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Research

Nutritive value and the nitrogen dynamics of *Trifolium subterraneum* and *Phalaris aquatica* under warmer, high CO_2 conditions

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Summary

• Will changes in nutritive values and N-relations offset initial gains in forage productivity under potential climate change observed for grass–legume pastures of south-eastern Australia?

• Herbage nutritive value and symbiotic nitrogen fixation were investigated for pure and mixed swards of subterranean clover (*Trifolium subterraneum*) and phalaris (*Phalaris aquatica*) in field tunnels at ambient and 690 μmol mol^{-1} CO_2 concentrations and at ambient and warmed (+3.4°C) air temperatures.

• Elevated CO_2 increased the nonstructural carbohydrate content of herbage whereas warming tended to decrease it. These effects were mainly on soluble carbohydrates in phalaris and starch in clover herbage. The N concentration of both species was decreased by elevated CO_2 but unaffected by warming. The proportion of clover-N derived from N_2 fixation was increased by 12% under elevated CO_2 but decreased by 6% under warming.

• Concurrent warming and high-CO_2 conditions are expected to lead to improved herbage nutritive value for ruminants due to increased nonstructural soluble carbohydrate content. Longer term effects on nutritive value and N-dynamics via species persistence and competition require further study.

Key words: carbohydrate, carbon dioxide, climate change, nitrogen fixation, nutritive value, phalaris (*Phalaris aquatica*), subterranean clover (*Trifolium subterraneum*), temperature.

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Introduction

Temporary and permanent pastures commonly represent between 35 and 60% of the land utilized for agriculture in many parts of the world (Peoples *et al.*, 1995; Whitehead, 1995). There are several studies of the effects of temperature or elevated CO_2 on individual forage species and communities (for example, Manderscheid *et al.*, 1997; Schenk *et al.*, 1997a,b; Carter *et al.*, 1999), however, relatively little is known about the likely impact of concurrent changes in atmospheric CO_2 concentrations and ambient temperature regimes on pasture productivity and herbage nutritive value, or interspecies competition and species persistence in mixed pasture swards. Most of the pasture legume data currently available have been derived from studies evaluating the effect of elevated CO_2 on white or red clover (*Trifolium repens*, *T. pratense*, Zanetti *et al.*, 1996; Manderscheid *et al.*, 1997; Meier & Fuhrer, 1997; Schenk *et al.*, 1997a,b). It is not clear from these studies what effect elevated CO_2 and temperature may have on biological nitrogen (N) fixation or herbage nutritive value. For example, Schenk *et al.* (1997b) found increases in biomass and the legume content of perennial ryegrass (*Lolium perenne*)/white clover swards with elevated CO_2, but crude protein (N × 6.25) either decreased or increased according to time of season, and fibre content was decreased. Such differential responses to changes in CO_2 concentration by different components of the pasture, and by different aspects of forage quality make it difficult to speculate about the likely impacts on the nutritive value and N relations of other grass-legume mixes. It will be particularly important to

be able to predict the response of legumes to environmental change before any long-term implications for livestock or crop production can be determined since the N dynamics of many farming systems are dominated by inputs of fixed N by pasture legumes (Whitehead, 1995; Peoples & Baldock, 2001).

This paper reports a study of the effects of elevated CO_2 and increased temperature on pasture nutritive value, soil N uptake and N_2 fixation by the most important and widely distributed pasture legume in Australia, subterranean clover (*Trifolium subterraneum*, Pearson *et al.*, 1997), either grown separately or in combination with the perennial grass, phalaris (*Phalaris aquatica*). Experiments were conducted in the field, using temperature gradient tunnels under realistic daily and annual temperature and radiation fluctuations. In the same experiment, Lilley *et al.* (2001) found that pure clover and the clover-grass mixture had similar forage productivity, but pure phalaris accumulated less biomass. Elevated CO_2 increased clover growth relative to the ambient CO_2 treatment; however, warming reduced clover herbage in monoculture at ambient CO_2 by 28% and reduced the growth enhancement by elevated CO_2 to 8%. In contrast, growth of the phalaris monoculture was not significantly affected by elevated CO_2 or by higher temperature. Clover dominated the mixture except in the warm temperature and ambient CO_2 treatment. Growth of the mixed sward was increased by 34% in response to higher CO_2, but was unaffected by warming, while elevated CO_2 combined with warming increased herbage yield by 23%.

Materials and Methods

Description of the experiment

The experiment was conducted in six square-section tunnels (1.25 m wide × 1.25 m high × 11.8 m long) described by Lilley *et al.* (2001). The tunnels operated with a step change in temperature and no discernible CO_2 gradient. Along the length of each tunnel there was: a short (0.8 m) air inlet section where, in the case of the elevated CO_2 tunnels, pure CO_2 was injected and mixed; a 'field temperature' experimental section (4.9 m), where air temperature was close to ambient; a controlling zone (3 m) which contained the electronic controls and data logging equipment, fans, and heaters in a heating compartment; and a final 'warmed' experimental section (3.1 m) in which the air outlet was located. Temperatures were monitored using well-shielded, aspirated thermocouples located in each experimental section of the tunnel, 50 cm above the soil surface, so that they were close to the centre of the tunnel and above the pasture canopy at all times (see Lilley *et al.*, 2001 for further details). Soil temperatures were measured using a thermocouple buried 5 cm below the surface in the middle of the 'field temperature' and 'warmed' experimental sections. Irrigation was applied regularly so that water was not limiting to growth at any stage of the experiment.

Two temperature and two CO_2 regimes, were imposed in a split plot design with CO_2 as main plots (tunnels), temperature as subplots (within tunnels), with three replications. Each temperature × CO_2 subplot (2.45 × 1.25 m) contained three sward types. These were phalaris monoculture, clover monoculture, and a 50 : 50 mixture of these species on an area basis. Details of average environmental conditions for each harvest period are presented in Lilley *et al.* (2001). Briefly, average CO_2 concentration was 380 μmol mol^{-1} in the ambient CO_2 tunnels and 690 μmol mol^{-1} in the elevated CO_2 tunnels. Total solar radiation, average daily air and soil temperatures were maximal early in the experiment during summer (15.4 MJ m^{-2} d^{-1}, 20.5°C and 21.5°C, respectively, in the field treatment), declined through autumn and winter (to 6.0 MJ m^{-2} d^{-1}, 7.1°C and 8.6°C, respectively), and increased again during spring and summer. For the duration of the experiment the average difference between the 'field temperature' and 'warmed' sections was 3.4 ± 0.3°C. Temperatures in the field treatment were 0.3–1.4°C above that outside the tunnels.

Pure and mixed swards of phalaris (cv. Holdfast) and subterranean clover (cv. Mt Barker) were established in 'field' and 'warmed' sections of each of the tunnels at the Ginninderra Experiment Station (149°06′ E, 35°12′ S), near Canberra, Australian Capital Territory, Australia. Seeds were sown into a fine seedbed on 14 December 1995, in rows 8.5 cm apart. Seed density was selected to obtain an equal plant density of clover and phalaris. The target population density was 235 plants m^{-2}. The mixture was sown in a checkerboard pattern made up of alternating rows and a row length of 23 cm for each species. A basal fertilizer application of superphosphate and molybdenum (28 S, 23 P, and 0.075 Mo kg ha^{-1}) was applied at sowing. Swards were well watered and hand weeded for the duration of the experiment.

The sampling area for each plot was 0.34 m^2 with guard areas of at least 15 cm in each direction. Swards were harvested on Jan 12, Jan 30, Feb 15, Mar 6, Apr 2, May 15, July 25, Sept 4, Oct 3, Oct 25, and Nov 26 1996 (29, 47, 63, 83, 110, 153, 224, 265, 294, 316, and 348 days after sowing.) At harvest, swards were cut 7 cm above ground level to ensure adequate regrowth, so our nutritive value measurements refer to herbage above this height. Cut herbage was separated by plant species and oven dried at 60°C to a constant weight. At harvests 10 and 11, plant bases between the soil surface and the cutting height of 7 cm were also collected, and roots were recovered from the top 15 cm of soil at the final harvest (see Lilley *et al.*, 2001 for further details).

Tissue analyses

In vitro digestibility Oven-dried plant material from harvests 3, 7, and 10 of clover and phalaris monocultures was coarsely ground through a Wiley mill fitted with a 1-mm sieve and were analysed for *in vitro* digestibility using the method of McLeod & Minson (1978, 1980). Briefly, 0.5 g of ground tissue was

placed in a Falcon tube, acid-pepsin solution was added and the sample was incubated at 50°C for 72 h. A cellulase-buffer solution was added to each sample and they were incubated at the same temperature for a further 48 h. The contents of each tube were then filtered, the dry weight of the residue recorded and the percentage digestibility of the original sample was calculated.

Nonstructural carbohydrate For harvests 3–11 a portion of the dried biomass was puck-milled to a powder in preparation for carbohydrate analysis. For harvests 3–6, oven-dried cut herbage samples were analysed and for harvests 7–11 a subsample was collected separately the afternoon before the harvest and the material was freeze-dried. Soluble carbohydrate and starch analyses were adapted from the procedures described by Smouter & Simpson (1989). Between 50 and 60 mg of powdered sample was weighed into an Eppendorf 2.2 ml microfuge tube and the sample was mixed in 1–1.5 ml of 80% ethanol (v/v). After incubation at 75°C for one hour the supernatant was removed and the ethanol extraction was repeated. This was followed by two water extractions at 60°C for 1 h each. All supernatants removed were combined and made up to a known volume. Anthrone solution was added to an aliquot and after incubation at 100°C for exactly 15 min the absorbance at 625 nm was determined with a spectrophotometer.

The starch assay was conducted on the dried and weighed pellets remaining from the soluble carbohydrate assay. An enzymatic assay was conducted using a Total Starch Assay procedure kit (Megazyme International Ltd., Bray, Co. Wicklow, Ireland). Unmodified wheat starch standards were included to check yield. Water was added to the pellets and they were incubated at 100°C for 30 mins. Then 0.5 ml (100 U) of thermostable α-amylase solution in MOPS buffer (pH 7) was added and tubes were returned to the boiling water bath for 5 min, 0.7 ml of sodium acetate buffer (200 mM, pH 4.5) was then added, followed by 0.1 ml amyloglucosidase (20 U, undiluted). The solution was vortexed and then incubated at 50°C for 1 h. After centrifuging, the supernatant was combined with glucose oxidase/peroxidase reagent, incubated at 50°C for at least 20 min and the absorbance at 510 nm was measured within 60 min. Soluble carbohydrate and starch content were calculated as a percentage of dry weight (d. wt) and total nonstructural carbohydrate (TNSC) was calculated as the sum of these.

Total N and 15N composition Finely ground samples of cut herbage, plant bases and roots were analysed for total N and ^{15}N using an automatic N and carbon analyser (ANCA–SL) interfaced to a 20–20 stable isotope mass spectrophotometer (Europa Scientific, Crewe, UK). The natural abundance of ^{15}N present was expressed as $\delta^{15}N$ (parts per thousand, ‰) with reference to air (Shearer & Kohl, 1986). By definition, the $\delta^{15}N$ of atmospheric N is zero.

Determination of N_2 fixation Estimates of N_2 fixed were obtained by comparing the ^{15}N concentrations of the clover against the non-N_2 fixing reference plant (phalaris) growing together in the case of the mixture or immediately adjacent for the monoculture. The reference plant was used to provide a measure of the isotopic composition of plant-available soil N. It is assumed that the legume and reference plant explore a soil N-pool of identical $^{15}N : {}^{14}N$ concentration (Peoples *et al.*, 1997). The proportion of the clover N derived from N_2 fixation (P_{fix}) was calculated according to the following equation (after Shearer & Kohl, 1986):

$$P_{fix} = 100(x - y)/(x - c) \qquad \text{Eqn 1}$$

(x, the mean $\delta^{15}N$ of the phalaris shoot N; y, the $\delta^{15}N$ of the clover shoot N; and c, the isotopic fractionation that occurs during N_2 fixation.) It was assumed that where the legume fixes no atmospheric N_2, the isotopic composition of the legume will be the same as for the reference plant (x). As the legume increasingly fixes greater proportions of its N, its ^{15}N concentration will approach the ^{15}N composition of a fully symbiotic plant (c). In this study the value of c. used was −0.85‰ based on a previous glasshouse study of subterranean clover (Peoples *et al.*, 1998).

Over the duration of this study $\delta^{15}N$ values in clover ranged from 1.73 to 3.52‰ in the monoculture and 0.67–2.66‰ in the mixture, while those in phalaris ranged from 7.23 to 8.50‰ in the monoculture and 6.62–8.47‰ in the mixture (SE of mean $\delta^{15}N$ determinations ranged from ±0.06‰ to ±0.45‰). These levels of difference between $\delta^{15}N$ values of the legume and the reference plant suggest a relatively precise estimate of P_{fix} (10% or less, as a proportion of a given P_{fix} value) in this study (Unkovich *et al.*, 1994). Calculations of N_2 fixation in the mixture were based on total sward area and were not corrected for plant density or the proportion of ground area (50%) sown to each species.

Nonenclosed comparison plots, outside the tunnels were also established, to examine the effect of the plastic tunnel on the pasture at ambient CO_2 and ambient temperature. Tissue N concentration, P_{fix}, digestibility, soluble carbohydrate content and starch content were similar to that of plants in the field, ambient CO_2 treatment (data not shown), despite slower growth rate in these plots up until March 6 (Lilley *et al.*, 2001). To simplify description of the results, nonenclosed comparison data are not presented.

Statistical analysis Data were analysed by ANOVA using a split plot design (CO_2 as whole plot and temperature as subplot treatments) and incorporating harvest as a repeated measure (Steel & Torrie, 1980), using General Linear Model Procedure (PROC GLM) of the SAS statistical software package (SAS, 1989). Sward types were analysed separately. *F*-tests were used to detect significant differences between treatments.

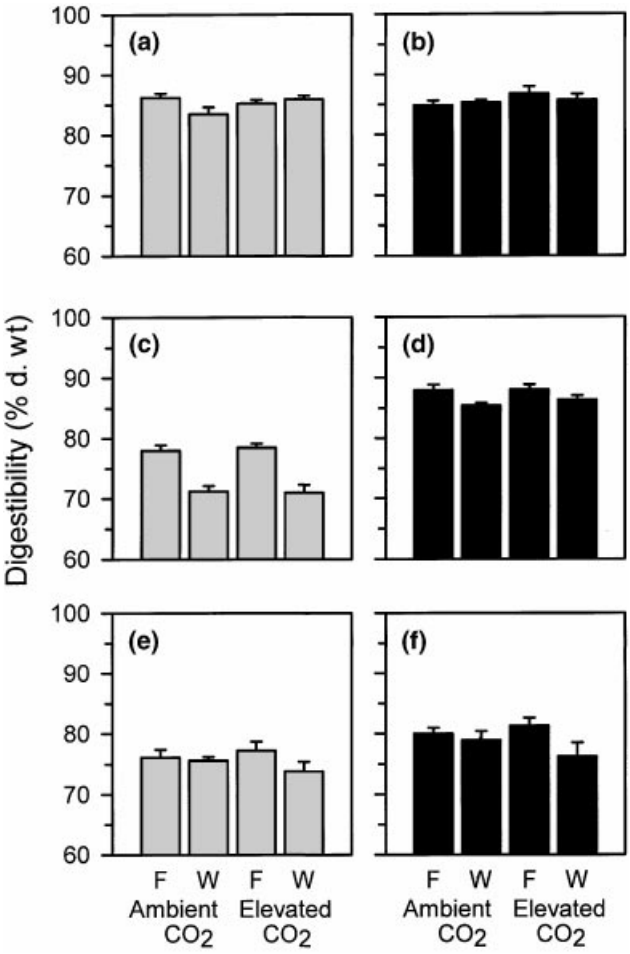

Fig. 1 *In vitro* digestibility of clover (grey columns) and phalaris (black columns) leaf and stem material from swards harvested on a 15 February (a, b): 25 July (c, d); and 25 October (e, f). Swards were grown at ambient or elevated CO_2 and under field temperatures (F) or with warming (W). Bars indicate SE of the mean ($n = 3$).

Results

Changes in plant characteristics

Digestibility *In vitro* digestibility of harvested herbage declined with age of the sward in both species from an average of 85% 9 wk after sowing (February 15) to an average of 76% for clover and 79% for phalaris in late spring (Oct 25; Fig. 1). Digestibility was not significantly effected by environmental treatment, except in clover at the July 25 harvest, where warmer temperatures reduced digestibility from 78 to 71%.

Nonstructural carbohydrate

Soluble carbohydrate and starch content in herbage above 7 cm was similar in monocultures and mixtures for each species, so mean species responses to treatment and seasonal changes are shown in Fig. 2. Over the course of the experiment, average total nonstructural carbohydrate (TNSC; soluble carbohydrate plus starch) content of clover was increased by 28% under elevated CO_2, but decreased by 10% with warming at ambient CO_2 (Table 1). TNSC was also 16% greater in phalaris herbage under elevated CO_2 and was reduced by 22% with warming at ambient CO_2. TNSC content of the plant bases (0–7 cm above the soil surface) was similar to the cut herbage for clover and slightly lower than the cut herbage of phalaris plants (Table 1). Similarly to cut herbage, elevated CO_2 increased TNSC content of both species and warming significantly decreased TNSC content of plant bases of clover.

The major seasonal and treatment effects on TNSC content of clover were changes in starch content, with little change in soluble carbohydrate content (Fig. 2a,c). The starch content of the clover was low in autumn and winter (0.5–2.4%), but increased rapidly during spring and summer to 6–15%. In contrast, almost all of the TNSC found in clover bases (0–7 cm above the soil surface) was soluble carbohydrate rather than starch. Both starch and soluble carbohydrate content of clover tended to be higher under elevated CO_2.

Soluble carbohydrate was the dominant (15–28% of DW) nonstructural carbohydrate in herbage and plant bases of phalaris. Elevated CO_2 significantly increased both starch and soluble carbohydrate content, while warming reduced soluble carbohydrate but not starch content of phalaris. Soluble carbohydrate content of phalaris stem bases increased between harvest 10 and 11, in line with the increases observed in herbage above 7 cm.

N content Shoot N content remained relatively constant for all swards through the growing season, and average values during the experiment are presented in Table 2a. For both species, N concentration was lower under elevated CO_2, while there was a smaller effect of warming resulting in a reduction of N concentration for clover (significant in the mixture). Nitrogen fertilizer was not applied in this experiment and for phalaris, a greater N concentration was observed in the mixture relative to the monoculture, while there was no difference between sward types for clover. Nitrogen content of plant bases and roots were not significantly affected by environmental treatment and averaged data are presented in Table 2b, although as with the cut herbage, there was a tendency for lower N concentration in the elevated CO_2 treatment. The plant bases had a lower N concentration than cut herbage (Table 2b), with the N concentration of clover being higher than phalaris. Root material had intermediate N composition between cut herbage and plant bases, and conformed to the same species ranking as in above-ground plant material.

Change in N dynamics The total amount of N recovered in phalaris herbage tended to increase under warming and decrease under elevated CO_2 in both the monoculture and mixture (Table 3), although these effects were not statistically significant. By contrast, warming reduced total N yield in clover monocultures and the clover-grass mixture. Elevated CO_2

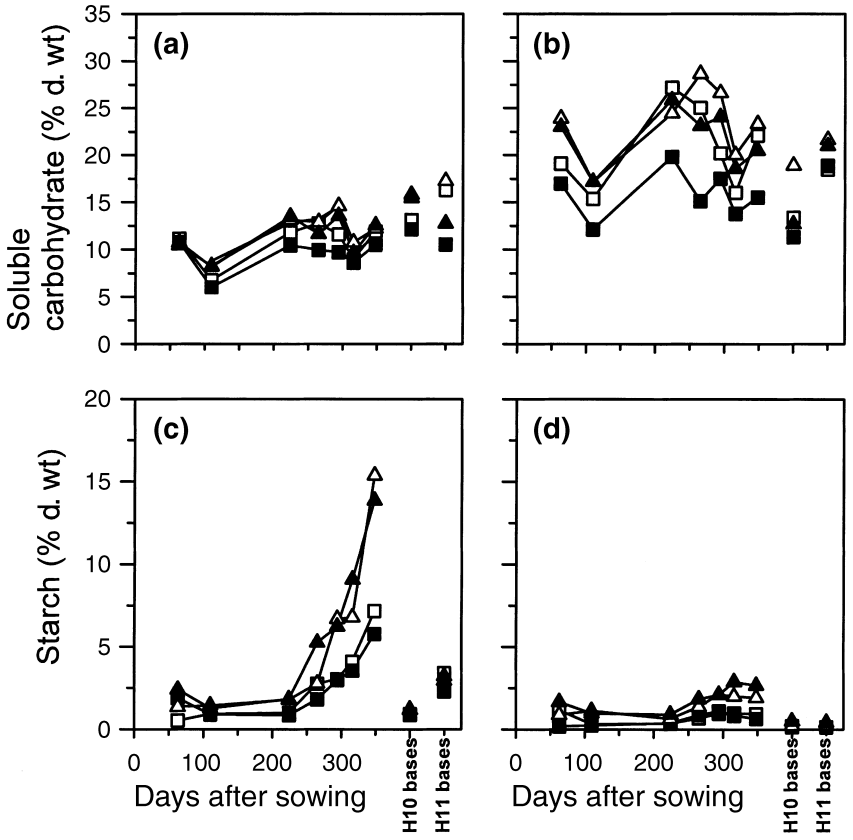

Fig. 2 Soluble carbohydrate content of (a) clover and (b) phalaris, and starch content of (c) clover and (d) phalaris for pasture herbage at seven harvests and plant bases at two harvests (at right). Values are average of monocultures and mixtures. Swards were grown under field temperatures with ambient (open squares) or elevated (open triangles) CO_2 supply, or with warming and ambient (closed squares) or elevated (closed triangles) CO_2 supply. For herbage, *F*-tests for the following main effects and interactions were significant ($P < 0.05$): (a) CO_2, harvest, $CO_2 \times$ harvest; (b) CO_2, temperature, harvest, $CO_2 \times$ temperature; (c) CO_2, harvest, $CO_2 \times$ harvest; (d) CO_2, harvest, $CO_2 \times$ temperature, $CO_2 \times$ harvest. For bases: (a) CO_2, temperature, temperature $\times$ harvest; (b) harvest, temperature $\times$ harvest; (c) harvest; (d) nil.

Table 1 (a) Average total nonstructural carbohydrate (TNSC) content of clover and phalaris herbage harvested between 63 and 348 days after sowing ($n = 42$) and (b) plant bases harvested at 316 and 348 days after sowing ($n = 12$). Values shown are the average of the monoculture and mixture and SE of the mean

		Clover		Phalaris	
	Treatment	Field	Warm	Field	Warm
(a) Herbage (> 7 cm)	Ambient CO_2	13.3 ± 0.7	12.0 ± 0.5	21.1 ± 0.9	16.5 ± 0.6
	Elevated CO_2	17.0 ± 1.0	17.1 ± 1.0	24.5 ± 1.2	23.5 ± 0.9
CO_2 effect		**		**	
Temperature effect		n.s.		**	
CO_2–temperature interaction		n.s.		n.s.	
(b) Plant bases (0–7 cm)	Ambient CO_2	16.9 ± 1.1	12.9 ± 0.9	16.1 ± 1.0	15.2 ± 1.8
	Elevated CO_2	18.7 ± 0.8	15.9 ± 0.8	20.5 ± 1.3	17.3 ± 1.5
CO_2 effect		**		*	
Temperature effect		**		n.s.	
CO_2–temperature interaction		n.s.		n.s.	

Significance of the main effects of CO_2 and temperature and their interaction are shown (* and ** indicate significance at the $P < 0.05$ and 0.01 levels, respectively).

compensated for the effect of warming, and the reduction in clover N yield caused by warming was smaller under elevated CO_2. These differences in clover N yield were largely associated with differences in biomass production (Lilley *et al.*, 2001), rather than any major change in N concentration of the tissue (Table 2). Total N removed in herbage (above 7 cm) was much greater for pure clover (equivalent to 390–580 kg N ha^{-1}) than for pure phalaris (150–240 kg N ha^{-1}, Table 3). Stem bases contained around 23% and 12% of total N harvested in the clover and phalaris monocultures, while the roots recovered

Table 2 (a) Nitrogen content (% of DW) of phalaris and clover shoots. Values are the average of cut herbage (above 7 cm) for harvests 3–11 and their standard errors ($n = 27$). (b) Nitrogen content (% of DW) of plant bases (0–7 cm above ground, average of harvests 10 and 11) and roots (harvest 11) of clover and phalaris

(a)

	Clover				Phalaris			
	Monoculture		Mixture		Monoculture		Mixture	
Treatment	Field	Warm	Field	Warm	Field	Warm	Field	Warm
Ambient CO_2	3.9 ± 0.06	3.8 ± 0.06	3.8 ± 0.06	3.7 ± 0.09	2.5 ± 0.07	2.6 ± 0.11	2.7 ± 0.07	9 ± 0.11
Elevated CO_2	3.5 ± 0.08	3.3 ± 0.08	3.4 ± 0.07	3.3 ± 0.07	2.0 ± 0.06	2.0 ± 0.06	2.6 ± 0.11	2.3 ± 0.08
CO_2 effect	*		**		*		**	
Temperature effect	n.s.		*		n.s.		n.s.	
CO_2–temperature interaction	n.s.		n.s.		n.s.		*	

(b)

	Clover		Phalaris		
Tissue	Monoculture	Mixture	Monoculture	Mixture	Clover + phalaris mixture
Plant bases	1.9 ± 0.03	1.9 ± 0.04	0.7 ± 0.02	0.8 ± 0.02	–
Roots	2.7 ± 0.05	–	1.2 ± 0.02	–	1.9 ± 0.08

Significance of the main effects of CO_2 and temperature and their interaction are shown (* and ** indicate significance at the $P < 0.05$ and 0.01 levels, respectively). (b) Values are averages of all CO_2 and temperature treatments and their standard errors (bases n = 24; roots n = 12) since main effects of CO_2, temperature and their interaction were not significant.

New Phytologist

Table 3 Total N yield (g m^{-2}) harvested in the (a) accumulated herbage (above 7 cm) and (b) cumulative total plant (cut herbage, plant bases and root) for pasture swards grown for 348 days

Pasture component	Species										
		Clover				Phalaris				Clover + phalaris Mixture	
	Sward type	Monoculture		Mixture		Monoculture		Mixture			
	Treatment	Field	Warm	Field	Warm	Field	Warm	Field	Warm	Field	Warm
(a)	Ambient CO_2	54 ± 4	39 ± 2	35 ± 4	16 ± 4	20 ± 3	24 ± 5	11 ± 2	24 ± 3	46 ± 3	40 ± 3
	Elevated CO_2	58 ± 2	51 ± 3	40 ± 3	35 ± 3	15 ± 2	18 ± 3	15 ± 0.1	13 ± 1	55 ± 2	48 ± 3
> 7 cm herbage N yield	CO_2 effect		*		*		n.s.		n.s.		*
	Temperature effect		*		*		n.s.		*		*
	CO_2–temperature interaction		n.s.		n.s.		n.s.		*		n.s.
(b)	Ambient CO_2	76 ± 5	55 ± 3	–	–	27 ± 3	30 ± 5	–	–	62 ± 3	48 ± 3
	Elevated CO_2	81 ± 3	70 ± 3	–	–	24 ± 2	25 ± 2	–	–	73 ± 3	61 ± 5
Total plant N yield	CO_2 effect		*		–		n.s.		–		**
	Temperature effect		*		–		n.s.		–		*
	CO_2–temperature interaction		n.s.		–		n.s.		–		n.s.

Significance of the main effects of CO_2 and temperature and their interaction are shown (* and ** indicate significance at the $P < 0.05$ and 0.01 levels, respectively). Treatment means and SE ($n = 3$) are shown.

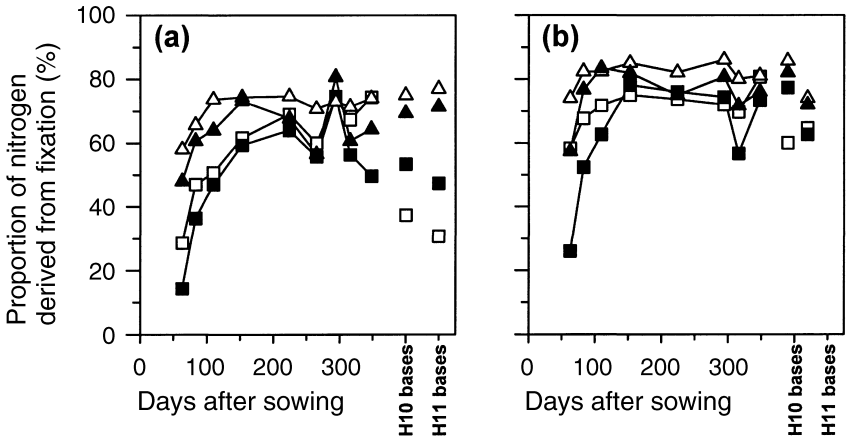

Fig. 3 Proportion of shoot N derived from symbiotic N_2 fixation in (a) clover monoculture swards, and (b) clover in a mixed sward. Swards were grown under field temperatures with ambient (open squares) or elevated (open triangles) CO_2 supply, or with warming and ambient (closed squares) or elevated (closed triangles) CO_2 supply. For herbage, *F*-tests for the following main effects and interactions were significant ($P < 0.05$): (a) harvest, $CO_2 \times$ harvest; (b) CO_2, temperature, harvest, $CO_2 \times$ harvest, temperature × harvest. For bases, nil.

Table 4 Estimates of the proportion (%) of clover N derived from N_2 fixation (P_{fix}) in: (a) accumulated herbage (cut above 7 cm), and (b) plant bases at H11 (0–7 cm), from pasture swards grown for 348 days

Pasture component	Sward type	Monoculture		Mixture	
	Treatment	Field	Warm	Field	Warm
(a) > 7 cm	Ambient CO_2	59 ± 6	53 ± 3	71 ± 5	64 ± 6
	Elevated CO_2	71 ± 4	64 ± 3	82 ± 1	76 ± 2
CO_2 effect		*		n.s.	
Temperature effect		n.s.		n.s.	
CO_2–temperature interaction		n.s.		n.s.	
(b) 0–7 cm	Ambient CO_2	70 ± 2	47 ± 9	65 ± 6	63 ± 15
	Elevated CO_2	77 ± 9	71 ± 8	74 ± 1	72 ± 2
CO_2 effect		n.s.		n.s.	
Temperature effect		n.s.		n.s.	
CO_2–temperature interaction		n.s.		n.s.	

Significance of the main effects of CO_2 and temperature and their interaction are shown (* and ** indicate significance at the $P < 0.05$ and 0.01 levels, respectively). Treatment means and SE ($n = 3$) are shown.

at final harvest contained the equivalent of 5% and 16%, respectively, of the total N recovered over the duration of the experiment (data not shown). However, since these estimates of root N were based on the physical recovery of root tissue it is likely the contribution of below-ground plant N to the N economy of the pasture swards was underestimated (McNeill *et al.*, 1997; Peoples & Baldock, 2001).

The proportion of clover N derived from N_2 fixation (P_{fix}) increased during the growth period (Fig. 3). Elevated CO_2 stimulated a greater reliance on N_2 fixation for growth earlier in the experiment while warmer temperatures reduced P_{fix} throughout. The cumulative estimates of P_{fix} over the duration of the experiment were higher in clover grown in the mixture than in pure swards (Table 4).

During the experiment between 240 and 310 kg N ha^{-1} of mineral N was estimated to have been assimilated from the soil by the various swards (Fig. 4). Soil N recovery was not significantly affected by elevation of CO_2 or warming and no significant differences were observed in mineral N content of the soil at final harvest (mean was 83 kg ha^{-1}). The amount of N_2 fixed by clover (Fig. 4) was increased by CO_2 elevation and decreased under warming where clover biomass was reduced (Lilley *et al.*, 2001). The proportion of the total N uptake that was fixed increased up to 12% by CO_2 elevation and decreased 6–23% by warming (Table 4) in all above-ground components of the clover monoculture.

Discussion

Nonstructural carbohydrates

Elevated CO_2 produced an average increase in TNSC of 28% for clover and 16% for phalaris. The greatest difference was observed at the 3 October harvest during peak spring growth

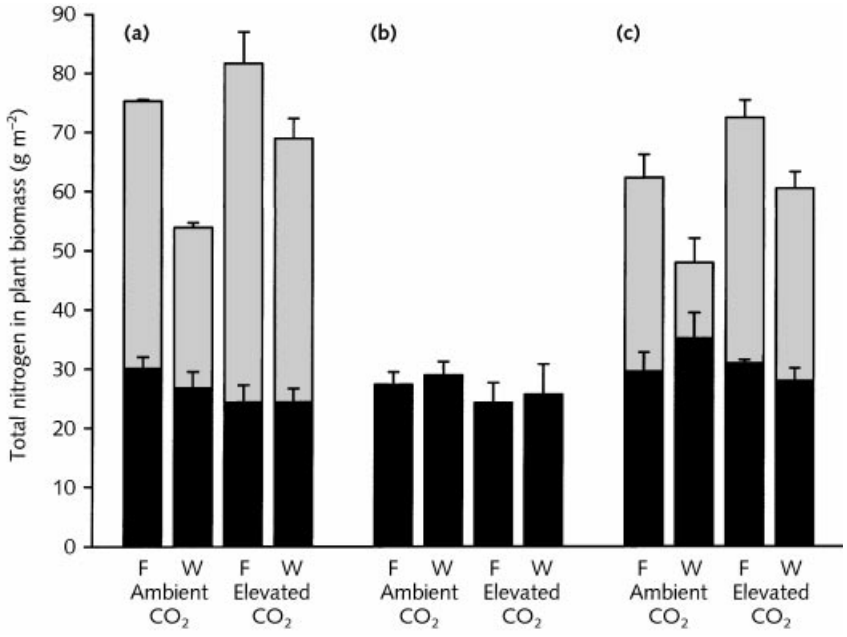

Fig. 4 Total nitrogen (g m^{-2}) in cut herbage, plant bases and roots of (a) clover monoculture (b) phalaris monoculture and (c) mixed pasture swards grown for 348 days under ambient and elevated CO_2. Temperature treatments: F, field temperature; W, warmed air. N source: soil acquired N, black columns; atmospherically fixed N, grey columns. SE (n = 3) are shown for soil acquired N and fixed N data.

when CO_2 elevation produced a 46 and 37% increase in clover and phalaris, respectively (Fig. 2). Several others have observed similar increases in TNSC levels under conditions of elevated CO_2 (Korner & Miglietta, 1994; Fischer *et al.*, 1997; Poorter *et al.*, 1997). The TNSC levels tended to be higher in spring and summer, when radiation was also higher; however, warming at ambient CO_2 decreased TNSC content at all times of the year, by an average of 10 and 22% in clover and phalaris, respectively. Modelling of nonstructural carbohydrate levels in forage grasses in relation to meteorological conditions predicts a decline in TNSC with increasing temperature, but suggests that the relationship can be complicated by the existence of low temperature thresholds, high temperature 'saturation' of the response, and the interacting effects of radiation, soil water status and photoperiod (Wulfes *et al.*, 1999), confirming the complexity of interactions with other variables. Under elevated CO_2 conditions, warming had little impact on TNSC in either species. To the extent that the TNSC concentration is an expression of a pool experiencing loss from a sink and a gain from the source, this result suggests that the enhancement of the sink by warming was matched by the CO_2 enhancement of the source. It is a result and interpretation that is compatible with there being little net change in plant growth when the warming and elevated CO_2 treatments were combined (Lilley *et al.*, 2001).

Potential impacts on livestock Although forage quality can be influenced by many factors (Whitehead, 1995), changes in CO_2 concentration and temperature can potentially influence three of the most important determinants of herbage nutritive value for ruminant livestock: digestibility, TNSC and N or protein content. Herbage digestibility was generally high in this study, ranging from 71 to 90%, and was not affected by elevated CO_2. Whether changes in herbage digestibility occur in elevated CO_2 conditions will depend on what happens to other components of herbage nutritive value, such as TNSC and N. However, it is important to note that the amount of metabolisable energy a ruminant animal gains from herbage is a direct function of the digestibility, and utilization of protein is usually also better in more highly digestible herbage (Standing Committee on Agriculture, 1990).

In the current experiment TNSC increased in response to elevated CO_2, from 13.5 to 17% in clover and 21.5–25% in phalaris. Changes in TNSC of this order may result in only small (< 1% unit) and perhaps undetectable changes in digestibility (Ciavarella *et al.*, 2000b). The relationships between TNSC, digestibility and animal performance are complex and are not easy to predict. Increases in TNSC may be associated with increases in digestibility, but usually only if there is a concomitant decrease in the herbage structural components. On the other hand, if increases in TNSC are associated with decreased N concentration in herbage, then changes in digestibility would likely be small or nonexistent. However, increased TNSC can lead to better utilization of N in the rumen, even if digestibility is unaffected (Dove & Milne, 1994).

There is usually excess soluble N in the rumen, especially when the N concentration of herbage exceeds 2.0% (Standing Committee on Agriculture, 1990), so a decline in N concentration of about 0.5% units at elevated CO_2 is unlikely to have a negative impact on livestock performance. Indeed, the net result of greater rumen N utilization and greater preference and intake of higher TNSC herbage is likely to be increased livestock performance (Hight *et al.*, 1968; Dove & Milne, 1994; Ciavarella *et al.*, 2000a) under elevated CO_2 conditions.

Nitrogen – tissue concentration and yield The lower N concentration observed in the cut biomass of both clover and

phalaris under elevated CO_2 (Table 2a) is consistent with observations from many other studies (Luo *et al.*, 1994). The reduction in N concentration was less in clover than phalaris presumably due to the capacity of clover to fix atmospheric N_2. The lower N concentration led to a 20% lower total N yield of phalaris herbage in the monoculture under elevated CO_2 (Table 4). Similar reductions in response to elevated CO_2 have been reported elsewhere for grass pastures (Soussana *et al.*, 1996; Zanetti *et al.*, 1997), however, Soussana *et al.* (1996) also observed increased N concentration of ryegrass under a 3°C warming at elevated CO_2. In the present study, increases due to warming were not significant for N concentration or total N yield. Warming did not affect N concentration of clover tissue, although the negative effect on biomass resulted in an overall reduction in total N yield (Table 3). On average, tissue N concentration of phalaris was 17% greater in the mixture than the monoculture, suggesting either a N sparing effect by the legume (*sensu* Herridge *et al.*, 1995), and/or phalaris was more competitive than clover for soil N. Certainly the clover growing in the mixed sward derived a much higher proportion of its N requirements from N_2 fixation than when growing in monoculture (Table 4) (see also Dear *et al.*, 1999). The reduction in $\delta^{15}N$ of phalaris in the mixture compared with the phalaris monoculture under both ambient and elevated CO_2 implies a possible transfer of fixed N from clover to phalaris representing between 8 and 18% of the phalaris shoot N. However, such ^{15}N data should be interpreted with caution (Chalk & Smith, 1994).

Elevated CO_2 stimulated greater N_2 fixation by the clover (Fig. 4), and this has also been reported for several other legume species (Murphy, 1986; Manderscheid *et al.*, 1997; Zanetti *et al.*, 1998). Total N yield of the clover monoculture increased by 8% while a 16% increase was observed for the mixture under elevated CO_2 (Table 3). Warmer temperatures reduced P_{fix}, N content of the tissue and total N yield.

In our study and those of Dear *et al.* (1999) and Zanetti *et al.* (1996), tissue N concentration of clover did not differ between clover grown in monoculture or clover-grass mixture. Total N yield of cut biomass of the mixture was similar to that of the clover monoculture, except in the ambient CO_2 field temperature treatment where the monoculture had a 20% greater total N yield than the mixture (Table 3a). When all plant components were taken into account, the clover monoculture yielded the greatest total N; however, the mixture tended to derive more N from the soil (Fig. 4).

A number of investigations in southern Australia have shown that under a variety of management systems, subterranean clover commonly fixes 20–30 kg N for every tonne of above-ground legume dry matter accumulated (Bolger *et al.*, 1995; Peoples *et al.*, 1998; Dear *et al.*, 1999; Peoples & Baldock, 2001). Our results are in agreement with these studies, with N_2 fixation ranging from 20 to 28 kg fixed N t^{-1} clover DM above 7 cm, or 18–24 kg fixed N t^{-1} clover DM above ground-level. Year-to-year variability in productivity and clover content of pasture can result in large fluctuations in the total amount of N_2 fixed by subterranean clover – from 20 to 238 kg N ha^{-1} (Peoples *et al.*, 1998; Peoples & Baldock, 2001). Typically, subterranean clover does not germinate and begin fixing N until April or May (autumn); it flowers and sets seed in spring and completes its annual life-cycle. However, our experiment was sown in mid-December (early summer) and received irrigation throughout the next 11 months, therefore N_2 fixation and N uptake from the soil was much greater than would normally be expected in farmers' paddocks. During our one-year study an equivalent of 320 kg fixed N ha^{-1} was harvested in the clover monoculture and 250 kg fixed N ha^{-1} where clover was grown in association with grass under ambient CO_2. This was two- to threefold greater than that observed for dryland, subterranean clover-based pastures by Dear *et al.* (1999) in the same region and the same year. Under conditions of elevated CO_2 we observed fixation yields of 420 and 320 kg fixed N ha^{-1} in the clover monoculture and mixture, respectively. These values are comparable with those of Zanetti *et al.* (1997), who measured fixation of 300–335 kg N ha^{-1} y^{-1} at elevated CO_2 and 218–236 kg N ha^{-1} y^{-1} at ambient CO_2 in ryegrass/white clover mixtures.

In conclusion, this study indicates that elevated CO_2 conditions should result in increases in TNSC which, in turn, should lead to improved herbage nutritive value for ruminants and increased livestock performance. Symbiotic N_2 fixation by subterranean clover appears to be enhanced by elevated CO_2, but decreased by warmer temperatures. Total N yield of grazed herbage may be slightly higher for subterranean clover-phalaris mixtures, although a warming of 3–4°C may negate this advantage to the N economy of the pasture. Longer-term studies are required to determine whether higher soluble carbohydrate content of phalaris stem bases leads to greater persistence of phalaris in pastures of the region.

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