Evidence for a combination of pre-adapted traits and rapid adaptive change in the invasive plant *Centaurea stoebe*

Martin L. Henery[1]*, Gillianne Bowman[1], Patrik Mráz[1], Urs A. Treier[1,2], Emilie Gex-Fabry[1], Urs Schaffner[3] and Heinz Müller-Schärer[1]

[1]*Department of Biology, Unit of Ecology and Evolution, University of Fribourg, Chemin du Musée 10, CH-1700 Fribourg, Switzerland;* [2]*Department of Biological Sciences, Ecoinformatics & Biodiversity, Aarhus University, Ny Munkegade 114, Building 1540, DK-8000 Aarhus C, Denmark; and* [3]*CABI Europe – Switzerland, Rue des Grillons 1, CH-2800 Delémont, Switzerland*

Summary

1. Introduced plants have the potential to rapidly evolve traits of ecological importance that may add to their innate potential to become invasive. During invasions, selection may favour genotypes that are already pre-adapted to conditions in the new habitat and, over time, alter the characteristics of subsequent generations.

2. Spotted knapweed (*Centaurea stoebe*) occurs in two predominantly spatially separated cytotypes in its native range (Europe–Western Asia), but currently only the tetraploid form has been confirmed in the introduced range (North America), where it is invasive. We used several common garden experiments to examine, across multiple populations, whether tetraploids and diploids from the native range differ in life cycle, leaf traits and reproductive capacity and if such differences would explain the predominance of tetraploids and their advance into new habitats in the introduced range. We also compared the same traits in tetraploids from the native and introduced range to determine whether any rapid adaptive changes had occurred since introduction that may have enhanced invasive potential of the species in North America.

3. We found tetraploids had lower specific leaf area, less lamina dissection and fewer, narrower leaves than diploids. Diploids exhibited a monocarpic life cycle and produced few if any accessory rosettes. Diploids produced significantly more seeds per capitulum and had more capitula per plant than tetraploids. In contrast, the vast majority of European tetraploids continued to flower in both seasons by regenerating from multiple secondary rosettes, demonstrating a predominantly polycarpic life cycle.

4. During early growth tetraploids from North America achieved greater biomass than both tetraploids and diploids from the native range but this did not manifest as larger above-ground biomass at maturity. In North American tetraploids there was also evidence of a shift towards a more strictly polycarpic life cycle, less leaf dissection, greater carbon investment per leaf, and greater seed production per capitulum.

5. *Synthesis.* Our results suggest that the characteristics of tetraploid *C. stoebe* pre-adapted them (compared to diploid conspecifics) for spread and persistence of the species into habitats in North America characterized by a more continental climate. After the species' introduction, small but potentially important shifts in tetraploid biology have occurred that may have contributed significantly to successful invasion.

Key-words: biological invasion, *Centaurea stoebe,* plant life cycle, plant traits, ploidy, rapid adaptive change, reproductive potential

*Correspondence author. E-mail: martin.henery@csiro.au

Introduction

The invasiveness of a plant species may be derived from a combination of pre-adapted traits and rapid adaptive changes in the species following introduction (Müller-Schärer & Steinger 2004). Interspecific studies examining traits of invasive plants have identified that traits associated with reproductive capacity and leaf traits associated with rapid carbon capture, such as high specific leaf area (SLA), leaf area ratio, photosynthetic rate, and net assimilation rate, may pre-adapt species to become invasive (Pyšek & Richardson 2007). Once introduced to a new habitat and released from co-evolved natural enemies (Liu & Stiling 2006) these species may be able to exploit resources to a greater extent than co-occurring native species (Blumenthal 2006; Blumenthal *et al.* 2009). Recent studies have highlighted the importance of co-determinants such as residence time, range size and propagule pressure that interact with plant traits in the invasion process and show that the relative importance of traits is context-dependent and differs between species (Pyšek, Křivánek & Jarošík 2009b; Pyšek *et al.* 2009a). This partly explains the difficulty in generalizing across species about the importance of plant traits in the process leading from a species' introduction to invasion.

In addition, an increasing number of studies comparing various traits of species from their native and invasive ranges have shown that evolutionary adaptive changes can occur that alter traits, which may contribute to these species becoming invasive (Ellstrand & Schierenbeck 2000; Lee 2002; Müller-Schärer, Schaffner & Steinger 2004; Bossdorf *et al.* 2005; Barrett, Colautti & Eckert 2008; Whitney & Gabler 2008). Such trait shifts can increase the relative fitness of invasive species over native species in the same plant communities. These changes, however, may occur over several generations which may partly explain the lag time commonly seen between the first records of a species in a new region and its subsequent development into a highly successful invasive species (Sakai *et al.* 2001).

The most commonly tested hypothesis related to evolutionary change is whether plants in the introduced range, once released from their co-evolved natural enemies, have been able to reallocate resources previously used on defence, to achieve increased growth, the so-called Evolution of Increased Competitive Ability (EICA) hypothesis (Blossey & Nötzold 1995). Whilst increased growth and/or reduced defence has commonly been detected in invasive conspecifics (Siemann & Rogers 2003a,b; Jakobs, Weber & Edwards 2004; Wolfe, Elzinga & Biere 2004; Agrawal *et al.* 2005; Blumenthal & Hufbauer 2007; Zou, Rogers & Siemann 2008a; Zou *et al.* 2008b; Abhilasha & Joshi 2009), such reallocation of resources may be minor and not always manifest as increased above-ground vegetative biomass (Maron *et al.* 2004a; Genton *et al.* 2005; Barney, Whitlow & DiTommaso 2009). Changes in traits other than size, e.g. numbers or size of propagules and SLA, may be equally important for promoting invasiveness (Bossdorf *et al.* 2005). Higher SLA and associated increased rates of carbon capture could be allocated to a more extensive root system or more propagules per plant rather than in greater leaf biomass or shoot size. Whilst SLA has been found to be generally higher in invasive plants relative to co-occurring native species (Leishman *et al.* 2007), a shift in this trait within a species could be the result of selection for adaptation to a new environmental niche during invasion.

Recent studies indicate that interspecific variation in ploidy level in species is associated with invasiveness (Pyšek *et al.* 2009a) and that within species, polyploids rather than diploids are the dominant invasive forms (Lafuma *et al.* 2003; Pandit, Tan & Bisht 2006; Schlaepfer *et al.* 2008; Treier *et al.* 2009). Hence, the potential for becoming invasive not only depends on the variation in ploidy of a species but also on the ploidy level of a cytotype. Although the reason for this is currently unclear, it has been proposed that a broader tolerance of environmental conditions in polyploids, their greater potential to rapidly evolve via an ability to carry greater genetic diversity and the potential for duplicated genes to evolve new functions in the longer term, may explain their success (Soltis & Soltis 2000). It is also possible that, in a similar interaction to that proposed for resource availability and release from natural enemies (Blumenthal 2006), some polyploids may possess traits that give them advantages over diploids when herbivores/pathogens are absent, such that they are able to increase their spread and maintain occupancy of colonized sites (Müller-Schärer, Schaffner & Steinger 2004).

In the invasive plant *Centaurea stoebe* L. tetraploids and diploids occur primarily in discrete populations across the native range in Europe whilst tetraploids dominate the non-native range in North America entirely (Treier *et al.* 2009). Such a pronounced shift in cytotyope frequency could be due to founder events or be derived from a larger degree of environmental niche overlap between the native and introduced ranges of tetraploids than in diploids (Treier *et al.* 2009). In the latter instance, assuming both cytotypes were introduced to North America, the combination of traits possessed by tetraploids may have made them inherently more invasive than diploids. Moreover, tetraploid *C. stoebe* have been shown to be polycarpic which, in the absence of specialist herbivores, could give them greater lifetime fecundity than monocarpic diploids thus facilitating their rapid spread (Müller 1989; Broz *et al.* 2009).

In conjunction with changes in life cycle associated with ploidy, a significant shift in the climatic niche has occurred between *C. stoebe*'s native and introduced ranges (Broennimann *et al.* 2007). Tetraploids in North America now occupy areas with drier and more continental conditions than do tetraploids in Europe, whereas the niche of native diploids and tetraploids is largely overlapping (Treier *et al.* 2009). In addition, North American tetraploid genotypes have reduced expression of genes related to constitutive defence, consistent with reduced defence costs in the absence of herbivores resulting in rapid adaptive change (Broz *et al.* 2009). Such a reduction of defence costs may have contributed to the observed increased vegetative growth and competitiveness of invasive

tetraploid genotypes relative to native genotypes in this species (Ridenour *et al.* 2008; He *et al.* 2009). The aforementioned findings indicate that *C. stoebe* may have undergone rapid evolutionary change in North America.

In this study, we sought to compare *C. stoebe* leaf traits, growth, life cycle and reproductive capacity of: (i) diploids with tetraploids from the native range in Europe and (ii) tetraploids from the native range (Europe) with tetraploids from the introduced range (North America). The first comparison is aimed at assessing what traits, possibly associated with previously identified life cycle differences between diploid and teraploid cytotypes, may potentially explain the dominance of the tetraploid cytotype in North America and the spatial separation of cytotypes in Europe. The second comparison is aimed at determining whether rapid adaptive changes, e.g. in reproduction, leaf traits or growth, have occurred in tetraploids from the introduced range relative to tetraploids from the native range. We chose to address this using a series of common garden experiments. In the absence of detailed population genetics information, our approach used a large number of populations from each cytotype and geographic region to average effects across multiple genotypes and to avoid confounding population level differences.

Materials and methods

STUDY SPECIES

Centaurea stoebe L. (syn. *Centaurea maculosa* Lam., Asteraceae) is native to Europe and western Asia. In North America, to where it was introduced 120 years ago (Maddox 1979; Roché, Piper & Talbott 1986), it has become highly invasive. Although currently considered a single species (Ochsmann 2000; Španiel *et al.* 2008), it consists of diploid and tetraploid forms with only the latter cytotype unambiguously identified as *C. stoebe sensu stricto* thus far in the introduced range (Treier *et al.* 2009). All seeds used in this study were collected in summer and autumn 2005 from over 100 populations as part of a distribution-wide field survey (for collection methods and locations, see Treier *et al.* 2009). When these seeds were germinated for experiments, any populations with individuals that exhibited morphological characters that we associated with other closely related taxa or hybrids were excluded from subsequent analyses. Using seed sourced from multiple open-pollinated families in each population and from multiple populations throughout the range of the species was intended to ensure robust comparison of the species traits at different ploidy levels and between different continents. The three geographic and cytotype combinations European diploids, European tetraploids and North American tetraploids are referred to hereafter as geo-cytotypes. As outlined below, several cohorts of plants were grown at different times over 3 years in separate experiments (see descriptions of Experiment 1, 2 and 3 below). The first cohort of plants was used to assess differences between the two geo-cytotype comparisons of interest by destructively measuring above-ground biomass in developing rosettes and mature plants and testing our ability to predict biomass using variables measured non-destructively. The second cohort was used to assess the differences in leaf traits between geo-cytotypes, and the third cohort used to compare life cycle and fecundity. Although the exact populations used in each cohort

differed slightly, all plant material originated from the seed collections described above and thus any comparisons across populations identified are based on genotypes that are reasonably consistent between experiments. In addition, all plants were grown in similar conditions at the same location, in a single glasshouse and in potting media made at the site. In all experiments, the locations of plants or populations (if seedlings) were randomized to avoid confounding position effects.

EXPERIMENT 1: ALLOMETRIC GROWTH IN YOUNG ROSETTES AND FINAL PLANT BIOMASS

For each geo-cytotype, 15 populations were selected (i.e. a subset of 45 of the original 108 populations), from which 10 seeds, each of 10 different open-pollinated families (i.e. 10 mother plants) were selected for sowing. Single seeds were planted into individual 2×2 cm cells in 10×15 cell seedling trays filled with sterilized and sieved compost on 9 and 13 May 2008. The seedling trays (each tray containing the seeds for one population and half the seeds for another) were then watered and the seeds left to germinate in the glasshouse located at the University of Fribourg, Switzerland (46°47′34.5″ N/7°9′20.6″ E, 18–30 °C, natural photoperiod and humidity) for 4 weeks, after which c. 15 plants per population (where available) were re-potted into 11 cm diameter × 13 cm deep, 1 L plastic pots in a 1 : 2 mix of coarse sand and sterilized compost. The remaining seedlings that were not transplanted were left in the germination trays for approximately two further weeks. After this period, a subsample of 76 seedlings representing all three geo-cytotypes was removed from the trays, the number of leaves and the length of the longest leaf recorded, the soil carefully washed from the roots, the roots and shoot separated, dried for minimum 48 h at 50 °C and then weighed. This was performed to determine whether the number of leaves, the length of the longest leaf, and the SLA could be used as effective non-destructive predictive variables for plant biomass.

Two months after sowing, between 5 and 8 of the plants potted into 1 L pots from each population were selected from the 15 available populations for each geo-cytotype. The number of leaves, the length of the longest leaf, the number of accessory rosettes and the number of accessory rosette leaves (generally much smaller than main rosette leaves) were recorded for each plant. The pots remained in the glasshouse and were watered as required until 3 November 2008 at which point they were moved outside and buried to ¾ of the pot depth in sand to overwinter. On 20 February 2009, the plants were returned to an unheated glasshouse under 16:8 light-dark cycle. After initially producing some new rosette leaves, all plants started bolting but were allowed to grow until the plants were dissected to measure biomass between the 10 and 26 March 2009. Prior to dissection, all of the above plant parameters were again measured with an additional parameter, the height of the highest flowering shoot, included. Due to the particular cold winter, plant mortality resulted in slightly uneven numbers in each geo-cytotype group but in total 170 surviving plants were measured. We also took some more detailed measurements on a subset of plants ($n = 28$), where the flowering stem had only just started to form. In a similar manner to that described above for young rosettes, using this subgroup we investigated how closely the parameter length of the longest leaf was correlated with median or mean leaf length in a rosette and whether these variables, combined with the number of leaves and mean SLA for each plant, were able to significantly account for any variation in above-ground biomass of mature second-year rosettes. *Centaurea stoebe* is tap-rooted but in the experiment plants commonly formed many fibrous roots with the

notable absence of a large central tap root. During plant dissection, soil was removed from the roots to collect the tap root to measure root biomass, but because of the aforementioned root architecture only a few larger roots could be separated from soil sufficiently for measurement. After soil removal, the roots were separated from the above-ground biomass, both plant parts dried for a minimum of 48 h at 50 °C, and then weighed.

EXPERIMENT 2: LEAF TRAITS AND EARLY ROSETTE DEVELOPMENT

A second set of seeds was sown at the beginning of October 2007. This set comprised of seeds from 86 of the original 100 populations, and the number of seeds sown per population was variable as a result of the exhaustion of seed stocks. Usually five seeds, each from 16 maternal plants, per population were sown directly into 1 L pots filled with the soil mix as described above. After germination, only one seedling was left in each pot for further cultivation and extra seedlings were removed. The seedlings were grown in the glasshouse supplemented with artificial light for a 16 h day period, with temperature control conditions of 23 °C day and 15 °C night, and watered when needed (i.e. approximately every 3 days). Leaf measurements were taken 2 months after sowing as leaves emerging after this become much more extensively dissected and thus difficult to accurately scan for area measurements. The harvest lasted 1 week in late December 2007. Numbers of plants sampled for each population varied between 1 and 21 individuals as a result of the limited availability of seed and variable germination but the total sample size was large, comprising 957 scanned leaf images. To standardize measurements between plants, the 6th true leaf (not counting cotyledons) was cut from an individual rosette. In some rare cases, where the 6th true leaf was damaged by fungi or insects, the 5th, 7th or 8th true leaf was collected. Immediately after being cut from the plant, a binary image of each leaf was captured using a flat bed scanner. The leaves were then dried for minimum 48 h at 50 °C and the leaf dry weight recorded. Leaf scans were analysed with the software Image J (Rasband 1997–2009). For each leaf image leaf length, the maximum leaf width, leaf perimeter and leaf area were measured. SLA was calculated by dividing the scanned leaf area by the leaf dry weight (Garnier *et al.* 2001). A subset of these leaves ($n = 200$, representing 23 native diploid populations, 17 European tetraploid and 26 North American tetraploid populations with plants per population ranging between 1 and 6) were also analysed for leaf nitrogen content using Mass Spectrometry in the Isolab at the Institut für Pflanzenwissenschaften ETH, Zürich, Switzerland. We removed a second set of leaves from the plants to calculate leaf dry matter content (LDMC) as the ratio of leaf dry weight to fresh weight (Garnier *et al.* 2001). This set of leaves were also scanned for corresponding area measurements and we calculated leaf thickness as $(SLA \times LDMC)^{-1}$ (Vile *et al.* 2005).

EXPERIMENT 3: REPRODUCTIVE CAPACITY AND LIFE CYCLE

In May 2006, five seeds each from 10 maternal plants from each of 100 populations were germinated in trays as described previously (Experiment 1). After 8 weeks, one plantlet per mother plant (1000 plants in total) was transferred to a 1 L pot (15 cm diameter, 13 cm height) with a soil mix as given in Experiment 1. The plants were grown in a naturally lit glasshouse supplemented with artificial light for 16 h daytime. The glasshouse was unheated but temperatures stayed above 0 °C in winter 2006/07. Plants were watered regularly,

but were not given nutrient solution. An additional 60 plants (five plants from four populations of each geo-cytotype) were grown to be used for biomass measures. Only four plants flowered out of the 550 surviving into summer 2006. The length of the longest leaf and the number of leaves were assessed three times (10–14 July 2006, 2 months after sowing; 7–11 August 2006, 3 months; 27 April–3 May 2007, 12 months) before the plants started bolting. At this stage, all plants were relocated outside on a bed of coarse sand in the Botanical Garden of Fribourg, and the positions of the pots randomized. When the first flower opened (6 July–23 August 2007, *c.* 15 months), each plant was rescored for the number of flowering stems, the height of the highest flowering shoot and the number of inflorescence buds larger than 5 mm. The latter was used as an estimate of the total number of capitula (flower heads) per plant. Seed production per capitulum was determined by averaging the number of seeds for the first three capitula to mature per plant and the total number of capitula that bore seed was counted once the stem had dried out at the beginning of October 2007. At this time, plant survival and number of accessory rosettes (newly formed) were also assessed. During flowering, which was roughly synchronous in all individuals that flowered, pollinating insects were able to access all the plants. This meant that pollen from diploids and tetraploids were probably exchanged, with the likely associated compatibility problems and potential interference in fertilization. Sufficient pollen movement occurred, however, such that successful pollination was achieved. The plants remained outside for an additional year with supplemental watering and survival, number of flowering stems, number of accessory rosettes and total number of capitula assessed again in October 2008.

During the week of the 7–11 August 2006, we sampled 60 plants for biomass. These plants were removed from their pots and the soil was washed away from the roots. The roots were cut from the rosette and both parts dried for 48 h at 60 °C before being weighed. Immediately prior to harvest, the aforementioned measurements of plant size were taken.

STATISTICAL ANALYSIS

We used a multiple regression to determine if the variables number of leaves, length of the longest leaf, population mean SLA and the fixed factor geo-cytotype could be used to predict above-ground biomass at both the young rosette stage (6–7 weeks old) (Experiment 1), 3-month-old rosettes (Experiment 3) and in mature plants entering their second growing season (Experiment 1). We used step-wise removal with comparison of the variance ratio for each variable with a test criterion of one to determine significance of variables to the model.

For our data set on leaf traits, the line best describing the allometric scaling relationships between pairs of log-transformed leaf variables for each geo-cytotypes were determined using the standardized major axis (SMA) estimation and testing routines (SMATR ver 2.1) (Warton *et al.* 2006) for fitting bivariate lines in R (R Core Development Team 2005). If the separate SMA lines for each geo-cytotype were found to not differ significantly in slope then a common SMA slope was estimated and the cloud of points describing the pairwise trait values for each geo-cytotype were tested for shifts in elevation (i.e. a shift in one dimension relative to the other clouds of points).

Differences in plant growth and reproductive parameters amongst the three geo-cytotypes were analysed using a generalized linear mixed model (GLMM) with geo-cytotype as a main effect and 'plant' nested within 'population' nested within geo-cytotype as a random effect. Dependent variables in this analysis included number of leaves,

above- and below-ground biomass, number of capitula per plant, seed production per capitulum and total seed output. Length of the longest leaf, SLA, LDMC and leaf thickness were analysed using linear mixed models (LMM) with restricted maximum likelihood (REML) estimation using the same model structure as before but assuming a normal distribution. Differences between tetraploids from invasive and native ranges were examined by excluding diploid plants from the analyses. Differences in probability of flowering and mortality for each geo-cytotype were investigated using a LMM with a binomial distribution and a logit link with geo-cytotype as a main factor. In all LMM, dependent variables were transformed as to improve normality where required.

Results

GROWTH AND DEVELOPMENT OF YOUNG ROSETTES

Tetraploid *C. stoebe* from North American accumulated significantly greater biomass during early growth than both diploid and tetraploid conspecifics from the native range of the species (Experiment 1, Table 1, 1.5 months, higher intercept than both European diploids and tetraploids). The LMM explained 68.7% of variation in above-ground biomass in young rosettes with length of the longest leaf and number of leaves accounting for the majority, and differences in SLA between populations accounting for a minor component. The interactions between all significant linear variables and the fixed factor geo-cytotype were non-significant indicating equal slopes for the linear relationships of the three geo-cytopes. Subsequent analyses using LMM with geo-cytotype as a fixed effect revealed that the significantly greater biomass of North American tetraploids is not due to greater number of leaves (Poisson distribution, deviance ratio = 1.08, d.f. = 2, $P = 0.341$) or different length of the longest leaf (normal distribution, $F = 0.06$, d.f. = 2, $P = 0.940$) in any geo-cytotype.

Similarly, in 3-month-old first-year plants (Experiment 3, Table 1), the majority of variation in above-ground biomass was accounted for by a combination of number of leaves and length of the longest leaf (89.0%). There was, however, no

detectable difference amongst geo-cytotypes in rosette biomass at this stage (Table 1, 3 months). The variables number of accessory rosettes and number of accessory rosette leaves were not significant explanatory variables. We did not have mean population SLA data for several of the populations used in these particular biomass measurements and thus could not assess its effect on biomass at this plant stage. As for 1.5-month-old young rosettes, there was no effect of geo-cytotype on number of leaves (GLMM, Poisson distribution, $F_{2,35} = 0.05$, $P = 0.954$) or length of the longest leaf (using the latter as a correlate of mean leaf length) (LMM, $F_{2,38} = 0.26$, $P = 0.775$).

As the plants from Experiment 3 matured further, we found significantly more leaves on diploid plants than both North American and European tetraploids (Fig. 1a), whilst initial differences in leaf length levelled out between geo-cytotypes (Fig. 1b). Similarly, in Experiment 1, 2.5-month-old diploid plants had significantly more leaves than tetraploids (Fig. 1a). Although North American tetraploids at this age tended to have more leaves than European tetraploids in this cohort, this does not indicate greater total leaf area deployed because length of the longest leaf of North American tetraploid plants was found to be significantly less than both other cytotypes (Fig. 1b). Leaf length was considerably greater in these plants (Experiment 1), relative to plants of equivalent age from Experiment 3, due to the fact that Experiment 3 plants were germinated in January in the glasshouse and thus initially grew much more slowly than plants from Experiment 1 which germinated in May.

LEAF TRAITS

We found that leaves from developing rosettes of the three geo-cytotypes differ in structure. SLA of diploid plants was significantly higher than that of North American and European tetraploids which did not differ significantly (Fig. 2a). This difference appears to be driven by an increasing trend in LDMC from diploids to invasive tetraploids (Fig. 2b). The calculated surrogate for leaf thickness $(SLA \times LDMC)^{-1}$ also showed

Table 1. Generalized linear model of the effect of geo-cytotype on shoot dry weight of *Centaurea stoebe* young rosettes whilst accounting for variation in plant size with three standardized variables. Different superscript letters on estimated intercepts for different geo-cytotypes indicate groups that significantly differ based on least significant differences (5% level). Geo-cytotypes are European diploids (2×EU), European tetraploids (4×EU) and North American tetraploids (4×NA)

| Plant age (months)	n	R^2	Independent variable	Estimated parameters	t	P
1.5	67	0.69	Length of longest leaf	0.050	7.16	< 0.001
			Number of leaves	0.034	4.87	< 0.001
			Population mean SLA	0.017	1.96	0.054
			2×EU	0.133[a]	11.71	< 0.001
			4×EU	0.153[a]	14.30	< 0.001
			4×NA	0.192[b]	14.39	< 0.001
3	41	0.66	Length of longest leaf	0.259	5.49	< 0.001
			Number of leaves	0.247	5.72	< 0.001
			2×EU	1.647	30.48	< 0.001
			4×EU	1.651	39.13	< 0.001
			4×NA	1.687	38.68	< 0.001

SLA, specific leaf area.
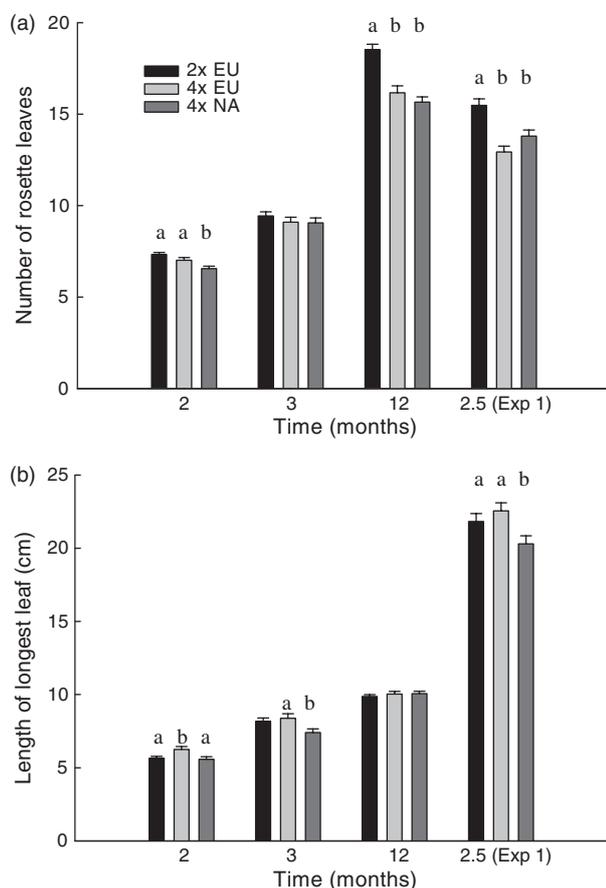
Fig. 1. Means and standard errors for: (a) total number of leaves and (b) length of the longest leaf for *Centaurea stoebe* plants from three different geo-cytotypes European diploids (2×EU), European tetraploids (4×EU), and North American tetraploids (4×NA). Measurements were made twice in the first year of growth in 2006 (after 2 and 3 months) and then again the following spring in 2007 (12 months) for Experiment 3 (see Materials and Methods). Numbers of individuals for the three measurements at different ages were: 2×EU = 344, 248, 219; 4×EU = 191, 138, 126; 4×NA = 204, 157, 140. The fourth group of bars on each chart (a & b) is the cohort of plants grown for Experiment 1 (*n* = 105, 104, 104 for 2×EU, 4×EU and 4×NA, respectively). Different letters above the columns indicate geo-cytotypes that differ significantly (*P* < 0.05).

European tetraploids to have significantly thicker leaves than diploids, with North American tetraploids showing intermediate values (Fig. 2c). We additionally found a small but significant positive relationship between SLA and leaf N content that accounted for 7.2% of the variation (*F* = 6.09, d.f. = 3, 195, *P* < 0.001). However, leaf N content did not differ between ploidy levels (*F* = 0.6262, d.f. = 198, *P* = 0.4297) or between European and North American tetraploids (*F* = 1.689, d.f. = 124, *P* = 0.1961), neither when including or excluding SLA as a covariate in a linear model.

European tetraploids had a greater increase in width for a given increase in leaf length (steeper SMA regression slope) than the other two geo-cytotypes (Fig. 3a, Table 2), which did not differ from each other. Variance in leaf width was significantly greater in European tetraploids than in the other two groups (data not shown) and the greater spread of data in this

dimension may explain the steeper slope of the relationship with leaf length (Fig. 3a, Table 2). In addition, maximum leaf length of plants of equivalent developmental age was slightly but significantly longer in European tetraploid plants than the other two geo-cytotypes which again did not differ (Fig. 1b, 2 months). In contrast, for a given leaf length, leaf width was greater in leaves from European diploids than the other geo-cytotypes (Fig. 3a).

The lamina was significantly more dissected in diploids than both tetraploid geo-cytotypes (in some tetraploid genotypes, the lamina is lobed rather than finely dissected) as shown by the elevation of allometric SMA relationship between leaf perimeter and leaf area (Fig. 3b, Table 2). The regression was, however, only marginally significant indicating high variability in the extent of leaf dissection in diploids (Table 2). Lamina dissection appears further reduced in the invasive range with consequently significantly greater leaf area for given leaf length in North American tetraploids than European tetraploids, and even greater than diploids (Fig. 3b, Table 2). This pattern is consistent with the results of the analyses on the relationship between leaf length and leaf area or dry weight (Fig. 3c,d, Table 2). Invasive tetraploids with least dissected leaves had the greatest leaf area and highest leaf dry weight at a given leaf length, whilst native diploids with the most dissected leaves produced leaves with the lowest area and biomass at a given length.

MATURE PLANT BIOMASS

All surviving plants from Experiment 1 that were returned to the glasshouse after overwintering outside immediately produced new main rosette leaves, developed multiple accessory rosettes and initiated bolting within a few weeks. We wished to examine the usefulness of various plant parameters in estimating biomass of second-year rosettes in a regression model. Unfortunately, the rapid onset of bolting after revival of the plants from winter dormancy limited the number of plants we could use in this analysis. Multiple regression analysis on a subset of these plants showed that using the most representative leaf size (i.e. median or mean leaf length) accounted for more variation in above-ground biomass than using length of the longest leaf (Table 3). There was, however, moderately close correlation between length of the longest leaf and either mean or median leaf lengths (*r* = 0.65, *P* < 0.0001 and *r* = 0.69, *P* = 0.0002, respectively) justifying the use of length of the longest leaf as a surrogate, when maximizing efficiency of measurement for large sample sizes. In addition, we determined that number of accessory rosettes and total leaves (sum of accessory rosette leaves and main rosette leaves) were significant terms in the model but SLA was not a significant explanatory variable (Table 3). In first-year rosettes, main rosette leaves comprise most of the above-ground biomass of the plant and the leaves of accessory rosettes are small and contribute relatively little to above-ground biomass (result not shown). After resprouting in the second year, leaves of accessory rosettes were larger and more numerous. Consequently, number of accessory rosettes accounted for a significant proportion
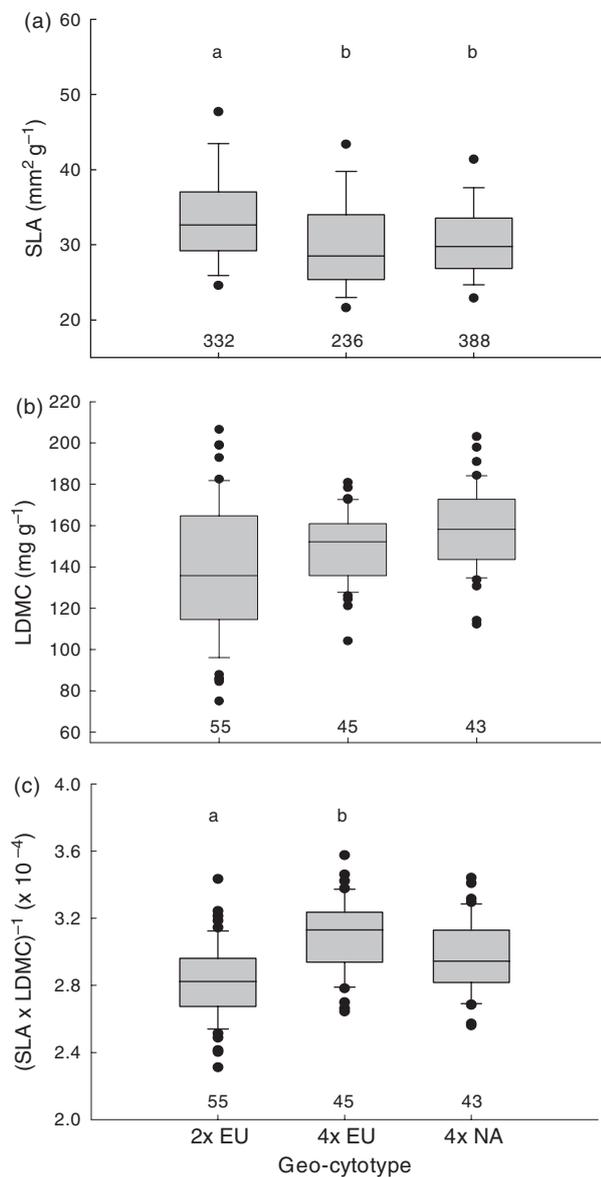
Fig. 2. Boxplots of: (a) specific leaf area (SLA), (b) leaf dry matter content (LDMC) and (c) leaf thickness (as calculated by the inverse of SLA × LDMC) for three geo-cytotypes European diploids (2×EU), European tetraploids (4×EU), and North American tetraploids (4×NA). Numbers below each boxplot indicate sample sizes. Different letters above the boxplots indicate geo-cytotypes that differ significantly (*P* < 0.05) as determined by linear mixed models using restricted maximum likelihood estimation. The upper and lower boundary of the box indicates the 75th and 25th percentile, the central line indicates the median, and whiskers above and below indicate the 90th and 10th percentiles, respectively. Outliers are shown as dots except in (a) where, for clarity, they indicate 95th and 5th percentile values due to large samples sizes and numerous outliers.

of above-ground biomass. The maximum proportion of variance accounted for by the complete model was relatively small (33.3%) compared to that in young first-year rosettes (68.7%), although small sample size is likely to have contributed to this (Table 3).

Following the previous analyses, and due to the presence of flowering shoots, we used the additional variables height of the

highest flowering shoot and number of accessory rosettes in a LMM to account for variation in dry shoot weight of adult second-year plants (from Experiment 1) (Table 4). With these additional covariates, this model accounted for a similar proportion of variation in shoot weight (70.0%) as that explained for biomass of young *C. stoebe* rosettes, but there was no longer a detectable difference in shoot weight between geo-cytotypes (Table 4). Root dry weight was not found to differ between geo-cytotypes (Wald statistic = 0.93, d.f. = 2, 199, *P* = 0.628).

LIFE CYCLE AND REPRODUCTIVE CAPACITY

There were great differences in life cycle associated with ploidy level. Diploid *C. stoebe* that survived to reproductive age flowered either during the second growing season (2007, 52%, Fig. 4a), or in the third growing season (2008, 48% Fig. 4b). The majority (83%) of the diploid plants that flowered in 2007 died after flowering (Fig. 4c) with 99.5% of the surviving diploid plants flowering in the subsequent year (Fig. 4d), indicating a monocarpic life cycle in diploid *C. stoebe* plants. Consequently, in diploids there were very few individuals that were common to flowering data collected in both years and results for each season can thus be considered largely independent.

In contrast, most of the tetraploid plants flowered in the second growing season, with a significantly reduced proportional mortality in North American tetraploids than European tetraploids after flowering (11% vs. 26%, respectively, Fig. 4c). The proportion of surviving plants that flowered in the third year was again high (98% and 92%, Fig. 4d) with only 11% of the plants flowering for the first time in the third year (Fig. 4b).

In the second growing season (2007), we found no significant effect of ploidy level or geographic origin of tetraploid plants on the number of capitula or number of flowering stems produced per flowering plant (Fig. 5a,b, respectively). Also, geo-cytotypes did not differ in the proportion of capitula that were pollinated and that developed achenes (Fig. 5c). Diploids did, however, produce significantly more seeds per capitulum than tetraploid plants (Fig. 5d) resulting in a significantly higher number of seeds per plant with diploids producing, on average, 1.7 times as many seeds as European tetraploids (Fig. 5e). Although the total seed output per plant was not significantly different between European and North American tetraploids (Fig. 5e), plants from the introduced range had significantly greater seed output per capitulum than native tetraploids (Fig. 5d).

In the third growing season (2008), diploid plants produced significantly more capitula per flowering plant than both native European and North American tetraploids (Fig. 5a). This difference arose despite the production of fewer flowering stems (generally only one) per plant on diploids (Fig. 5b). Greater number of flowering stems in tetraploids was due to the production of flowering stems by multiple secondary rosettes that developed the previous year, whilst the main rosette senesced. Diploids rarely produced accessory rosettes,
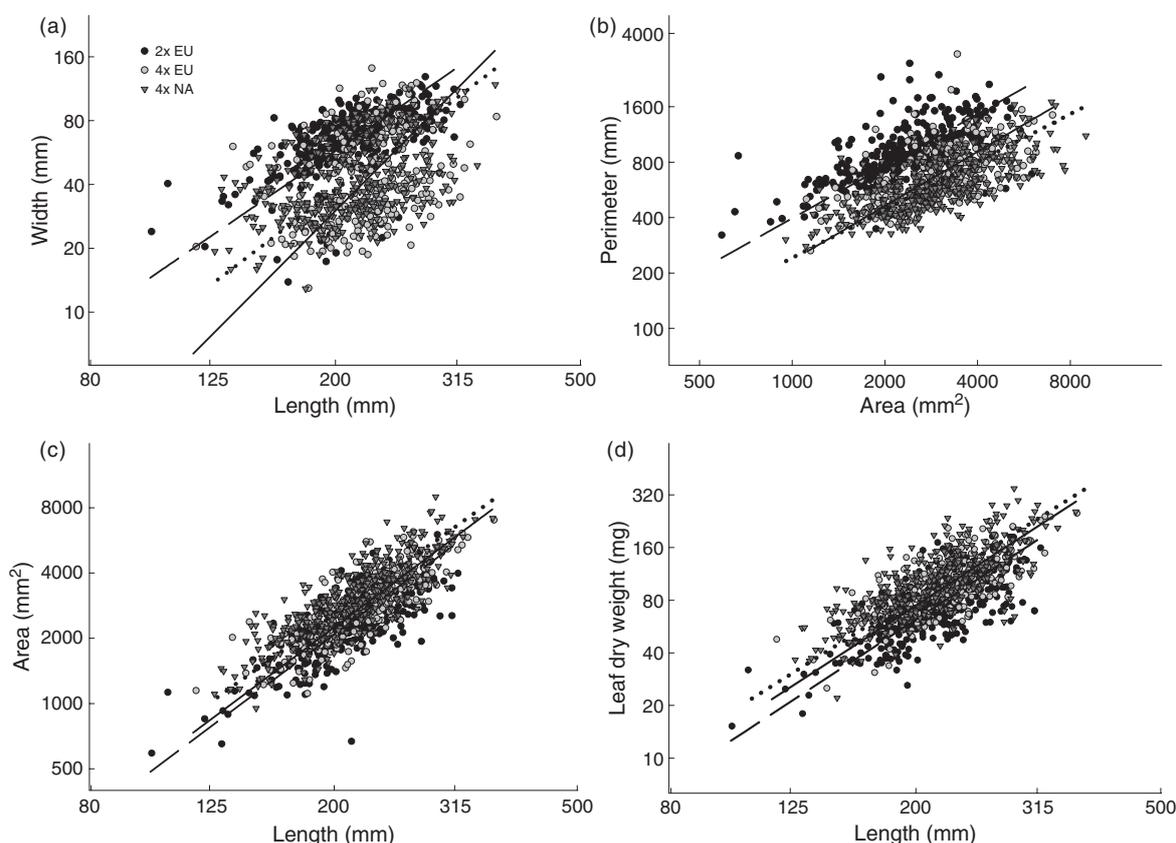
Fig. 3. Standardized major axis regression (SMA) relationships (log–log scaled) between: (a) leaf length and leaf width, (b) leaf area and leaf perimeter, (c) leaf length and leaf area and (d) leaf length and leaf dry weight measured on leaves of similar developmental age from 2-month-old *Centaurea stoebe* plants. Separate regressions are for European diploids (2×EU, circles and dashed line) European tetraploids (4×EU, grey circles and solid line) and North American tetraploids (4×NA, triangles and dotted line).

whilst invasive tetraploids tended to produce a greater number of accessory rosettes than native tetraploids (Fig. 5f).

Discussion

Previous investigations of *C. stoebe* (syn *C. maculosa*) from the native range have indicated significant life cycle differences associated with ploidy level (Broz *et al.* 2009; Treier *et al.* 2009). Directly comparing traits of native and invasive genotypes without accounting for cytotype differences could therefore lead to spurious conclusions about adaptive changes that arose after introduction to North America. In this study, we have compiled a substantial data set detailing early vegetative growth and mature reproductive stages in all geographical and cytotype combinations. The trait data for each geo-cytotype was averaged across a large number of populations in a common garden environment to minimize the effect of individual populations on the results. We found that, for several of the traits we examined in early growth, as well as in life cycle and reproduction, there were significant differences between diploids and tetraploids. In addition, within the tetraploid cytotype there were more subtle differences between plants from the native and invasive range indicating shifts in traits that may have been selected for the new environment since introduction.

LIFE CYCLE AND LEAF TRAIT DIFFERENCES OF
TETRAPLOIDS AND DIPLOIDS

We found that tetraploid *C. stoebe* tended to have higher LDMC, thicker leaves with lower SLA than diploids. This was accompanied by a reduction in dissection of the lamina although the extent of dissection was variable. We also found that diploids had significantly wider leaves than tetraploids, but the higher level of dissection of the lamina in this cytotype and consequent reduction in area in combination with a higher SLA, resulted in an overall lower dry weight per leaf for an equivalent leaf length. These differences in leaf construction did not, however, result in significantly different above-ground biomass between the two cytotypes.

The lack of environmental niche differentiation between European diploids and tetraploids (Treier *et al.* 2009) suggests that differences in leaf traits are not selected by the abiotic environment but are associated with a shorter, monocarpic life history in diploids, where maximizing leaf area and carbon assimilation into stored reserves for reproduction is most advantageous. Greater leaf dissection and lower carbon construction cost per leaf in diploids are possibly associated with a shorter monocarpic life span. With less dry mass invested per leaf, diploid plants can deploy more leaves per plant (Fig. 1a). As in other species (e.g. Feng, Fu & Zheng 2008), the higher

Table 2. Results of standardized major axis regression (SMA) analysis of pairwise combinations of leaf traits from three *Centaurea stoebe* geo-cytotypes, European diploids (2×EU), European tetraploids (4×EU) and North American tetraploids (4×NA). The separate SMA regression lines for each geo-cytotype were compared for differences in slope and if found to be not significantly different were examined for shifts in elevation. Slopes or elevations of regressions that were significantly different between geo-cytotypes are shown by different letters in the final column

Traits (X and Y)	Geo-cytotype	n	R^2	P	Slope	Intercept	Homogeneity of slope	Shift in elevation	Significant difference
Length and width	2×EU	332	0.59	< 0.001	1.99	−2.81	< 0.001	–	a
	4×EU	237	0.62	< 0.001	2.92	−5.23			b
	4×NA	388	0.69	< 0.001	2.21	−3.50			a
Area and perimeter	2×EU	332	0.41	0.097	0.940	−0.223	0.101	< 0.001	a
	4×EU	236	0.36	0.002	0.980	−0.569			b
	4×NA	388	0.50	0.053	0.865	−0.210			c
Length and dry weight	2×EU	332	0.51	< 0.001	2.31	−3.52	0.690	< 0.001	a
	4×EU	237	0.50	< 0.001	2.30	−3.40			b
	4×NA	388	0.56	< 0.001	2.21	−3.16			c
Length and area	2×EU	332	0.59	< 0.001	2.10	−1.52	0.587	< 0.001	a
	4×EU	237	0.62	< 0.001	2.10	−1.49			b
	4×NA	388	0.69	< 0.001	2.02	−1.23			c

Table 3. Results of linear models examining the usefulness of standardized variables to predict above-ground biomass in mature second-year *Centaurea stoebe* plants. The variables SLA and height did not significantly account for variation in above-ground dry weight and were excluded from the models

F	Total d.f.	P (model)	R^2	Independent variable	Estimated parameters	t	P
3.72	27	0.025	0.23	Constant	1.169	34.20	< 0.001
				Length of longest leaf	0.069	1.96	0.061
				Number of leaves	0.151	2.95	0.007
				Number of accessory rosettes	−0.123	−2.41	0.024
5.49	27	0.005	0.33	Constant	0.704	4.22	< 0.001
				Mean leaf length	0.004	2.84	0.009
				Number of leaves	0.164	3.41	0.002
				Number of accessory rosettes	−0.121	−2.56	0.017
5.41	27	0.005	0.33	Constant	1.169	36.59	< 0.001
				Median leaf length	0.094	2.81	0.010
				Number of leaves	0.166	3.42	0.002
				Number of accessory rosettes	−0.126	−2.65	0.014

SLA, specific leaf area.

Table 4. Results from generalized linear mixed model analysis examining the effect of geo-cytotype on shoot dry weight of *Centaurea stoebe* mature second-year plants whilst accounting for variation in plant size using standardized variables as covariates. Significance of geo-cytotypes European tetraploids (4×EU) and North American tetraploids (4×NA) are in comparison to European diploids as the reference level

F	Total d.f.	P (model)	R^2	Independent variable	Estimated parameter	t	P
62.82	159	< 0.001	0.70	Constant	1.438	46.52	< 0.001
				Length of longest leaf	0.030	1.73	0.086
				Number of leaves	0.142	6.34	< 0.001
				Height of flowering stem	0.250	14.78	< 0.001
				Number of accessory rosettes	−0.052	−2.44	0.016
				4×EU	−0.040	−0.97	0.334
				4×NA	0.017	0.41	0.684

SLA of diploid *C. stoebe* is likely aligned with higher rates of photosynthesis, gas exchange and net assimilation relative to tetraploids.

The functional role of leaf dissection, however, is less clear. Highly dissected leaves, as seen in diploid *C. stoebe* in this study, track ambient air temperatures more closely than more
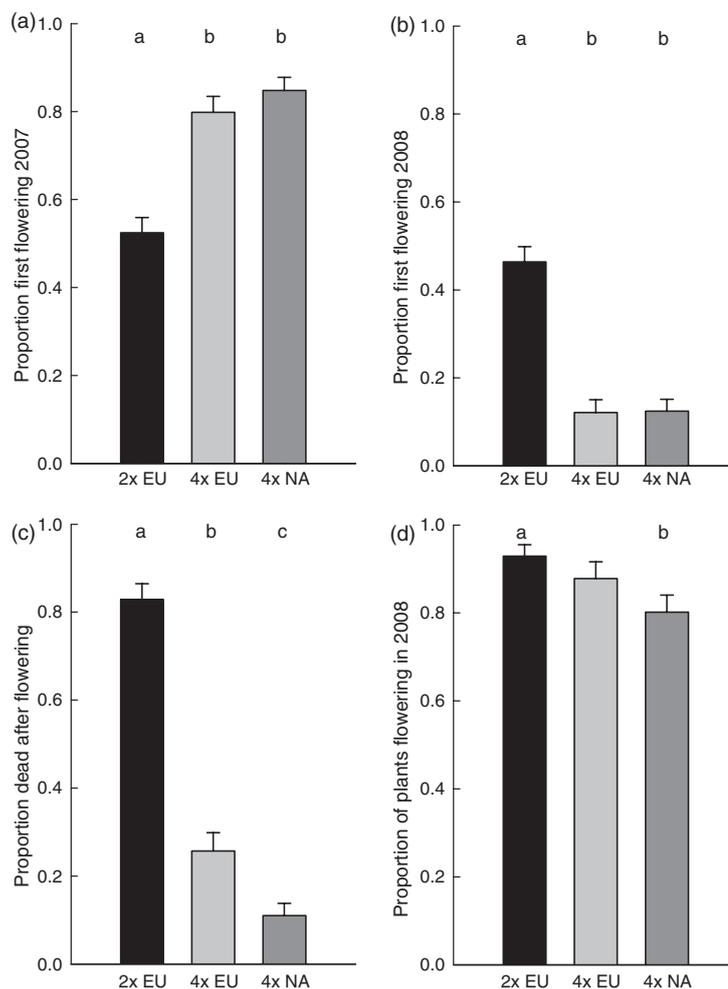
Fig. 4. Estimated mean proportions (with standard errors) of: (a) plants that first flowered in the second year, (b) plants that first flowered the third year (c) plants that died following flowering in the second year, and (d) plants that survived into the third year and then flowered, from three different *Centaurea stoebe* geo-cytotypes [European diploids (2×EU), European tetraploids (4×EU), and North American tetraploids (4×NA)]. Proportions are estimations from linear mixed models for a binomial distribution with a logit link and with geo-cytotype as a fixed factor. Different letters above the columns indicate geo-cytotypes that differ significantly ($P < 0.05$).

entire leaves (see Schuepp 1993 and references therein). This may provide more dissected leaves with potential for greater rates of photosynthesis in higher temperatures when water is available. Such effects of leaf dissection on photosynthesis have been demonstrated in studies using phylogenetically independent contrasts within the genus *Pelargonium* (Nicotra *et al.* 2008) and in other intraspecific studies comparing ecotypes (Gurevitch 1988, 1992), morphotypes (Lynn & Waldren 2002), and cultivars (Pettigrew, Heitholt & Vaughn 1993). In contrast, in North American tetraploids a shift to less dissected leaves than observed in the equivalent European cytotype might indicate both a pre-adaptation and an evolutionary shift to a leaf form that is advantageous in dryer and more continental climatic conditions east of the Rocky Mountains. Such a cline in decreased dissection of leaves with increasing altitude and concomitant decrease in ambient temperature within a species has been reported previously (Gurevitch 1992). Intriguingly, in the invasive teraploid form of *C. stoebe* a lower SLA rather than a higher SLA may have been advantageous for colonizing new environments following its introduction to North America. A higher SLA has been demonstrated in invasive vs. native conspecifics (e.g. Zou, Rogers & Siemann 2007) and in phylogenetically controlled comparisons of invasive and non-invasive species (e.g. Grotkopp, Rejmánek & Rost 2002; Grot-

kopp & Rejmánek 2007), and is thus commonly thought to be associated with invasiveness. In contrast, our results and others (Caño *et al.* 2008; Leishman, Thomson & Cooke 2010) suggest that rapid carbon capture via high SLA may not be always advantageous in invasive plants and highlight the importance of ecological context for each individual species in determining whether particular plant traits will confer an advantage (Pyšek, Křivánek & Jarošík 2009b; Pyšek *et al.* 2009a).

The generally parallel SMA regression lines fitted for three out of the four pairwise leaf trait combinations (Fig. 3b–d) indicate that allometric relationships did not vary over leaf size between geo-cytotypes. It seems unlikely that these relationships would differ for plants of different developmental stages but as we collected leaves of approximately the same age and only on young plants of even age we cannot verify this. A change in leaf trait relationships with plant age could partly explain why using mean population SLA or leaf dry weight per unit length values from young plants in a multiple regression did not account for a significant proportion of variation in shoot biomass of second-year plants. It seems more probable, however, that the increasing complexity in plant structure (presence of accessory rosettes and flowering shoots) and inherent variability in accumulated biomass with increasing age reduces the importance of subtle differences
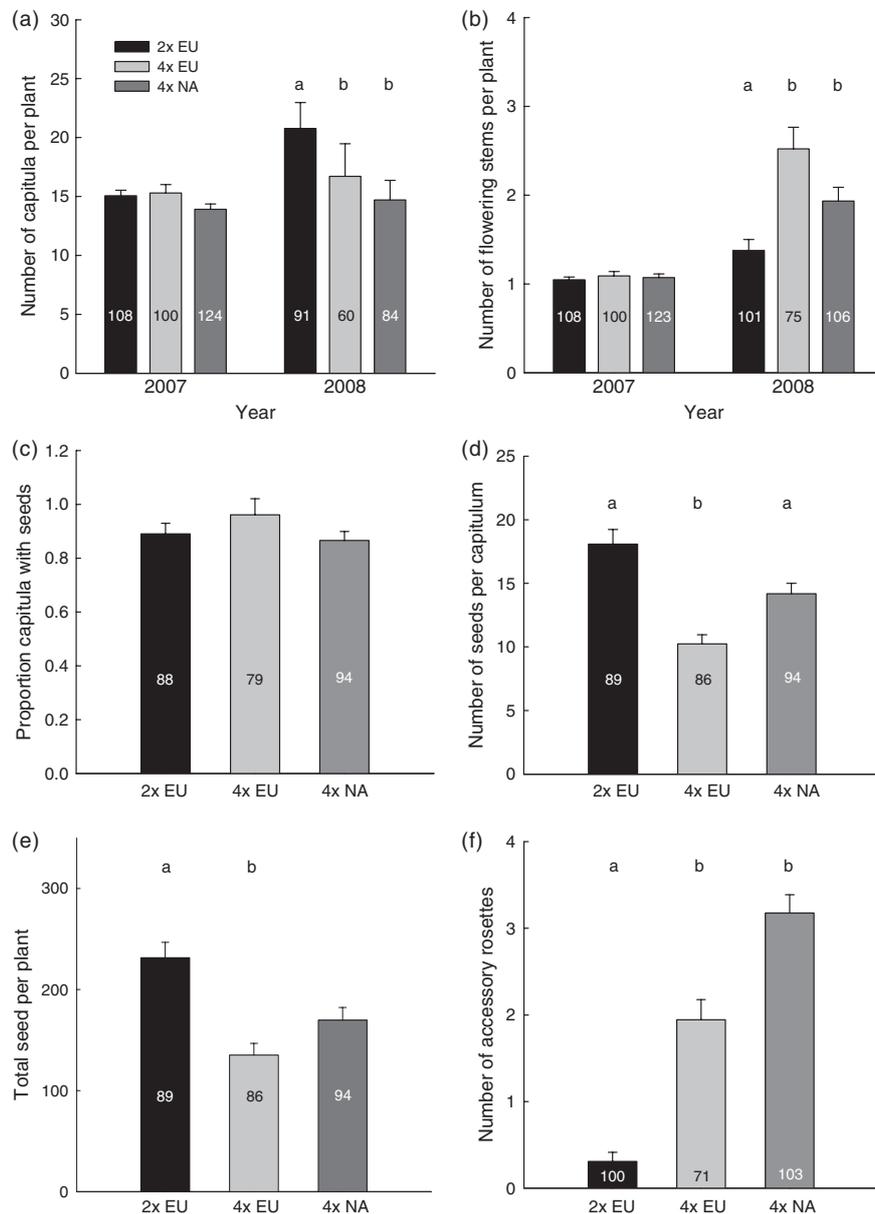
Fig. 5. Reproductive traits for three different *Centaurea stoebe* geo-cytotypes European diploids (2×EU), European tetraploids (4×EU), and North American tetraploids (4×NA) grown for three summers in a common garden experiment. Bar charts are means and standard errors for: (a) number of capitula per plant for two separate years, (b) number of flowering stems per plant for two separate years (c) proportion of capitula that were successfully pollinated and produced seeds in 2007, (d) number of seeds per capitulum in 2007 estimated from 3 to 5 dissected seed heads, (e) calculated total seed per plant in 2007, and (f) number of accessory rosettes per plant at flowering in 2008. Numbers on bars are sample sizes. Different letters above the columns indicate geo-cytotypes that differ significantly ($P < 0.05$).

in leaf construction in accounting for variation in plant weight.

In accordance with our expectations we found that, in the first year of flowering (second year of life), diploids produced significantly more seeds per flowering plant. This difference was primarily derived from a greater number of seeds produced per capitulum than tetraploids, whilst the proportion of capitula bearing seeds did not differ between the cytotypes. This finding concurs with previously published results demonstrating that diploid *C. stoebe* produce more flowers per capitulum than tetraploid conspecifics (Španiel *et al.* 2008).

Roughly 50% of the diploid plants flowered in the first season with the remainder flowering the next summer. The plants flowering in the second flowering season produced larger inflorescences with significantly more capitula per plant (average of 15 capitula in 2007 vs. 20 in 2008) suggesting that diploids that had not flowered in 2007 were able to invest more in reproduction due to greater stored reserves. In tetraploids, a polycarpic life cycle dominated and investment in reproduction in the first season may potentially have reduced reproduction in the second year. Differences in propagule production between ploidy levels must be considered in light of this result. This consider-

ation aside, the average seed production in diploids would consequently be considerably greater than that estimated from the previous seasons' data alone. This would reduce differences between cytotypes in their projected population growth rates that were derived in a previous model based on number of capitula only (Broz *et al.* 2009). Whether this manifests in lifetime fecundity differences is currently a subject of further study.

EVIDENCE FOR POST-INTRODUCTION EVOLUTION IN TETRAPLOIDS

During early growth (rosettes < 3 months old), we found that North American tetraploids tend to accrue above-ground biomass faster than European tetraploids (and diploids). This extra biomass is not derived from having a greater number or longer leaves but results primarily from a combination of small differences in SLA, LDMC and area per unit leaf length that amount to greater total dry weight invested per leaf.

In North American tetraploids, greater accumulation of biomass in rapidly growing young plants may represent evidence of a trade-off between growth and defence because it compliments previous findings on lower defence-related gene expression in North American tetraploids (Broz *et al.* 2009). SLA is generally positively correlated with both leaf nitrogen and photosynthetic rate (both across (Niinemets 1999; Reich *et al.* 1999; Wright *et al.* 2004) and within (Li *et al.* 2008; Tanaka *et al.* 2008) plant species) yet North American tetraploid genotypes are able to accumulate biomass faster than diploids and European tetraploids, despite lower SLA than diploids and no significant increase in leaf N. These results suggest that genotypes in the invasive range have reallocated resources to growth via reduced expression of biochemical pathways involved in plant defence and the consequent reduction in associated energy costs, thus generally supporting the EICA hypothesis. As has been shown for other invasive species, however, evidence of reduction in defence investment does not necessarily carry over to significantly greater biomass in mature plants (Maron, Vilá & Arnason 2004b). Although it may not translate to greater final biomass, more rapid early growth may still confer a competitive advantage during the critical life stage following germination.

Our ability to detect small shifts in plant biomass and reproductive capacity between European and North American tetraploids may have been confounded by parallel changes to their respective life cycles. In a common garden, European tetraploids are less distinctly mono- or polycarpic with different populations exhibiting variation in life history and leaf traits, whilst North American tetraploids are exclusively polycarpic (Fig. 4). This matches previous field observations (Treier *et al.* 2009) and may indicate a selection towards greater persistence/longevity with concomitant increases in reproductive capacity in North America. Thus, although North American tetraploids may gain an initial growth advantage during establishment, more rapid growth and assimilation in some shorter-lived European tetraploid genotypes (and diploids) probably

results in equivalent average above-ground biomass in mature second-year plants. The design of our experiment was not ideal to compare allocation to root systems between geo-cytotypes but it is possible that long term persistence in polycarpic tetraploid plants may well have selected for relatively greater allocation to an extensive root system rather than to leaves, particularly in North American genotypes where individual plants may survive for many years in drier habitats than those occupied by the species in Europe (Broennimann *et al.* 2007).

In summary, there is evidence of higher growth rate during the early vegetative stage in invasive genotypes vs. the equivalent cytotype from the native range. Whilst more rapid early growth is potentially important during establishment of seedlings, the change appears to be subtle and is not evident in older plants where increased variability with time and differences in life history between the two tetraploid groups may affect our ability to detect such a difference.

We also observed a small but significant increase in reproductive capacity in North American tetraploids. There was a trend towards higher total seed production in tetraploids from the invasive range compared with tetraploids from the native range that derived from a significant increase in the number of seeds per capitulum. This difference did not result in a significant difference in total seed production between these two groups because tetraploids in the native range tended to have more capitula per plant. As suggested above, changes in allocation to defence or traits that relate to persistence (e.g. deep woody roots, resprouting potential) may also be manifest in greater/lesser reproductive effort. Thus, differences in reproductive output may be confounded by differences in life cycle as shorter-lived genotypes (amongst European tetraploids) are likely to invest more in reproduction earlier in their life span. Therefore, it is possible that the trend towards greater seed production per plant by North American tetraploids would be greater over a longer time frame if, particularly in the absence of natural enemies, North American genotypes are better competitors (Ridenour *et al.* 2008), survive longer and continue to produce more seeds per capitulum. An increased ability to form accessory rosettes in North American tetraploids supports a hypothesized shift towards a greater ability to persist at a locality. It is clear from studies of other invasive species that shifts to greater vegetative propagation, growth and sexual reproduction are equally possible outcomes of evolutionary change in invasive genotypes (e.g. van Kleunen & Fischer 2008; Abhilasha & Joshi 2009; Hull-Sanders *et al.* 2009). Thus it is likely that measurement of just one group of traits related to one aspect of the life cycle may not detect the additive effects of potentially subtle changes to a plant's structure and function that could result in significant changes to the ecology of an invasive species.

In conclusion, evidence from this and other studies on *C. stoebe* (Broz *et al.* 2009; Treier *et al.* 2009) support the hypothesis that prominent life cycle differences and changes in leaf traits associated with an increase in ploidy may have given tetraploids both an advantage in colonizing potential via increased lifetime fecundity (due primarily to greater inherent longevity once established), and a leaf structure that may be

better adapted to the dryer and more continental habitats the species has colonized in North America. In addition, our findings suggest that tetraploids might have undergone further evolutionary changes which increased the rate of spread during colonization of North America. Given the relatively minor changes to individual traits between invasive and native forms, it will be a considerable challenge to determine the relative importance of pre-adaptation vs. rapid evolutionary change during the invasion process in this species and in others.

Acknowledgements

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