Report to the Stapledon Memorial Trust

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THE EFFECT OF POLYPHENOL OXIDASE ON LIPOLYSIS IN RED CLOVER

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The Fellowship:

The fellowship was carried out at the USDA-Agricultural Research Service, US Dairy Forage Research Center, Madison, Wisconsin, USA to investigate the effect of polyphenol oxidase (PPO) on lipolysis in red clovers which have been genetically modified to silence the PPO gene. The use of transgenic methodology will further our understanding of both the action of PPO during lipolysis and the mechanism for polyunsaturated fatty acid protection within the rumens of animals fed red clover. The trip also involved attendance at the American Society of Animal Science annual conference in Minnesota, where two papers were given on work carried out on the PPO and lipid projects (Appendix 1 and 2).

The enzyme polyphenol oxidase (PPO) is a copper metalloprotein involved in a number of browning reactions in plants, including that of red clover leaves when cut or crushed and exposed to the air. PPO catalyses the oxidation of endogenous phenols to quinones in the presence of oxygen. The PPO-generated quinones are highly reactive, electrophilic molecules which covalently modify and crosslink a variety of nucleophilic cellular constituents, such as proteins, amines and amides, leading to the formation of melanin pigments. It has been shown that this browning reaction is associated with a reduction in the extent of red clover proteolysis both *in silo* and in the rumen. This could be due to the complexing of leaf proteins and/or the denaturing

of plant proteases. The reduction in protein breakdown through this mechanism results in over 80% of red clover silage protein being retained as true protein resulting in improved N-use efficiency and final product quality. Lipolytic activity has also been shown to be reduced in red clover resulting in a significant reduction in lipolysis both *in silo* and *in vivo*. Lipolysis (the splitting of the ester bond in glycerol based lipids) is a prerequisite for the microbial hydrogenation (biohydrogenation) of unsaturated fatty acids. This addition of hydrogen to unsaturated fatty acids in the rumen is thought to increase the saturated fat content of ruminant products and is considered to reduce the healthiness of ruminant fat.

The USDA team of Hatfield and Sullivan in Madison, Wisconsin has been working on the genetics of PPO in red clover (Sullivan et al., Plant Physiology 136: 3234-3244, 2004) and have developed genetically modified lines of red clover with the PPO gene silenced. This material, although difficult to work on in the UK due to GM restrictions, was an extremely valuable tool. It allowed us to determine a mechanism for the reduced lipolytic activity observed with red clover and so produce a breeding strategy for forages which exhibit this trait. We carried out an experiment to determine the role of PPO on lipolysis in batch culture, this is reported in appendix 3. In addition we developed of two rapid PPO assays for red clover, with the help of their experienced biochemists (Appendix 4 and 5). This allowed us to show that the activities of the normal and transgenic lines are comparable and it is indeed PPO activity which results in the differences observed in lipolytic and proteolytic activity. We also were in the process of developing methods to visualise and/or measure phenol/lipid complexes produced by PPO activity to help understand the mechanism of PPO on lipolysis and, ultimately, biohydrogenation.

Links and follow-up to the Fellowship:
Initial discussions on collaboration between the IGER and the Madison groups took place at the International Grassland Congress in Dublin, June 2005. This visit followed up on these discussions and contributed to the development of an extremely beneficial and productive link between IGER and the USDA, US Dairy forage research centre. It is intended for this to lead to further collaboration between the institutes, with the specific aim of developing collaborative research under a memorandum of understanding.

The material readily available at Madison helped determine the role of polyphenol oxidase in the reduction of proteolysis and lipolysis both in silo and in the rumen. This in turn gave plant breeders targets for producing plants with enhanced levels of these quality traits to the commercial benefit of the producer, who could demand a premium for products with an improved fatty acid profile, and the health benefits of the consumer. The visit and future collaboration would strengthen and add value to existing BBSRC, DEFRA and EU research at IGER focused on dietary manipulation of the fatty acid composition of ruminant products. IGER has an excellent international profile in this area and this activity will further enhance the reputation of the Institute (IGER) and the BBSRC in the United States of America. The collaboration will enhance UK science via involvement in international research.

This visit established joint research initiatives and objectives. The collaboration will be consolidated further through additional work leading to further joint publications.

This will provide a positive foundation on which to develop collaborative research proposals. It is envisaged that postgraduate research students and fellows are likely to be an important route forward in the collaboration. Once a collaboration agreement is signed between the USA and EC for the Framework 7 programme, then scope for exchanges of staff via the Marie Currie initiatives will be investigated. Otherwise it's simultaneous applications by IBER to BBSRC in the UK and by US Dairy Centre to USDA, with scope in both applications for travel of staff between the two institutes.

Appendix 1.

Lipolysis of red clover with differing Polyphenol oxidase activities in batch culture. M. R. F. Lee^{1}^, L. J. Parfitt^{2}^, and F. R. Minchin^{1}^, ^{1}^ Institute of Grassland and Environmental Research, Aberystwyth, UK, ^{2}^ Institute of Rural Studies, University of Wales, Aberystwyth, UK.

Polyphenol oxidase (PPO) oxidises endogenous phenols to quinones, which react with nucleophilic sites of other compounds such as proteins. In red clover, this complexing reaction has been shown to reduce both plant mediated proteolysis and lipolysis. This experiment investigated the role of red clover PPO on lipolysis in the presence and absence of rumen micro-organisms. Triplicate macerated shoot samples of two red clover lines, a wild type with a basal level of PPO activity (High PPO) and a mutant with reduced PPO activity (Low PPO) were incubated in anaerobic buffer, with and without strained rumen liquor inoculum (I+ and I-), at 39ºC, over six time points (0, 1, 2, 4, 6 and 24 h). At each time point the samples were destructively harvested and lipolysis measured as percentage lose of membrane lipid. Lipolysis data was analysed using a general analysis of variance with repeated measurements (Genstat 8®). The table shows the reducing effect of PPO on lipolysis (High vs Low) but also the elevated level of lipolysis when micro-organisms are present (I+ vs I-). If the PPO effect was solely due to the deactivation of plant lipases this difference should be neutralised through the addition of microbial lipases. The retention of the PPO effect in the I+ treatments suggests that PPO exerts some form of protection on the membrane lipids, in a similar manner to the complexing of protein. The lipid in forages is mainly in the form of polar membrane lipids, and polar lipid – phenol complexes could form due to the highly electrophilic nature of the PPO-produced quinones.

	High PPO		Low PPO			Sig.		
	I+	I-	I+	I-	s.e.d	PPO	I	PPO×I
Lipolysis (%) at 1h	10.6	6.6	37.4	8.5	2.97	*	*	NS
2h	16.7	8.9	37.5	17.2	5.10	*	*	NS
4h	36.0	14.6	44.6	21.9	3.68	*	***	NS
6h	35.0	23.8	57.5	25.6	6.43	*	**	NS
24h	71.5	28.3	82.4	41.9	5.94	*	***	NS

*P<0.05, **P<0.01, ***P<0.001

Key Words: Polyphenol oxidase, lipolysis, red clover

Appendix 2

The effect of fish oil supplementation on ruminal C18 PUFA metabolism in beef steers offered either grass or red clover silage. M. R. F. Lee[1], K. J. Shingfield[2], and N. D. Scollan[1], [1]Institute of Grassland and Environmental Research, Aberystwyth, UK, [2]MTT Agrifood Research, Jokioinen, Finland.

Red clover and fish oil have been shown to alter ruminal lipid metabolism increasing PUFA and conjugated linoleic acid (CLA), respectively, in ruminant products. This study investigated the additive effect of these two feeds on C18 PUFA metabolism in beef steers. Eight Hereford × Friesian steers prepared with rumen and duodenal cannulae were offered either grass or red clover silage at 90% *ad libitum* with one of three levels of fish oil 0, 1, 2, or 3 % DMI. The experimental design consisted of four 2 × 2 Latin squares within each oil level with an extra period. Flows of fatty acids at the duodenum were assessed using the dual phase-indigestible-marker technique. DMI was significantly ($P < 0.001$) higher for red clover silage (5.98) than grass silage (5.09 kg/d). Oil level had no effect on DMI with the exception of red clover at 3% oil which was significantly ($P < 0.01$) lower. C18:2 *n-6* and C18:3 *n-3* intakes averaged 13.2 and 25.1 for grass silage and 17.9 and 36.2 g/d for red clover silage, respectively. Biohydrogenation of C18:2 *n-6* and C18:3 *n-3* were significantly lower ($P < 0.001$) on red clover silage than grass silage with oil level increasing the extent of biohydrogenation in both diets ($P < 0.05$; 0.81 and 0.85 to 0.91 and 0.92 for grass silage and 0.76 and 0.73 to 0.87 and 0.83 for red clover silage at 0 and 3 % oil, respectively). C18:1 trans was significantly increased by oil level for both diets (4.6 to 15.0 and 9.4 to 22.5 for grass and red clover silage at 0 and 3 % oil, respectively). Oil level increased the proportion of C18:1 trans 11 in the duodenal digesta in both diets from 0.47 and 0.31 with no oil to 0.52 and 0.51 at 3 % oil for grass silage and red clover silage, respectively. CLA was also significantly increased on both diets by oil level (0.21 and 0.27 to 0.48 and 0.57 g/d for grass and red clover silage at 0 and 3 % oil, respectively). The results of this study show that red clover and fish oil have the potential to beneficially alter the fatty acid profile of ruminant products.

Appendix 3

Red clover polyphenol oxidase reduces ruminal lipolysis in *in vitro* batch culture.
M.R.F. Lee[1], F. R. Minchin[1], R.D. Hatfield[2] and M. L. Sullivan[2]
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Introduction It has been shown that the rate of lipolysis and proteolysis differ significantly between red clover genotypes with different levels of polyphenol oxidase (PPO) activity (Lee *et al.* 2004). Sullivan and Hatfield, (2006) reported the development of genetically modified lines of red clover with the PPO1 gene silenced. This material was used to examine the role of the red clover PPO enzyme on lipolysis and ultimately C18 polyunsaturated fatty acid biohydrogenation in batch culture. If the role of PPO is proven in reducing ruminal lipolysis of plant lipids it may influence breeding strategies for forages which exhibit this trait in an attempt to increase the levels of beneficial PUFA and decrease detrimental trans and saturated fatty acids in animal products.

Materials and methods Anaerobic incubation medium was prepared as described by Goering and Van Soest (1970) and 20ml dispensed under CO_2 into 24 flasks

maintained at 39°C. Fresh red clover from silenced PPO1 gene plants (S) and active PPO1 gene plants (A) were cut 3 cm above soil level. The tissue was crushed and cut into 5 mm strips, with a sample retained at -20°C to measure PPO activity. Four grams of fresh material was loaded into each incubation flask. Three bottles were allocated to each time point (0, 1, 6 and 24 h) for each treatment (S and A). The flasks were then inoculated with 10 ml of strained rumen liquor (from two rumen fistulated cows and strained through a double layer of muslin). The flasks were sealed and incubated at 39°C in the dark with continuous CO_2 purging. At each time point the appropriate incubation flasks were removed and 40 ml of isopropanol : chloroform (1:1 v/v) along with 1 ml of internal standard (2.5 mg C23:0 / ml chloroform) added and the lipid extracted and fractionated by TLC as described by Lee et al. (2004). Lipolysis was calculated by expressing the decrease in the proportion of membrane lipid between the initial time point T_0 and incubation time point T_x, and then analysed using a repeated measures analysis of variance (Genstat 8.1, Lawes, Agricultural Trust, 2005).

Results PPO activity for the PPO1-silenced red clover (S) and the active PPO1 red clover (A) were 0 and 11.4 nkat/mg protein. Fig. 1. shows the extent of lipolysis of S and A. At all time points lipolysis in the S treatments were higher than the A treatments with 6 and 24 h being significantly higher ($P<0.001$) than S.

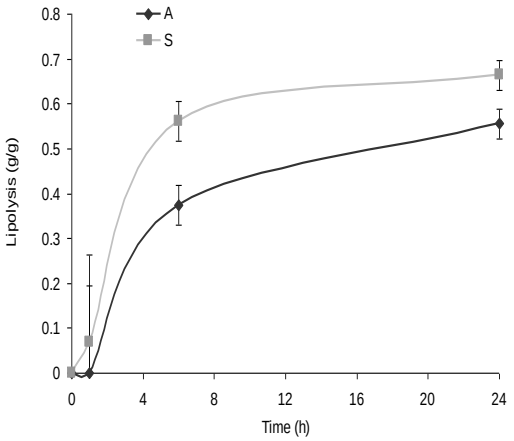

Figure 1. Lipolysis of the genetically modified red clover (S) and the non-genetically modified red clover (A) incubated in batch culture with rumen liquor at 39°C for 24 h.

Conclusions The PPO1-silenced red clover had significantly higher lipolytic activity in batch culture than the red clover with the active PPO1 gene. This implies a role of PPO in reducing the extent of lipolysis in the presence of rumen micro-organisms. Mechanistically this may be due to a binding of quinones to the lipid or/and the formation of protected protein/lipid complexes

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Note: Appendices 4 and 5 not included, but available from Michael.lee@bbsrc.ac.uk or office@britishgrassland.com