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Defining Climate Adaptive Forage Traits and Genetic Resources – Final Report on MPI Contract AGR30811

November 2014

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Report prepared for MPI

November 2014

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1. Executive Summary

Responses to elevated CO_2 were recorded for eight perennial ryegrass cultivars grown in the FACE site at Flock House. Genomic analyses were carried out on ryegrasses from the FACE treatment rings and the surrounding pasture. Growth of the ryegrasses was not changed under elevated CO_2 but flowering dates were altered substantially. Early-flowering types flowered earlier than expected and later types were later than expected. Concentrations of the endophyte alkaloid epoxy-janthitrem were increased markedly with higher CO_2. The potential risks to animal health imposed by elevated epoxy-janthitrem will need to be managed through plant breeding in future cultivars. None of the other endophyte alkaloids measured were changed by the CO_2 treatment. The genomic analyses showed very little genetic variation in the old pasture ryegrass population at the FACE site after 17 year's exposure to elevated CO_2. This indicates that breeding for ryegrass performance in future CO_2 conditions should initially be very widely based to optimise adaptation.

2. General Introduction

Perennial ryegrass is the most important component of New Zealand's low-cost forage supply to pastoral farming, and there is a long history of plant breeding to improve its adaptation and productivity as farming systems have evolved (Hunt & Easton 1989, Stewart 2006). This cultivar development has always been conducted under contemporary ambient CO_2 concentrations.

Perennial ryegrass genotypes acclimated to enriched CO_2 typically show improved response to increasing CO_2 (Newton et al. 2001). There is very little evidence available of the potential for genetic adaptation to increased CO_2 within this species, and consequently no strategy for enhancing the potential for positive yield responses in the mid and long-term future under higher CO_2 regimes.

The present genetic resource base used in New Zealand to develop ryegrass cultivars is comparatively narrow. It may not therefore be the optimum source for future cultivar development to best realise the potential benefits afforded by CO_2 enrichment. Understanding how elevated CO_2 (and related system changes) impact on ryegrass at the genetic level will enable development of future plant breeding strategies.

This short term project addressed Impacts of Climate Change and Adaptation, Research

Theme 1 by carrying out underpinning research identifying potential land-based business opportunities for adaptation, Priority 4. The specific aims were to test the genetic potential for perennial ryegrass adaptation to elevated atmospheric CO_2

concentrations and to assess whether there is sufficient variation within this species to sustain the profitability of low cost locally produced feed systems under future climates.

3. Experimental

3.1 Effects of elevated CO_2 on ryegrass growth and endophyte metabolites

Introduction

There is strong evidence that perennial ryegrass responds to increased CO_2 by increasing its photosynthetic rate, accumulating greater dry weight (DW), and decreasing its crude protein concentration (Isopp et al. 2000, Ryle et al. 1992). The effect of elevated CO_2 concentrations on the *Neotyphodium* endophyte symbiosis in perennial ryegrass has been much less studied. Marks & Clay (1990) reported that CO_2 enrichment did not alter the interactions between perennial ryegrass and its endophytic fungal symbionts. More recently it has been shown that endophyte infected (E+) ryegrass had higher carbohydrate levels than endophte free (E-) plants, and the difference was greatest in ambient CO_2 conditions (Hunt et al. 2005). E- plants had 40% lower concentrations of soluble protein under elevated CO_2 than under ambient CO_2 but this endophyte effect was largely absent in E+ plants. Concentrations of the endophyte alkaloids peramine and ergovaline declined with increasing nitrogen supply under ambient CO_2 but remained roughly constant across nitrogen levels at elevated CO_2.

In this experiment the effect of elevated CO_2 on growth and endophyte metabolites was tested for different ryegrass populations in a field experiment.

Methods

The experiment was conducted at the Free-air CO_2 Enrichment (FACE) site at Flock House, near Bulls in the Rangitikei. The site consists of six experimental 12m diameter areas that are exposed to either ambient CO_2 or the CO_2 concentration expected in 2050 (500 ppm), without any enclosure. A further description is provided by Newton et al. (2006). Seven ryegrass cultivars and one breeding line (collectively referred to as cultivars) were used (Table 1).

Table 1. Cultivar name, ploidy status, endophyte strain and heading date classification of the experimental ryegrasses

Cultivar	Ploidy	Endophyte	Heading date
Alto	2n	AR37	Late
Avalon	2n	AR1	Late
Banquet II	4n	Endo 5	Late
Bealey	4n	NEA2	Late
Commando	2n	AR37	Early
GA194	2n	AR37	Mid
One50	2n	AR37	Late
Trojan	2n	NEA2	Late

Plants were raised from endophyte infected seed, and when they had about 10 tillers they were split into two clonal replicates. One clonal replicate was converted to endophyte free status using fungicides (Latch & Christensen 1982), and the endophyte status of all the material was confirmed by immunodetection (Simpson et al. 2012). Both the E+ and E- plants were cloned further to provide the experimental material. Plants with approximately 20 tillers were transplanted into bare soil within each FACE ring, on 15/07/2013, in a completely randomised row-column design at 350 mm spacings. The design was optimised to avoid clonal replicates from appearing twice in the same column or row. Each plant was planted in a 200mm deep × 110 mm diameter PVC pipe to restrict lateral movement of nutrients. The rings were irrigated at a rate of 10 mm/ring each morning using a computer-controlled sprinkler installed in the middle of each ring.

At planting, phosphate was applied to the plants as a solution of KH_2PO_4 at a rate equivalent to 0 or 35 kg P ha^{-1} and all plants received urea solution equivalent to 50 kg N ha^{-1}. From 03/09/2013, the fertiliser treatments were repeated at 56 day intervals.

Ryegrass shoot dry weights (DW) were recorded on 11 occasions between 02/09/2013 and 29/07/2014, by clipping, oven drying and weighing. Data were analysed over all harvests using REML for repeated measurements with time. For individual harvests data were analysed using a mixed model anova. Samples for endophyte alkaloid analyses were collected in February 2014. Analysis of samples for lolitrem B, epoxy-janthitrems, ergovaline and peramine were conducted by HPLC following established methods (Rasmussen et al. 2012), on selected cultivar-endophyte combinations.

Results

Analysis of the shoot DW data over all harvests revealed no significant effect of CO_2 concentration, but highly significant (P<0.001) effects of cultivar type, phosphate treatment and endophyte status (Table 2). The only significant interaction among the main effects was for cultivar × endophyte status (P<0.001). Six of the ryegrasses showed improved growth in the presence of endophyte, but for GA194 and Trojan, the E+ plants were smaller on average.

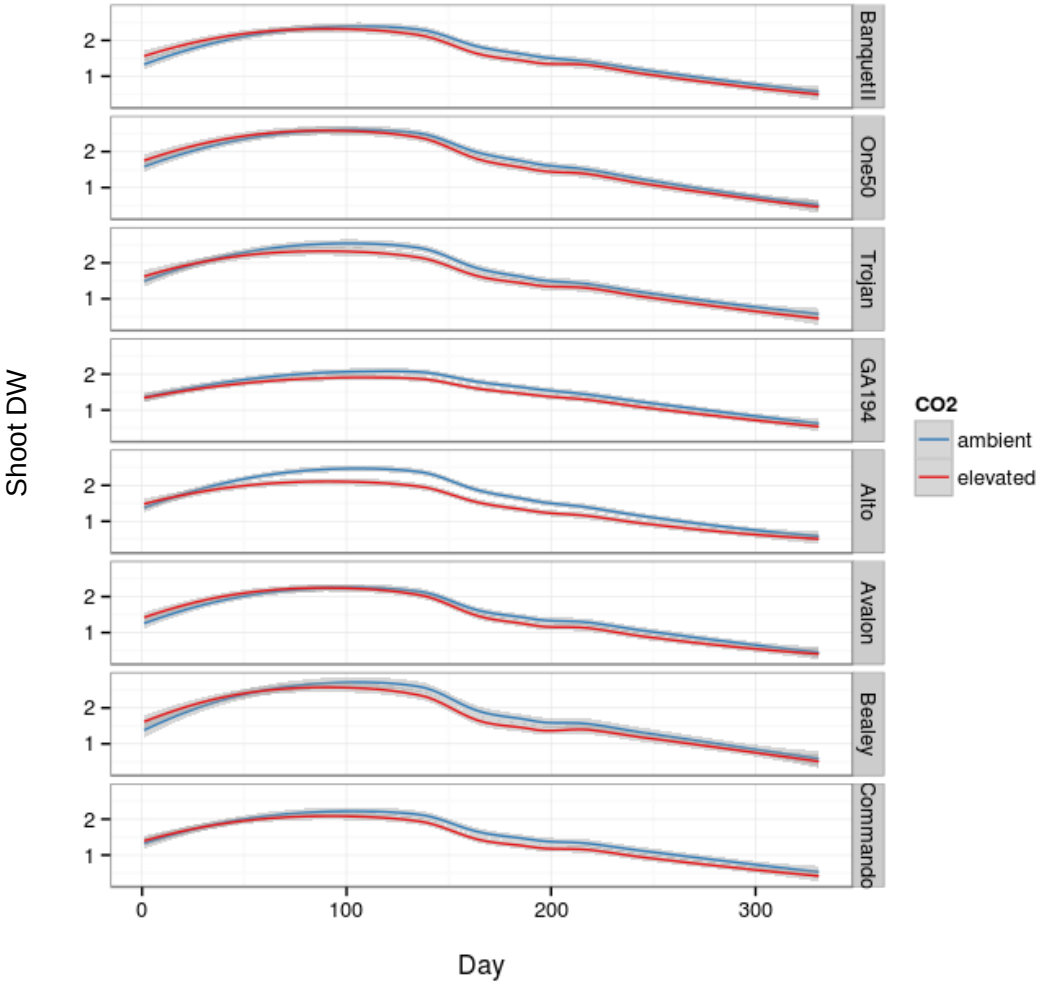

Figure 1. Shoot DW (log g plant^{-1}) for eight perennial ryegrasses grown at ambient and elevated CO_2 commencing on 2/09/13

Table 2. Effect of experimental variables on average shoot DW (g plant^{-1}) over all harvest dates.

Cultivar	DW	CO_2	DW	Phosphorus	DW	Endophyte	DW
Alto	0.25	398	0.30	0	0.231	E+	0.22
Avalon	0.16	500	0.20	35	0.30	E-	0.29
Banquet	0.28						
Bealey	0.33						
Commando	0.16						
GA194	0.24						
One50	0.33						
Trojan	0.27						
SED	0.03		0.10		0.01		0.01

Analysis of the individual harvest date data for shoot DW showed that the CO_2 effect was significant ($P<0.05$) on only one of the 11 dates (Table 3) and cultivar effects were consistently highly significant. The phosphate treatments did not differ for the first two harvests but differed at $P<0.05$ for harvest three and four, and at $P<0.001$ for the remaining harvests. There were significant differences in shoot DW between the E+ and E- plants at all harvests except the second one.

Table 3 Significance levels for treatment effects on plant dry weight at 11 harvest dates.

Table 3:

Date	CO_2	Cultivar	Phosphorus	Endophyte
02-09-13	ns	0.001	ns	0.01
30-09-13	ns	0.001	ns	ns
18-12-13	ns	0.001	0.05	0.01
15-01-14	ns	0.001	0.05	0.001
12-02-14	ns	0.001	0.001	0.001
11-03-14	ns	0.001	0.001	0.01
08-04-14	ns	0.001	0.001	0.01
06-05-14	ns	0.001	0.001	0.01
03-06-14	0.05	0.001	0.001	0.01
01-07-14	ns	0.001	0.001	0.001
29-07-14	ns	0.001	0.001	0.01

There were significant effects of CO_2 level on total epoxy-jathitrems in the ryegrasses, and the individual epoxy-janthitrems except for epoxy-janthitrem IV (Table 4, Table 5). There was no effect of CO_2 concentration on peramine, lolitrem B or ergovaline. There were significant differences among the ryegrass cultivars for all the alkaloids, but no cultivar × CO_2 interactions. The phosphorus treatment had no effect on alkaloid concentrations.

Table 4. Significance levels for main treatment effects on endophyte alkaloid concentration (ppm) of perennial ryegrasses.

Alkaloid	CO_2	Cultivar	Phosphorus
Total epoxy-janthitrems	0.01	0.001	ns
Epoxy-janthitrem I	0.001	0.001	ns
Epoxy-janthitrem II	0.05	0.001	ns
Epoxy-janthitrem III	0.01	0.001	ns
Epoxy-janthitrem IV	ns	0.01	ns
Epoxy-janthitrem V	0.05	0.001	ns
Peramine	ns	0.001	ns
Lolitrem B	ns	0.05	ns
Ergovaline	ns	0.001	ns

Table 5. Effect of CO_2 concentration on total epoxy-janthitrem content (ppm) of Alto, Commando and GA194 perennial ryegrasses.

CO_2	Total epoxy-janthitrems					
	Alto	SEM	Commando	SEM	GA194	SEM
398	7.76	2.06	13.65	2.05	14.49	2.06
500	14.32	2.06	20.53	2.10	19.25	2.06

Discussion

The lack of any effect of CO_2 concentration on ryegrass shoot growth has been previously reported (Clark et al. 1997). For ryegrass, the magnitude of the growth response to elevated CO_2 can be limited by the supply of available soil nitrogen N (Daepp et al. 2001). In the present experiment the fertiliser N inputs were equivalent to 50 kg N ha^{-1} applied every eight weeks. The total N input over the course of the

experiment was equivalent to 350 kg N ha^{-1} so it seemed unlikely that N supply limited the plants' responses to elevated CO_2. However the daily irrigation at the rate of 3650 mm yr^{-1} in addition to 900 mm rainfall may have leached significant quantities of N and other mobile nutrients below the rooting zone, resulting in conditions that were less nutrient-rich for plant growth than the inputs might suggest. This may explain the very limited growth response to elevated CO_2.

The shoot DW yields were significantly higher in the plus P treatments but the response required three applications of P before it was statistically significant. It has been suggested that future farming systems under elevated CO_2 may require greater inputs of P fertiliser to prevent yield suppression and to stimulate any response to N (Gentile et al. 2012). The total fertiliser P input was equivalent to 245 kg P ha^{-1} which is high by New Zealand standards. The results do suggest that the P status of soils could be an important factor determining the growth response of ryegrass in future farming under elevated CO_2.

The effects of endophytes on shoot DW were relatively small and varied between harvest dates. This suggests that the plants were not under significant pressure from insect herbivory, and the irrigation treatment meant they were not under moisture stress. Under these circumstances we would not expect to see major effects of endophyte on plant performance (Young et al. 2013).

The lack of response of lolitrem B, ergovaline and peramine concentrations to elevated CO_2 confirms previous research (Hume et al. 2004). The substantial rise under elevated CO_2 in total epoxy-janthitrem concentration of Alto, Commando and GA194 ryegrasses infected with AR37 endophyte was unexpected. It is thought that variation in endophyte alkaloid concentrations is largely the product of variation within the plant in endophyte mycelial mass (Ball et al. 1995, di Menna & Waller 1986). In this experiment the concentrations of peramine, lolitem B and ergovalene did not change between the CO_2 treatments, suggesting that the endophyte mycelial mass was also unchanged. If this is the case, the epoxy-janthitrem results suggest that the biosynthesis of this metabolite is being directly influenced by some aspect of the host plant's biochemical response to elevated CO_2. The increased levels of epoxy-janthitrems under elevated CO_2 would provide additional protection from herbivory by some insect pests but a greater risk of the occurrence and severity of ryegrass staggers. It will be important to confirm the CO_2 effect on epoxy-janthitrems in ryegrass, and determine its cause, so that breeders can select the best endophyte/host combination for future pastures.

3.2 Effects of elevated CO_2 on heading date in perennial ryegrass

Introduction

Ryegrass heading date is an important agronomic trait for farmers because the change from vegetative to reproductive growth is associated with a decrease in nutritive value for grazing animals. Daylength and temperature are the main controllers of the switch to flowering in temperate species. Elevated CO_2 has been shown to advance flowering in some species, and delay it in others (Springer & Ward 2007). The only reported research with ryegrass showed that elevated CO_2 delayed flowering in annual ryegrass (*Lolium multiflorum*) (Cleland et al. 2006).

Methods

Floral characteristics were measured on the cultivars in the experimental system described above. Heading date was monitored from the time of appearance of the first reproductive tillers until the majority of the plants had reached full maturity. Plants were checked every second day for seed head emergence. The date when there were three seed heads per plant was recorded as the heading date.

Results

There was a significant ($P<0.001$) cultivar x CO_2 level interaction for the time of heading. The cultivars that headed earlier under ambient CO_2 tended to head even earlier under elevated CO_2, but the later heading cultivars at ambient delayed heading further under elevated CO_2. This increased the span of heading dates across the cultivars from about 10 days to 26 days.

Discussion

This is the only research we are aware of that examined the effect of elevated CO_2 on flowering date of a range of germplasm within a forage grass, and the observation that flowering date could be advanced or delayed depending on the cultivar is unique. Heading date is a heritable trait in perennial ryegrass and cultivars are bred with different heading dates to suit different farm systems and climates. The increase in the span of heading dates recorded is very substantial by normal breeding standards.

Choice of cultivars with different heading dates is important for farmers depending on the regional environment and farming system. For example, early heading is advantageous in regions where early summer rainfall is low, and late heading is beneficial in irrigated systems. The broadening of the range of heading dates in response to elevated CO_2 that was recorded in this study will provide additional possibilities for cultivar choice to suit specific environments in future faming systems.

Publication

Maw BR, Jones CS, Newton PCD, Hatier J-HB 2014. Elevated atmospheric CO_2 alters heading date of perennial ryegrass. Proceedings of the New Zealand Grassland Association 76: 217-220.

3.3 Genotypic variation in perennial ryegrass after long-term exposure to elevated CO_2

Introduction

FACE site field trials with natural ecotypes of the model plant species *Arabidopsis thaliana* indicate within-species genetic variability in plant responses to elevated CO_2, suggesting the potential for adaptation in some species from standing genetic variation (Li et al. 2006, 2008). Evolutionary responses to variation in CO_2 are thought to have occurred over geologic timescales; however, as yet no strong evidence has been found for genetic changes within plant species in response to either the global increase in CO_2 since the industrial revolution (~200 years) or to experimental manipulation of CO_2 levels (Gienapp et al. 2008, Leakey & Lau 2012).

In an effort to characterise the effects of elevated CO_2 on pastoral agricultural systems, AgResearch have been running a FACE trial site since 1997. As a major temperate grassland species globally, *L. perenne* is a well-characterised C3 grass in terms of its acclimation response to changes in atmospheric CO_2. Phenotypic changes include stimulation of higher photosynthetic rates (Ainsworth et al. 2003), variation in metabolite allocation in interaction with endophyte and nitrogen availability (Hunt et al. 2005), and changes in root biomass (Suter et al. 2002). Notably, in common garden experiments *L. perenne* plants collected as seed from enriched CO_2 rings at the FACE site show an increase in potential soil nitrification rate compared with plants raised from seed collected from the ambient CO_2 rings (Bowatte et al. 2013). One possible explanation for

this observation is that genetic differentiation of *L. perenne* has occurred in the CO_2 treatment plots (Bowatte et al. 2013).

Here, we take a population genetics approach, using both traditional microsatellite genotyping and genotyping by sequencing (GBS), to investigate whether perennial ryegrass exhibits changes in genotypes grown under enriched CO_2 over the sixteen-year FACE experiment. Genotyping by sequencing (Elshire et al. 2011) and related, high throughput, high marker density technologies (Davey et al. 2011) are an emerging tool in population genetic analyses, with the potential to greatly increase our power to detect weak population structure and genetic structure at fine scales (Narum et al. 2013). A second, independent aim was to investigate how genetic structure varies within pasture at the microsite-scale.

Such information can be of valuable assistance in developing cultivars suited to the challenges posed by our predicted future climate, and can aid in understanding how pastures develop and persist over time. Addressing these aims stands to directly benefit NZ's pastoral economy, but may also shed light on how - and how quickly - natural systems might adapt to climate change, tackling more fundamental questions in evolutionary and conservation genetics.

Methods

Plant Collection

Fresh ryegrass leaf tissue was collected from the FACE site at Flock House. The FACE site was established on naturalized grass pasture in 1997; prior to establishment, the pasture had been in constant grass since at least 1940 with grazing by sheep, cattle and goats (Edwards et al. 2001).

Within-plot and pasture-wide collections were undertaken over two stages in May and September 2013. The initial collection, over three days in May 2013, included 48 individuals from each of the six circular plots within the FACE site pasture (288 individuals, total). Three plots represented ambient atmospheric CO_2 plots and three represent enriched atmospheric CO_2 plots. Individuals were collected in a grid arrangement such that collections within the plots were made at least 1.5m apart. A pasture-wide collection of 279 individuals was made in September 2013. These individuals were collected from across the majority of the FACE site following an 8m x 8m grid layout, and included additional sampling of 2-6 individuals within each of the three ambient CO_2 control and three enriched CO_2 treatment plots.

DNA Extraction and Genotyping

50mg of fresh leaf tissue was arrayed into Corning 1ml 96-well plates and freeze-dried prior to DNA extraction. Extractions were carried out at AgResearch (Grasslands campus) following a modified Whitlock DNA extraction protocol (Whitlock et al. 2008). Briefly, frozen tissue was ground using 3.2mm stainless steel beads in a Qiagen TissueLyser. A sodium sulphite/SDS homogenization buffer containing proteinase K and RNase A was added, and samples were incubated for one hour at 55^{o}C. An ammonium acetate/acetic acid precipitation buffer was then added, samples mixed and cooled on ice for 15 minutes, and then centrifuged for 30 minutes. The supernatant was mixed with guanidinium chloride binding buffer and applied to a filter plate, which was then centrifuged to bind the DNA. The filters were washed three times, using the guanidinium chloride binding buffer, followed by a NaCl/Tris/ethanol wash buffer, and finally with 100% ethanol. Finally, DNA was eluted from the membrane using a 10mM tris solution. DNA quantity was determined using PicoGreen fluorescence. DNA quality was tested using overnight incubations at 37^{o}C in TE and high salt buffers, and by digestion with the restriction enzyme *Hind*III. 30µL of each sample was shipped to the Institute for Genomic Diversity at Cornell University for genotyping by sequencing (GBS) library preparation and sequencing, and the remainder stored for microsatellite marker genotyping.

Genotyping by Sequencing

The methylation-sensitive restriction endonuclease *Pst*I was used for reduced-representation library construction, following the method of Elshire et al. (2011). A barcode of between five and eight unique nucleotides was ligated to the digestion products of each sample, using a set of 96 unique barcodes to identify individual samples within the plate. 96 samples were then pooled together and run in a single lane of an Illumina HiSeq run, generating approximately 200,000,000 short sequence reads per run.

Microsatellite marker genotyping

A second genotype dataset was generated for all ryegrass samples using a set of 15 multi-allelic microsatellite markers derived from an initial screen of 20 markers, and selected based on distribution across linkage groups, amplification robustness, allele size and number. Microsatellite genotyping was performed using an M13 primer-tailing polymerase chain reaction scheme; for details, see Bloomer et al. (2014). Briefly, microsatellite markers were amplified by PCR using marker-specific forward and reverse primers, with an M13-tail sequence on the forward primer. One of three fluorescently-labelled complementary M13 primers was used to tag each marker; three markers from the same individual, each tailed with a different fluorescent label, were then pooled for

screening by capillary separation on an ABI3730 sequencer at the Massey Genome Service.

Data analysis

Genotyping by Sequencing

In total, 558 individuals (279 within-ring individuals and 279 pasture-wide individuals) representing six lanes of Illumina run data were submitted for genotyping-by-sequencing. The data were initially processed at AgResearch to take advantage of the draft reference genome for ryegrass, which is not yet publically available. The draft reference genome comprises some 10,000 sequence contigs; for the purposes of the GBS pipeline, these were ordered into thirteen pseudo-"chromosomes" based on homology with the rice (*Oryza sativa*) genome, with a string of 100 'N' nucleotides as padding between sequence contigs.

Initial SNP genotypes were generated using the Tassel4 Discovery Pipeline (Glaubitz et al. 2014). Briefly, sequence tags were processed to remove barcodes and assign to individuals, then merged into a master Tag Count file, with a given tag being observed at least five times across the six plates to be included. Unique tags were aligned against the ryegrass reference genome using BWA to build a Tags On Physical Map file (TOPM). All tags in the original fastq files were then used to call SNPs, with a given SNP requiring a minor allele frequency of 0.01 and presence across a minimum 10% of individuals to be included. Duplicate SNPs (arising from tags on both the forward and complementary DNA strand) were merged with the callHets option invoked, and a mismatch tolerance of zero.

Secondary filtering was performed using vcftools v0.1.12 (Danecek et al. 2011). Individuals with greater than 10% missing data or where contamination during DNA preparation was suspected were removed from the dataset; allele scoring in the microsatellite dataset (below) were also used to inform the selection of individuals. Genotypes were filtered to remove all allele calls with a genotype quality score of <98%, and loci were filtered to ensure all were biallelic with a minor allele frequency greater than 0.01 and no more than 10% missing data. Loci with greater than 70% heterozygosity were removed as they may represent misaligned, paralogous reads. Finally, a thinning step was performed to ensure SNPs were no closer than 32bp apart; as such, no more than two SNPs were retained for any given tag. Following an initial round of data analysis, we identified one plate with a strong genetic differentiation signal based on Illumina flowcell; these individuals were subsequently pulled from the analyses to avoid biasing the results. Ultimately, this resulted in a dataset of 406 individuals and 7007 SNPs for subsequent analyses.

Microsatellite marker filtering

Microsatellite marker quality analysis and allele calls were made using Genemapper v3.7 (Applied Biosystems). Ambiguous allele calls were coded as missing data. Individuals with more than two alleles at a given locus were removed from the analysis as suspected contamination, and individuals with missing data at three or more microsatellite loci were removed before subsequent analyses. Individuals removed from the GBS dataset for reasons of suspected contamination or missing data were also removed from the microsatellite dataset in order to maximize the overlap between datasets; however, those individuals pulled from the GBS dataset due to suspected variation in Illumina run quality were maintained within the microsatellite dataset to maximise available information about genetic variation across the wider pasture. Ultimately, 473 individuals (Table 6) were genotyped in the microsatellite dataset.

Table 6. Number of samples genotyped with GBS (7007-SNP dataset) and microsatellites

Population	**Treatment**	**Individuals genotyped**	
		GBS	**Microsatellite**
R1	Enriched CO_2	44	43
R2	Ambient CO_2	45	45
R3	Enriched CO_2	30	27
R4	Ambient CO_2	45	45
R5	Enriched CO_2	45	45
R6	Ambient CO_2	29	27
Pasture	NA	168	241
Total		**406**	**473**

Conversion of SNP data from a VCF format to population genetics software-specific file formats was performed using PGDSpider v2.0.7 (Lischer & Excoffier 2012) and GenAlEx v6.5 (Peakall & Smouse 2012). Population pairwise F_{ST} and AMOVA were calculated in Arlequin v3.5 (Excoffier & Lischer 2010) for both the 7007-SNP and microsatellite datasets. Samples were partitioned and tested for genetic differentiation in several ways (Table 7).

Table 7. Data partitioning for population genetic analyses

Partition	Sample partition/population definitions for F_{ST} and AMOVA analysis
A	Each ring (1-6) and pasture-wide samples defined as seven separate populations; ambient CO_2 rings, enriched CO_2 rings and pasture-wide samples hierarchically defined as three separate groups
B	Rings (1-6) defined as six separate populations; ambient CO_2 rings and enriched CO_2 rings hierarchically defined as two separate groups
C	Each ring (1-6) and pasture-wide samples defined as seven separate populations; ambient CO_2 rings; rings and pasture-wide samples defined as two separate groups

STRUCTURE analyses

The GBS dataset was analyzed using fastSTRUCTURE (Raj et al. 2014) using the simple prior analysis option, for values of K (number of ancestral populations inferred) from one to eight. fastSTRUCTURE uses the admixture and independent loci assumptions of STRUCTURE (Pritchard et al. 2000) for inference. The Python script whichK.py was used to determine the values of K best describing the dataset.

The microsatellite dataset was analyzed with STRUCTURE v. 2.3.4 (Pritchard et al., 2000). Analysis settings consisted of: Admixture; Allele frequencies correlated; Infer alpha with an initial value of 1; Identical Alphas for all populations. The number of ancestral populations inferred (*K*) ranged from one to eight, with 100,000 generations of burn-in and 3,000,000 generations of data collection. Five analytical replicates of each *K* value were run.

Correlation of allele frequencies

To explore the possibility of genetic differentiation between individuals in elevated CO_2 vs. control plots explicitly, allele frequencies for each locus were compared for all individuals from the three elevated CO_2 rings vs. all individuals from the control rings. The frequencies of each allele in each treatment were plotted against one another to identify alleles at significantly different frequencies in the two treatments. Ultimately, allele frequencies were sorted by locus to reveal the most extreme allele frequency differences between the two treatments.

Results

Population differentiation

Pairwise F_{ST} and an analysis of molecular variance (AMOVA) were calculated in Arlequin v3.5 (Excoffier & Lischer 2010) for both the 7007 SNP dataset and the 15-microsatellite dataset. Pairwise F_{ST} values were calculated between each of the six individual treatment plots, and between each treatment plots and the wider pasture sample (Table 8); average pairwise F_{ST} was also calculated for each ring. In addition, pairwise F_{ST} and AMOVA were calculated comparing all individuals in the ambient and enriched CO_2 treatments, and comparing individuals collected within treatment rings with those individuals collected in the pasture-wide sample (Table 9). Significance of the F_{ST} values obtained was determined via permutation test. For both SNP and microsatellite marker datasets, most pairwise F_{ST} values obtained were statistically significant; however, all values were exceptionally low and are not likely to indicate meaningful differentiation either between individual treatment plots or between aggregate treatments and the wider pasture sample.

Table 8. Pairwise F_{ST} by plot in the 7007-SNP dataset and the microsatellite dataset (bracketed)

		Enriched			Ambient			Pasture
		R1	R3	R5	R2	R4	R6	
Enriched	R1	0						
	R3	0.00737* (0.00348)	0					
	R5	0.00535* (0.00223)	0.00255* (-0.000)	0				
Ambient	R2	0.00601* (0.00674*)	0.00359* (0.00434)	0.00134 (0.00309)	0			
	R4	0.00525* (0.00813*)	0.00349* (0.00351)	0.00215* (0.00302)	0.00310* (0.00637*)	0		
	R6	0.00845* (0.00571)	0.00137 (-0.00181)	0.00522* (0.00746*)	0.00562* (0.00446)	0.00537* (0.01012*)	0	
Pasture		0.00793* (0.00304)	0.00332* (-0.00116)	0.00465* (0.00102)	0.00596* (0.00431*)	0.00561* (0.00328)	0.00380* (0.00363)	0
Average Pairwise F_{ST} §		0.00673 (0.00489)	0.00362 (0.00139)	0.00354 (0.00280)	0.00427 (0.00489)	0.00416 (0.00574)	0.00497 (0.00493)	0.00521 (0.00235)

* Denotes F_{ST} significant at $p<0.05$ after 110 permutations

§ Calculated by averaging F_{ST} values across all pairwise comparisons for a given ring (or pasture)

Similarly, the AMOVA results suggest very little genetic variation, if any, distinguishes the treatments (Tables 9 and 10). SNP genotyping data analysis indicates that just 0.03% of genetic variation was found at the between-treatment level (Table 9) when considering all samples (data partition A, Table 7), while 95.77% was found within individuals and 3.81% among individuals within specific treatment plots. A similar result is found with the microsatellite dataset, with 91.29% variation found within individuals and 8.48% among individuals within treatment plots; -0.08% of variation is found at the between-treatment level (negative values are an artefact of the analysis and are equivalent to 0). Comparing ambient and enriched CO_2 treatments (data partition B, Table 7), no variation within the 7007-SNP dataset distinguishes the treatments (Table 10). Similarly the ring-sampled individuals are distinguished from the pasture-wide sampled individuals (data partition C, Table 7) by only 0.14% of variation in the 7007-SNP dataset (Table 10).

Table 9. Analysis of molecular variance (AMOVA) by plot for the 7007-SNP and microsatellite datasets

	Percentage of variation Data partition A	
Source of variation	**7007-SNP**	**Microsatellite**
Among treatments (Enriched, Ambient, Pasture-wide)	0.05	-0.08
Among plots within treatments	0.42	0.31
Among individuals within plots	3.91	8.48
Within individuals	95.61	91.29

Table 10. AMOVA and pairwise F_{ST} by CO_2 treatment and by plot in the 7007-SNP dataset and the microsatellite dataset (bracketed)

Source of variation	**Percentage of Variation**	
	Ambient v Enriched	Pasture v All Rings
	Data partition B	Data partition C
Among groups	-0.07 (0.04)	0.14 (-0.13)
Among populations within groups	0.43 (0.32)	0.38 (0.33)
Among individuals within populations	4.00 (9.38)	3.91 (8.49)
Within individuals	95.64 (90.25)	95.57 (91.31)
Pairwise F_{ST}	0.00089*	0.00368*

* Denotes FST significant at p<0.05 after 110 permutations

A Principal Coordinates Analysis (PCoA) based on genetic distance was performed in GenAlEx for both the 7007-SNP and microsatellite datasets. In the 7007-SNP dataset (Figure 2) the first two principal coordinates explained only 1.45% and 0.97% of variation, respectively. Minimal clustering of individuals, primarily from within the rings located along the south-eastern edge of the pasture, was observed but no clustering of individuals by plot or treatment was identified. The first two principal coordinates of the microsatellite dataset PCoA explained slightly more variation, at 4.66% and 3.70% for components 1 and 2 respectively, although again no clustering by plot or treatment was observed.

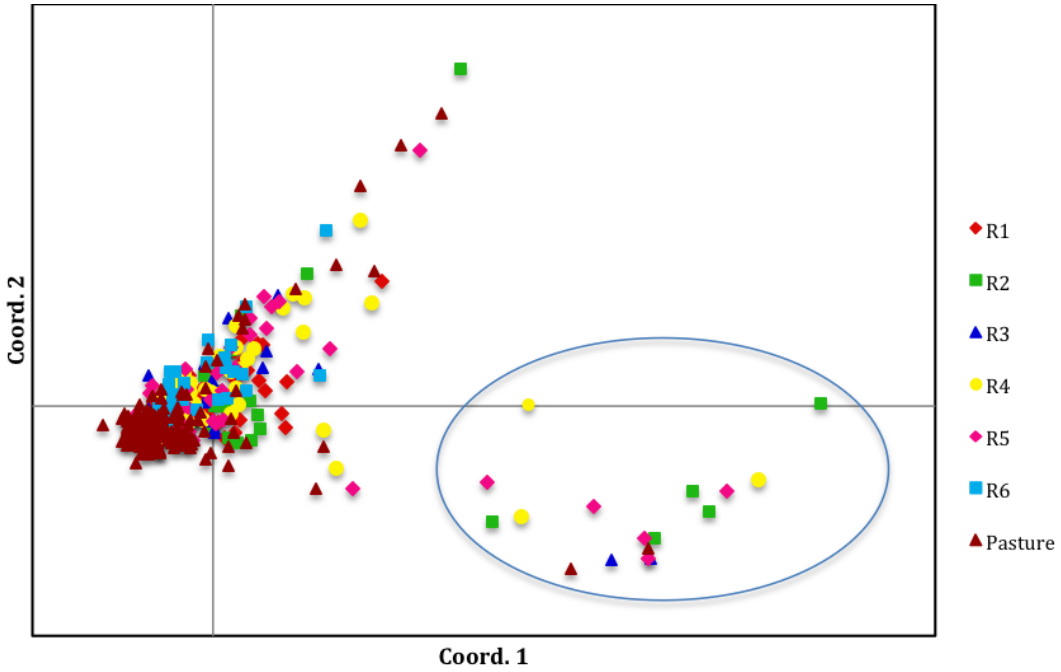

Figure 2: Principal Coordinates Analysis (PCoA) based on genetic distance between all individuals in the treatment ring (R1-R6; R1, R3, R5 ambient CO_2, R2, R4, R6 enriched CO_2) and pasture-wide (Pasture) collections, using the 7007-SNP dataset. Coordinate 1 (x axis) explains 1.45% of variation, while Coordinate 2 (y axis) explains 0.97% variation. Circled individuals are differentiated from all other samples in the fastSTRUCTURE analysis of 7007-SNP data

Structure analyses were performed with both the 7007-SNP and microsatellite datasets, considering values of K (underlying population groupings) from one to eight. The chooseK function of fastSTRUCTURE identified K=2 and K=3 as best fitting the data for the 7007-SNP dataset. No evidence is found in either the 7007-SNP (Figure 3a) or the microsatellite dataset (not shown) STRUCTURE analysis to support clustering of individuals according to ambient or enriched CO_2 treatment within the pasture and rings.

A pattern at K=3 and K=5 in the 7007-SNP dataset distinguishes the majority of the ring treatment samples, collected and sequenced in June 2013, from pasture-wide sampling completed in October 2013 (this appears to be a by-product of GBS library preparation). At K=2 and K=3 there is weak evidence of clustering of individuals collected within treatment plots from the pasture's southeastern edge (Rings 2, 3, 4, and 5; Figure 3b). At K=4, a cluster of individuals is identified that largely reflects sampling from within Ring 1 (Figure 3b). Interestingly, the microsatellite dataset STRUCTURE results identify no evidence of clustering in any individuals in the pasture; instead, at all values of K, all individuals share an approximately equal genomic contribution from all hypothetical populations.

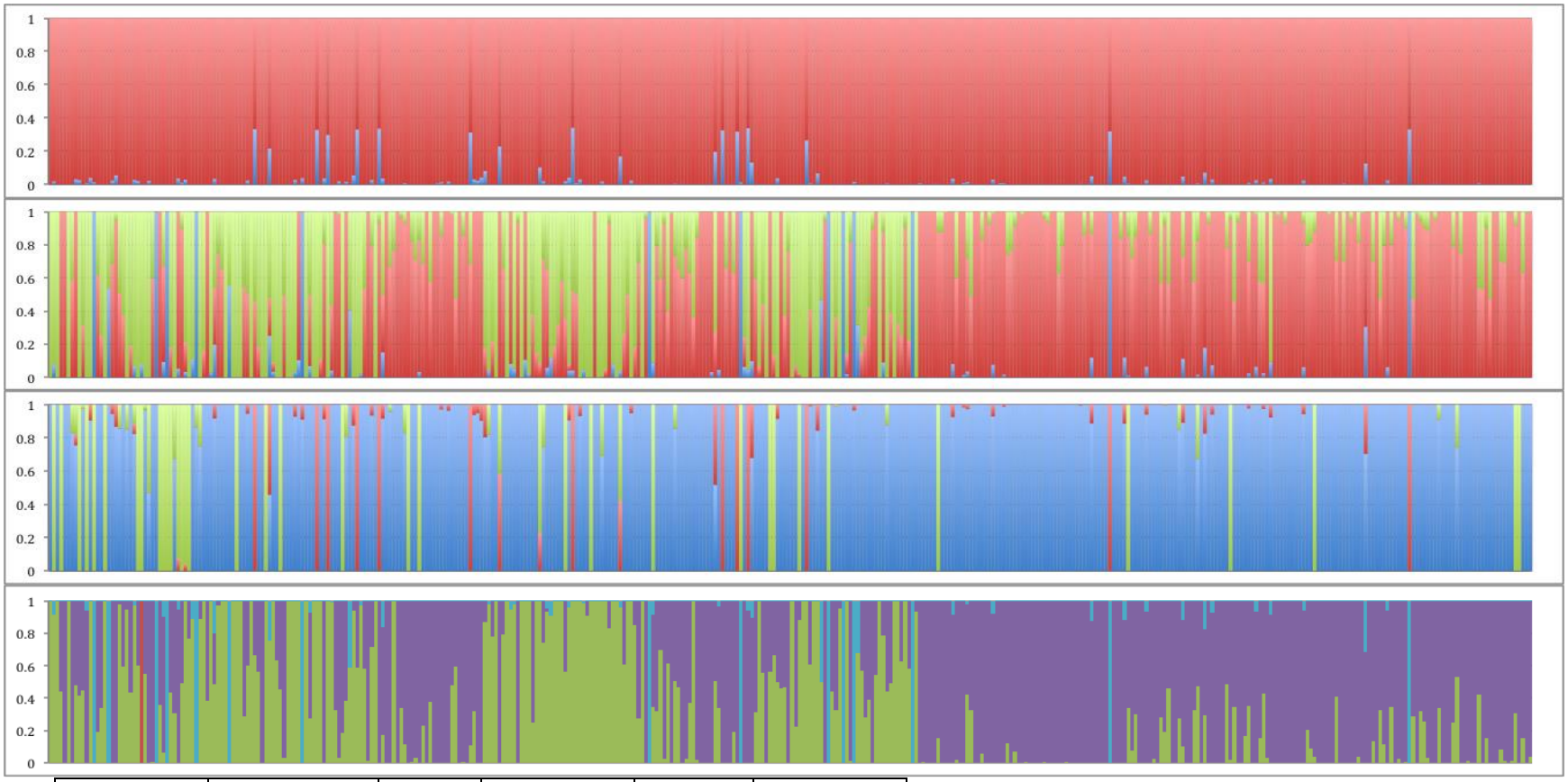

Figure 3a: fastSTRUCTURE analysis of 7007-SNP dataset, showing meanQ plots for K=2-5. Individuals are sorted by collection site (x axis). The fourth grouping at K=4 and fifth grouping at K=5 make no significant contribution to population structure and are negligible on the barplots

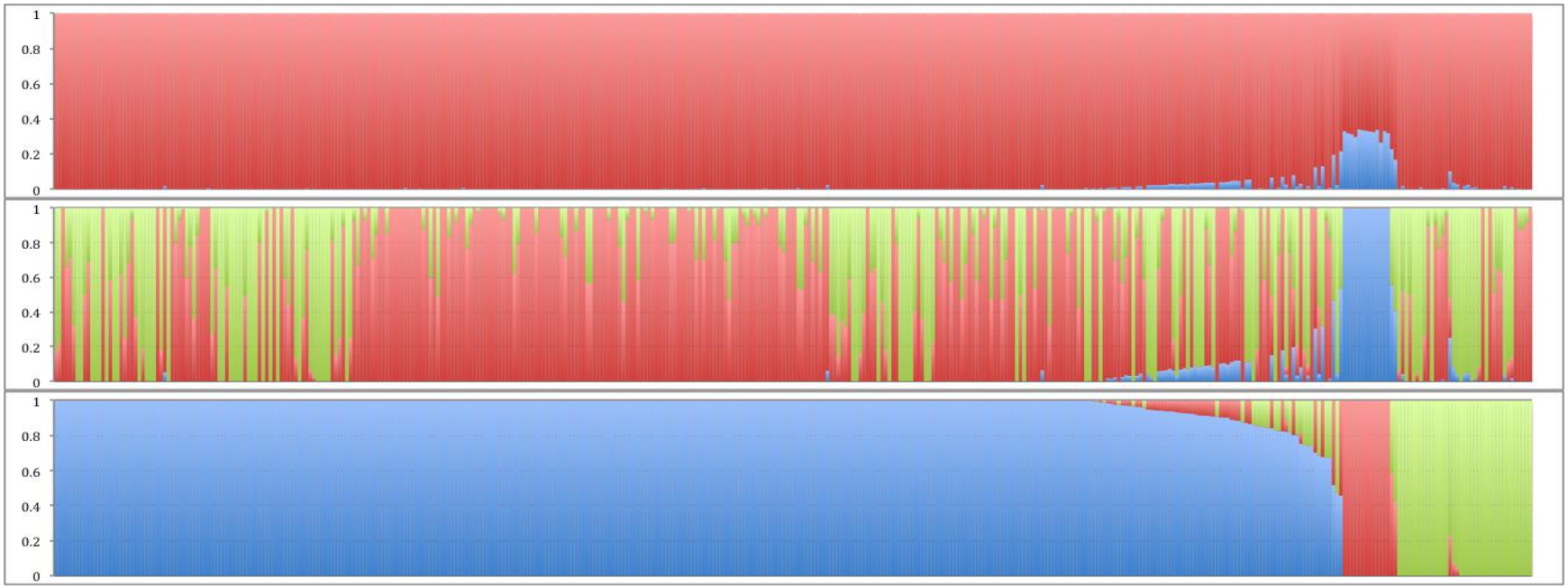

Figure 3b: fastSTRUCTURE analysis of 7007-SNP dataset, showing meanQ plots for K=2-5. Individuals (x axis) are sorted by proportion of population groupings at K=4. Individuals coloured completely red at K=4 were collected primarily from within the rings located along the south-eastern edge of the pasture (Rings 2, 3, 4 and 5), and represent a distinct cluster in the PCoA (Figure 2). Individuals coloured partially or completely green at K=4 are primarily collected from within Ring 1

The 7007-SNP dataset suggests a very weak pattern of isolation by distance across the pasture-wide samples (Figure 4), although this relationship is not significant (P=0.06). In contrast, significant evidence of an inverse relationship between genetic and geographic distance was detected using the microsatellite dataset.

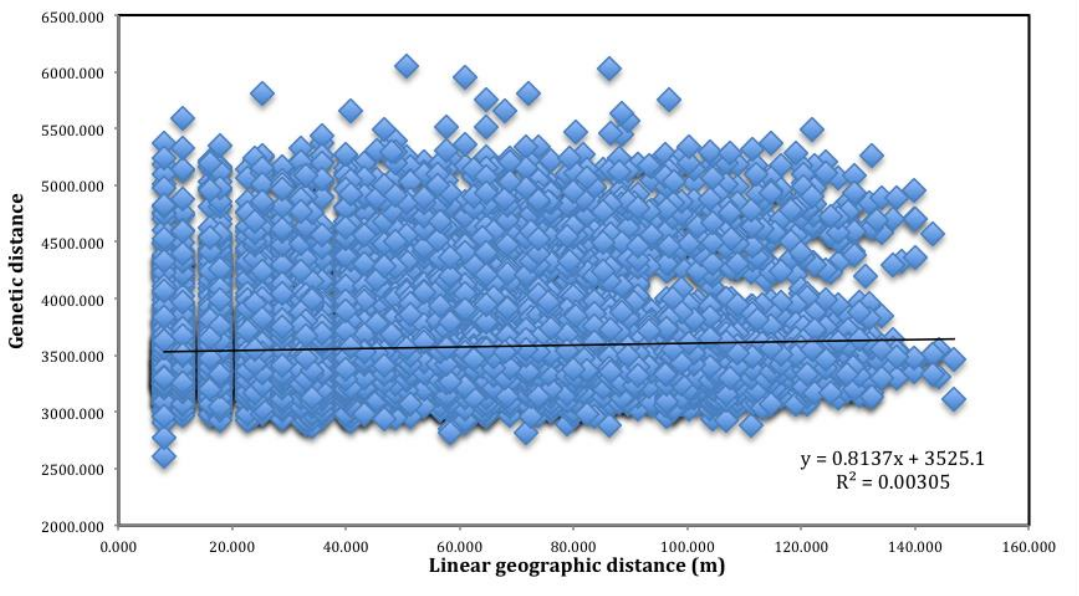

Figure 4. Isolation by distance among the pasture-wide sample

In agreement with all other analyses, allele frequencies were strongly correlated between individuals in elevated CO_2 rings and control rings ($R^2 > 0.99$). The greatest allele frequency difference observed among treatments was less than 0.2. These data further suggest no genetic differentiation in response to elevated CO_2.

Discussion.

Early work at the site identified potential for changes in plant community composition (Edwards et al., 2001), in agreement with findings at other global FACE sites; it is similarly possible that, within *Lolium perenne*, some genotypes may prove better suited to enriched CO_2 conditions. Here, we investigate the possibility that genetic differentiation may have occurred in response to enriched CO_2 over the sixteen-year duration of the FACE experiment. Despite the use of two complementary, high-resolution genotyping approaches, we have identified no evidence of differentiation in response to enriched CO_2 at the FACE site; indeed, there is only limited evidence of genetic differentiation across the pasture as a whole.

Populations do not appear differentiated in response to enriched CO_2

Neither microsatellite data, nor the higher resolution GBS (7007-SNP) dataset, have identified evidence of genetic differentiation within the FACE site in response to enriched CO_2 conditions. While mostly statistically significant, pairwise F_{ST} values between individual treatment rings and between the wider pasture are extremely low, ranging from 0.00137 to 0.00845. For comparison, in a similar, longer-running grassland experiment weak but meaningful differentiation was inferred from pairwise F_{ST} values of around 0.1 (Freeland et al. 2010). Pairwise F_{ST} between all enriched and all ambient CO_2 individuals in the current study is still lower, at 0.00089. Broadly, the AMOVA analysis of enriched and ambient CO_2 rings supports these findings; ~0.43% of molecular variation observed distinguishes rings within a CO_2 treatment in the 7007-SNP dataset, but no genetic variation (-0.07%) distinguishes the CO_2 treatments from one another. Both pairwise F_{ST} and AMOVA analysis indicate that, while individual rings may be very slightly differentiated from one another, no meaningful differentiation exists between enriched and ambient CO_2-grown plants in aggregate. Similarly, neither the fastSTRUCTURE analysis nor the PCoA identify any evidence of clustering of genotypes based on CO_2 treatment.

Such limited genetic differentiation is perhaps unsurprising, in particular given the relatively short duration of the FACE experiment and the limited opportunities for seed set afforded to the pasture under grazing management. Although early work at the FACE site indicated variation in seed set and seedling recruitment for some species under ambient and enriched CO_2 conditions (Edwards et al. 2001), no consistent trend was identified in ryegrass over the two years surveyed. The strength of enriched CO_2 as a selective pressure is also unknown (particularly at the levels present at the FACE site), but may be extremely low or non-existent; as such, changes in either seedset or persistence of particular genotypes may not have occurred or may need a much longer timescale to become apparent. Compounding both limited selective pressure and few, if any, generations of seed set, gene flow (when seed set has occurred) within the pasture has the potential to be relatively high. *Lolium perenne* is an obligate outcrosser, and as it is wind-pollinated, pollen is likely to be dispersed widely. Similarly, movement of stock within the pasture could result in movement of any seeds produced out of rings in which they were produced and into the wider pasture or *vice versa*. Rapid, within-pasture genetic differentiation in the face of high gene flow has been demonstrated previously in *Anthoxanthum oderatum*, but over a considerably longer ~160 year period and with soil fertiliser inputs as the selective pressure (Freeland et al. 2010). Given that we observe no genetic differentiation between ambient and enriched CO_2-grown individuals here, phenotypic differences observed between the enriched and ambient plants under common garden conditions (Bowatte et al. 2013) likely reflect maternal effects rather

than heritable genetic differences. Such effects would be due to phenotypic plasticity within existing genotypes in response to variation in atmospheric CO_2. This highlights the point that the FACE site may or may not possess standing genetic variation for response to elevated CO_2.

While known to have been in pasture and under mixed grazing management since at least 1940, the exact history of the FACE site prior to 1997 is ambiguous. It is thus unclear what ryegrass genotypes, or how much genetic variation, relative to the variation found across the species, may have been present in the pasture at the beginning of the FACE experiment. Particularly if the genetic variation present is fairly limited, it is possible that no variation for CO_2 response is segregating within the pasture. Further analysis of our dataset alongside GBS or microsatellite data for known ryegrass accessions would be of considerable interest as a starting point for understanding both what genotypes are present and how much genetic variation exists within the pasture.

Genotyping By Sequencing detects weak signals of genetic structuring across the wider pasture

While no genetic variation is observed segregating between ambient and enriched CO_2 treatment rings, analyses of the 7007-SNP dataset provide some evidence of genetic differentiation in the wider pasture. In particular, the fastSTRUCTURE and principal components analyses identify a number of individuals, primarily located in rings along the southeastern edge of the pasture, as genetically distinct from the largely homogeneous majority of individuals collected. These individuals remain clustered at all values of K, providing reasonable evidence of meaningful differentiation relative to the wider pasture. These individuals may represent (1) migrants from other pastures, perhaps as a consequence of stock movement between periods of grazing in the pasture, (2) the product of isolated genetic drift or (3) the result of adaptation to microsite variation in the pasture. Again, concurrent analysis of this dataset with known ryegrass accessions may clarify the likely origin of these genotypes and the mechanism(s) of their persistence.

Similarly, at K=4 fastSTRUCTURE identifies a cluster of individuals, primarily those collected within Ring 1, as a genetically distinct group. While not reflected in the PCoA, the individuals identified may account for the nearly twice as high average pairwise F_{ST} found for Ring 1 compared with other individual rings or the wider pasture. While Ring 1 appears to be located on sandier topsoil than the majority of the pasture, weak isolation by distance, as identified using the pasture-wide data collection, could be sufficient to explain the apparent differentiation of Ring 1 relative to others. Notably, Ring 1 is located the greatest distance from any other ring within the pasture. However, the

pasture has been established for approximately 75 years, which may be long enough for genetic differences to arise in response to microsite variations such as water availability or soil type within the pasture or as a consequence of genetic drift.

Genotyping By Sequencing and microsatellite genotyping for the detection of within-pasture genetic variation

A direct comparison of results from both the microsatellite and 7007-SNP datasets clearly highlights the greater resolution afforded by genotyping by sequencing-derived data for studies of within-pasture genetic differentiation. While both microsatellites and SNP data identified a similar degree of population differentiation and broadly similar levels of population hierarchy in the AMOVA, the PCoA and STRUCTURE analyses with microsatellite data were unable to detect the low level variation observed with SNPs. A consequence of this high resolution, however, is the risk of confounding signal due to run-to-run variation in Illumina sequencing. This is likely to be the case particularly in studies where actual genetic structure or differentiation is weak. The signal of sequencing run variation identified here will be analysed further and can inform development of quality control protocols for data handing as AgResearch continues to develop the GBS pipeline for further studies.

As greater interest pours into pastoral genetics, appropriate tools for dissecting current and future issues will need to be identified. Here, GBS, when wielded with considerable caution, has demonstrated its potential utility in detecting even very weak signals of genetic differentiation. Further studies that examine the partitioning of genetic variation within and among pastures, particularly in a cultivar context, hold great promise in aiding cultivar selection, identifying mechanisms that promote or erode genotype persistence, and future-proofing the pastoral industry.

<u>Manuscripts in preparation</u>

Symonds V et al. Pasture genetics: Genotyping-by-Sequencing reveals cryptic genetic variation in *Lolium perenne* (Poaceae) across a mixed management pasture (Theoretical and Applied Genetics)

Symonds V et al. Genotyping-by-Sequencing: data quality and experimental design are of key importance in detecting weak population structure (Molecular Ecology)

4. Project Conclusions

- The substantial increase in epoxy-janthitrems under elevated CO_2 has potentially negative implications for animal health. The response need to be confirmed, and the causative mechanisms elucidated so the potential risk can be mitigated.
- Shifts in heading date of perennial ryegrass under elevated CO_2 will be naturally accounted for in future cultivar development programmes done under the ambient CO_2 conditions at the time.
- The genomic analyses showed very little genetic variation in the ryegrasses within the FACE rings or the wider pasture sample. This suggests that a very wide genepool should be explored for breeding ryegrasses adapted to future CO_2 conditions.

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