

Research Paper

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Seed traits and germination of native grasses and invasive forbs are largely insensitive to parental temperature and CO₂ concentration

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Abstract

The structure and function of grassland ecosystems can be altered by a changing climate, including higher temperature and elevated atmospheric CO₂ concentration. Previous studies suggest that there is no consistent trend in seed germination and seedling recruitment as affected by these conditions. We collected seeds of two native and two invasive species over 6 years from a field study with elevated CO₂ (600 p.p.m.) and temperature (1.5/3.0°C day/night) on the mixed-grass prairie of Wyoming, USA. Seed fill, viability and mass were evaluated and germination tests were conducted under alternating temperatures in growth chambers. Thermal time requirements to reach 50% germination (θ_{50}) and base temperatures (T_b) for germination were determined using thermal time models. Climate change conditions had limited effects on seed fill, viability and mass. The combination of CO₂ enrichment and warming increased germination of *Bouteloua gracilis*. *Centaurea diffusa* and *Linaria dalmatica*, two invasive species in this study, had the lowest θ_{50} and T_b required for germination among all the species studied. Although final germination percentages of these invasive species were not affected by treatments, previous studies reported increased seed production under future climate conditions, indicating that they could be more invasive at the regeneration stage in the future. We conclude that projected future temperature increases will have little effect on seed reproductive traits of native species. In addition, the distribution and abundance of *B. gracilis* and invasive species may be favoured by global climate change due to enhanced germination or seed production traits caused by elevated parental CO₂ and temperature conditions.

Introduction

Rising atmospheric CO₂ concentration and temperature are two main components of global climate change. Species sensitivities to CO₂ and temperature are driven by traits that influence growth, such as photosynthetic metabolism and water use efficiency (Poorter and Navas, 2003; Morgan *et al.*, 2004b; Ainsworth and Long, 2005), or reproduction, such as seed production and seedling recruitment (Morgan *et al.*, 2004a; Hovenden *et al.*, 2008; Williams *et al.*, 2007). Compared with reproductive responses, the vegetative responses of functional groups to climate change are more consistent and distinctive, with C₃ plants usually achieving greater productivity under elevated atmospheric CO₂ concentrations due to directly stimulated photosynthesis (Sage and Kubien, 2003); while C₄ plants are more sensitive to temperature and in general have higher thermal optimum than that observed for C₃ plants (Sage and Kubien, 2007).

Seed germination as a critical stage of the life cycle reflects adaptation to local habitats and population dynamics (Paula and Pausas, 2008), and is strongly dependent on environmental filters (Donohue *et al.*, 2010; Walck *et al.*, 2011; Fraaije *et al.*, 2015; Mondoni *et al.*, 2015). The contribution of seed traits including germination to plant community dynamics has received increasing attention in recent years (Jimenez-Alfaro *et al.*, 2016). Understanding seed germination responses to climate change is important for predicting changes in species composition, and therefore in the structure and function of ecosystems. Seed traits, such as seed mass, fill rate, viability and germination have strong adaptive implications for species distribution and abundance under future climates (Hovenden *et al.*, 2008).

Effects of CO₂ enrichment during plant growth and seed production and the influence on the germination of those seeds have been reported in many species. Newton (1991) observed a trend towards higher seed production per plant and greater individual seed mass but a variable response in seed numbers at elevated CO₂. A meta-analysis on 79 species shows that in general, CO₂ enrichment resulted in more flowers (+19%), more fruit (+18%), more seeds (+16%), greater individual seed mass (+4%), and greater total seed mass (+25%) (Jablonski *et al.*, 2002).

Thürig *et al.* (2003) found that elevated CO₂ concentrations significantly increased seed number of graminoids, but not that of forbs and legumes, in a natural, nutrient-poor calcareous grassland. In the same study, elevated CO₂ concentrations also tended to increase seed mass and shorten the time for germination.

Warming is also expected to influence seed traits. Greater seed viability under artificial warming has been reported in alpine and high-latitude ecosystems (Wookey *et al.*, 1995; Kullman, 2002; Lantz *et al.*, 2009). Positive effects of warming on seed mass have been reported in herbaceous plants in forests (De Frenne *et al.*, 2010), alpine herbaceous species (Sandvik and Eide, 2009), and a native perennial grass (Gao *et al.*, 2012). However, among 15 species studied in a calcareous grassland, seed mass was not significantly affected by warming after 3 years (Hovenden *et al.*, 2007). Parental warming during plant growth and seed development increased subsequent seed germination in shrubs and forbs (Graae *et al.*, 2008), forest tree and herbaceous species (Chidumayo, 2008; De Frenne *et al.*, 2010), and a grass species *Themeda triandra* (Williams *et al.*, 2007). It reduced seed germination percentage in a dwarf shrub species in the subarctic (Graae *et al.*, 2008), three savanna woody species in Africa (Chidumayo, 2008), and grass species in the Eurasian grassland (Gao *et al.*, 2012) and Australian temperate grassland (Williams *et al.*, 2007), but did not affect *Leymus chinensis* (Gao *et al.*, 2012) and 20 species in the subarctic (Milbau *et al.*, 2009).

Despite the high number of studies conducted, most of the previous studies have focused on a single factor in global climate change, either elevated CO₂ concentration or parental warming but rarely on the combination of the two. Germination of *Austroanthonia caespitosa*, a dominant C₃ species from Australia, was affected by an elevated CO₂ and warming interaction, such that elevated CO₂ or warming reduced germination if applied alone, but not when applied together (Hovenden *et al.*, 2008). Information is also limited regarding temperature thresholds for germination such as base temperature and thermal time requirement in changing environments (Jimenez-Alfaro *et al.*, 2016). Filling these research gaps can facilitate better predictions of ecosystem structure and function under a changing climate.

To address these issues, we used the Prairie Heating and CO₂ Enrichment (PHACE) experiment to determine the effects of elevated CO₂ and warming and their combination on various seed properties. The PHACE project is located on the mixed-grass prairie of Wyoming, USA. We used thermal time models to identify temperature thresholds during germination, and to link these thresholds to seed properties under climate change conditions. Objectives of this research were: (1) to identify treatment effects on seed fill rate, seed viability, individual seed mass and germination in selected species, including two invasive species; (2) to identify the shifts in germination thresholds of those species as affected by treatments; and (3) to identify seed properties that may affect exotic plant invasion under future climatic conditions. We hypothesized that: (1) seed fill percentage is reduced under parental elevated CO₂ concentration and warming, due to the disruption of normal seed development (Spears *et al.*, 1997; Young *et al.*, 2004); (2) parental warming and elevated CO₂ would increase and decrease seed viability, respectively, due to altered seed protein content for embryo growth (Andalo *et al.*, 1996; Bai *et al.*, 2003); (3) parental warming, CO₂ enrichment, and their combination, which increased growth of several of the studied species (Blumenthal *et al.*, 2013; Reeves *et al.*, 2015), would also increase individual seed mass; and (4) future climatic

conditions benefit invasive species over native species at the regeneration stage, mirroring responses of vegetative growth and overall seed production.

Materials and methods

The PHACE experiment

The Prairie Heating and CO₂ Enrichment (PHACE) experiment was set up at the United States Department of Agriculture–Agricultural Research Service, High Plains Grasslands Research Station, Cheyenne, WY, USA (41°11'N, 104°54'W) in a northern mixed-grass prairie community (Dijkstra *et al.*, 2010). Mean annual precipitation is 384 mm and mean temperatures are 17.5°C in July and –2.5°C in January. The plant community consists of about 55% C₃ grasses with *Pascopyrum smithii* (Rydb.) A. Löve and *Hesperostipa comata* (Trin. and Rupr.) Barkworth dominating; C₄ grasses, almost solely *Bouteloua gracilis* Lag. ex Griffiths, contribute about 25% of the composition, and sedges, forbs and small shrubs contribute about 20% (Morgan *et al.*, 2011). The PHACE experiment was initiated in spring 2016. Twenty circular experimental plots (diameter 3.4 m) were established and split into two sections. One half of each plot was retained as the northern mixed prairie plant community, while the other half was designed to study plant invasion. The plant invasion area consisted of three, 80 × 70 cm subplots. The toadflax subplot was planted with 20 *Linaria dalmanica* (L.) P. Miller seedlings in June 2006 to study its invasion into undisturbed prairie (Blumenthal *et al.*, 2013).

The PHACE experiment used Free Air CO₂ Enrichment (FACE) technology to elevate ambient CO₂ concentrations (Miglietta *et al.*, 2001), and infrared heater arrays to warm the canopy (Kimball *et al.*, 2008). Two concentrations of atmospheric CO₂ were applied starting in April 2006: ambient [385 p.p.m.v. (c)] and elevated [600 p.p.m.v. (C)]. Two temperature regimes were applied starting in April 2007, control (t) and heated (T) (1.5/3.0°C warmer day/night). The atmospheric CO₂ application was shut off during the winter and turned on in the spring in each experimental year. The warming treatment ran year round.

Twenty plots were used for the elevated CO₂ concentrations and warming treatments, consisting of a full factorial design with five replicates of each of the four combinations [ambient CO₂ and ambient temperature (ct); ambient CO₂ and warming (cT); elevated CO₂ and ambient temperature (Ct); and elevated CO₂ and warming (CT)].

Seed collection and germination tests

Four species from the mixed-grass prairie were studied (Table 1). Seeds from two native perennial grasses [*Bouteloua gracilis* and *Koeleria macrantha* (Ledeb.) Schult] were harvested from the Northern Mixed Prairie area inside each plot. Two invasive species [*Centaurea diffusa* and *Linaria dalmanica*] were harvested from the invasive species area of each plot. Because species only produced enough seeds to harvest in some years, the collection years differed by species (summarized in Table 1). Seeds were given at least 4 weeks after-ripening at room temperature (about 20°C) before being stored at 4°C in darkness for approximately 6 months. Seeds were cleaned and the numbers of filled and empty seeds were recorded. Seed fill percentage was then determined using the portion of filled seeds in the total seeds for each treatment. Dry seed mass of 10 to 30 seeds per unit was

Table 1. Seed collections between 2007 and 2012 from the Prairie Heating and CO₂ Enrichment experiment plots, located in Cheyenne, Wyoming

Species	Collection					
	2007	2008	2009	2010	2011	2012
<i>Bouteloua gracilis</i>	x	x				
<i>Koeleria macrantha</i>				x	x	
<i>Centaurea diffusa</i>			x		x	x
<i>Linaria dalmatica</i>			x			

Table 2. Alternating (12 h/12 h) temperature regimes with temperature amplitude of 10°C for germination tests of seeds from inside the Prairie Heating and CO₂ Enrichment experiment plots, located in Cheyenne, Wyoming, determined by availability of seeds

Species	Alternating temperature (°C)						
	10/0	12.5/2.5	15/5	20/10	25/15	30/20	35/25
<i>Bouteloua gracilis</i>	x		x		x		x
<i>Koeleria macrantha</i>	x		x	x	x		
<i>Centaurea diffusa</i>			x		x	x	
<i>Linaria dalmatica</i>	x	x	x	x			

determined for each species. Seed mass was not recorded for *K. macrantha* in 2010 and 2011 due to seed limitation.

Filled seeds were used for germination and viability tests. Germination tests were conducted using Sanyo growth chambers (Sanyo Versatile Environment Chamber MLR-350H, Sanyo Scientific, USA). One of seven alternating temperatures (12 h/12 h) with a temperature amplitude of 10°C was randomly assigned to each chamber (10/0, 12.5/2.5, 15/5, 20/10, 25/15, 30/20, 35/25°C). Three or four temperature regimes were chosen for the seeds of each species, depending on seed availability (Table 2). Germination tests were conducted under 24 h darkness for all species. A randomized complete block design (RCBD) with three or five replicates was used and replicates were put into growth chambers at 7 day intervals. Each field replicate consisted of a Petri dish with moist filter paper and 25–100 seeds (depending on seed availability) of a single species. Petri dishes were randomly placed in growth chambers using the middle shelves to minimize temperature differences within chambers. Petri dishes were sealed in clear plastic bags to reduce water evaporation and distilled water was added when necessary to keep the filter paper moist. Petri dishes were wrapped by zip-lock bags with two layers of aluminum foil to keep the seeds in darkness. Germination was checked under a green safe light. Seeds were sprayed with 0.05% benomyl solution when growth of microorganisms was observed. Germinated seeds were counted and removed at 1 day intervals. Seeds were considered germinated when the radicle was ≥ 2 mm. Germination tests were terminated when no seeds germinated for 14 consecutive days.

Tetrazolium testing was conducted at the end of the germination test to determine seed viability of the remaining, un-germinated seeds of *B. gracilis* and *K. macrantha*. Seeds were stained with 0.1% tetrazolium chloride solution for 24 h at room temperature and then checked under the microscope. Seeds were assumed viable when embryos were stained evenly red (Grabe, 1970). Seeds from *C. diffusa* and *L. dalmatica* were considered viable at the end of the germination test if the embryos were firm when pressed (Ren

and Bai, 2016). Seed viability was determined by calculating the portion of viable seeds (germinated and non-germinated) in total filled seeds under each temperature regime for a germination test. The maximum viability obtained under all the tested temperature regimes was used as the viability ratio for this species.

Thermal time models were developed according to Qiu *et al.* (2006). A seed population was considered to be composed of subpopulations because of differences in their relative germination rate (Garcia-Huidobro *et al.*, 1982). Germination rates for subpopulations were calculated with the reciprocal of germination time. Base temperature (T_b) and thermal time requirements for 50% subpopulation germination (θ_{50}) were calculated for each replicate of each treatment and species. Base temperature and thermal time for the germination of subpopulations were estimated using extrapolation (graphical) methods.

Data analysis

Data on seed mass, seed fill, seed viability, T_b and θ_{50} for *B. gracilis*, *K. macrantha* and *C. diffusa* were analysed as a RCBD with a two-way analysis of variance (ANOVA) using the mixed model procedure in SAS version 9.3 (SAS Institute Inc., USA). For each species, seed mass, seed fill, seed viability, T_b and θ_{50} were taken as the dependent variable, and in each case the main effects and possible interactions of year and climate change treatments were used as independent variables. Replicates and blocks were factored into the model as random effects. When the interaction of year \times treatment was significant ($P \leq 0.05$), data were analysed within each year for treatment effects. A one-way ANOVA was used to test warming treatment effect on seed mass, seed fill, seed viability, T_b and θ_{50} for *L. dalmatica*, for which multiple replicates were only available in elevated CO₂ plots.

Because different temperature regimes were used for germination, interaction of year, growth chamber temperature, and climate change treatments on seed germination of *B. gracilis*, *K. macrantha* and *C. diffusa* were analysed as a RCBD with a

Table 3. ANOVA table (*P*-value) for seed fill, seed mass and seed viability of *Bouteloua gracilis* (2007 and 2008), *Koeleria macrantha* (2010 and 2011), *Centaurea diffusa* (2009, 2011 and 2012), and *Linaria dalmatica* (2009), harvested from PHACE plots, located in Cheyenne, Wyoming

Parameter	Species	Effect	numDF	<i>F</i> -value	<i>P</i> -value	
Seed fill	<i>Bouteloua gracilis</i>	Year	1	1.02	0.322	
		Trt	3	0.93	0.442	
		Trt×Year	3	1.42	0.262	
	<i>Koeleria macrantha</i>	Year	1	24.84	<0.001	
		Trt	3	1.05	0.389	
		Trt×Year	3	2.94	0.054	
	<i>Centaurea diffusa</i>	Year	2	5.63	0.012	
		Trt	3	0.15	0.929	
		Trt×Year	4	1.00	0.428	
	<i>Linaria dalmatica</i>	Trt	3	0.30	0.826	
	Seed mass	<i>Bouteloua gracilis</i>	Year	1	1.36	0.262
			Trt	3	1.10	0.377
Trt×Year			3	0.33	0.806	
<i>Centaurea diffusa</i>		Year	2	8.77	0.100	
		Trt	3	4.03	0.016	
		Trt×Year	5	1.44	0.239	
<i>Linaria dalmatica</i>		Trt	3	2.66	0.184	
Seed viability		<i>Bouteloua gracilis</i>	Year	1	0.03	0.954
			Trt	3	1.25	0.314
			Trt×Year	3	0.12	0.948
		<i>Koeleria macrantha</i>	Year	1	9.61	0.005
			Trt	3	0.65	0.593
	Trt×Year		3	1.01	0.409	
	<i>Centaurea diffusa</i>	Year	2	1.32	0.289	
		Trt	3	0.35	0.787	
		Trt×Year	4	1.58	0.217	
	<i>Linaria dalmatica</i>	Trt	3	0.42	0.751	

Field treatments (Trt) include ambient CO₂, ambient temperature; ambient CO₂, warming; elevated CO₂, ambient temperature; and elevated CO₂, warming.

three-way ANOVA using the mixed model procedure in SAS version 9. A two-way ANOVA was conducted to analyse the germination temperature and climate change treatments effects as well as their interaction on germination of *L. dalmatica*. Treatment means were separated using Tukey's test at $P \leq 0.05$.

Results

Seed fill of *K. macrantha* averaged 84.8 and 94.5% in 2010 and 2011, respectively (data not shown). Seed fill of *C. diffusa* averaged 99.5 and 95.2% in 2011 and 2012, which were significantly higher than that in 2009 (averaged at 86.0%) (data not shown). Seed fill for *L. dalmatica* and *B. gracilis* were 90.6 and 18.3%, respectively (Table 5). Seed fill rate was not affected by the interaction of year and climate change treatments (Table 3). Seed fill for *K. macrantha* and *C. diffusa* was significantly different between years. There was no significant treatment effect on seed fill in any species (Tables 3 and 5).

Individual seed mass varied among species, ranging from 0.1 mg in *L. dalmatica*, to 1.5 mg in *C. diffusa* (Table 5). Seed mass was not recorded for *K. macrantha* (2010 and 2011). There was no significant difference in individual seed mass between climatic change treatments within species, with the exception of *C. diffusa*. The climatic treatment cT increased seed mass of *C. diffusa* compared with other treatments. Seed mass of *B. gracilis* and *C. diffusa* was not affected by the interaction of year and climatic change treatments (Table 3).

Seed viability of *K. macrantha* fluctuated between years (Table 3), averaging 67 and 88% in 2010 and 2011 (data not shown). Seed viability was lowest in *B. gracilis* (62%) and highest in *L. dalmatica* (99%). Seed viability was not affected by the interaction of year and climatic change treatments, or by treatment (Table 3).

A wide range of temperature regimes was used for the germination tests. High variation in total germination was observed among species, ranging from 44% in *B. gracilis* to 90% in *C.*

Table 4. ANOVA table (*P* value) for total germination of *Bouteloua gracilis* (2007 and 2008), *Koeleria macrantha* (2010 and 2011), *Centaurea diffusa* (2009, 2011 and 2012) and *Linaria dalmatica* (2009), harvested from PHACE plots, located in Cheyenne, Wyoming

Species	Effect	numDF	denDF	<i>F</i> -value	<i>P</i> -value
<i>Bouteloua gracilis</i>	Year	1	96	3.27	0.074
	Trt	3	96	6.99	<0.001
	Tem	3	96	0.08	0.971
	Year×Trt	3	96	2.58	0.058
	Year×Tem	3	96	0.50	0.686
	Trt×Tem	9	96	0.28	0.978
	Year×Trt×Tem	9	96	0.08	0.999
<i>Koeleria macrantha</i>	Year	1	88	32.33	<0.001
	Trt	3	88	2.00	0.119
	Tem	3	88	4.40	0.006
	Year×Trt	3	88	6.21	<0.001
	Year×Tem	3	88	0.40	0.751
	Trt×Tem	9	88	0.22	0.991
	Year×Trt×Tem	9	88	0.24	0.988
<i>Centaurea diffusa</i>	Year	2	60	3.13	0.051
	Trt	3	60	0.36	0.785
	Tem	2	60	2.29	0.110
	Year×Trt	4	60	1.26	0.295
	Year×Tem	4	60	1.19	0.326
	Trt×Tem	6	60	0.08	0.998
	Year×Trt×Tem	8	60	0.31	0.960
<i>Linaria dalmatica</i>	Trt	3	16	0.57	0.645
	Tem	3	16	5.69	0.008
	Trt×Tem	9	16	0.33	0.954

Field treatments (Trt) include ambient CO₂, ambient temperature; ambient CO₂, warming; elevated CO₂, ambient temperature; and elevated CO₂, warming; germination temperature treatments (Tem) are shown in Table 2. The bold font signify the *P* value that less than 0.05, indicating significant treatment effect.

diffusa. We found no interactive effects among year, temperature regime for germination, and climatic change treatment on seed germination (Table 4). High temperatures enhanced and low temperatures reduced germination of *K. macrantha* and *L. dalmatica* (data not shown). Germination of *B. gracilis* and *C. diffusa* was not affected by germination temperature regime.

Significant treatment effects on total germination were observed in *B. gracilis* (Tables 4 and 5). Neither CO₂ enrichment nor warming alone affected total germination of *B. gracilis*, but their combination (CT) significantly increased germination from 36% in the control (ct) to 63% (Table 5). Inconsistent treatment effects were observed between 2010 and 2011 for *K. macrantha* (interaction *P* = <0.001). Warming alone significantly reduced germination of *K. macrantha* compared with the control in 2010 but not in 2011. The combination of CO₂ and warming significantly reduced germination of *K. macrantha* compared with the control in 2011 but not in 2010 (Table 5). None of the treatments significantly affected germination of *C. diffusa* and *L. dalmatica* (Tables 4 and 5).

Base temperatures (*T*_b) varied among species, with the lowest value in *L. dalmatica* (0°C) and the highest value in *C. diffusa* (6.2°C) (Fig. 1). *T*_b was not affected by the interaction of year

and climate change treatments (Table 6). Climate change treatments had no effect on *T*_b.

Centaurea diffusa had the smallest thermal time requirement to reach to 50% germination (θ_{50}) (14.2°C day) among all species (Fig. 2). θ_{50} was not affected by the interaction of year and climate change treatments, except for that of *C. diffusa* (Table 6). Climate change treatments had a significant effect on θ_{50} of *C. diffusa* in 2012, where CT treatment significantly increased θ_{50} (Figs 1 and 2).

Discussion

Seed traits as affected by climate change

Contrary to our hypotheses, CO₂ enrichment and warming exerted limited effects on seed fill and seed mass. The only significant response was that warming alone increase per-seed mass of *C. diffusa*, an invasive species (Table 5). Previous studies reported that increased temperature can disrupt normal seed development, increasing the proportion of shrivelled and abnormal seeds, and reducing seed fill (Spears *et al.*, 1997; Young *et al.*, 2004). In addition, plants grown in warmer environments tended to produce smaller seeds due to quicker seed filling and faster ripening

Table 5. Seed fill, seed mass, seed viability and total germination for *Bouteloua gracilis* (2007 and 2008), *Koeleria macrantha* (2010 and 2011), *Centaurea diffusa* (2009, 2011 and 2012) and *Linaria dalmatica* (2009), harvested from PHACE plots, located in Cheyenne, Wyoming

Tested parameter	Species/year	Treatment			
		ct	cT	Ct	CT
Seed fill (%)	<i>Bouteloua gracilis</i>	17.1 ± 2.7	17.4 ± 2.6	21.3 ± 2.9	17.3 ± 2.5
	<i>Koeleria macrantha</i>	90.8 ± 2.6	88.0 ± 4.0	91.9 ± 2.1	88.0 ± 2.0
	<i>Centaurea diffusa</i>	95.0 ± 3.2	99.3 ± 0.4	92.0 ± 3.5	94.0 ± 3.8
	<i>Linaria dalmatica</i>	97.5	86.9	92.9 ± 1.3	85.3 ± 10.8
Seed mass (mg/seed)	<i>Bouteloua gracilis</i>	0.30 ± 0.02	0.36 ± 0.01	0.33 ± 0.02	0.36 ± 0.04
	<i>Centaurea diffusa</i>	1.24 ± 0.04 ^b	1.47 ± 0.10 ^a	1.13 ± 0.05 ^b	1.15 ± 0.08 ^b
	<i>Linaria dalmatica</i>	0.11	0.11	0.1 ± 0.01	0.1 ± 0.01
Seed viability (%)	<i>Bouteloua gracilis</i>	56.9 ± 10.3	68.8 ± 7.7	47.1 ± 12.1	73.6 ± 6.2
	<i>Koeleria macrantha</i>	80.9 ± 6.9	73.5 ± 10.2	81.7 ± 5.8	71.9 ± 9.3
	<i>Centaurea diffusa</i>	94.0 ± 2.2	99.0 ± 0.7	96.0 ± 1.2	95.0 ± 1.4
	<i>Linaria dalmatica</i>	100.0	100.0	100.0 ± 0.0	96.4 ± 3.6
Seed germination (%)	<i>Bouteloua gracilis</i>	35.8 ± 4.1 ^b	41.7 ± 4.6 ^b	36.2 ± 5.7 ^b	62.8 ± 3.3 ^a
	<i>Koeleria macrantha</i> (2010)	54.8 ± 6.4 ^a	35.3 ± 7.8 ^b	64.1 ± 5.5 ^a	58.7 ± 6.8 ^a
	<i>Koeleria macrantha</i> (2011)	78.4 ± 4.1 ^a	84.6 ± 2.2 ^a	78.0 ± 3.6 ^a	60.7 ± 5.3 ^b
	<i>Centaurea diffusa</i>	87.7 ± 2.3	94.0 ± 2.3	88.9 ± 1.4	88.4 ± 1.4
	<i>Linaria dalmatica</i>	82.0 ± 10.6	81.0 ± 9.9	70.0 ± 6.7	75.0 ± 5.5

Treatments include ambient CO₂, ambient temperature (ct); ambient CO₂, warming (cT); elevated CO₂ concentrations, ambient temperature (Ct); and elevated CO₂, warming (CT). Germination data were not significantly affected by the interaction between treatments and temperatures (Table 4) and was averaged across different temperature regimes. The values are means ± standard error. Mean comparisons were done using Tukey's test. Different superscript letters indicate significant differences at $\alpha = 0.05$.

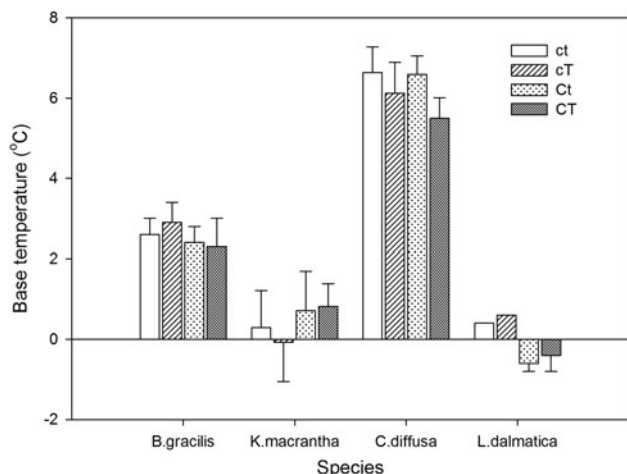


Figure 1. Base temperature (T_b , °C) for *Bouteloua gracilis* (2007 and 2008), *K. macrantha* (2010 and 2011), *Centaurea diffusa* (2009, 2011 and 2012), and *Linaria dalmatica* (2009) from PHACE plots, located in Cheyenne, Wyoming. Treatments include ambient CO₂, ambient temperature (ct); ambient CO₂, warming (cT); elevated CO₂ concentrations, ambient temperature (Ct); and elevated CO₂ concentrations, warming (CT). The bars are means ± standard error. Mean comparisons were done using Tukey's test at $P \leq 0.05$.

(Fenner, 1992), although positive effects of warming on seed mass have been reported in *Beta vulgaris* L. (Wood *et al.*, 1980) and *Glycine max* (L.) Merr. (Seddigh and Jolliff, 1984). However, some previous experiments use extremely high temperatures to simulate heat stress, which is bound to harm seed yield. Our result agrees with Hovenden *et al.* (2007), who reported that seed mass of most species was not affected in a global climate change

experiment in a grassland in Tasmania. The 1.5/3.0°C (day/night) rise in temperature in the present study is within the range of prediction for temperature increase in the Great Plains by 2090 (Loehman, 2009). This study found no evidence for an effect of increased temperature, indicating that global warming might have little effect on seed fill and mass for native species in northern mixed-grass prairie. However, the data for *C. diffusa* may be an indication that some invasive species in these grasslands could benefit from higher temperature by producing heavier seeds.

Although CO₂ enrichment did not affect seed mass in this study, greater individual seed mass under elevated CO₂ concentrations has been observed in many species, including crops (Bai *et al.*, 2003), pasture and old-field species (Newton, 1991), and grasses (Huxman *et al.*, 1998). For 79 crops and wild species, individual seed mass increased an average of 4% under CO₂ enrichment (Jablonski *et al.*, 2002). This is probably due to increased photosynthesis (Sage and Kubien, 2003), water use efficiency (Morgan *et al.*, 2011), and plant assimilate availability (Jablonski *et al.*, 2002). It should be noted, however, that earlier climate change studies were often conducted in growth chambers or glasshouses (Huxman *et al.*, 1998), within one growing season (Garbutt and Bazzaz, 1984), and with very high CO₂ concentrations (Bai *et al.*, 2003). General conclusions or hypotheses based on responses observed in controlled environment conditions should be observed with caution until validated through field experiments (Drake *et al.*, 1985). Climate change studies using Open-top Chambers (OTC) or Free Air CO₂ Enrichment (FACE) systems enabled field studies under manipulated CO₂ concentrations with relatively stable performance (Newton, 1991), and have often yielded different results from previous studies, such as species-specific effects on individual seed mass, rather than a general pattern of enhanced mass (Thürig

Table 6. ANOVA table for base temperature (T_b) and thermal time requirement for 50% subpopulation germination (θ_{50}) of *Bouteloua gracilis* (2007 and 2008), *Koeleria macrantha* (2010 and 2011), *Centaurea diffusa* (2009, 2011 and 2012) and *Linaria dalmatica* (2009), harvested from PHACE plots, located in Cheyenne, Wyoming

Parameter	Species	Effect	numDF	F-value	P-value	
T_b	<i>Bouteloua gracilis</i>	Year	1	0.34	0.573	
		Trt	3	1.26	0.329	
		Trt×Year	3	1.00	0.425	
	<i>Koeleria macrantha</i>	Year	1	0.19	0.668	
		Trt	3	0.18	0.906	
		Trt×Year	3	0.24	0.867	
	<i>Centaurea diffusa</i>	Year	2	3.52	0.040	
		Trt	3	0.73	0.541	
		Trt×Year	4	1.93	0.126	
	<i>Linaria dalmatica</i>	Trt	3	2.57	0.192	
	θ_{50}	<i>Bouteloua gracilis</i>	Year	1	1.38	0.264
			Trt	3	1.98	0.115
Trt×Year			3	1.28	0.327	
<i>Koeleria macrantha</i>		Year	1	0.44	0.514	
		Trt	3	0.61	0.617	
		Trt×Year	3	0.02	0.995	
<i>Centaurea diffusa</i>		Year	2	13.84	<0.001	
		Trt	3	1.55	0.219	
		Trt×Year	4	4.87	0.003	
<i>Linaria dalmatica</i>		Trt	3	1.22	0.410	

Treatments include ambient CO₂, ambient temperature; ambient CO₂, warming; elevated CO₂, ambient temperature; and elevated CO₂, warming. The bold font signify the P value that less than 0.05, indicating significant treatment effect.

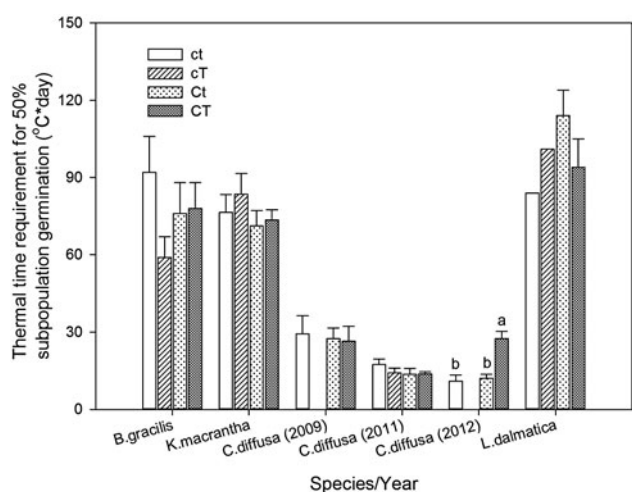


Figure 2. Thermal time requirement for 50% subpopulation germination (θ_{50} , °C day) for *Bouteloua gracilis* (2007 and 2008), *K. macrantha* (2010 and 2011), *Centaurea diffusa* (2009, 2011 and 2012), and *Linaria dalmatica* (2009) from PHACE plots, located in Cheyenne, Wyoming. Treatments include ambient CO₂, ambient temperature (ct); ambient CO₂, warming (cT); elevated CO₂ concentrations, ambient temperature (Ct); and elevated CO₂ concentrations, warming (CT). The bars are means \pm standard error. Means comparisons were done using Tukey's test. Different lower case letters indicate significant differences at $\alpha = 0.05$.

et al., 2003; Stiling *et al.*, 2004; Hovenden *et al.*, 2007; Williams *et al.*, 2007).

Surprisingly, seed viability was not affected by climate change treatments. C/N ratio in vegetative tissues and seed is believed to affect seed protein content for embryo growth, which in turn can influence seed viability (Andalo *et al.*, 1996; Bai *et al.*, 2003). Previous PHACE reports have found elevated CO₂ to decrease and warming to increase soil and plant N (Dijkstra *et al.*, 2010; Mueller *et al.*, 2016). We did not test C/N ratios in the seeds in this study due to seed number limitation.

Impacts of CO₂ enrichment, warming, and the combination of these two on seed germination were consistent across a wide range of temperature regimes (Table 4). Seed germination response to warming and/or elevated CO₂ concentrations is species specific, agreeing with previous studies (Huxman *et al.*, 1998; Williams *et al.*, 2007; Gao *et al.*, 2012; Marty and BassiriRad, 2014). Future climate condition (CT) favoured seed germination of C₄ grass *B. gracilis* but not the three C₃ species in this study. In contrast, CT reduced seed germination of *K. macrantha* in 2011. C₄ plants generally have a higher thermal optimum for photosynthesis (Ehleringer *et al.*, 1997; Sage and Kubien, 2007). The promoting effects of warming on C₄ plants could be augmented by CO₂ by offsetting warming-induced desiccation (Morgan *et al.*, 2011; Blumenthal *et al.*, 2013). In the early years of the PHACE study, when our

seeds were sampled, CT also stimulated the above-ground growth of *B. gracilis* (Morgan et al., 2011). Our results indicate that *B. gracilis* may benefit from combined warming and CO₂ enrichment in terms of seed germination. However, it should be noted that seedlings of *B. gracilis* are very susceptible to drought (Briske and Wilson, 1980). The adventitious root growth for seedling establishment of this species is affected by the combination of humidity and temperature (Briske and Wilson, 1978). When the humidity requirement is met, higher temperature could favour the seedling growth of *B. gracilis* (Briske and Wilson, 1978). There are important regional differences in terms of precipitation in the USA. It is projected that the northern states would experience more precipitation in the winter and spring, except for the Northwest in the spring, while the Southwest is projected to experience less precipitation, particularly in the spring (National Climate Assessment; <http://nca2014.global-change.gov/report/our-changing-climate/precipitation-change#state-ment-16555>). Hence, the impact of the higher seed germination will depend on these combined temperature/precipitation patterns in different regions.

After 3 years exposure to CT, C₃ graminoids were favoured, mainly due to the vegetative regeneration of perennials, reversing the initial shift towards C₄ grasses (Mueller et al., 2016). Shaw et al. (2002) reported that no effect of CO₂ on net primary production of an annual grassland was observed until the third year. Therefore, long-term studies are needed to determine the contribution of regeneration from seeds on community dynamics of grasslands.

Seed regeneration of invasive species in the mixed-grass prairie affected by global climate change

Climate change treatments had limited effects on seed characteristics including germination percentage of *C. diffusa* and *L. dalmatica*, the two invasive species in our study. The combination of the highest T_b and the lowest θ_{50} enables *C. diffusa* to finish germination quickly once it starts at relatively higher temperature. In contrast, *L. dalmatica* (2009) had the highest θ_{50} but lowest T_b , indicating that the initiation of germination for this species may occur early in the spring when temperatures are low, but it takes longer to germinate. T_b and θ_{50} of *B. gracilis* were intermediate among species (Figs 1 and 2).

Blumenthal et al. (2013) reported that *L. dalmatica* was favoured under elevated CO₂ with a 13-fold increase in biomass and a 32-fold increase in seed production. Similarly, elevated CO₂ increased number of open flowers, seed heads and seeds of *C. diffusa* 5-, 3- and 3-fold, respectively (Reeves et al., 2015). Hence, elevated CO₂, both with and without warming, seems to favour seed reproduction of *C. diffusa* and *L. dalmatica* quantitatively rather than qualitatively. Invasive species are often opportunistic and able to respond to environmental changes (Dukes and Mooney, 1999; Bradley et al., 2010; Shine, 2011; Walck et al., 2011), but our results suggest that seed traits and germination play little role in this opportunism for these two grassland invaders.

Our results also indicated that *C. diffusa* and *L. dalmatica* had different recruitment strategies. *Centaurea diffusa* had lowest θ_{50} and *L. dalmatica* had lowest T_b required for germination among the four species studied, respectively. Species with a lower T_b can germinate early in spring, giving it competitive advantage during seedling establishment. Vujnovic and Wein (1997) also reported that *L. dalmatica* can germinate at low temperatures. Species with a lower θ_{50} can finish seed germination more quickly, reducing the likelihood of predation or damage from adverse environmental conditions.

In conclusion, the combination of warming and CO₂ enrichment enhanced seed germination of the C₄ species *B. gracilis* which could promote its ability to regenerate over long time-frames in mixed-grass prairie under climate change. However, the timing of precipitation in conjunction with spring warming could determine in large part whether or not *B. gracilis* seedlings would be successful. Invasion by non-native species such as *L. dalmatica* and *C. diffusa* could increase under future climate conditions, possibly due to increased seed production but not seed regeneration *per se*, which might reduce biodiversity and reduce the ecosystem health potentially.

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