

Enemies lost: parallel evolution in structural defense and tolerance to herbivory of invasive *Jacobaea vulgaris*

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Abstract According to the Shifting Defense Hypothesis, invasive plants should trade-off their costly quantitative defense to cheaper qualitative defense and growth due to the lack of natural specialist enemies and the presence of generalist enemies in the introduced areas. Several studies showed that plant genotypes from the invasive areas had a better qualitative defense than genotypes from the native area but only a few studies have focused on the quantitative defenses and tolerance ability. We compared structural defenses, tolerance and growth between invasive and native

plant populations from different continents using the model plant *Jacobaea vulgaris*. We examined several microscopical structure traits, toughness, amount of cell wall proteins, growth and root-shoot ratio, which is a proxy for tolerance. The results show that invasive *Jacobaea vulgaris* have thinner leaves, lower leaf mass area, lower leaf cell wall protein contents and a lower root-shoot ratio than native genotypes. It indicates that invasive genotypes have poorer structural defense and tolerance to herbivory but potentially higher growth compared to native genotypes. These findings are in line with the Evolution of Increased Competitive Ability hypothesis and Shifting Defense Hypothesis. We also show that the invasiveness of this species in three geographically separated regions is consistently associated with the loss of parts of its quantitative defense and tolerance ability. The simultaneous change in quantitative defense and tolerance of the same magnitude and direction in the three invasive regions can be explained by parallel evolution. We argue that such parallel evolution might be attributed to the absence of natural enemies rather than adaptation to local abiotic factors, since climate conditions among these three regions were different. Understanding such evolutionary changes helps to understand why plant species become invasive and might be important for biological control.

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Introduction

Invasive species are defined as non-native species that successfully establish and spread when introduced beyond their native range (Williamson 1996; Pyšek et al. 2004). The spread of invasive species often poses serious threats to the native biodiversity, the ecosystem services of the invaded area, the local agricultural productivity, and human health. Invasive species therefore cause significant environmental damage and economic losses. (Hobbs and Mooney 1998; Kark and Antonio 2002; Pimentel et al. 2005; Pejchar and Mooney 2009; Pyšek and Richardson 2010). Only 2 % of introduced plant species eventually become invasive weeds although they are not necessarily pest species in their native areas (Williamson and Fitter 1996; Williamson 1996; Reichard and White 2001; Bell et al. 2003). Knowledge of what makes species become invasive has a great importance for predicting potentially invasive species before and after introduction and controlling existing invasive species.

The first theories on why plants become invasive emphasize the absence of natural enemies after introduction of plants in a new area (Williamson 1996; Keane and Crawley 2002; Mitchell and Power 2003). When plants are introduced into a new habitat, they leave their co-evolved natural enemies behind. This release from detrimental herbivore pressure potentially resulting in a fast increase in distribution and abundance (the enemy release hypothesis (ERH), (Keane and Crawley 2002). Further hypotheses were proposed on the basis of evolutionary changes during invasion. The Evolution of Increased Competitive Ability hypothesis (EICA) predicts that the escape from specialist herbivores leads to an evolutionary change in invasive plants in energy allocation from defense to growth which can give invasive plants a higher competitive ability to outcompete local plant species (Blossey and Nötzold 1995).

Defense is often divided into two types related to its effect on herbivores: quantitative defenses and qualitative defenses (Feeny 1976; Rhoades and Cates 1976). Quantitative defenses (e.g. tannins and trichomes) act against specialist as well as generalist herbivores and are costly to produce (Poorter and De Jong 1999). Qualitative defenses (toxins such as alkaloids and glucosinolates) act against generalist herbivores and are cheaper to produce, but specialist herbivores are often adapted to these defenses

(Berryman 1988; Strauss et al. 2002). Specialist herbivores often use these compounds as a cue to locate their host plant, as an oviposition and feeding stimulant, and may sequester the qualitative plant defense compounds for their own defense (van der Meijden 1996; Hay et al. 1990; Müller et al. 2001; Bernays et al. 2003; Macel and Vrieling 2003; Nieminen et al. 2003). In the invasive area where specialist herbivores are absent, plant can shift their allocation to produce more of the cheap qualitative defenses against generalist herbivores without having the side effect of attracting the specialist herbivores. By doing so they can decrease their costly quantitative defenses against the absent specialist herbivores. As a net result the plants in the invasive area are well defended and resources can be saved for growth and reproduction which can give these plants a competitive edge over the local plant species (Doorduyn and Vrieling 2011). This evolutionary shift of quantitative defense to qualitative defense in the invasive area is called the Shifting Defense Hypothesis (SDH) (Müller-Schärer et al. 2004; Joshi and Vrieling 2005; Doorduyn and Vrieling 2011).

To study the role of the defense mechanisms involved in the evolutionary shift of invasive plant species, most studies have focused on the qualitative defenses while only a few of them have examined quantitative defenses (Rogers and Siemann 2005; Zou et al. 2008; Feng et al. 2009; Alba et al. 2011; Gard et al. 2012; Zhang et al. 2012). Quantitative defenses can be based on morphology or on chemical compounds that occur in higher concentrations (Feeny 1976; Rhoades and Cates 1976). As an important part of quantitative defenses, morphological defenses are based on anatomical or structural traits, such as leaf structures (see Fig. 3), leaf toughness, hairiness and thorns. Such traits confer a fitness advantage to the plant by directly deterring both generalist and specialist herbivores from feeding on it but tend to be costly in terms of resources (Hanley et al. 2007; Kurokawa and Nakashizuka 2008). Several invasive species were recorded to have evolved lower amounts of quantitative defenses after invasion (Willis et al. 1999; Feng et al. 2009; Siemann and Rogers 2001; Huang et al. 2010).

Plant cell walls contain several kinds of proteins, such as hydroxyl-proline-rich glycoproteins, proline-rich proteins and glycine-rich proteins, which play a role in defense, growth, development, signaling,

intercellular communication and environmental sensing (Showalter 1993). Besides protein, plant cell walls also consist of carbohydrates, lignin, water, and encrusting substances which could serve as part of the structural defenses as well (Bradley et al. 1992; Showalter 1993). Feng et al. (2009) found that invasive *Ageratina adenophora* had 45 % lower cell wall protein content than native populations which resulted in a poorer structural defense. Another important structural defense is leaf toughness. It can reduce the suitability of leaves as a food source for herbivores through indigestible polymers such as cellulose and lignin in plant tissues (Raupp 1985). Furthermore, leaf hardness is a defense property which acts as a physical barrier to normal feeding or oviposition by phytophagous insect herbivores (Wright et al. 1989; Kogan 1994). In studies on leaf morphological traits leaf hardness was found to be strongly positively correlated with leaf mass per area (the ratio between the dry mass and the area of plant leaves, LMA). Therefore LMA is used as an easy-to-assess index of structural biomass (Groom and Lamont 1999; Wright and Cannon 2001; Hanley et al. 2007). Moreover, the reciprocal of LMA, the specific leaf area (SLA), is often associated with plant growth rates (Castro-Díez et al. 2000). It is argued that a lower LMA/higher SLA is one of the most important traits associated with high relative growth rate, small seed mass, and invasiveness (Grotkopp and Rejmánek 2007; Hanley et al. 2007; Feng et al. 2008). Several invasive plant species were recorded to have lower LMA/higher SLA than their native congeners (Baruch and Goldstein 1999; Durand and Goldstein 2001; Nagel and Griffin 2001; Burns 2006; Feng et al. 2008). According to above, cell wall proteins, the mechanical properties of cell walls as well as leaf toughness and LMA can be considered as important traits for evaluating plant structural defenses.

Instead of deterring herbivores, plants can also reduce the negative fitness effects of herbivores through being tolerant to damage (van der Meijden et al. 1988). Tolerance is defined as the ability of a plant to vegetatively or reproductively overcome the damage caused by herbivores (Agrawal et al. 1999; Strauss and Agrawal 1999). A variety of plants suffer from high levels of herbivory or even frequent defoliation during their life time. In order to survive from such attacks, some plant species are likely to develop compensating mechanism: regrowth (van der

Meijden et al. 1988). As one of the most common tolerance strategies of plants, regrowth ability implies the saving and storage of nutrients and energy in plant parts that are relatively free from herbivore attack (McNaughton 1983; Rosenthal and Kotanen 1994; De Jong and Van Der Meijden 2000; Anten and Pierik 2010). Depending on the sink–source relationships, the reallocation of resources in different plant organs could result in a compensatory regrowth to replace damaged tissue after herbivory (Utsumi and Ohgushi 2007). Since root-shoot ratio was found to be directly correlated with regrowth in *Jacobaea vulgaris* (van der Meijden et al. 1988), we considered the root-shoot ratio as a proper proxy for tolerance to herbivory in this study. Although structural defenses and tolerance play such significant roles against herbivore attack, they have been rarely studied together in the light of the EICA and SDH hypothesis.

It is worth to point out that so far evidence that the escape from specialist herbivores is the selective force leading to changes in allocation patterns in invasive plants is largely circumstantial and other biotic or abiotic factors can also play important roles. We therefore set out a study system where multiple invasive regions are compared that differ in climatological conditions. If the change in the herbivore guild is the main selective force, parallel evolutionary changes in traits related to tolerance, structural defenses and growth are expected in each of the geographically and climatologically differing invasive ranges.

In this study we ask if the invasive plant genotypes have evolved decreased amounts of structural defenses and tolerance compared to the plant genotypes from the native area. We examined the microscopical structures of leaf traits, the amount of cell wall proteins, leaf toughness, leaf thickness (LMA) and tolerance (root-shoot ratio) in native and invasive plant genotypes of common ragwort, *Jacobaea vulgaris*, our model plant. We compared native and invasive *J. vulgaris* genotypes and expect invasive genotypes to have (1) lower structural defense against herbivory (2) have a decreased root-shoot ratio which leads to a lower tolerance and regrowth ability. Moreover, we evaluated these traits in introduced populations from geographically and climatically distinct invaded regions (New Zealand, Australia, and North America). As such, we were able to evaluate whether structural defense and tolerance evolved in

parallel across these different locales following a shared history of release from specialist natural enemies. To our knowledge, this study is one of the first that focuses on the comparison of both the tolerance and structural defense between the same plant species from invasive and native areas. Hence the result would contribute to the critical evaluation of the role of tolerance and structural defense involved in the evolutionary shift mechanism behind invasion success.

Materials and methods

Study species

Jacobaea vulgaris formerly known as *Senecio jacobaeae*, is a monocarpic perennial plant that belongs to the family of the *Asteraceae*. It is native to Eurasia and was introduced into parts of New Zealand (first recorded at 1874) (Poole and Cairns 1940), Australia (first recorded at 1890) (McLaren et al. 2000), and North America (first recorded at 1913) (Harris et al. 1971). In the native range it is attacked by more than 70 herbivores and most herbivory is by the two main specialist herbivores: *Tyria jacobaeae* (Cinnabar moth) and *Longitarsus jacobaeae* (Fleabeetle) (Joshi and Vrieling 2005). In the introduced ranges, *J. vulgaris* was recorded to be fed by more than 40 species of generalist arthropods in North America but no specialists herbivore was observed (Frick 1972). *Jacobaea vulgaris* contains pyrrolizidine alkaloids (PAs) which are toxic to horses and cattle (Johnson et al. 1985; Stegelmeier et al. 1999; Gardner et al. 2006). Therefore it received a pest status because infestations have resulted in significant livestock losses due to alkaloid poisoning and decreased pasture yields (Coombs et al. 1996). Because of its weediness and toxicity, it has been intensively studied to discover how selection has changed these traits in the invasive range (Willis et al. 2000; Joshi and Vrieling 2005; Stastny et al. 2005). PA's act as oviposition and feeding stimulants for the specialist herbivore *T. jacobaeae* (Macel et al. 2004; Potter et al. 2004) and both *T. jacobaeae* and *L. jacobaeae* sequester PAs for their own defense against predators (Aplin and Rothschild 1972; Zoelen and Meijden 1991; Dobler et al. 2000). In a common garden experiment Joshi and Vrieling (2005) found that invasive *J. vulgaris* had on

average 90 % more PAs (a qualitative defense) and 37 % higher reproductive biomass than genotypes from the native areas. Furthermore invasive *J. vulgaris* were more vulnerable to the specialist herbivores *T. jacobaeae*, *L. jacobaeae* and *Platyptilia isodactylus* but better protected against the generalist herbivores *Mamestra brassicae* and *Spodoptera exigua* (Joshi and Vrieling 2005). These findings are in line with the SDH hypothesis and indicated an evolutionary shift from lower protection against specialist towards increased growth and reproduction as well as higher protection against general herbivores in *J. vulgaris*. Furthermore, *J. vulgaris* in the native area is regularly defoliated by *T. jacobaeae* and shows a strong regrowth after defoliation (Islam and Crawley 1983; van der Meijden et al. 1988).

Recently, *T. jacobaeae*, *L. jacobaeae* and other specialist herbivores have been introduced into to the invasive areas as biological controls for combating the invasion of *J. vulgaris* for several years (McEvoy et al. 1991; McEvoy and Coombs 1999). However, Rapo et al. (2010) only found small differences between traits of invasive *Jacobaea vulgaris* populations (New Zealand and North America) with and without biological control history of *L. jacobaeae* but larger differences between native and invasive populations. It suggests that the recent introduction of the biological control agents did not yet cause a rapid evolutionary adaptation of *J. vulgaris* populations in the introduced range towards the native phenotype.

Further, Doorduyn et al. (2010) found that the amount of neutral genetic variation of *J. vulgaris* in the invasive habitats was equal to the native habitat. This suggests that multiple introductions from different source populations have occurred as the native populations are significantly different from each other with respect to neutral genetic variation. This indicates that the filtered introduction of pre-adapted genotypes is not a likely explanation and that indeed evolutionary changes occurred in the invasive *J. vulgaris* plants at the introduced areas than pre-adaptation. In addition, Joshi and Vrieling (2005) found that the studied traits in the three different invasive areas all showed the same significant trends, a phenomenon not easily explained by pre-adaptation.

Plant material and growth conditions

Seeds were collected from 10 to 15 plants and bagged individually from 19 native populations in Europe and

from 20 invasive populations in New Zealand, Australia and the USA (Table 1; Fig. 1). Seeds were germinated in petri dishes with moistened filter paper and from each population five seedlings from five different maternal lines were potted in 0.5 L pots with 5 % potting soil (Slingerland potground, Zoeterwoude, The Netherlands), 95 % sandy soil (collected from Meijndel, The Netherlands, 52°13'N, 4°34'E) and 0.75 g Osmocote slow release fertilizer (Scott[®], Scotts Miracle-Gro, Marysville, Ohio, USA; N:P:K:MgO 15:9:11:2.5). Plants were grown in a climate room for 17 weeks at 20 °C, 70 % humidity, 16 h daylight with a light intensity of 113 $\mu\text{mol PAR m}^{-2}\text{s}^{-1}$. They were watered when needed. After 10 weeks 50 mL Pokon solution NPK 7-5-6 (8 mL L⁻¹) and Fe-EDTA of 3.2 g L⁻¹ was given to the plants twice a week. At the end, two genotypes per population were randomly picked to be used for the microscopic analysis (after 14 weeks), for toughness measurements (after 16 weeks) and for cell wall measurements one of these two genotypes were randomly chosen (after 12 weeks). After 17 weeks all of the five genotypes per population were harvested for dry weight (Table 1). For practical reasons the number of replicates per population for microscopic analysis, toughness measurements and cell wall measurements are low (1 or 2). However as we are not interested in differences between populations within a range but to differences between ranges we chose to have a broad sampling of populations to estimate differences between ranges.

Structural defenses

Leaf microscopic measurements

For microscopic measurements, sections were cut from the tip of the middlemost leaf of each plant after 14 weeks of growth using a hand microtome (Fig. 2). Sections were then stained using propidium iodide for 15 min. Propidium iodide stains DNA as well as cell wall material. Images were acquired by using a Zeiss LSM exciter on an Axio observer microscope (Exc. with a HeNe 534 laser and em. LP 560). A full cross section of the leaf was obtained by tile scanning the specimen with a 40 × 1.2 NA Plan APO water immersion objective. This gave an image size of 321.43 $\mu\text{m} \times 482.14 \mu\text{m}$ with a resolution of 80 nm per pixel. As leaf structure parameters, we measured

the upper and lower cuticle thickness and epidermis cell wall thickness, the palisade parenchyma layer thickness, the sponge parenchyma layer thickness and leaf thickness, which are all considered to contribute to leaf toughness and structural defense. Measurements were made using ImageJ[®] 1.42q and each measurement of cell wall thickness and leaf layers was made five times on different parts of the cross-section as outlined in the Fig. 3. In total 14 cell traits were measured as indicated in the Fig. 3. For statistical analysis the average of the five measurements was taken.

Leaf toughness measurements

Leaf toughness was measured at the middlemost leaf of each plant after 16 weeks of growth using a punch and die method on an Instron 4000 according to Onoda et al. (2008) (Fig. 2). A flat ended sharp-edged cylindrical steel punch (diameter = 1.345 mm) and a steel die with a sharp-edged hole (0.2 mm) were used. The punch and die were installed into a general testing machine (5542, Instron, Canton, MA, USA), and the punch was placed to go through the middle of the hole of the die without any friction. When the punch started to compress the leaf, a sharp increase in force is observed. Maximum force (N) was recorded just before the leaf fractured. The speed of the punch was constant (24.7 mm min⁻¹) and the machine recorded the load was applied to the sample simultaneously. Work (μJoule) was also recorded during the whole process and the total work to penetrate the leaf was calculated as the area under the force displacement curve. Punch strength and punch toughness were calculated from a force–displacement curve (Aranwela et al. 1999). Punch strength ($\text{MN} \cdot \text{m}^{-2}$ or MPa) = Maximum force/A, and punch toughness ($\text{kJ} \cdot \text{m}^{-2}$) = work/A, where A is the area of the punch (1.42 mm²). Leaf mass per area (LMA) was calculated as the ratio between the dry mass and leaf area from the same leaf used for toughness measurements. Each plant was measured two times on the same leaf and for statistical analysis the average of the two measurements was taken.

Cell wall protein measurements

From each population one plant was randomly selected for cell wall proteins extraction using the protein

Table 1 Origin of *Jacobaea vulgaris* populations used in this study and number of plants used for the different measurements

Origin	Country	Location	Longitude	Latitude	Number of plants used in measurements				
					Growth 17 weeks	Microsc. 14 weeks	Tough. 16 weeks	CW 12 weeks	
Invasive	Australia	Barramunga	143°41' E	38°33' S	4	2	2	1	
		Beech forest	143°33' E	38°38' S	4	2	3	1	
		Dairy Plains	146°31' E	41°34' S	4	1	1	1	
		Franklin	147°00' E	43°05' S	5	2	2	1	
		Mayberry	146°18' E	41°33' S	4	2	2	1	
		Turton's Creek	146°15' E	38°33' S	5	2	1	1	
	New Zealand	Craigieburn	174°13' E	39°25' S	4	2	2	1	
		Landsborough	169°02' E	43°53' S	5	2	2	1	
		Maruia	172°13' E	42°11' S	2	2	2	1	
		Opunake	173°51' E	39°27' S	5	2	2	1	
		Southland	167°55' E	45°28' S	1	1	1	1	
		Whatipu	174°31' E	37°01' S	4	2	2	1	
	USA	Corvallis	123°19' W	44°58' N	5	2	2	1	
		Spur Road, Conrad	111°35' W	47°48' N	5	2	2	1	
		Six Rivers National Forest	123°57' W	41°42' N	4	2	2	1	
		Kootenai National Park	114°53' W	48°17' N	4	2	2	1	
		No Bear road	120°00' W	43°00' N	5	2	2	1	
		South Cooper mountain	122°50' W	45°40' N	4	2	2	1	
		Salem	123°02' W	44°56' N	4	2	2	1	
		Surprise Hill	115°00' W	48°15' N	4	1	1	1	
Native		Belgium	Brussels	04°25' E	50°51' N	5	2	2	1
			Spa	05°50' E	50°29' N	4	2	2	1
	Denmark	Sundstrup	09°18' E	56°36' N	5	2	2	1	
	England	Deal	01°24' W	51°13' N	4	2	2	1	
	Finland	Kirkkonummi	24°31' E	60°09' N	5	1	1	1	
	France	Mt. St. Michel	01°32' W	48°37' N	4	2	2	1	
	Germany	Holzlarhen	11°43' E	47°53' N	4	2	2	1	
		Lubeck	10°42' E	54°05' N	3	2	2	1	
	Hungary	Csokvaomany	20°22' E	48°10' N	4	2	2	1	
	Ireland	Near Caherdaniel	08°02' W	53°07' N	4	2	2	1	
	Netherlands	Veluwe	06°00' E	52°19' N	5	2	2	1	
		Wageningen	05°34' E	52°10' N	4	2	2	1	
	Norway	Malvik	10°37' E	63°25' N	5	2	2	1	
	Poland	Near Warsaw	19°25' E	51°52' N	5	2	2	1	
	Scotland	Dundee	03°02' W	56°29' N	2	2	2	1	
	Spain	Puerto de San Glorio	03°37' W	40°01' N	5	2	2	1	
	Sweden	Lund	13°13' E	55°43' N	5	2	2	1	
	Switzerland	l'Himelette	07°00' E	47°07' N	5	2	2	1	

Table 1 continued

Origin	Country	Location	Longitude	Latitude	Number of plants used in measurements			
					Growth 17 weeks	Microsc. 14 weeks	Tough. 16 weeks	CW 12 weeks
		Rothenthurm	08°04' E	47°06' N	1	1	1	1

Growth, measurement of shoot and root dry mass after 17 weeks of growth; Microsc, measurement of leaf structural parameters after 14 weeks of growth; Tough, measurement of leaf toughness and LMA after 16 weeks of growth; CW, measurement of cell wall proteins after 12 weeks of growth

extraction protocol of Takashima et al. (2004). Two 1 cm diameter punches were extracted from the middlemost leaf after 12 weeks of growth, avoiding the main leaf veins (Fig. 2). Water soluble material and SDS soluble material were removed. The remaining cell wall material was oven dried at 60 °C for 18 h and weighed as cell wall proteins. Each plant was measured three times on the same leaf and for statistical analysis the average of three measurements was taken. Therefore each population is only represented by one sample but they represent a random estimate of what is present in each distribution range.

Growth measurements and tolerance

After 17 weeks all plants were harvested, dry weight of shoots and roots were measured after oven drying at 60 °C for a minimum of 48 h. From leaves that were used in the previous measurements, fresh and dry mass were measured and added to the shoot mass. Root-shoot ratio of each plant was calculated and was considered to be trait associated with tolerance.

Statistical analysis

As the main interest of this study was to find differences in invasive versus native areas, statistical analysis was performed by a nested ANOVA, with origin as a fixed factor and population nested within origin as a random factor. Normality of the residuals was checked with a Kolmogorov–Smirnov test. For the cell wall protein measurements a one-way ANOVA was performed for analyzing the data since from each population only one plant was measured. Leaf thickness, leaf mass ratio, cell wall protein, shoot mass and root-shoot ratio, were compared between invasive populations from the three geographic

regions (North America, New Zealand and Australia) and the native populations. The differences among this four regions were analyzed by a post hoc LSD test. All analyses were carried out using SPSS 18.0 (SPSS: An IBM Company).

To exam the difference in local climate among the four geographic regions (Europe, USA, Australia and New Zealand), 19 bioclimatic variables of the current conditions (ca 1950–2000) at the collected site of each sampled population were downloaded from the WorldClim dataset (<http://www.worldclim.org/current>) in 5 arc-min resolution. A partial least square-discrimination analysis (PLS-DA) was performed with the SIMCA-P software (v.11.0, Umetrics, Umea, Sweden) for classifying all sampled populations based on the 19 bioclimatic variables. The scaling method for PLS-DA was unit-variance and the model was validated by using the permutation method through 20 iterations.

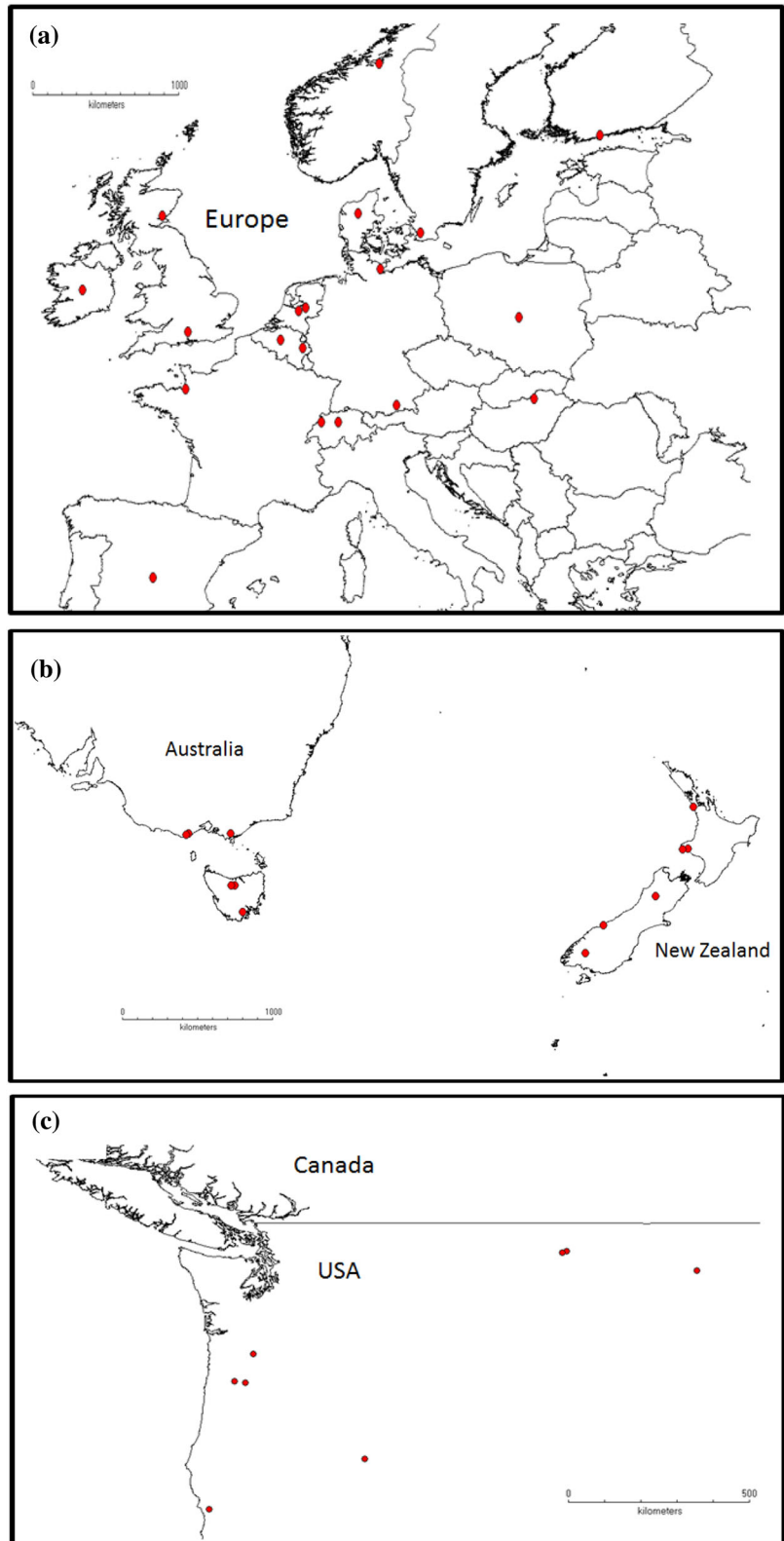
Results

Structural defense

Leaf microscopy

Out of the seven measurements on *J. vulgaris* leaf cross sections, only two showed a difference between native and invasive populations: the lower epidermis cell wall thickness and leaf thickness (Table 2). Lower epidermis cell walls from the invasive origin were 12.1 % thicker ($p = 0.024$) than that of the native populations. The invasive populations had significant thinner leaves than native populations ($p = 0.023$). Furthermore, there was a trend towards a thinner (7.1 %) palisade parenchyma layer in the invasive populations ($p = 0.065$).

Fig. 1 Distribution map of native and invasive *Jacobaea vulgaris* populations from Europe (a), Australia and New Zealand (b) and USA (c) used in this study. For the native range, there are 19 populations from Europe. For the invasive range, there are six populations from Australia, six populations from New Zealand and eight populations from USA



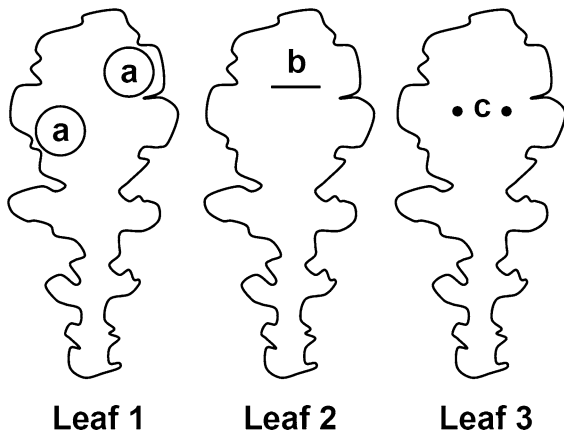


Fig. 2 Locations of the different leaf measurements. *Leaf 1* location of the punches for cell wall material analysis. *Leaf 2* Location of the coupes for microscopic measurements. *Leaf 3* Location of the spots for toughness measurements

Leaf toughness, LMA and cell wall proteins

Leaf punch strength and punch toughness did not differ significantly between native and invasive *J. vulgaris* populations (Table 3). LMA was 8.3 % lower in the invasive *J. vulgaris* populations compared to the native ones ($p = 0.038$). No significant differences were found among populations. Invasive populations contained on average 10.8 % lower amounts of cell wall protein per unit leaf area than the native populations on the basis of leaf area ($p = 0.037$), but

there was no significant difference on the basis of leaf mass (Table 4).

Growth traits and tolerance

After 17 weeks of growth, plants from invasive populations had 13.7 % more shoot mass ($p = 0.029$) than that of native populations (Table 5). No significant difference ($p = 0.089$) in the root mass was found but there is a tendency that invasive genotypes had smaller roots. Furthermore, the root-shoot ratio of the invasive *J. vulgaris* populations were 18.7 % lower than those of the native populations ($p = 0.030$). Furthermore, invasive populations tend to have a higher total biomass than native populations but this difference was not statistically significant. Significant differences were found among the populations for all the growth traits ($p = 0.002$ for shoot mass, $p = 0.006$ for root mass, $p = 0.003$ for shoot mass and $p = 0.034$ for root-shoot ratio, respectively).

Parallel evolution: comparisons of three invasive regions with native region

The PLS-DA plot showed that the sampled populations of the four geographic regions were clearly separated based on the 19 bioclimatic variables (Fig. 5). It shows the local climate conditions differed between the native and invasive range but also among the three invasive regions.

Fig. 3 Measurements made on leaf cross section of native and invasive *Jacobaea vulgaris* plants. Arrows indicate measurements made. Sample cross section is a cropped, modified and cleaned up version of a coupe from Landsborough, New Zealand sample

- (a) Upper cuticle thickness
- (b) Upper epidermis cell wall thickness
- (c) Lower epidermis cell wall thickness
- (d) Lower cuticle thickness
- (e) Palisade parenchyma layer thickness
- (f) Sponge parenchyma layer thickness
- (g) Leaf thickness

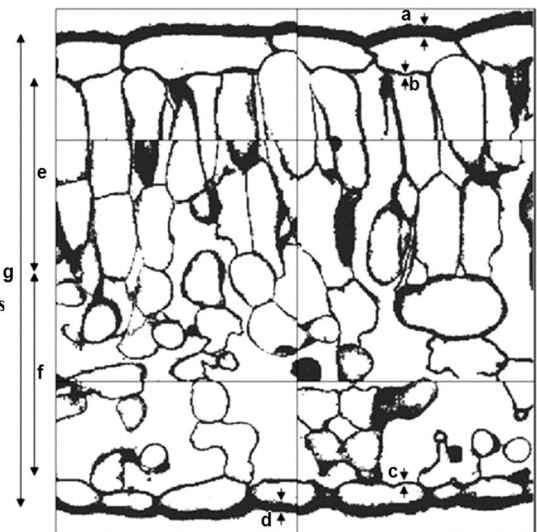


Table 2 Average leaf microscopical traits in μm of invasive and native *Jacobaea vulgaris* populations

Measurements (μm)	Native	Invasive	<i>P</i> (origins)	<i>P</i> (populations)
Upper cuticle thickness	3.44 \pm 0.14	3.49 \pm 0.13	NS	NS
Upper epidermis cell wall thickness	1.11 \pm 0.04	1.11 \pm 0.03	NS	NS
Lower epidermis cell wall thickness	0.99 \pm 0.04	1.11 \pm 0.03	0.024	NS
Lower cuticle thickness	2.16 \pm 0.08	2.28 \pm 0.10	NS	NS
Palisade parenchyma layer thickness	119.31 \pm 3.61	110.79 \pm 3.31	NS (0.065)	NS
Sponge parenchyma layer thickness	120.86 \pm 3.69	120.21 \pm 3.62	NS	NS
Leaf thickness	276.62 \pm 2.95	264.53 \pm 3.08	0.023	NS

Averages were tested with a nested ANOVA, with origin as a fixed factor and population nested within origin as a random factor. Values are mean values \pm SE ($n = 20$ for invasive populations and $n = 19$ for native populations). *P* (origins): significance level of nested ANOVA between invasive and native origins. *P* (populations): significance level of nested ANOVA among populations. NS = not significant

Table 3 Average punch strength, punch toughness and LMA of invasive and native *Jacobaea vulgaris* populations

Measurements	Native	Invasive	<i>P</i> (origins)	<i>P</i> (populations)
Punch strength (MPa)	0.636 \pm 0.015	0.636 \pm 0.012	NS	NS
Punch toughness ($\text{kJ} \cdot \text{m}^{-2}$)	0.210 \pm 0.007	0.227 \pm 0.009	NS	NS
LMA ($\text{g} \cdot \text{m}^{-2}$)	62.74 \pm 1.88	57.55 \pm 1.50	0.038	NS

Averages were tested with a nested ANOVA, with origin as a fixed factor and population nested within origin as a random factor. Values are mean values \pm SE ($n = 20$ for invasive populations and $n = 19$ for native populations)

P (origins): significance level of nested ANOVA between invasive and native origins. *P* (populations): significance level of nested ANOVA among populations. NS = not significant

We further investigated the five traits (leaf thickness, leaf mass ratio, cell wall protein, shoot mass and root-shoot ratio) which are significantly differed between invasive and native genotypes and compared them among invasive populations from three geographic regions (North America, New Zealand and Australia) and native populations (Fig. 4). The results showed that invasive populations from the three regions were all different from native populations in all the traits. Moreover, none of these traits from invasive populations differed significantly among the three geographic regions (ANOVA, root-shoot ratio: $F_{2,19} = 0.265$, $p = 0.768$; shoot dry weight: $F_{2,19} = 2.578$, $p = 0.082$; LMA: $F_{2,19} = 0.062$, $p = 0.940$; Leaf thickness: $F_{2,19} = 0.706$, $p = 0.501$; cell wall proteins per leaf area: $F_{2,19} = 2.853$, $p = 0.072$).

Discussion

Structural defense

According to the Enemy Release Hypothesis (ERH) (Keane and Crawley 2002) plants experience less

herbivore pressure when they are introduced into a new habitat and leave behind their old, co-evolved, natural enemies. Escape from specialist herbivores, often requiring costly deterrents to keep them at bay, is thought to allow for an evolutionary shift in energy allocation from defense to growth (Feng et al. 2009; Ru et al. 2011). This shift can give invasive plants increased competitive ability, outcompeting local plant species (Blossey and Nötzold 1995). In this study we did not find strong evidence from microscopic analysis supporting our hypothesis that invasive genotypes have evolved lower structural defense against herbivory. The most interesting anatomical finding was that invasive *J. vulgaris* had significantly thinner leaves. Leaf thickness plays an important role in plant anti-herbivore defense and Peeters (2002) found it was negatively associated with densities of external chewers. Thus leaf thickness could potentially be used as one of the leaf structural traits to predict the functional composition of herbivorous insect assemblages.

Leaf toughness, however, was not different between invasive and native types. We did not find that

Table 4 Average amount of cell wall proteins on the basis of leaf mass and of leaf area of invasive and native *Jacobaea vulgaris* populations

Measurements	Native	Invasive	<i>P</i>
Cell wall proteins ($\text{g} \cdot \text{g}^{-1}$ dry weight)	0.291 ± 0.011	0.278 ± 0.012	NS
Cell wall proteins ($\text{g} \cdot \text{m}^{-2}$)	10.63 ± 1.45	9.48 ± 1.82	0.037

Averages were tested with a one-way ANOVA

Values are mean values \pm SE ($n = 20$ for invasive populations and $n = 19$ for native populations)

P: significance level of one-way ANOVA between invasive and native origins. NS = not significant

Table 5 Average shoot dry mass, root dry mass, total plant dry mass and root-shoot ratio of invasive and native *Jacobaea vulgaris* populations

Measurements	Native	Invasive	<i>P</i> (origins)	<i>P</i> (populations)
Shoot mass (g)	6.13 ± 0.25	6.97 ± 0.24	0.029	0.002
Root mass (g)	5.61 ± 0.35	5.23 ± 0.31	NS (0.089)	0.006
Total mass (g)	11.74 ± 0.52	12.20 ± 0.49	NS	0.003
Root-shoot ratio ($\text{g} \cdot \text{g}^{-1}$)	0.922 ± 0.046	0.750 ± 0.037	0.030	0.034

Averages were tested with a nested ANOVA, with origin as a fixed factor and population nested within origin as a random factor

Values are mean values \pm SE ($n = 20$ for invasive populations and $n = 19$ for native populations)

P (origins): significance level of nested ANOVA between invasive and native origins; *P* (populations): significance level of nested ANOVA among populations. NS = not significant

invasive *J. vulgaris* genotypes had significant lower leaf punch strength and punch toughness than native genotypes. Yet, we did find that the invasive genotypes showed smaller LMA, which is consistent to our hypothesis. Low LMA facilitates efficient light capture and such plants tend to have productive but short-lived leaves and high growth rates (Poorter and Evans 1998). Conversely high LMA is associated with leaf structural traits that confer toughness and thus resistance against herbivores (Hanley et al. 2007). Therefore a lower LMA could be associated with faster growth of invasive plants which in turn may contribute to their ability to outcompete local plants in the introduced habitats, but this lower LMA may also entail a lower level of structural defense. In this study, we did find that the LMA tended to be positively correlated with punch toughness and strength but this trend was not significant (Pearson correlation one-tailed test, $r = 0.032$, $p = 0.065$ and $r = 0.022$, $p = 0.107$, respectively).

Leaf cell walls constitute a substantial amount of nitrogen and account for 30–50 % of leaf dry mass, therefore a large amount of cell walls could increase leaf structural toughness which, in turn, would contribute to a greater tolerance to physical damage

(Onoda et al. 2004). In this study we found that the invasive *J. vulgaris* genotypes had a 10.8 % lower amount of cell wall proteins per leaf area than the native genotypes, which resulted in a significantly poorer structural defense to herbivory. In addition, Feng et al. (2009) also found invasive *Ageratina adenophora* plants to have lower cell wall protein contents than native populations. They argued that selection for invasiveness could in this species be associated with preferential allocation of nitrogen to photosynthetic functions at the expense of allocation to cell walls, as this reallocation gives the invasive plants a competitive advantage at the cost of a poorer structural defense. Since the specialist natural enemies in the introduced habitats are absent, the invasive plants could gain benefits from such trade-offs between defense and growth for their invasion success.

Growth traits and tolerance

On average there were no significant differences in total mass and root mass between native and invasive *J. vulgaris* genotypes after 4 weeks of growth. However, we found that invasive *J. vulgaris* genotypes

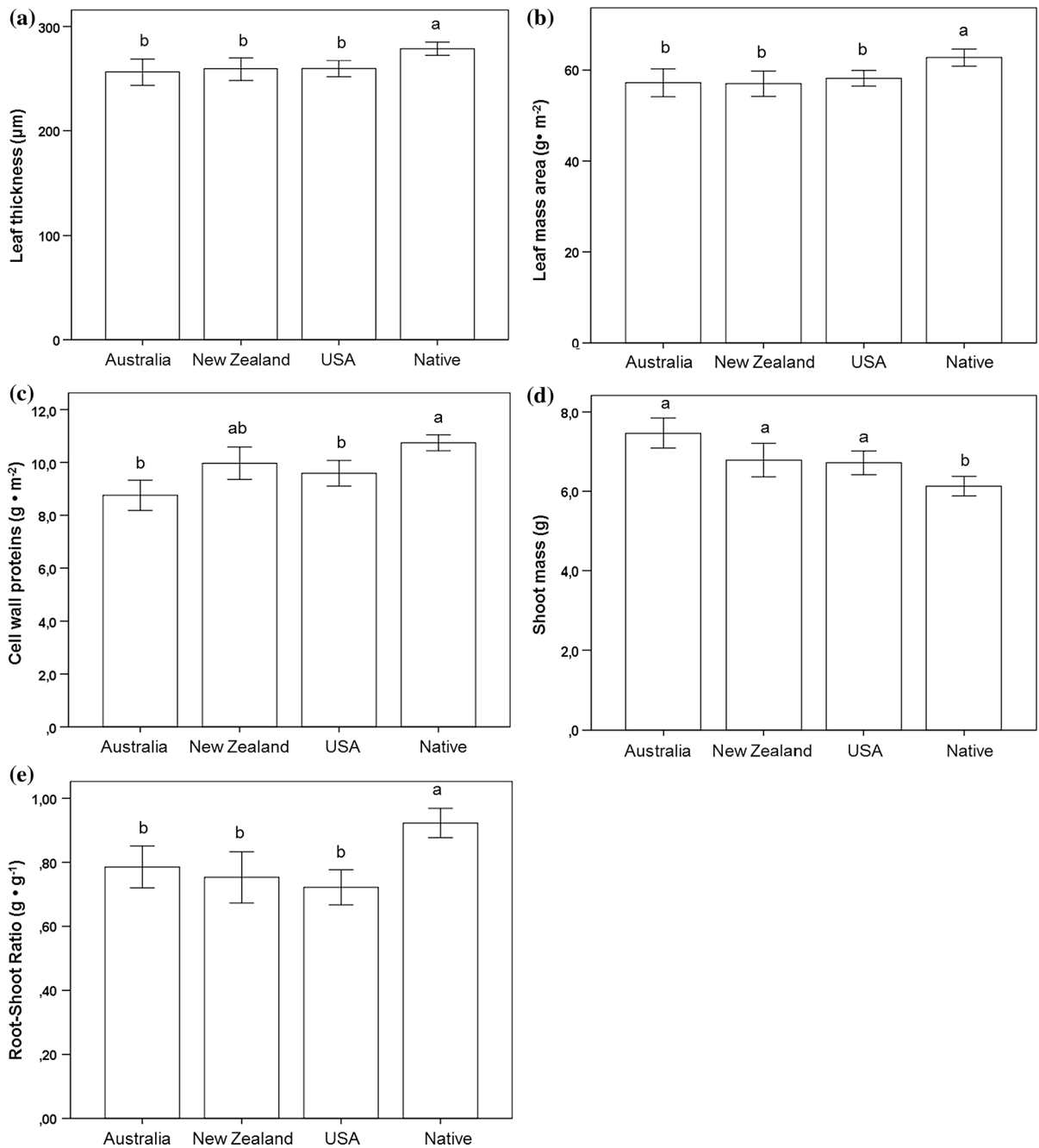


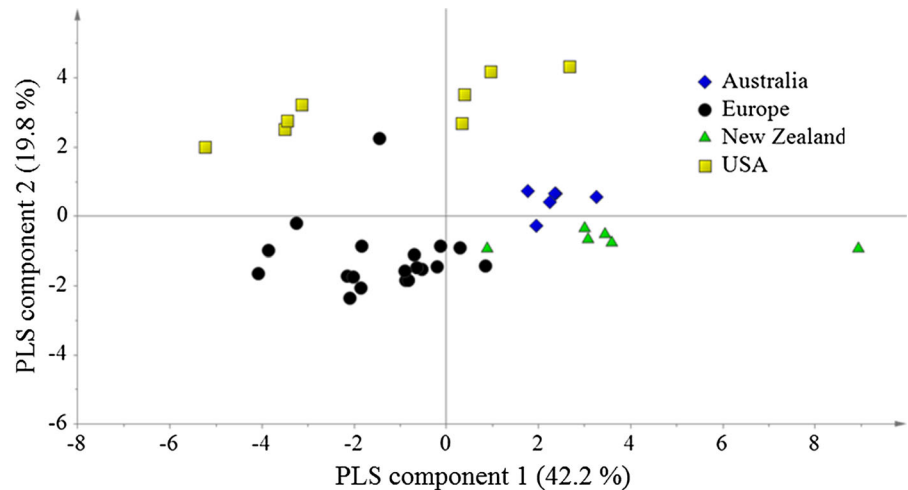
Fig. 4 The comparisons of leaf thickness (a), leaf mass ratio (b), cell wall protein (c), shoot mass (d) and root-shoot ratio (e) among invasive *Jacobaea vulgaris* populations from three geographic regions (Australia, New Zealand and USA) with

native populations. Values are mean \pm SE. Different letters indicate significant differences among treatments at $p < 0.05$ according to a post hoc LSD test (ANOVA)

invested more in the aboveground parts than in underground parts resulting in a significantly larger shoot mass and, as already noted, had lower LMA values compared to the native genotypes. Having

larger shoots and thinner leaves may enable invasive genotypes to grow faster inherently (Lake and Leishman 2004; Leishman and Thomson 2005; Grotkopp and Rejmánek 2007), and, independent of this growth

Fig. 5 Partial least square-discrimination analysis (PLS-DA) plot classifying the four geographic regions of native and invasive ragwort based on 19 bioclimatic variables from the collected sites of each sampled population (N = 19 for Europe, N = 6 for Australia, N = 6 for New Zealand and N = 8 for USA)



potential, also enable them to compete more effectively for light (Schieving and Poorter 1999). Indeed Joshi and Vrieling (2005) found that invasive *J. vulgaris* plants produced significantly higher biomass and had 37 % higher reproductive output compared to native plants in a common garden experiment after 8 months growth. Therefore the relatively small and only marginally significant difference in final biomass between native and introduced is probably associated with the short duration of the experiment and the difference would likely have amplified had the experiment been longer.

On the other hand one could also expect that a decreased allocation to the root might lead to an increased allocation to the shoot in order to increase the amount of photosynthetic tissue and/or to be a better competitor for light. Indeed invasive *J. vulgaris* populations had a significantly lower root-shoot ratio (Fig. 4). Furthermore, the root-shoot ratio is also associated with plant regrowth ability after damage. Consistent with our hypothesis, we found that invasive *J. vulgaris* genotypes have lower regrowth ability and tolerance than native genotypes. This is further supported by the finding of Joshi and Vrieling (2005) who found that native *J. vulgaris* genotypes had a 12 % higher regrowth ability after full defoliation.

It is worth to point out that there are not many studies specifically focusing on differences plant underground development between native and invasive plants, but among the among the available findings there appears to be no consistent trend towards invasive plants having lower root-shoot ratios

(D'Antonio and Mahall 1991; Pattison et al. 1998; Marler et al. 1999; Claridge and Franklin 2002; Wilsey and Polley 2006; Kumschick et al. 2013). Root-shoot ratio can be influenced by variable factors such as soil condition, local competition and herbivory (Monk 1966; Ågren and Ingestad 1987; van der Meijden et al. 1988. Hutchings and John 2004; Poorter et al. 2012). We argue that this allocation-to-root phenomenon we found in the native *J. vulgaris* is species specific, which is due to the selection pressure of its specialist herbivore *Tyria jacobaeae* in the natural habitats. The foliar-feeding larvae of this specialist herbivore can remove all the aboveground parts of *J. vulgaris* plants within a short time period (Dempster 1971). However, the main period of herbivory of this univoltine moth only lasts for 6 weeks with a peak in June. Therefore native *J. vulgaris* could develop a tolerance strategy in order to survive from such attacks. During the plant's development, resources from aboveground shoots are allocated to underground parts, which resulted in a larger root system for later regrowth after herbivory (Islam and Crawley 1983; van der Meijden et al. 1988). In contrast, it could be argued that the lower root-shoot ratio of invasive *J. vulgaris* genotypes represents a redistribution of resources from root storage (as in native genotypes) to growth of aboveground parts, and thus increasing potential growth.

Parallel evolution

Notably, we also investigated the difference among invasive populations of *J. vulgaris* from three

geographic regions (North America, New Zealand and Australia). The results showed that of all traits that significantly differed among invasive and native genotypes, none differed significantly between the three geographic regions (Fig. 4). It showed that the invasive *J. vulgaris* populations from those three geographically separated regions changed in the same direction suggesting a parallel evolution occurred (Joshi and Vrieling 2005). When plants are introduced into a new area, local adaptation to abiotic factors could also exert selective forces on invasive plants during evolution in addition to the absence of specialist herbivores (Bradley et al. 2009). Climate can exert a dominant control over the natural distribution of plant species (Pearson and Dawson 2003). In this study we found the local climate condition differed between the three invasive regions (USA, New Zealand and Australia, Figs. 1, 5). Climatic condition are considered as a potential selection force which in turn might shape the different defensive and growth traits in *J. vulgaris* populations among the three invasive regions. However, we found changes of the same magnitude and direction in quantitative defenses and tolerance in the three invasive regions. The absence of the change in traits correlated with climatic factors suggest that the disappearance of selection pressures from specialist herbivores rather than the adaptation to local abiotic factors caused the parallel changes in quantitative and tolerance traits.

In conclusion, invasive *J. vulgaris* was found to have thinner leaves, lower LMA, lower cell wall proteins contents and smaller root-shoot ratio, resulting in a poorer structural defenses and lower tolerance ability to herbivory but higher potential growth and competitive ability compared to native genotypes. These results support the EICA and SDH that due to the absence of adapted specialist herbivores, a net gain will be saved by the invasive plants by investing less in structural defense and tolerance for better growth. And all those traits that significantly different among native and invasive genotypes all changed in the same direction as predicted by the SDH in all three geographically separated invasive regions. This is in agreement with a parallel evolution occurred in those three different regions.

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