

Progress towards delivering high metabolizable energy ryegrass

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Abstract

A method developed at AgResearch to increase leaf fatty acids (FA) and plant growth, called ‘high metabolizable energy’ (HME), could elevate the nutritional quality and productivity of perennial ryegrass-based pastures. The industry strategy has been to perform HME ryegrass field trials offshore to test whether the herbage FA, gross energy (GE) and yield enhancing traits observed in containment translate into field swards. To complement the field-testing programme, growth and nutritional profile of HME miniswards were determined under simulated grazing in containment. The major changes measured in HME miniswards of increased FA (+0.8–1.4 %DM) and GE (+0.3–0.5 MJ/kg DM) and similar herbage dry matter (DM) production, closely mirrored data from field trials with hemizygous HME populations in the Midwest USA. Changes in other HME nutritional components were generally minor, although WSC was reduced late in the diurnal cycle. Secondary effects of HME on photosynthesis appear to be diminished in swards, suggesting that yield benefits may be limited to specific environment and management scenarios. The primary benefit of HME technology is expected to come from increasing the energy density of pasture, and animal feeding trials using homozygous HME material are now required.

Keywords: lipids, metabolizable energy, pasture quality

Introduction

There are strong economic and environmental incentives for New Zealand to enhance the metabolizable energy content of major forage species such as perennial ryegrass (*Lolium perenne* L. (PR); Barrett *et al.*, 2015). However, PR traits that contribute to metabolizable energy (ME) have received limited attention from breeders, as they are difficult to measure and manipulate. Previous successful targets of PR nutritional improvement through breeding include increased digestibility (Sampoux *et al.*, 2011; Wims *et al.*, 2017) and water-soluble carbohydrate (WSC) content (Edwards *et al.*, 2007). Lipids are a non-fermentable source of dietary energy which contain over twice the energy density of carbohydrates. The optimal amount of lipids in ruminant diets is known (6–8% DM) and supplementation trials have demonstrated a positive feed utilisation efficiency response (Cosgrove

et al., 2004; Schroeder *et al.*, 2004; Hess *et al.*, 2008).

A method developed at AgResearch Ltd. New Zealand, to increase the lipid content of PR leaves to above the levels found in conventionally-bred varieties has involved a GM technology called ‘high metabolizable energy’ or HME. Preliminary pot growth chamber trial data has been published, showing that HME technology enhanced PR leaf FA by +1.8 %DM and had a positive effect on shoot growth, especially under high N, H₂O and CO₂ supply (Beechey-Gradwell *et al.*, 2018). Detailed nutritional and *in vitro* rumen fermentation profile analysis of plants grown in a similar manner showed that HME PR with up to +2.5% DM higher leaf FA than controls also had +1.0–1.2 MJ kg/DM higher gross energy (GE), while decreasing the amount of total gas and methane as a proportion of total gas emitted during rumen fermentation (Winichayakul *et al.*, 2020).

The industry strategy has been to perform HME PR field trials in the Midwest of the United States in order to test whether the herbage fatty acid (FA), gross energy (GE) and yield (herbage DM) enhancing traits observed in containment translate into field swards under simulated grazing. The 2019 and 2020 field trials tested the performance of independent HME events expressed in the hemizygous condition in several genetic backgrounds, and data from the 2019 trial were recently made available (Beechey-Gradwell, 2020). HME expression enhanced end-of-season (autumn) herbage FA by up to +1.0 %DM and GE by up to +0.5 MJ kg/DM relative to control swards. HME herbage yield was similar to that of controls across the season (Beechey-Gradwell, 2020). The 2021 trial currently underway will be the first to field-test homozygous (*i.e.*, double positive) HME lines, and higher FA and GE levels are anticipated. Collectively, these data will inform discussions around the proposition of an HME field trial in New Zealand.

To complement the field-testing programme, simultaneous work has been conducted to understand the mechanism behind the HME growth advantage in spaced pots (Beechey-Gradwell *et al.*, 2020; Cooney *et al.*, 2021) and to profile HME PR in indoor sward experiments. The following experiment assessed the performance of a well-characterised high-expressing HME line in simulated miniswards. Herbage yield, FA, GE and other nutritional components were quantified over six defoliation cycles. Secondary aspects of HME

expression on plant physiology, including effects on photosynthetic gas exchange and diurnal sugar levels, were assessed. The data was contrasted with i) previously published indoor HME spaced pot work, and ii) field trial work using populations segregating for the HME transgenes. A number of scientific areas requiring further work leading up to animal feeding trials are discussed.

Materials and methods

Plant material and growth conditions

Replicates in this experiment consisted of isogenic vegetative clones of two plant genotypes. The wild type control genotype (WT) was untransformed. This WT genotype was used to create the HME genotype 'ODR6205' which was shown to test positive for incorporation of the HME construct into the genome. This HME genotype was selected on the basis of high 'expression levels' and is designated 'HL'. This HME genotype-WT model has been thoroughly studied in spaced pot growth trials designed to characterise the biochemical and physiological mechanisms behind the HME growth advantage (Beechey-Gradwell *et al.*, 2020; Cooney *et al.*, 2021). The following experiment was conducted in controlled temperature rooms set to 20/15°C day/night temperature. Plants were grown ~1 m below a series of 600 W NanaPro LED lights (LEDgrowlights, Hamilton, NZ) under a 12-hour photoperiod. Photosynthetically active radiation at plant height was recorded at $500 \pm 100 \mu\text{mol}/\text{m}^2/\text{s}$, depending on bench position. Plant nutrient and water limitations were removed by regularly flushing the pots with a complete basal nutrient solution containing N as 4 mM NH_4NO_3 . The volume of nutrient solution applied to the pots was regularly adjusted to meet plant demand.

Minisward design and establishment

Replicates were made by splitting established HL and WT plants into ramets, cutting the roots and shoots to a length of 5 cm, and transplanting into pots. A total of 108 WT and 108 HL ramets were generated and transplanted into 4 l pots (18 x 18 cm width, 20 cm depth) containing washed sand ($n=12$, nine ramets per pot). Black plastic sheeting was attached to the sides of these pots to exclude light up to the defoliation height of ~5cm. The ramets were given 3 weeks to establish a root system before defoliating. A subset of 18 ramets (two pots) were destructively harvested at this time, enabling the calculation of 'end of establishment' DM. End of establishment ramet DM was similar for WT and HL, which suggested that a good basis for comparison of the subsequent growth of the two genotypes was achieved. The remaining 'miniswards' ($n=10$) were subject to six further regrowth and defoliation cycles

to simulate grazing. The regrowth interval for harvests 2–7 was 21, 21, 17, 14, 21, and 21 days, respectively. Herbage DM per pot (shoot material harvested from 5 cm above the potting media) was determined after oven-drying for 4–5 days at 65 °C. Specific leaf area (leaf area per unit leaf DM) was also determined before each harvest for a 2–3 cm² leaf area subsample at each harvest.

Composition analysis

Oven-dried herbage material from duplicate swards from harvests 2–7 was pooled before mixing and grinding to a particle size <1 mm. Total fatty acids (FA) were extracted and analysed by FAMES GC-FID (Browse *et al.*, 1986). GE was determined using bomb calorimetry. Neutral detergent fibre (NDF), crude protein (CP), and dry organic matter digestibility (DOMD) were determined via wet chemistry as described in Winichayakul *et al.* (2020).

Leaf gas exchange

Gas exchange measurements were made shortly before harvest 5 and 6 with a LiCOR 6800 (LiCOR Ltd, Nebraska, USA) at saturating light (1500 photons m⁻² s⁻¹). Measurements were made on cut youngest, fully expanded leaves (total area 2–3 cm²) as described in Beechey-Gradwell, (2020), in order to facilitate uniform leaf orientation and light acclimation for all replicates. For 30 min prior to measurement, the leaves were illuminated with LED lights and then 5 min prior to measurement, were acclimated in the LiCOR chamber at 20°C, 70% RH, 400 ppm CO₂ and a flow rate of 600 μmol/s. The supply of CO₂ to the leaf was then adjusted in the following concentrations (ppm); 400, 300, 200, 100, 50, 400, 400, 400, 400, 600, 800, 1000, 1200, 1500, in order to generate an 'A/C_i' curve. At each CO₂ level, leaves were given 3 min to acclimate before logging net photosynthesis and stomatal conductance.

Diurnal sugar experiment

For harvests 6 and 7, rather than oven-dry the herbage from each pot, leaf material from one ramet within each sward was harvested at 3 h intervals throughout the diurnal cycle. These leaves were immediately placed in liquid N then stored at -80°C, then later freeze-dried and ground to a powder. Total water-soluble carbohydrate (WSC) content was determined for this material as the sum of low and high molecular weight carbohydrates as described in Beechey-Gradwell *et al.* (2020).

Statistical analysis

All statistical analyses were performed in R (Version 3.5.1, R foundation) and $P < 0.05$ was considered significant. The nutritional composition and yield data collected from swards at multiple time-points were

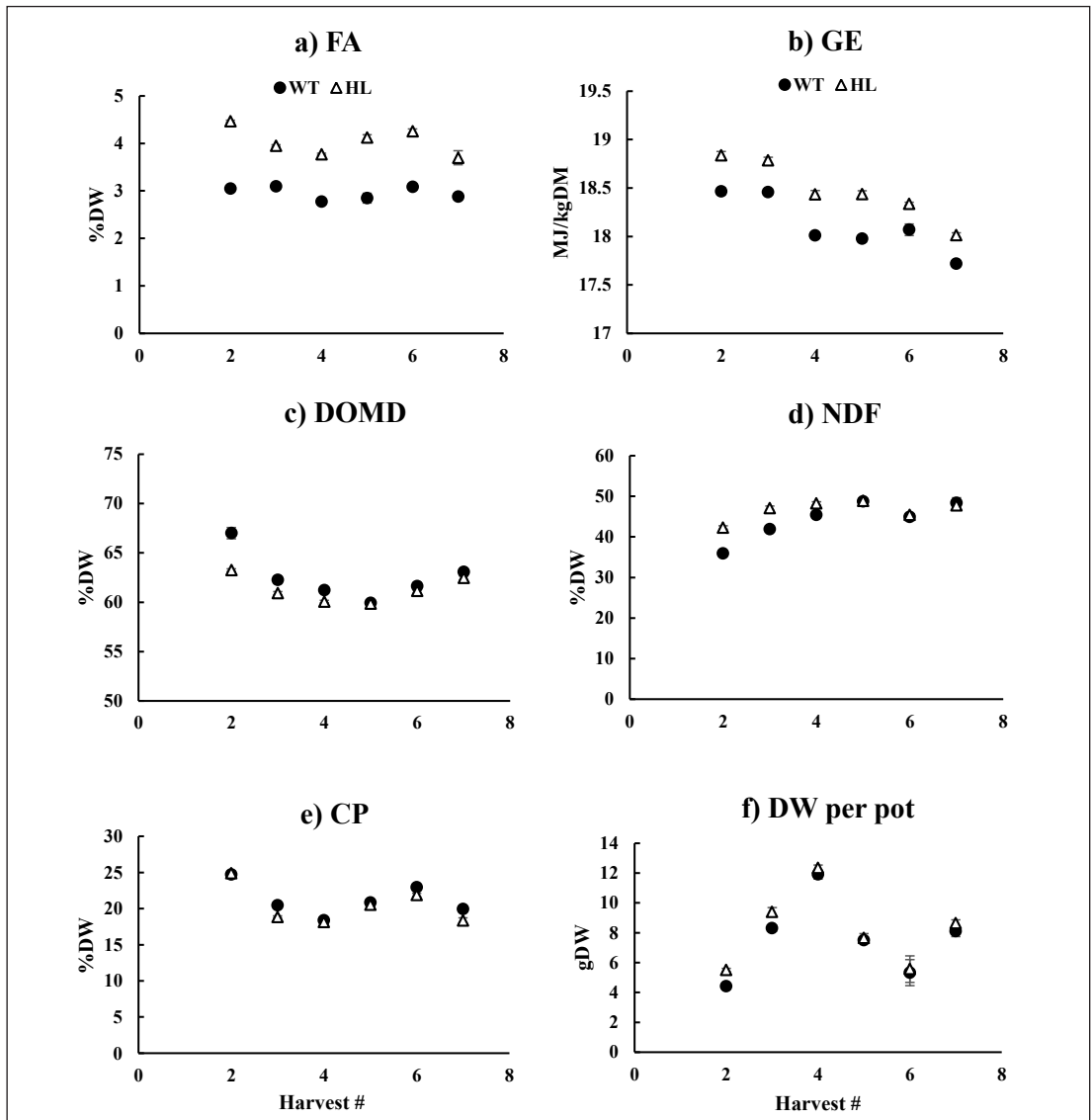


Figure 1 Herbage fatty acids (FA) (a), gross energy (GE) (b), dry organic matter digestibility (DOMD) (c), neutral detergent fibre (NDF) (d), crude protein (CP) (e) and yield (DW per pot) (f) for an HME perennial ryegrass transformant (HL, open triangles) and a wild-type control (WT, filled circles) grown in indoor miniswards under regular defoliation. Data points represent means \pm SE (for a-e, $n=5$, for f, $n=10$).

analysed using a linear mixed model (REML) with HME status and sward as fixed factors and harvest number (treated as categorical) as the repeated measure. The 'lmerTest' R package was used to estimate P values with Satterthwaite's method. This was followed by pairwise treatment comparisons, with P values adjusted according to the Benjamini-Hochberg (BH) procedure. Gas exchange data were collected at one timepoint and were analysed by Student's t test. For the diurnal experiment, a forward stepwise regression procedure was used to evaluate the relationship between leaf WSC

and FA and time of day. Interactions between HME status, time (treated as continuous) and quadratic \times time effects were tested in each of the models and variables and interaction terms where $P > 0.05$ were retained in the final model.

Results

HME nutritional profile and yield over time

Herbage fatty acid content (FA) was consistently enhanced by 28–46 % for HL (0.8–1.4 %DM) compared to WT (Figure 1a). Similarly, herbage gross energy (GE)

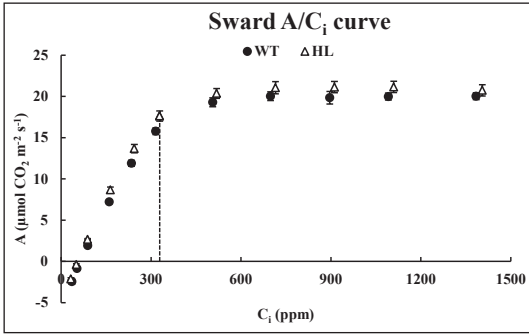


Figure 2 Response of net photosynthesis per unit leaf area (A) to intercellular CO_2 concentration (C_i) of an HME perennial ryegrass transformant (HL, open triangles) and a wild-type control (WT, filled circles) grown in indoor miniswards under regular defoliation. Measurements were made at saturating irradiance (1500 photons m^2/s). The dotted vertical line indicates the approximate intercellular CO_2 operating point at which plants were grown (corresponding to 400 ppm atmospheric CO_2). Data points represent means \pm SE ($n=8$).

was consistently enhanced by 0.3–0.5 MJ/kg DM for HL compared to WT (Figure 1b). HL had significantly higher herbage NDF and significantly lower herbage DOMD than WT at the beginning of the experiment (harvests 2–4), but these differences diminished with time and were not apparent at harvests 5–7 (Figure 1c, 1d). HL had 5–8% lower herbage CP (1.1–1.6 %DM) than WT at harvests 3, 6, and 7 (Figure 1e). HL herbage DM per pot was higher than for WT for harvests 2 and 3 but these differences gradually diminished and were not significant for harvests 4–7 (Figure 1f).

HME photosynthesis

Photosynthetic rate per unit leaf area was determined at saturating light (1500 photons m^2/s) shortly before harvests 5 and 6, and data from harvest 6 is shown in Figure 2. At ambient CO_2 supply (400 ppm), HL had a 12% higher photosynthetic rate than WT ($P=0.012$). Relative differences in photosynthetic rate between HL and WT were greater when leaves were supplied with low (100–300 ppm) CO_2 (15–39%) compared to high (600–1500 ppm) CO_2 (4–6%) (Figure 2).

HME diurnal WSC

For harvest 6 and 7, variation in WSC and FA over a day/night (diurnal) cycle were quantified, and data from harvest 7 is shown in Figure 3 (note values are mg/g DM). Over the course of the day herbage FA varied little (Figure 3b), while WSC varied substantially (Figure 3a) with a two-to-three-fold variation in total WSC primarily due to variation in low molecular weight WSC. HL consistently had 11–14 mg/g DM

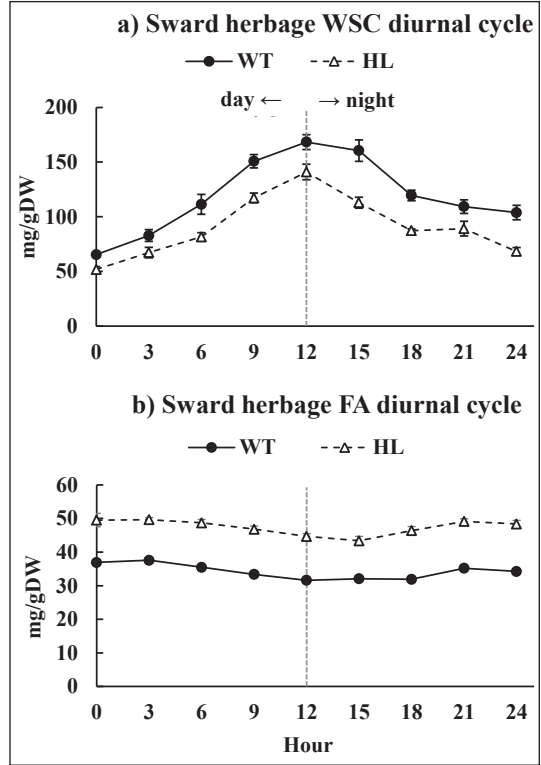


Figure 3 Diurnal variation in herbage water soluble carbohydrate (WSC) (a) and fatty acid (FA) (b) for an HME perennial ryegrass transformant (HL, open triangles) and a wild-type control (WT, filled circles) grown in indoor miniswards under regular defoliation. The dotted vertical line indicates the end of the photoperiod. Data points represent means \pm SE ($n=6$).

higher FA content than WT (Figure 3b). The slight drop in FA during the day (for both genotypes) could be explained by dilution due to WSC accumulation. The HL exhibited 14 mg/g DM lower WSC than WT at the beginning of the day and 34 mg/g DM lower WSC than WT by the end of the day. HL exhibited 20–46 mg/g DM lower WSC than WT during the night (Figure 3a).

Discussion

Quantifying the potential benefits of increasing FA

In the current study HME expression increased PR herbage FA by +0.8–1.4 %DM. Similar increases in HME FA (+0.8–1.0 %DM) were recorded at the fifth and final cut from two recent HME field trials (Beechey-Gradwell, 2020). Both experiments utilised HME lines with the transgenes expressed in the hemizygous (*i.e.*, ‘single positive’) condition, whereas an HME cultivar would ultimately consist of the HME transgenes expressed in the homozygous (*i.e.*, ‘double positive’) condition. There is now strong evidence

that the high HME FA trait is expressed under simulated grazing in a range of environmental conditions and plant genetic backgrounds. HME herbage FA differences were slightly diminished (+0.4–0.9% DM) in the field during summer (Beechey-Gradwell, 2020), but the relevance of this seasonal effect for New Zealand is unclear, given that average summer air temperatures at the field trial location (21–24°C) did not overlap with long-term summer air temperature averages across New Zealand (13–20°C) (NIWA).

Increasing HL herbage total FA by 0.8–1.4% DM increased herbage GE by 0.3–0.5 MJ/kg DM, in close agreement with HME field data (Beechey-Gradwell, 2020). GE rather than ME analysis was used because available proxies for forage ME do not explicitly account for the differences in energy density between different plant components – the scientific rationale for increasing plant lipids. To visualize the generality of the FA-GE relationship for HME PR, data were compiled from eight independent experiments, comprising all instances where HME and control PR FA and GE have been measured simultaneously (Figure 4). A positive slope was apparent, but there was unexplained variation. Surprisingly, most data points fell above the theoretical FA-GE line derived from a simple substitution equation where lipids (38 MJ/kg DM) displace sucrose (17 MJ/kg DM; Figure 4). This was true for both field and lab-grown HME samples. In the current study and in previous HME work (Winichayakul *et al.*, 2020; Beechey-Gradwell, 2020) increased FA and GE did not coincide with any consistent change in DOMD, which suggested that HME lipid accumulation does not penalize the accumulation of the total digestible plant fractions. However, increases in GE could mask, for example, changes in WSC (Figure 3), and this possibility should be field-tested.

Interest in breeding PR for higher levels of specific unsaturated FAs and total FA still exists in Europe and the UK (Palladino *et al.*, 2009; Hegarty *et al.*, 2013; Morgan *et al.*, 2020a; Morgan *et al.*, 2020b; Wilkinson

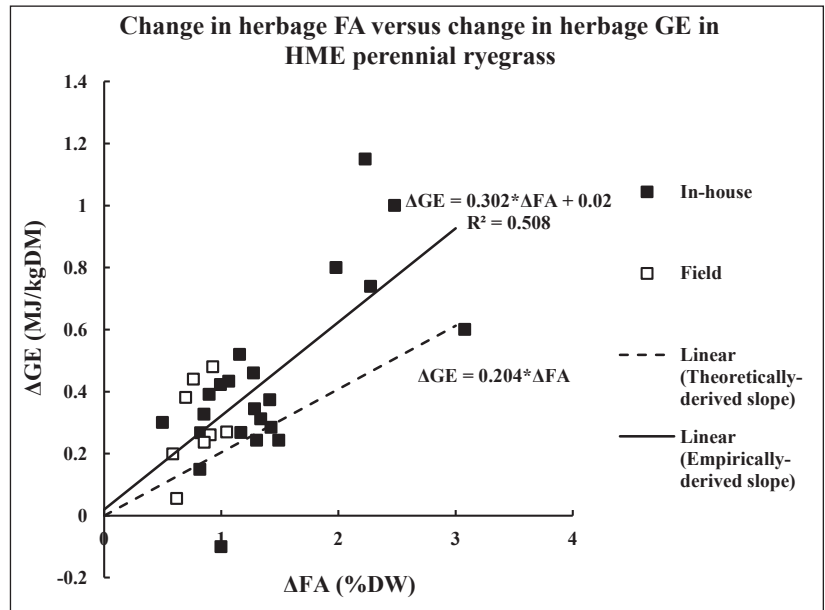


Figure 4 Relationship between the increase in mean herbage FA content (Δ FA) and the increase in mean GE (Δ GE) for HME compared to control perennial ryegrass across multiple experiments (some comprising of multiple harvests). The data are taken from three unpublished in-house experiments, the experiment described in this paper, Winichayakul *et al.*, (2020), and in-house and field data from Beechey-Gradwell, (2020). Δ for each parameter is calculated as the HME value minus the WT value.

et al., 2020), primarily due to the perceived human health benefits of consuming meat and milk products from pasture-fed animals (Morgan *et al.*, 2020a). For reference, a recent publication from Wales found that the difference in seasonal mean herbage FA between the lowest and highest genotypes from a PR breeding population was approximately 1.0% DM (Morgan *et al.*, 2020b). The FA-GE relationship has not been comprehensively scrutinised for conventionally-bred PR genotypes/populations differing in total FA content, but one publication showed that cultivar rankings on the basis of FA were quite different compared to GE (Palladino *et al.*, 2009). Increases in FA in the range of 2.0–2.5% DM have been regularly observed in HME PR in-house trials, which indicates that the increase in FA/GE achievable using HME may exceed the maximum achievable through selective breeding. Another major advantage of HME is that the basis of the high FA trait expression and transmission is understood and easily tested. However, major questions remain regarding the extent to which such high FA/GE levels will translate to a New Zealand growing season.

Secondary physiological effects of HME expression in swards

Observations from spaced pot HME experiments led us to propose a simple biochemical model accounting

for the intrinsic HME photosynthesis advantage, where HME lipid droplets behave as a carbon/energy sink which can cause partial depletion of leaf WSC reserves and remove end-product inhibition of photosynthesis (Beechey-Gradwell *et al.*, 2020; Cooney *et al.*, 2021). The major trait by which whole-plant photosynthesis was enhanced was greater specific leaf area, although a higher photosynthetic rate per unit leaf area was more important in some cases (Table 1; Cooney *et al.*, 2021). The intriguing question which this model did not account for was why the increase in HME photosynthetic carbon gain appeared to 'overcompensate' for the strict carbon requirement of additional leaf lipids, such that total plant growth, including root growth, increases.

A comparison of WT and HL noon leaf WSC levels and leaf photosynthesis traits from this experiment versus two previous spaced-pot trials showed that differences between the genotypes were diminished in swards (Table 1). This observation is consistent with the proposed photosynthesis model. Unlike the spaced pot experiments, increased HL photosynthesis (Figure 2) did not reliably deliver greater herbage yields (Figure 1), which suggested that changes in HL composition, biomass allocation or respiration may expend the additional fixed carbon. In field trials, a total of 16 comparisons of HME and control sward yield per ha have been made so far (four experiments comprising four harvests each). There was no indication of an HME yield penalty and an increase in HME yield has been observed once. Possible HME yield benefits may depend upon environment and management factors, which are not yet understood.

Future work

Overall, the results supported the potential for HME technology to deliver increased metabolizable energy.

In a comprehensive modelling exercise, the effect of an arbitrary 1 MJ/kg DM increase in PR ME was tested for two PR grazing-based Australian dairy systems (Ludemann *et al.*, 2015). Increases in farm operating profit in the range of 197–783 AUD per hectare of milking area occurred depending on pasture growth rates and on how the extra 1 MJ/kg DM was 'utilized' (*i.e.*, whether stocking rate was kept constant or increased, or the amount of purchased concentrate was reduced) (Ludemann *et al.*, 2015). The prospect of realizing such benefits *via* HME remains uncertain, due to the preliminary nature of the field data and technical and regulatory challenges ahead. Animal modelling relies heavily on assumptions about the partitioning of dietary energy to processes such as weight gain and milk solids production, but there is no data to quantify how these animal parameters respond to additional pasture ME delivered through higher lipids (Ludemann *et al.*, 2015).

The next step in the HME PR programme is animal feeding trials. The first trials will be performed using material grown in the field and subsequently ensiled, and will later incorporate methane and nitrous oxide emissions and N leaching measurements. Each trial will require approximately 20 kg of both homozygous HME and null control seed to be generated in containment facilities. In addition to animal nutrition considerations, the economic value of new PR varieties in New Zealand depends on seasonal yields, and the 'persistence' of novel traits under interactions with grazing animals, white clover and fungal endophyte (Parsons *et al.*, 2011). These factors as well as different environmental and management scenarios will eventually need to be considered to accurately assess the potential economic benefits of HME PR to New Zealand.

Table 1 Comparison of leaf parameters related to photosynthesis for an HME perennial ryegrass transformant (HL) and a wild-type control (WT) grown indoors in a) spaced pots and b) miniswards.

	a) Spaced pot			b) Sward		
	Genotype	Value	% change for HL	Genotype	Value	% change for HL
Noon leaf WSC (mg/g DW)	WT	203		WT	109	
	HL	75	-63	HL	82	-25
A_{area} ($\mu\text{mol CO}_2 \text{ m}^2/\text{s}$)	WT	13.9		WT	16.1	
	HL	17.3	24	HL	18.0	12
SLA (cm ² /g)	WT	222		WT	332	
	HL	324	46	HL	358	8

WSC = water soluble carbohydrate,

A_{area} = net photosynthesis per unit leaf area,

SLA = leaf area per unit leaf dry weight.

The data from spaced pots is an average from two independent experiments; an unpublished experiment and experiment 1 from Cooney *et al.*, (2021).

The data from swards is an average across harvests from the experiment described in this paper, for which WSC and A_{area} were determined before two of the harvests and SLA was determined before every harvest. '% change for HL' was calculated for each parameter as $(\text{HL}-\text{WT})/\text{WT} \times 100$.

Conclusions

Overall, the major changes measured in HL PR here (increased FA, increased GE, and generally similar herbage DM production per unit area) closely mirrored data from field sward trials with hemizygous HME populations in the Midwest USA. Changes in other nutritional components were generally minor, although WSC was reduced later in the diurnal cycle. Secondary effects of HME expression on photosynthesis appeared to be diminished in swards, which implied that yield benefits may be restricted to specific environment and management scenarios. This supported the suggestion that the primary benefit of HME technology will come from increasing the energy density of pasture.

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