantly higher level of stomatal conductance than North American tetraploids in the T2 treatment (Table 1, Fig. 2B), which was reflected in decreased carbon isotope discrimination in this treatment (Fig. 2C). This might suggest that the 4*x*EU plants were less stressed than the 4*x*NA (and 2*x*EU) plants, possibly due to their increased root:shoot ratio (higher in 4*x*EU in all treatments) and thus better water uptake:transpiration economy. Response to water stress was, however, similar for the two tetraploid geocytotypes in all other traits, indicating no post-introduction evolution of tolerance to drought in 4*x*NA plants.

Treatment effects and differences among populations within geocytotypes

Our data showed considerably larger trait variances and responses to drought among populations within geocytotypes than among geocytotypes, and these large among-population differences might have obscured potential among-geocytotype differences. Significant population effects, but not ploidy effects, have also been shown in a salt-tolerance experiment in the invasive *Phragmites australis* (Achenbach *et al.*, 2013), which have been explained in part by patterns of local adaptation. Here, we found significant latitudinal clines for European diploid and European tetraploid populations (Table 3), indicating adaptation to broader environmental conditions (Turesson, 1930; Neuffer and Hurka, 1986; Linhart and Grant, 1996; Maron *et al.*, 2004). Total and above-ground biomasses increased and root:shoot ratio decreased with increasing latitude in European diploid populations, while in European tetraploid populations a positive latitudinal association was only found in initial biomass. In contrast, no significant association between latitude and any of the tested traits was found in North American tetraploid populations, despite a similar magnitude of latitudinal ranges for all geocytotypes (Supplementary Data Table S1) and even larger multivariate climatic space occupied by the 4*x*NA populations compared with the European populations (Supplementary Data Fig. S4). Our data on the invasive 4*x*NA geocytotype of *Centaurea stoebe* s.l. are in line with results of other studies that did not find an indication of local differentiation in other invasive plants (e.g. Pahl *et al.*, 2013; Zhao *et al.*, 2013; but see e.g. Maron *et al.*, 2004, and Colautti *et al.*, 2009, who reported rapid latitudinal adaptive evolution in some introduced plant species). Our results confirm earlier findings by Mráz *et al.* (2011), who found a significant correlation between phenotypic differentiation (based on > 40 morphological and life history traits) and geographical distances in European diploid populations, but not in European and North American tetraploid populations. Similarly, strong regional differentiation in several phenological and reproductive traits were found within 2*x*EU populations, but were weaker in 4*x*EU and 4*x*NA populations (Hahn and Müller-Schärer, 2013). The most important climatic variables explaining clinal variation in performance traits in European populations were precipitation seasonality (coefficient of variation) and precipitation in the driest month. The significant among-population differentiation found in European diploids may be due to its longer evolutionary time (Mráz *et al.*, 2012*a*) and longer occupation of current habitats, in contrast to the recently spreading tetraploids in Europe (see above, Wells *et al.*, 2008 and Mráz *et al.*, 2012*b*) or the recently introduced North American tetraploids (Mráz *et al.*, 2011). This may also explain why among-population differences in traits were generally more pronounced within the 2*x*EU and 4*x*EU geocytotypes than within the 4*x*NA geocytotype (Table 2, Fig. 1).

Differences in plasticity among geocytotypes

We found either no or only statistically marginal differences among geocytotypes in plastic responses to drought, but nevertheless some clear trends emerged. In accordance with the hypothesis of trade-off between tolerance and plasticity (e.g. Lambers and Poorter, 2004), 4*x*EU, which is the most drought-tolerant geocytotype, demonstrated the lowest level of phenotypic plasticity in performance traits. Conversely, the 2*x*EU and 4*x*NA geocytotypes had higher accumulations of biomass and responded more plastically to water shortage. The only trait that was more plastic in the 4*x*EU geocytotype in the 2*x*EU versus 4*x*EU comparison was the root:shoot biomass ratio, which is a trait directly involved in water regime regulation. These non-significant differences in plasticity traits between 2*x* and 4*x* plants are in contrast to recently published data from a common garden experiment, which reported increased plasticity in traits associated with rapid growth and faster phenological development in 4*x* compared with 2*x* (Hahn *et al.*, 2012). This discrepancy could be due to the short duration of our experiment, assuming that the strongest effects of drought occur during early growth. Indeed, in the common garden experiment of

Hahn *et al.* (2012), increased plasticity in tetraploid plants was found only in the second year, whereas in the first year overall plasticity was similar between cytotypes.

Conclusions

In support of the pre-adaptation hypothesis, our data suggest slightly increased drought tolerance during early growth in European tetraploid plants compared with European diploid and North American tetraploid plants. Increased drought tolerance might provide European tetraploid plants with a selective advantage under drought stress and might contribute in part to their invasion success in North America and prevalence in southeastern Europe. In contrast to cytotype and range (native versus introduced tetraploid populations), the among-population differences, which were probably driven by local adaptations, were far better in explaining the response to drought stress and might have blurred the geocytotype effects.

SUPPLEMENTARY DATA

Supplementary Data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: origin of plant material. Table S2: effect of drought treatment on soil moisture in the pots. Table S3: comparison of relative plasticity indexes between European diploid and European tetraploid plants and between European tetraploid and North American tetraploid plants in response to water stress. Figure S1: linear relationship between soil humidity and stomatal conductance. Figure S2: stomatal conductance measured during water stress experiment in control treatment. Figure S3: block effect (position of experimental bench) on water loss in cultivated plants. Figure S4: principal component plot delineating the climatic space occupied by the populations of geocytotypes.

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