final report

Project code: **DAW.053**

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Date published: **November 1995**

ISBN: **9781741917932**

PUBLISHED BY
Meat & Livestock Australia Limited
Locked Bag 991
NORTH SYDNEY NSW 2059

Feeding systems for increasing marbling in cattle

Meat & Livestock Australia acknowledges the matching funds provided by the Australian Government to support the research and development detailed in this publication.

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Published and printed by Murdoch University, Western Australia, 6150

November, 1995

Abstract

Previous scientific and circumstantial evidence suggests that the amount of marbling can be influenced by the composition of the diet. This appears to be related to grains which can increase the availability of glucose to act as a substrate for marbling fat. The aim of this study was to use specific lipogenic enzymes in fat tissue as a marker to test the effect of glucose and diet in controlling the "glucose pathway" for lipogenesis. The study showed that increased glucose availability at constant energy intake increased the capacity for lipogenesis from glucose in fat tissue. The enzyme markers allowed comparisons among types of grain and processing methods which maximised glucose availability (maize = flaked sorghum > sorghum = wheat = barley > oats = lupins). The study has laid the foundation for field studies to access the effects of different grains on marbling scores in long fed cattle.

Acknowledgements

Thanks to the advice and guideness of Ian Johnsson for help in project design and execution. The members of the Technical Advisory Group group Ted Brockman, Mike Stevens, John Fry, Adrew Cabassi, David Roberts are thanked for their input during the design phase of the project. The skilled and dedicated technical assistance throughout the project of Mrs Barbara Waldoch and Mr Malcolm Boyce is greatfully acknowledged. Dr Leon Harris is thanked for his crutial role in developing the integrated procedures for enzyme assay. Helen Cockerill is also thanked for her skilled assistance in performing the acetylCoA carboxylase assay. The farm and research staff at Murdoch University, Medina Research Station (Agriculture Western Australia) and Vasse Research Station (Agriculture Western Australia) and the Karnet Prison Farm are thanked for their contributions.

Finally the authors thank Murdoch University and Agriculture Western Australia for supporting and allowing the research to proceed.

Executive Summary

Background

The factors controlling the expression of the economically important fat tissue, marbling, are poorly understood and need to be studied to allow Australian producers to compete on export markets in the Asia-Pacific area. For example cattle finished to similar age, weight and fatness specifications in Australian feedlots typically have lower marbling scores than genetically similar cattle in America and Japan. It is suggestedthat diet may be a factor in this difference.

Typical ruminant diets are heavily fermented in the reticulo-rumen and as a consequence acetate is a major substrate for fat synthesis rather than glucose. Despite this, fat depots show different rates of fat synthesis from glucose versus acetate. Marbling adipocytes show a preference for glucose while subcutaneous adipose tissue uses mainly acetate for fat synthesis. Consequently dietary regimes which increase the availability of glucose are likely to increase the rate of marbling.

The overall aim of this project was to use lipogenic enzymes in subcutaneous fat to assess the effects of glucose and nutrition on the capacity for fat synthesis. The approach was to use the activity of ATP citrate lyase and acetylCoA carboxylase as indices of fat synthesis from glucose and glucose plus acetate respectively. A further aim was to use the enzyme markers as an index of glucose availability within the animal under different dietary regimes.

Major findings

1. The enzyme marker ATP citrate lyase is stable for at least 24 hours post slaughter in carcasses kept at 4°C and so could be used as a marker for lipogenesis from glucose and perhaps the propensity for marbling under commercial conditions.

2. Glucose when administered at physiological doses as a nutrient under isoenergetic conditions increased the capacity for:

   (a) fat synthesis from glucose
   (b) total fat synthesis

3. Increasing feed intake increased the capacity for:

   (a) fat synthesis from glucose
   (b) total fat synthesis

4. Subcutaneous adipose tissue represents a convenient and sensitive site to monitor the effects of glucose and diet on the activity of the lipid enzymes.

5. Sheep and cattle respond in a similar manner with respect to control of lipogenic pathways by glucose availability.

6. The glucose pathway for fat biosynthesis in adipose tissue:

(a) varies markedly depending on the level of feeding and the type of grain in the ration.

(b) varies according to the predicted level of starch digestion in the small intestine.

(c) is sufficiently sensitive to glucose availability that it can be used to optimise grain processing to maximise digestion of starch in the small intestine.

(d) is more sensitive to glucose made available by the digestion of starch in the small intestine than to extra glucose made available by synthesis in the liver (gluconeogenesis) as a result of an increased extra feed.

7. Feed grains that result in a low rate of fat synthesis from glucose also result in a lower rate of total fat synthesis - that is diets promoting low rates of starch digestion in the small intestine allow for lower rates of fatty acid synthesis.

8. The extent of starch digestion in the small intestine for the grains tested was:

Maize > steamed flaked sorghum > sorghum = wheat = barley > oats = lupin

9. Pulse feeding of cereal grains (twice weekly) made safe with virginiamycin is feasible even at high grain intakes.

10. Pulse feeding of most cereal grains did not significantly increase the capacity for biosynthesis of fat from glucose.

11. Pulse feeding of wheat did increase the activity of ATP citrate lyase suggesting that more starch was digested in the small intestine.

12. It is possible that a less severe pulse feeding regime might have been more effective in increasing fat synthesis from glucose i.e. feeding every second day or feeding a base level of grain (0.8kg) and then pulsing an additional 1kg of grain every second day. This would reduce the period of low nutrition and so make the cycle between anabolic and catabolic states less severe. In the model used in the current study, any stimulation of the lipid enzymes due to increased glucose availability (via increased feed intake promoting starch digestion in the small intestine) may have been negated by the catabolic events associated with the subsequent relatively low period of nutrition.

When and how can industry benefit

This work has given the basis for a planned experiment to test the hypothesis that an increased glucose availability due to starch digestion in the small intestine will increase marbling scores in long fed cattle.

Who can benefit from the results

Increased marbling has huge financial implications for lot feeding enterprises and exporters with subsequent flow on to other sectors which form the backbone of the Industry.

In addition the powerful effects of grains which allow starch digestion in the small intestine should have significant implications for manipulating glycogen metabolism and so help to reduce dark cutting in beef. Further it likely that the production of soft fat (a desirable processing and quality trait) and marbling are co-controlled by glucose availability.

Main Research Report

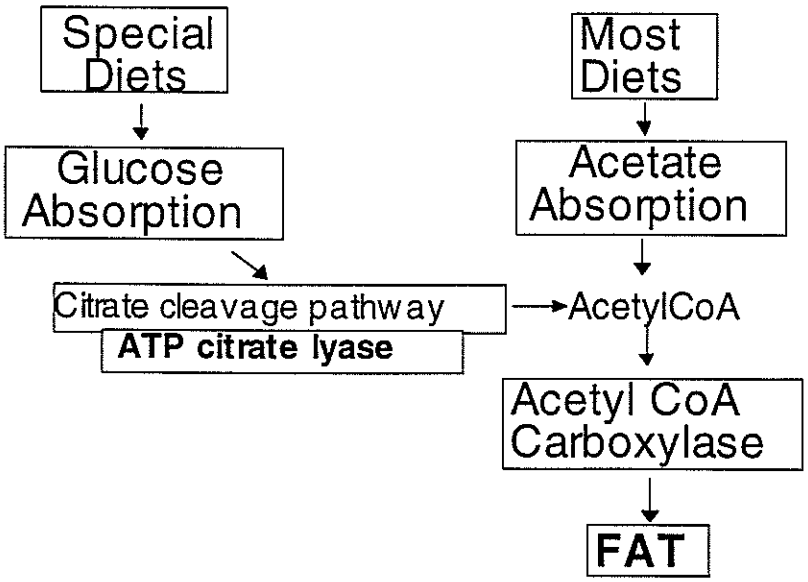
BACKGROUND

Marbling
The pattern of fat accretion is thought to be influenced primarily by the age of the animal and to some extent by genetics. The economically important depot fat, marbling, is one of the last depot sites to mature and becomes most pronounced as the carcass weight increases (around 400kg). Cattle finished under Australian dietary systems tend to have lower marbling scores than genetically similar cattle in America. It has been speculated that diet may be a factor in this difference.

Fat Accretion
The rate of fat accretion in finishing steers and lambs is determined primarily by (i) the metabolic state of the animal (ii) the intake of metabolisable energy and (iii) the age of the animal. Pathways for fat biosynthesis are either accretion of preformed fat in the diet or by synthesis *de novo*. The intake of fat by ruminants is limited since diets containing more than about 4% added fat tend to depress intake and this subject is not the focus of this study. During the fattening phase of growth the synthesis of fats *de novo* is dependent on the rate of supply of carbon, reducing power and glycerol.

The substrates for lipogenesis in ruminants are acetate and glucose. Diets which are extensively fermented in the rumen (i.e. most diets) promote acetate as the major source of carbon and reducing power for lipogenesis with a smaller contribution from glucose for some of the reducing power and all of the glycerol (Vernon,1981). An alternative pathway for lipogenesis with glucose as the primary substrate is typically seen in monogastric animals when glucose is a major end product of digestion. The lipogenic pathway using glucose is quite distinctive and utilises the citrate cleavage pathway as shown schematically in fig. 1.

Fig. 1. Pathways for lipogenesis *denovo* in ruminant animals



Key enzymes of the citrate cleavage pathway are ATP citrate lyase and NADP malate dehydrogenase. Both of these enzymes are induced by intravenous glucose infusion and consequently their activity in adipose tissue represents an estimate of the maximal capacity for lipogenesis from glucose (Ballard *et al.* 1972; Lindsay 1970)

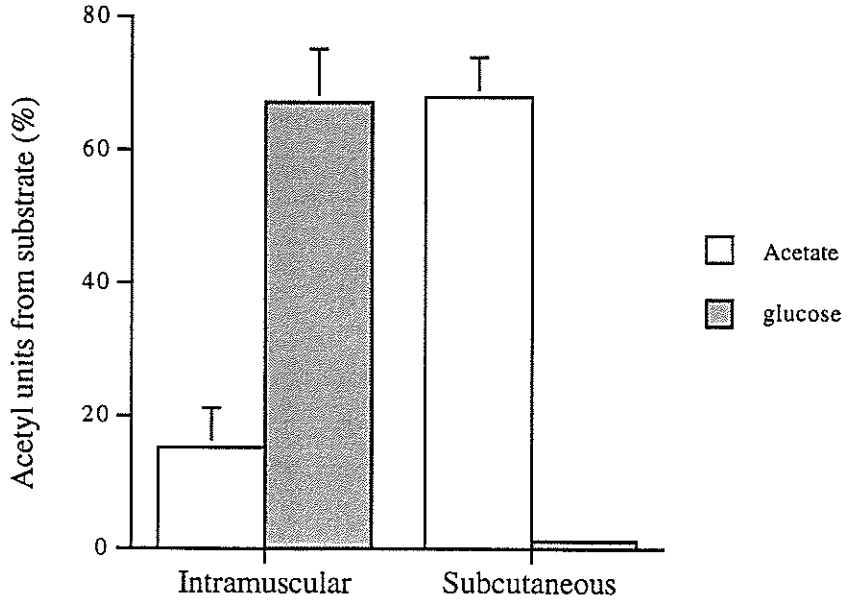
Regardless of the substrates used, the overall rate of lipogenesis in controlled by the activity of acetylCoA carboxylase which is under complex substrate, allosteric and hormonal control. Accordingly the activity of acetylCoA carboxylase is an indicator of the total capacity for lipogenesis - indeed Harris*et al.* (1994) have shown that fat accretion as determined by changes in body composition is closely correlated with the activity of acetylCoA carboxylase in adipose tissue.

Glucose availability and lipogenesis

The availability of glucose has long been thought a limiting factor for fat accretion in ruminants. Thus Preston and Leng (1987) speculated that diets high in roughage promote an excess of acetate with respect to glucose and so induce a reduced rate of lipogenesis for a given intake of metabolisable energy. Few studies have critically tested this hypothesis. Two groups (Ballard *et al.* 1972; Prior & Jacobson 1979) have found an increased rate of lipogenesis *in vitro* when glucose was infused into sheep or cattle - the results are equivocal however since glucose was infused in addition to the basal diet and so the experimental design was not isojoulic.

Different adipose tissue sites have been found to have different rates of lipogenesis from glucose versus glucose. Thus marbling adipocytes show a preference for glucose carbon while subcutaneous adipose tissue uses mainly acetate as a source of acetyl units for lipogenesis (Smith & Crouse 1984; Whitehurst *et al.* 1981). This difference is substrate preference is clearly shown in fig. 2 and exists even if the potential of lactate to act as a lipogenic substrate is included. The extent to which lactate can act as a substrate for lipogenesis is still controversial and most workers focus on glucose versus acetate.

Fig 2. The contribution of acetate and glucose carbon to lipogenesis in subcutaneous and intramuscilar fat tissue determined *in vitro* (adapted from Smith & Crouse 1984).



The results strongly suggest that the relative availability of glucose might influence the partitioning of fat between different depot sites.

The extent to which different cereal grains can result in the an increased capacity for lipogenesis from glucose is unknown. However a study by Smith *et al* (1992) clearly showed that as feeding level increased from 0.8x to 2.0x maintenance the capacity for glucose to act as a substrate for lipogenesis increased some 70 fold as determined from the activity of ATP citrate lyase or by direct incorporation of glucose carbon *in vitro*. This study suggests that either increasing feed intake and/or maize grain can allow for radical induction of the citrate cleavage pathway. Maize is atypical in that it allows for considerable quantities of starch to be digested in the small intestine (Janes 1985; Huntington 1994).

PROJECT OBJECTIVES

To assess the effect of nutrition on the capacity for lipogenesis from glucose and glucose plus acetate (total lipogenesis). The general approach was to use the activity of ATP citrate lyase as an index of the capacity for lipogenesis from glucose and to use the activity of acetylCoA carboxylase as an index of the total capacity for lipogenesis.

Hypothesis 1.

An increased glucose availability will increase the total capacity for lipogenesis as well as for lipogenesis from glucose.

Aims associated with hypothesis 1:

1. To establish that an intravenous glucose infusion given as an isojoulic supplement will increase the activity of ATP citrate lyase and acetylCoA carboxylase (i.e. the lipid enzymes) in adipose tissue.

2. To examine the time course and dose response of the lipid enzymes to glucose infusion.

3. To establish that increasing feed intake will increase the activity of the lipid enzymes.

4. To show that the response of the lipid enzymes to glucose infusion will be similar in sheep and cattle.

Hypothesis 2.

The response of the lipid enzymes in adipose tissue will differ between grains - in particular the capacity for lipogenesis from glucose will follow the same pattern as the predicted rate of starch digestion in the small intestines.

Aims associated with hypothesis 2:

To examine the effects of feeding oat, barley, wheat, sorghum, maize and lupin grain on:

(a) the activity of the lipid enzymes in adipose tissue
(b) the response of the lipid enzymes to increasing feed intake for the different grains

Hypothesis 3.

Pulse feeding of cereal grain will shift a proportion of starch digestion from the rumen to the small intestine and so stimulate the capacity for lipogenesis from glucose in adipose tissue

Aims associated with hypothesis 3:

To examine the effects of daily versus twice weekly (pulse) feeding of various grains on fed intake, growth rate, fat score and lipid synthesis as estimated using key enzymes of lipid biosynthesis.

METHODOLOGY

Enzyme assays

The general methodology was to use the activity of ATP citrate lyase and acetylCoA carboxylase as indices of the capacity for lipogenesis from glucose and total lipogenesis respectively. During the initial studies the assay systems for the enzymes were integrated into the one sample preparation procedure and then classical verification of the enzyme assays was performed. A key quality control point was the procedure for homogenisation as acetlyCoA carboxlyase was very sensitive to over homogenisation - that is its activity decreased if the fat sample was homogenised for too long. The next phase was to verify each assay and here linear responses with respect to homogenate volume and time of incubation were obtained. In addition the incubation time with citrate that was required to fully activate acetlyCoA carboxylase was established.

A detailed protocol for the assays is described in Appendix 1 and in Phillips (1994). The general procedure was to collect adipose tissue into liquid nitrogen and then store the samples at -80°C. The sample were then pulverised at the temperature of liquid nitrogen, mixed with homogenising medium and homogenised using a strict time-based protocol. The supernatant was then assayed for ATP citrate lyase using a modification of the spectrophotometric method described by Srere (1962). AcetlyCoA carboxylase was assayed using a modification of the isotopic method described by Smith and Prior (1981).

Glucose infusion studies

Merino or Merino Dorset cross wethers were used for the sheep experiments and Hereford cattle were used in the one cattle experiment. The diet consisted of a lupin/chaff/premix (75:24.4:0.6) pellet with a crude protein of 24% and a metabolisable energy (ME) of 11 MJ/kg dry matter - this diet was chosen so dietary starch was not a confounding factor; lupin grain contains virtually no starch. After 3-4 weeks acclimation to being housed individually and indoors, the sheep were fitted with indwelling jugular catheters. All animals were then connected to an infusion line. The groups receiving glucose were infused at a rate of 0.45-3.6gm glucose/kg body weight/day. The infusate was a 5-21% glucose solution made in sterile water and infused at 200ml/day. The control groups (i.e. not receiving glucose) did not receive an infusion. The feed offered to the sheep receiving the glucose infusion was reduced by 140gm (dry matter) for each 90gm of glucose (dry matter) infused based on the ME of the diet and glucose being 11 and 17.5MJ/kg dry matter respectively. The infusions lasted for 10-22 days.

Fat Biopsy

Fat was collected form beside the tail for both sheep and cattle. After local anesthesia an incision was made and subcutaneous fat (approx. 1gm) was dissected with scissors and placed immediately into liquid nitrogen. Swabs were used to control bleeding and when this subsided the wound was sutured.

Abattoir sampling

Sheep were transported for 1 hour to a local abattoir where they were slaughtered within 3 hours. Samples of the the tail fat pad were collected into liquid nitrogen within 30 minutes of death. Carcass weights and fat depth GR were measured 2 days after slaughter.

Grain feeding studies

All animals were housed individually and had access to two feed bins - one bin was used to feed cereal chaff and the other to feed the grain supplement. All cereal grains were supplemented with limestone (10kg/tonne), urea (10kg/tonne) and virginiamycin (20gm/tonne). Lupin grain was supplemented with limestone only. The chaff was supplemented with a commercial vitamin/mineral premix (2.5kg/tonne, Milne Feeds). Feed residues were weighed daily.

RESULTS & DISCUSSION

1. Quality Control

Enzyme stability

Samples of peri-renal fat were taken at the time of slaughter and then 30 minutes, 60 minutes, and 24 hours after slaughter from 5 carcasses kept in a chiller at 4°C. All samples were put into liquid nitrogen and assayed for the 3 enzymes in the lipogenic pathway: ATP-citrate lyase, NADP-malate dehydrogenase and acetyl CoA carboxylase. The results are summarised in Table 1. There were no significant changes in any of the enzymes during the first 60 minutes after slaughter. By 24 hours there were still no changes in ATP-citrate lyase and NADP-malate dehydrogenase but the activity of acetyl CoA carboxylase had declined significantly to approximately half of its original level. There were significant differences in the enzyme concentrations between sheep ($P<0.02$) for all of the enzymes.

The results indicate that samples of fat can be taken for analysis of lipid synthesis enzymes in post mortem samples provided that they are taken within 1 hour of slaughter. Samples may also be taken for analysis of ATP citrate lyase and NADP malate dehydrogenase even after 24 hours and this provides the opportunity to take samples in conjunction with chiller assessment of marbling in order to test whether the activity of the citrate lyase pathway is correlated with marbling.

Table 1. Activity of ATP-citrate lyase, NADP-malate dehydrogenase and Acetyl CoA carboxylase in samples of peri-renal fat in sheep carcasses at various times after slaughter.

Time after Slaughter	ATP-citrate lyase	NADP- malate dehydrogenase	Acetyl CoA carboxylase
0	45.5	283	75
30 min	36.2	283	67
60 min	35.8	290	79
24 hours	35.4	328	32*
Significance (P)	0.87	0.46	0.003

Effect of site of sampling

Samples of subcutaneous and peri-renal fat were taken from 32 of the sheep slaughtered at the end of the infusion experiment. There was a significant difference in activity of ATP citrate lyase and NADP malate dehydrogenase between peri-renal and subcutaneous fat. In the case of ATP citrate lyase there were higher levels of activity ($P<0.05$) in subcutaneous fat than in peri-renal fat (69 vs 51). Activity of NADP malate dehydrogenase was higher ($P<0.0001$) in kidney fat than in subcutaneous fat (161 vs 109). There was no significant difference between sites in the activity of Acetyl CoA carboxylase.

2. Glucose Infusion Studies

Effects of Glucose infusion and feed intake

An experiment was conducted in which 5 year old merino wethers (50kg) were given 5 levels of energy intake (of the lupin/chaff pellet described above) with or without an intravenous infusion of glucose (5 x 2 = 10 treatment groups each with 5 animals). The levels of energy intake were calculated to provide 0.8, 1.1, 1.4, 1.7 and 2.0 x the maintenance requirement. Glucose infusions supplied 90 g/d and were continued for 21 days before all animals were slaughtered. Samples of fat were taken at slaughter from subcutaneous and peri-renal depots. Samples of fat were again taken 24 hours after slaughter in order to examine the stability of enzymes post-mortem (see Quality control section above).

The results of this trial are summarised in Figs 3-6. The concentration of glucose in blood was significantly elevated ($P<0.0001$) in the glucose infusion treatment but importantly this elevation was relatively small indicating that the model was well within normal physiological limits.

Fig 3 The effects of glucose infusion on the concentration of glucose in blood.

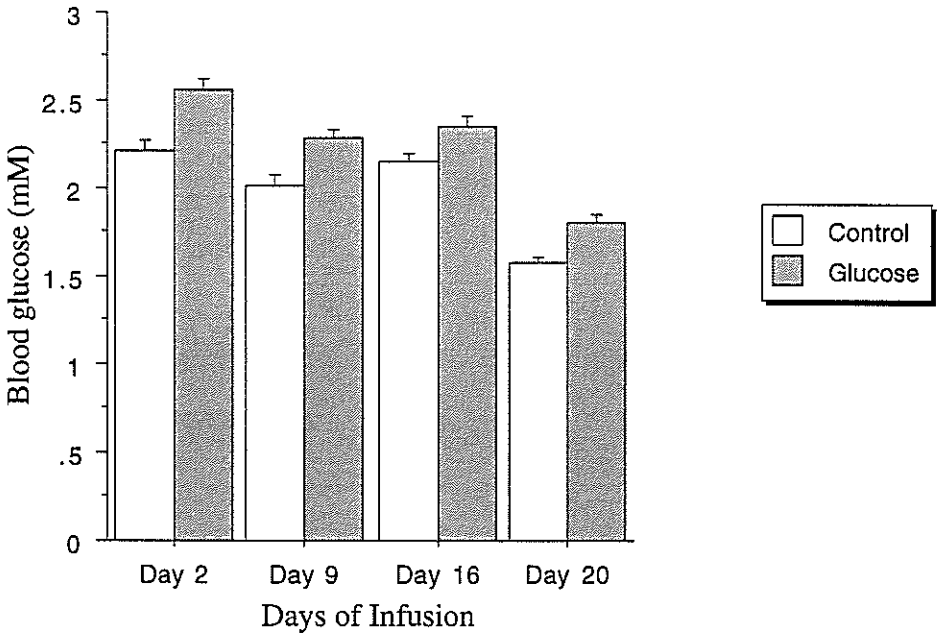
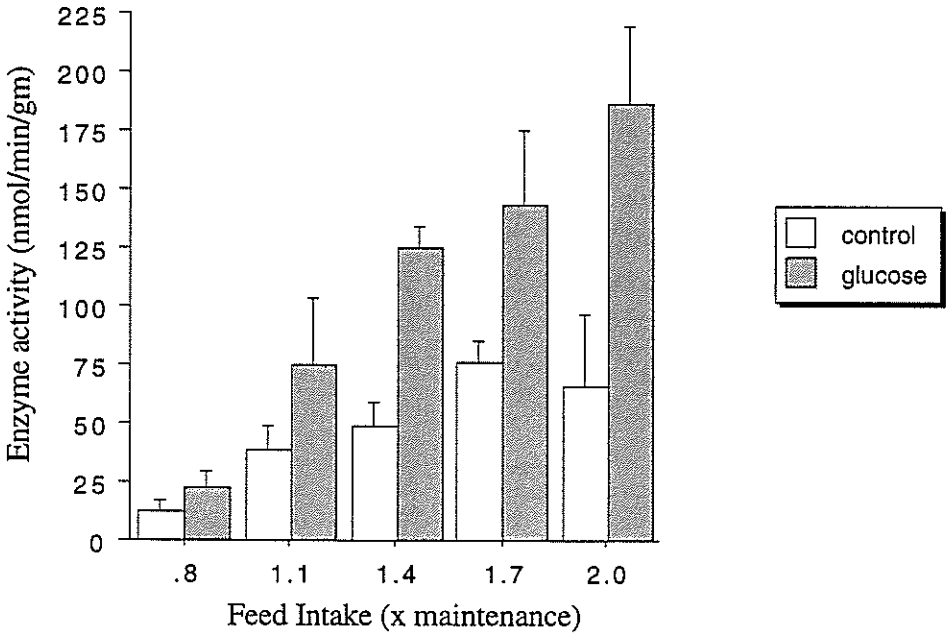


Fig 4 The effects of feeding level and glucose infusion on the activity of ATP citrate lyase in subcutaneous adipose tissue.



There was a clear response for all 3 enzymes to increasing levels of intake and to glucose infusion. The data for acetlyCoA carboxylase (fig. 6) is incomplete since it took some extra time to perfect this assay and accordingly sufficient fat tissue remained only in those

groups shown. The peri-renal fat depots showed a similar response to the subcutaneous adipose (see Mile Stone Report 1)

Fig. 5 The effects of feeding level and glucose infusion on the activity of NADP malate dehydrogenase in subcutaneous adipose tissue.

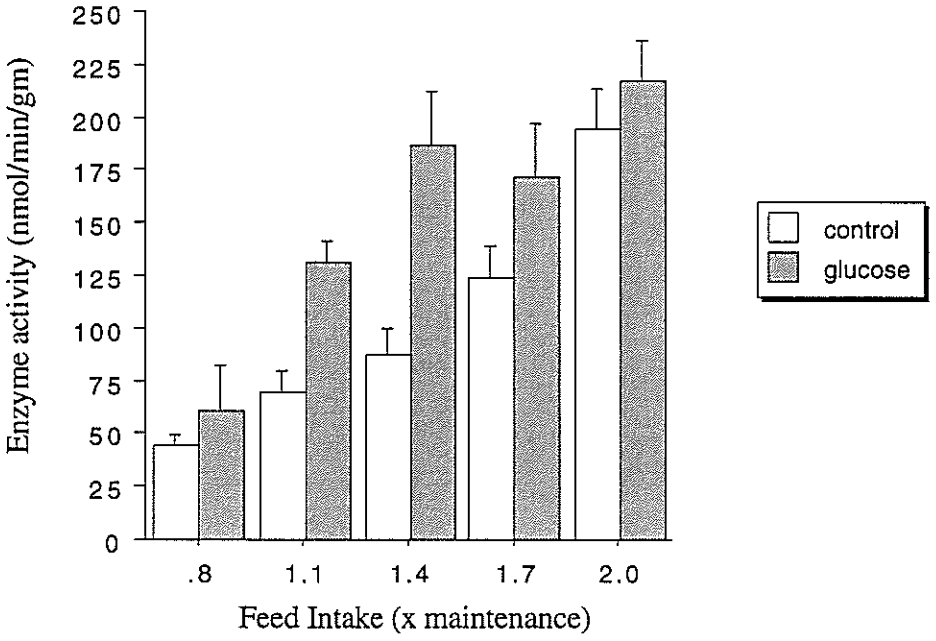
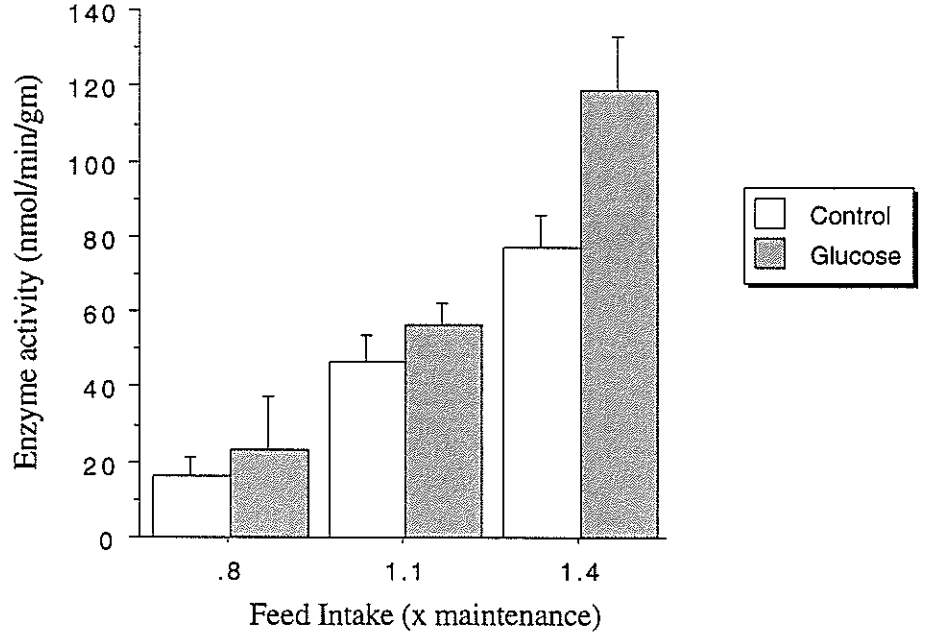


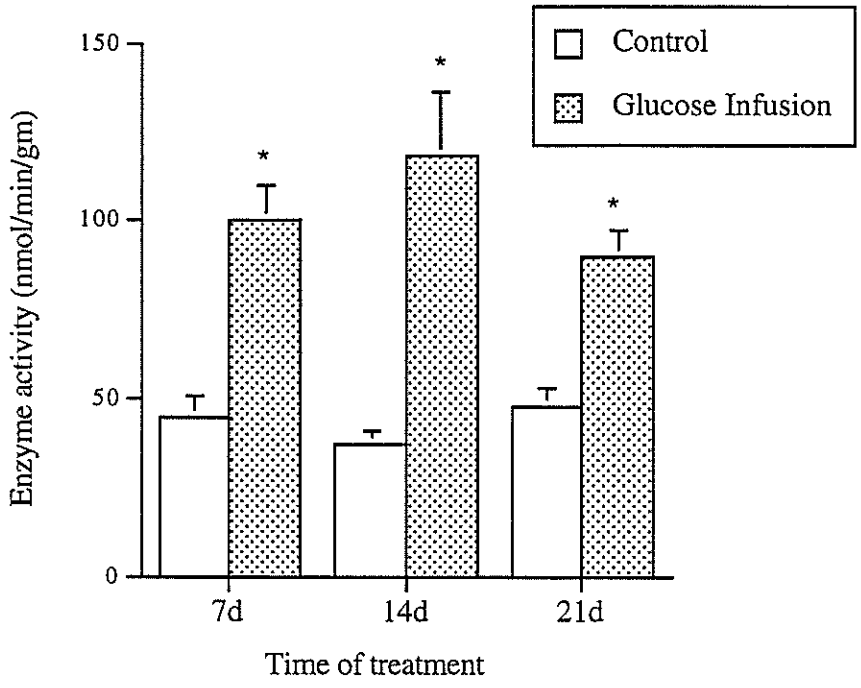
Fig. 6 The effects of feeding level and glucose infusion on the activity of acetylCoA carboxylase in subcutaneous adipose tissue



Time course

In this experiment 90gm of glucose was continuously infused intrajugularly and repeated biopsies of subcutaneous fat were taken for measurement of lipid enzymes. Ten Merino Dorset cross wethers (1 year old, 40kg) were fed a lupin based diet at 1.5 times maintenance with 5 control and 5 infused with glucose. The results for ATP citrate lyase are shown in fig.7. It is apparent that the response of the "glucose" pathway for fat biosynthesis was rapid with no further increases after 7 days. It was noted in this experiment that NADP malate dehydrogenase was not reliably influenced by glucose infusion - the activity remained constant and was always higher 2-3 times higher than ATP citrate lyase indicating that in the younger animals its activity was under different control and that it may have an additional function (see fig 9).

Fig 7. The effect of time on glucose infusion on the activity of ATP citrate lyase in subcutaneous fat (* significantly different to control, $P<0.05$)

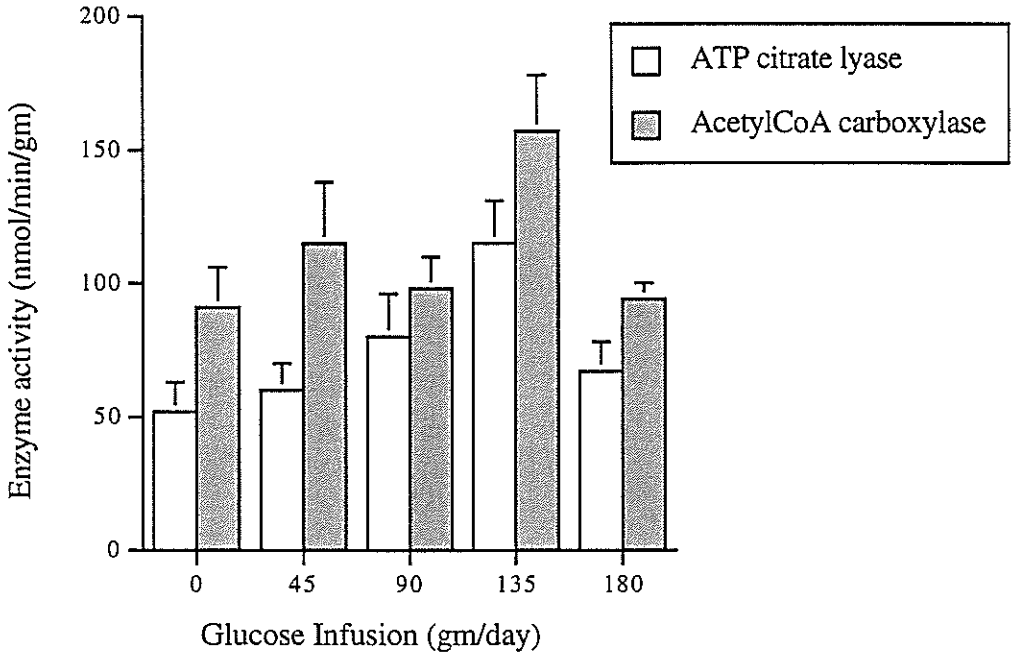


Dose response

The glucose infusion model was further validated over a range of doses from 45-180 gm/day in 1 year old Merino wethers (8 sheep per treatment, 40kg BW) fed a lupin based diet fed at 1.5 times maintenance. The results shown in Fig. 8 outline a significant linear response for ATP citrate lyase (P =0.034) and acetylCoA carboxylase (P =0.055) upto 135 gm/day. At 180 gm/day the response was reduced indicating the model may have become unphysiological. The model used in his study reduced the food intake as the glucose infused increased so all animals were receiving the same amount of metabolisable energy. Accordingly there was a relatively low feed intake at the 180 gm/day level and this may have reduced several important hormonal signals of gut origin which would normally stimulate lipogenesis. In the rat it has been shown that glucose delivered intrajugularly was less effective in altering fat and carbohydrate metabolism than when delivered intragastrically largely due to a reduced insulin response (McGarry *et al.* 1987). It was again noted

that the activity of NADP malate dehydrogenase was not reliably influenced by glucose infusion in this group of young sheep.

Fig 8. The effect of level of glucose infusion on the activity of ATP citrate lyase and AcetylCoA carboxylase in the subcutaneous adipose tissue of sheep.

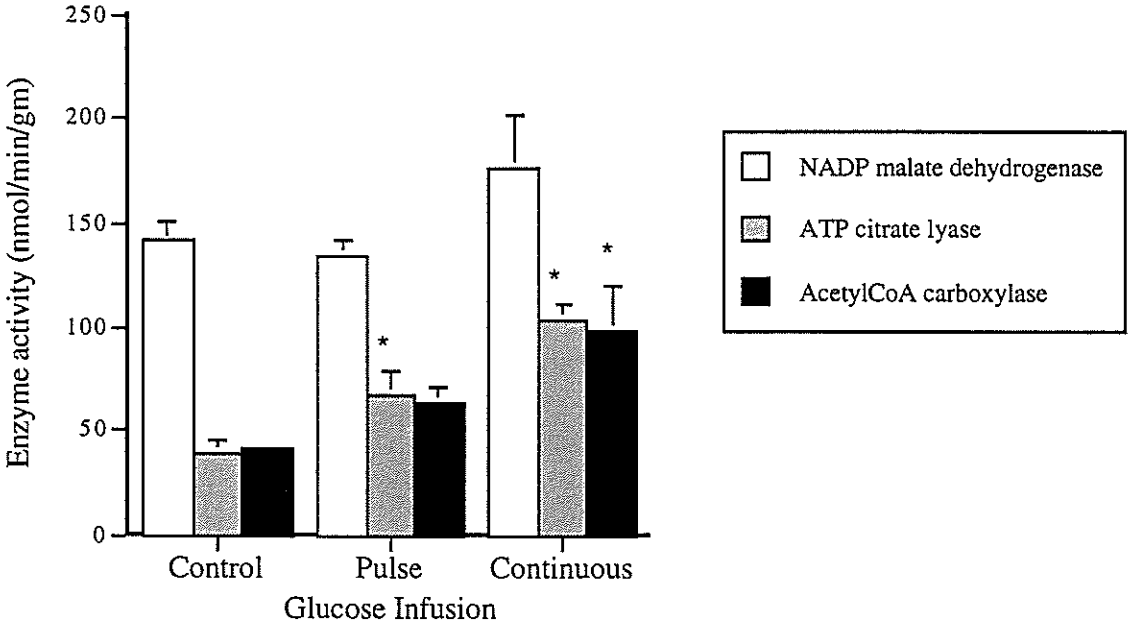


Pulse versus continuous infusion

Glucose was either not infused (n=8), infused continuously (n=8) or infused over a 4 hour period (n=4) at a rate of 90 gm/day in Merino Dorset cross wethers (1 year old, 40kg) fed a lupin based diet at 1.5 times maintenance. The continuous infusion of glucose significantly ($P<0.05$) increased the activity of the "glucose" pathway (ATP citrate lyase) and the overall capacity for fat biosynthesis (acetlyCoA carboxylase); a finding consistent with our previous work (Fig. 9). When the same quantity of glucose was infused over a 4 hour period the increase in lipid enzymes was reduced. The level of blood glucose was marginally elevated in the continuous infusion treatment (20%) however in the pulse infusion model the level of blood glucose increased to non physiological levels (i.e. from 3.5mM to 12-16mM). These very high levels of glucose in the blood were well above the renal threshold (10mM) and accordingly much of the infused glucose would have been lost in the urine and so the amount of glucose available to the animal would have been well below 90 gm/day. Consequently the lipid enzymes respond to the amount of glucose available at the tissue level.

It was again noted that the activity of NADP malate dehydrogenase was not reliably influenced by glucose infusion in this group of young sheep.

Fig 9. The effect of continuous versus pulse infusion of glucose on the activity of ATP citrate lyase, NADP malate dehydrogenase and AcetylCoA carboxylase in the subcutaneous adipose tissue of sheep (* significantly different to control, $P<0.05$).



Sheep as a model for cattle

Five Hereford steers (340kg) were trained to lead using a halter and were accustomed to experimental handling under confined and tethered conditions. Animals were fed a lupin based diet of pellets (5.0 kg/d) and hay (1.5 kg/d) estimated to provide approximately 1.4 x maintenance requirement for three weeks prior to a sample of fat being taken by biopsy from the base of the tail. The amount of pellets and hay were then reduced to 4.5 and 0.75 kg respectively and an intravenous infusion commenced supplying approximately 1.8 g/kg liveweight per day for 11 days. On day 11 of the infusion a further sample of fat was taken by biopsy and analysed for activity of the lipid enzymes.

Table 3. The effect of an infusion of an intravenous of glucose (720 g/d) on activities of ATP citrate lyase, NADP malate dehydrogenase and acetyl CoA carboxylase in samples of subcutaneous fat in cattle.

Enzyme activity	Control		Glucose infusion		Significance
	Mean	SE	Mean	SE	(*P*)
ATP-citrate	17	2.8	101	26.8	0.025
NADP - malate	97	20.6	199	33.2	0.002
Acetyl-CoA	48	11.7	116	21.3	0.009

It is clear that the enzymes associated with the citrate cleavage pathway responded to glucose infusions in both sheep and cattle experiments.

3. Major conclusions of glucose infusion studies

1. It is possible to take fat samples for enzymatic analysis in conjunction with chiller assessment of marbling in order to test whether the activity of the ATP citrate lyase is correlated with marbling.

2. Glucose when administered at physiological doses as a nutrient under isojoulic conditions increases the capacity for:

   (a) lipogenesis from glucose as assessed by the activity of ATP citrate lyase
   (b) total lipogenesis as assessed by the activity of acetylCoA carboxylase

3. Increasing feed intake increases the capacity for:

   (a) lipogenesis from glucose as assessed by the activity of ATP citrate lyase
   (b) total lipogenesis as assessed by the activity of acetylCoA carboxylase

4. ATP citrate lyase is a more reliable indicator for the capacity of lipogenesis from glucose than NADP malate dehydrogenase; especially in younger sheep.

5. Subcutaneous adipose tissue represents a convenient and sensitive site to monitor the effects of glucose and diet on the activity of the lipid enzymes.

6. Sheep and cattle respond in a similar manner with respect to control of lipogenic pathways by glucose availability.

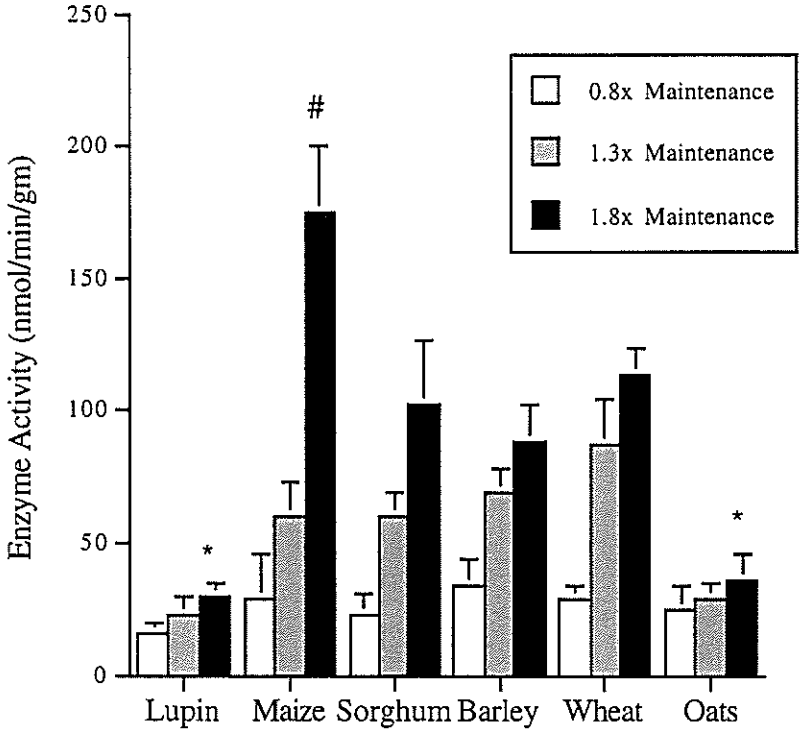
4. The effect of different grains on lipid enzymes.

One year old Merino wethers with a live weight of 35kg were randomly allocated to one of 18 treatments. The treatments were 6 grains (wheat, barley, maize, sorghum, lupin and oats) fed at three levels of intake (0.8, 1.3 and 1.8 times maintenance) with 8 sheep per treatment. The ration also included 200gm cereal chaff per day which was fed separately. Other dietary additions included 20gm/tonne virginiamycin, 1% urea, 1% limestone and a vitamin/mineral premix (Milne Feeds).

Sheep were acclimatised to individual pens for 3.5 weeks and then introduced to the full grain ration after receiving a drench of virginiamycin. They were fed the grain ration for 3 months and after feed intake was stabilised for a 30 day period the animals were slaughtered and subcutaneous fat collected and measured for the activity of the lipid enzymes.

There were significanct effects of grain type ($P<0.0001$), level of feeding ($P<0.0001$) and a level of feeding by grain type interaction ($P<0.0001$) on the activity of ATP citrate lyase in subcutaneous adipose tissue as shown in Fig. 10.

Fig 10. The effect of gain and level of feeding on the activity of ATP citrate lyase in the subcutaneous adipose tissue of sheep (* significantly lower than all other grains; # significantly higher than all other grains, $P<0.05$).

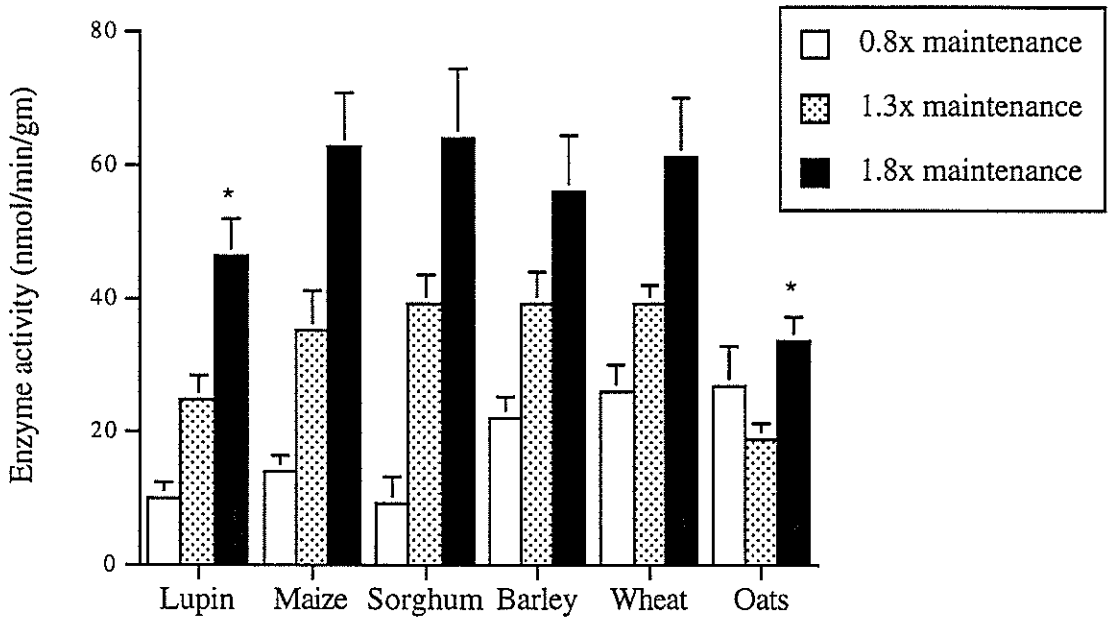


Even at the highest level of feeding (1.8 times maintenance) lupin and oat grain resulted in very low levels of the "glucose" pathway for fat biosynthesis. The likely reason is low levels of starch in the lupin grain and highly rumen degradable starch in the oats (Huntington,1994) resulted in little glucose absorption in the small intestine on these diets.

Feeding maize grain at 1.8x maintenance resulted in the highest level of ATP citrate lyase in subcutaneous fat and this reflects the high levels of starch escaping fermentation and becoming available for digestion and absorption in the small intestine (Huntington,1994). There was no significant difference in the activity of ATP citrate lyase in fat when either barley, wheat or sorghum were fed. This indicates that all 3 grains resulted in similar levels of starch being digested and absorbed in the small intestine. Perhaps the surprising result is for sorghum since this grain is known to allow starch to escape rumen fermentation. It is probable that the starch in sorghum was resistant to digestion both in the rumen and small intestine and that some processing of sorghum (steam flaking) might create the perfect balance between the ability to resist fermentation but still be digested in the small intestine.

The overall capacity for lipogenesis from all sources (glucose + acetate) in sheep eating different grains is shown as the activity of acetlyCoA carboxylase in adipose tissue in Fig. 11. The level of feeding significantly ($P<0.0001$) affected the activity of acetlyCoA carboxylase validating the use of this enzyme to estimate overall lipogenic rate. The type of grain also had a significant ($P<0.0001$) effect on the total lipogenic capacity with the inclusion of lupin or oaten grain reducing the overall rate of lipogenesis indicating that when little or no starch escapes fermentation lipogenesis is reduced. However when sheep consumed either sorghum, maize, barley or wheat the rate of lipogenesis was similar.

Fig.11. The effect of grain type and level of feeding on the activity of AcetylCoA carboxylase in the subcutaneous adipose tissue of sheep (* significantly lower than the other grains, $P<0.05$).



5. The effect of daily versus twice weekly (pulse) feeding of grain on animal performance and lipid enzymes

In this experiment 124 Merino wethers, 2 years oldwith a live weight of 49.5kg were randomly allocated to one of 10 treatments. The treatments were 5 grains (wheat, barley, maize, sorghum, steamed flaked sorghum) fed either daily or twice weekly (pulse fed) at 1kg (as fed, 90% dry matter) per head per day. The ration also included 200gm cereal chaff per day which was fed separately. Other dietary additions included 20gm/tonne virginiamycin, 1% urea, 1% limestone and a vitamin/mineral premix (Milne Feeds).

Sheep were acclimatised to individual pens for 3.5 weeks and then introduced to the full grain ration after receiving a drench of virginiamycin. They were fed the grain ration for 8 weeks and then slaughtered over 2 days at a local abattoir. The animals were transported to the abattoir at 6.30am and were slaughtered by 11.00am. The daily fed animals were slaughtered first and the pulse fed group on the following day. The pulse fed group were slaughtered 1 day after receiving their half weekly ration (i.e. 3.5kg grain). Samples of subcutaneous fat were collected for analysis of lipid enzymes and carcass weight, fat depth C, fat depth GR were measured 48 hours postmortem.

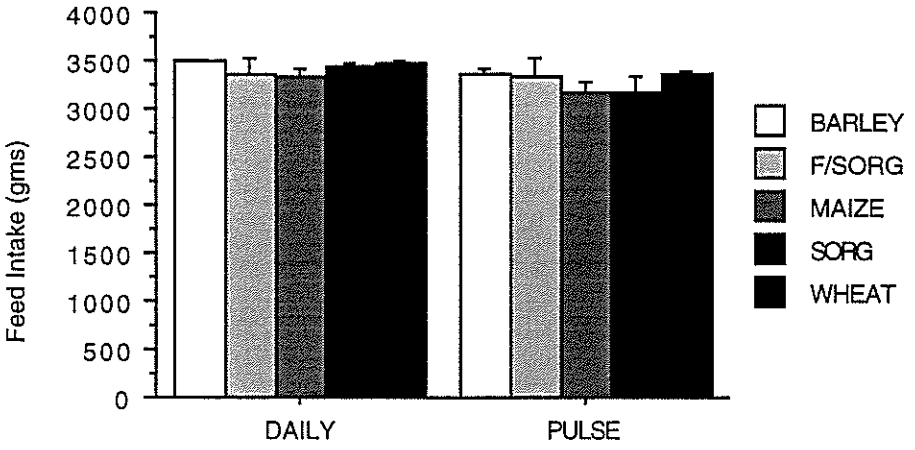
Feed Intake

The data for feed intake unless otherwise stated is based on the last 5 weeks of the trial when intake had stabilised.

The feed intake for daily and pulse fed sheep expressed as the total half weekly intake is shown in Fig.12. Full intake represents 3.5 kg. There was no significant effect of grain type but the pulse feeding group had a significantly lower intake (P=0.029). The

mean reduction was 4.1% (range 1.1-7.6%) with little change for flaked sorghum and the largest reduction for unprocessed sorghum.

Fig. 12. Total feed intake expressed as feed consumed over 3.5 days in daily versus pulse fed sheep (theoretical maximum intake = 3500gms)



The pattern of intake is shown below in Figs. 13-15 . Fig. 13. shows the consumption of grain during the first day after a pulse of 3.5kg of grain was offered; for comparison the intake of the daily fed group is also shown. There was a trend for grain type to influence intake (P=0.11) and this was due to a lower consumption of flaked sorghum in the pulse fed group. Pulse feeding resulted in a large increase (P<0.0001) in intake on the first day after the half weekly dose of grain was offered.

Fig. 13. Feed intake on day 1 in daily versus pulse fed sheep.

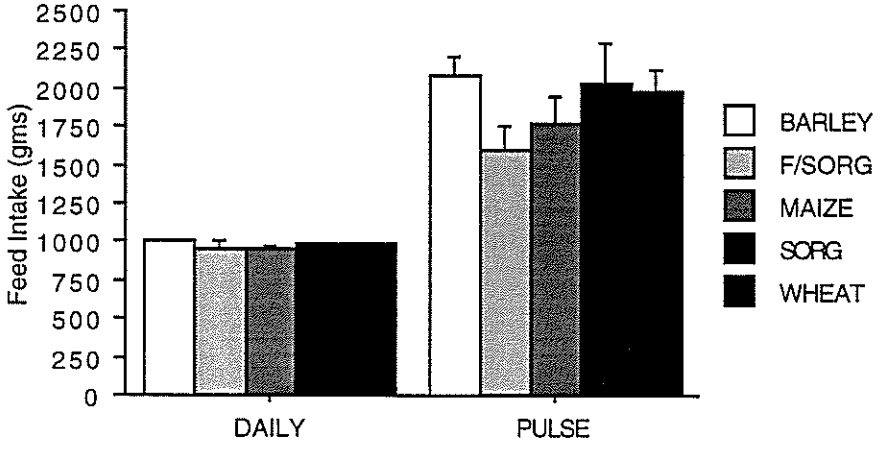


Fig. 14 Shows the consumption of grain in the second day after the pulse of 3.5kg of grain was offered. There was no effect of grain type but a significant reduction (mean of 166gm, P<0.0001) in intake when compared to the daily feeding regime.

Fig. 14. Feed intake on day 2 in daily versus pulse fed sheep

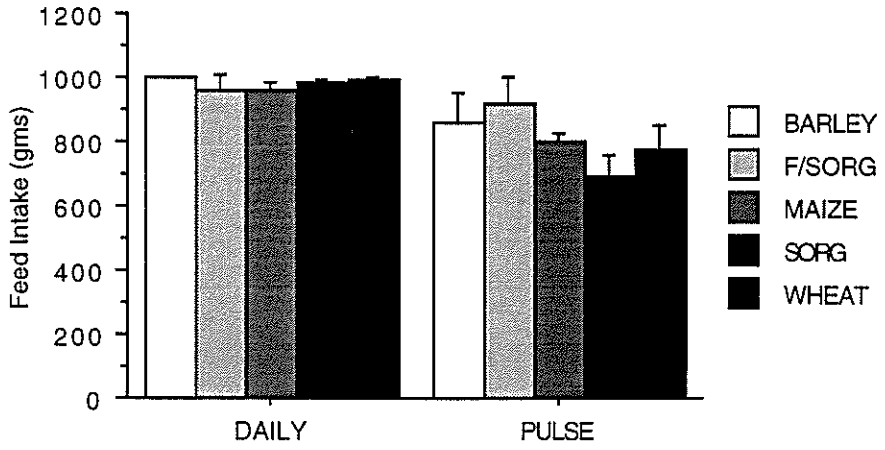
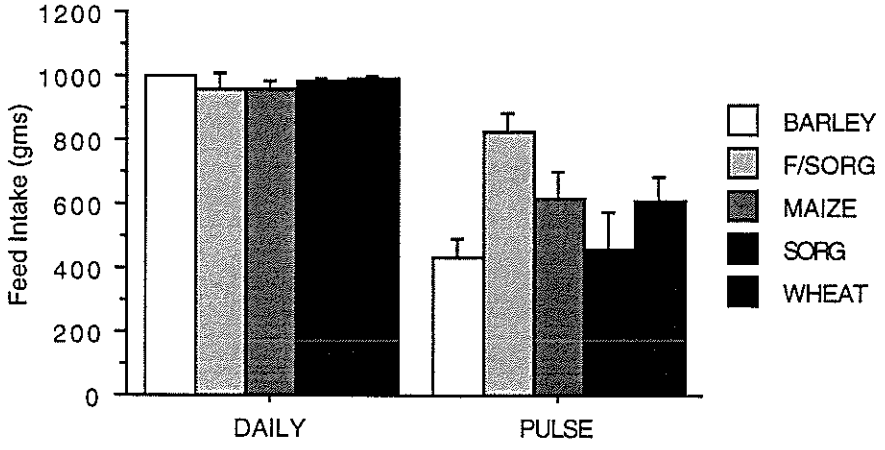


Fig. 15. Shows the consumption of grain in the third day after the pulse of 3.5kg of grain was offered. There was a significant effect of grain type (P=0.014) with the intake of flaked sorghum being higher than the other grains. There was a significant reduction (mean intake of 406gm, P<0.0001) in intake when compared to the daily feeding regime.

Fig. 15. Feed intake on day 3 in daily versus pulse fed sheep.

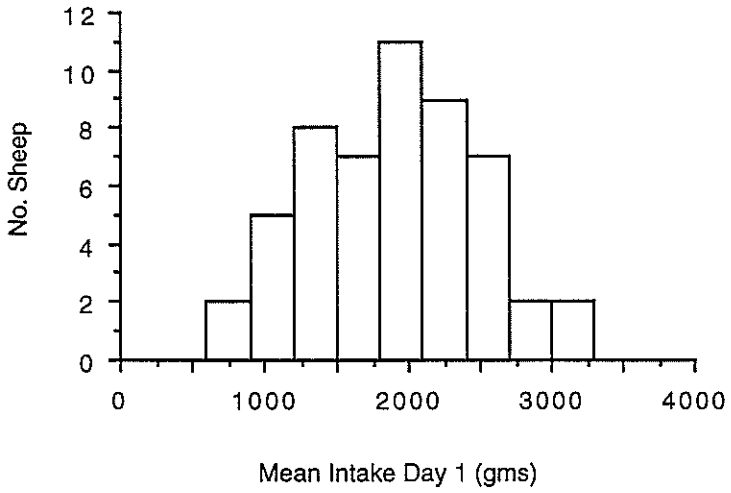


We can conclude for most diets that the pulse fed sheep were eating in a truly pulsatile manner with an intake of about 2, 0.75 and 0.5kg on days 1,2 and 3 after receiving the half weekly dose of grain. Steamed flaked sorghum was the exception with the pulse fed group being relatively similar to the daily fed group.

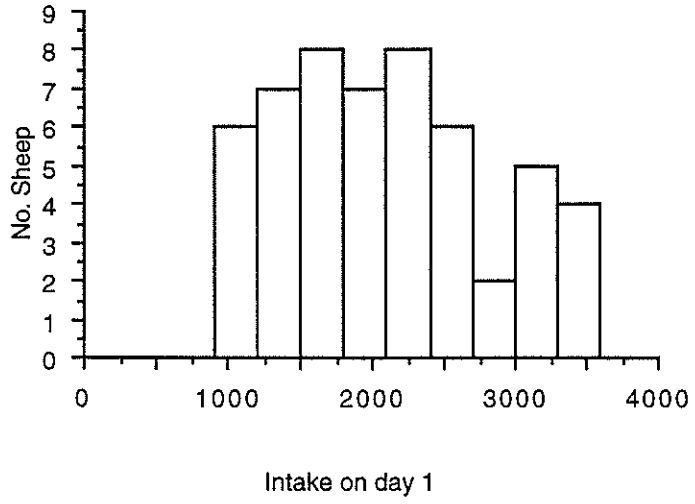
There was considerable variability in the pattern of intake for the pulse fed group. The frequency distribution of the amount of grain consumed on day 1 after a pulse is shown in figs. 16(a) - (c).

Fig. 16. The pattern of grain intake for the pulse fed sheep on day 1 after feeding: 16(a) mean over 5 weeks; 16(b) for the second last feeding before slaughter and 16(c) the last feed before slaughter.

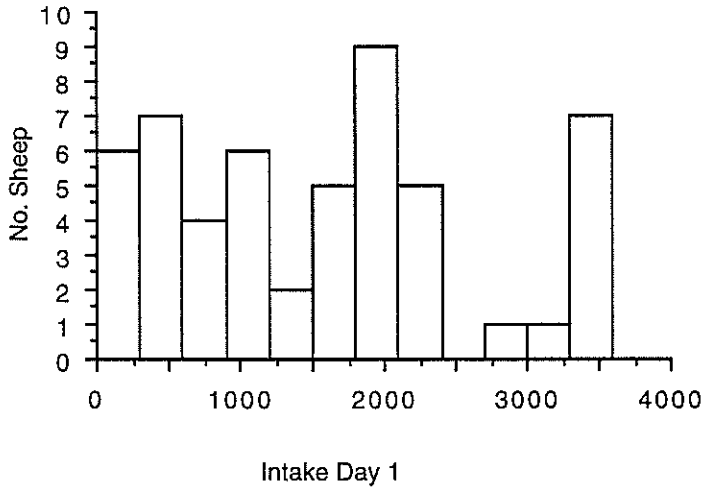
16(a) Mean pattern over 5 weeks



16(b) Pattern for the second last feeding before slaughter



16(c) Pattern for the last feed before slaughter

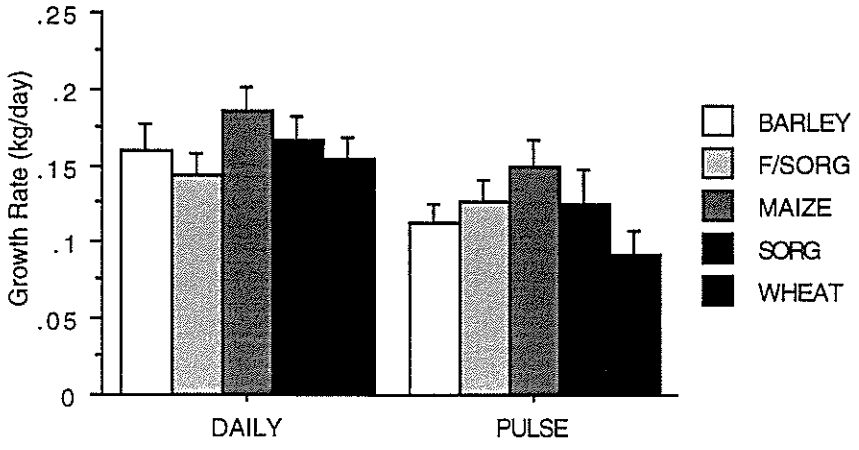


It is clear from the frequency distributions that on average (Fig. 16(a)) there was a near normal distribution of intake pattern with the mean intake of around 2kg and few sheep consuming over 2.5kg. However towards the end of the experiment (i.e. 4 days pre slaughter, Fig 16(b)) the pattern of intake was more wide spread with a large proportion of animals at either end of the distribution i.e. 1-1.5kg per day versus 2.5-3.5kg per day. On the day before slaughter there was a large proportion of sheep that ate poorly (Fig. 16(c)). The later fact is related to half the sheep being removed from the shed the day before i.e. the daily fed group of sheep were taken for slaughter. This animal movement clearly affected intake of those remaining.

Carcass Parameters -growth rate

The growth rate for all treatments is shown in Fig. 17. The pulse feeding of grain resulted in a significant ($P<0.0001$) decline in growth rate by 25% (mean 42 gm/day). There was a trend for an effect of grain type on growth rate ($P=0.07$) due to higher rates of growth on the maize based ration. The pulse fed group had slightly lower intakes (Fig 12) however the conversion of grain into body weight gain was still lower for the pulse fed group (6.4 versus 8.2 gm grain/gm gain).

Fig. 17 Growth rate in daily versus pulse fed sheep



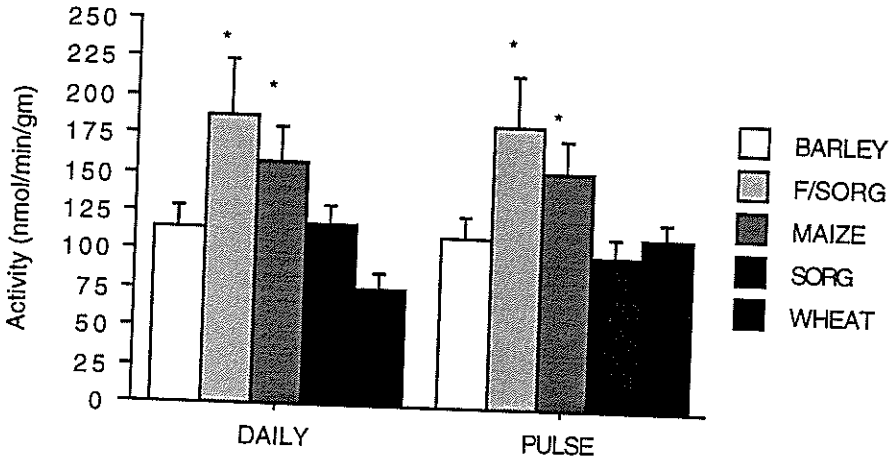
Carcass Parameters - carcass weight & Fat Depth

There were no effects of treatments on carcass weight or fat score. This indicates that gut weight (gut fill in particular) may have had an effect on liveweight gain such that the pulse fed group had lower gut fill and therefore a lower rate of liveweight gain.

Lipid enzymes

The activity of ATP citrate lyase for the treatments is shown in Fig. 18. There was no effect of pulse feeding on enzyme activity, however there was an effect of grain type ($P<0.0002$) with both steam flaked sorghum and maize resulting in elevated levels. This extends the findings of our previous experiments in that processing is essential to allow sorghum in the diet to fully influence the expression of ATP citrate lyase. Processed sorghum (i.e. steam flaking) has a much greater capacity to stimulate the potential rate of lipogenesis from glucose when compared to the unprocessed product . This is related to the very resistant nature of starch within sorghum such that in the raw state the starch escapes digestion in both the rumen and small intestines - appropriate processing still allows some starch to escape fermentation but then to be digested in the small intestine. Maize again showed an increased capacity to stimulate the potential rate of lipogenesis from glucose.

Fig. 18. The activity of ATP citrate lyase in daily versus pulse fed sheep (*significantly different to barley, sorghum and wheat)



A closer analysis of the results in Fig. 18 suggests an effect of pulse feeding wheat on the expression of of ATP citrate lyase - this is shown in more detail in table 2.

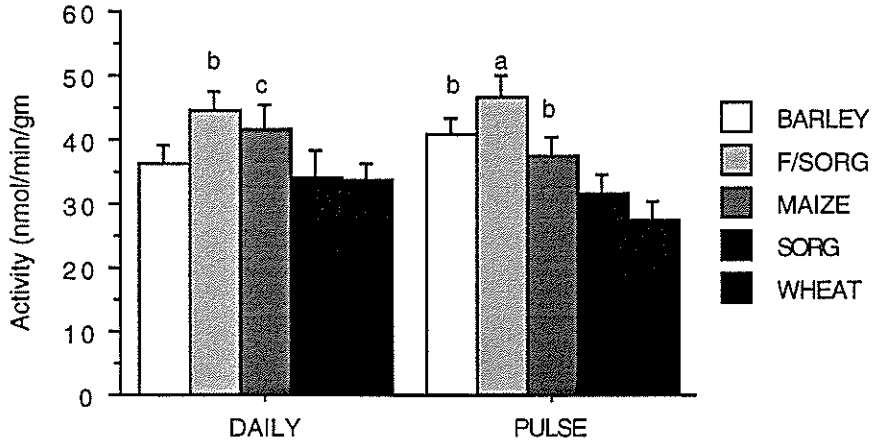
Table 2. The activity of ATP citrate lyase in subcutaneous fat in daily versus pulse fed sheep (* significantly lower than other values).

	Wheat		Barley	Sorghum
	Pulse	Daily	Daily	Daily
ATP citrate lyase (nmol/min/gm)	112±11	77±9*	115±14	117±13

It is clear that pulse feeding of wheat has increased the activity of ATP citrate lyase to equal that found for barley and sorghum. This suggests that pulse feeding can increase the proportion of starch escaping fermentation in the rumen. Wheat starch is known to be very soluble and rapidly fermented, so it is not surprising that wheat showed the biggest response to pulse feeding.

The activity of acetylCoA carboxylase (measure of maximal capacity for fat biosynthesis) in subcutaneous fat for the treatments is shown in fig. 19. There was no effect of pulse feeding but a significant effect of grain type ($P<0.0001$). Flaked sorghum and maize were associated with higher potential maximum rates of lipogenesis in both dietary regimes while in the pulse fed group barley also increased the capacity for lipogenesis.

Fig. 19. The activity of acetylCoA carboxylase in daily versus pulse fed sheep (*a* significantly different to maize, sorghum and wheat; *b* significantly different to sorghum and wheat; *c* significantly different to wheat)



6. Major conclusions of the grain feeding studies

1. The activity of ATP citrate lyase in subcutaneous adipose tissue

   (a) varies markedly depending on the level of feeding and the type of grain in the ration.

   (b) varies according to the predicted level of starch digestion in the small intestine.

   (c) is more sensitive to glucose made available by the digestion of starch in the small intestine than to glucose made available by gluconeogenesis as a result of increased feed.

   (d) is sufficiently sensitive to glucose availability that it can be used to optimise grain processing to maximise digestion of starch in the small intestine i.e. flaking sorghum makes a big difference.

2. Feed grains that result in a low activity of ATP citrate lyase in adipose tissue also result in a lower activity of acetlyCoA carboxylase in adipose tissue - that is diets promoting low rates of starch digestion in the small intestine allow for lower rates of fatty acid synthesis.

3. The extent of starch digestion in the small intestine for the grains tested was:

   Maize > steamed flaked sorghum > sorghum = wheat = barley > oats = lupin

4. Pulse feeding of cereal grains is feasible even at high grain intakes.

5. Pulse feeding results in a small reduction in overall intake perhaps due to a small amount of spoilage.

6. The pattern of grain intake for the pulse fed animals was variable with some animals showing intakes similar to daily fed animals while others consumed all their grain on day 1 and then fasted for 2.5 days. The intakes on day 1 tended to increase as the time frame of the experiment increased.

7. Pulse feeding had a relatively large negative impact on live weight gain but no effect on carcass weight. This suggests that difeerences in gut fill contibuted to the live weight response and feeding in a pulsatile manner did not induce inefficient use of nutrients for growth providing carcass weight is used as the parameter for measurement.

8. Pulse feeding of most cereal grains did not significantly increase the capacity for biosynthesis of fat from glucose as judged by the activity of ATP citrate lyase in adipose tissue in this study. When making this conclusion several reservations are needed because the pulse feeding model is inherently difficult to study since:

   (i) The variations in the pattern of intake become extreme in this model and so the appropriate time to sample adipose tissue to gain an average index of the potential for fat biosynthesis becomes difficult to predict. In this experiment fat samples were taken 1 day after a pulse of grain was fed. Ideally 2-3 biopsy samples are needed. Alternatively 12 month old animals could be kept on the daily versus pulse rations for several months and differences in fat score used to assess fattening potential.

   (ii) There was a bias specific to this experiment that could have reduced the activity of the lipogenic enzymes. Moving the daily fed animals away for slaughter 24 hours before the pulse fed group resulted in some psychological stress sufficient to depress intake (see figure 5(c)) meaning that some animals had a relatively lower intake for 3 days before sampling.

9. Pulse feeding of wheat did increase the activity of ATP citrate lyase suggesting that more starch was digested in the small intestine.

10. It is possible that a less severe pulse feeding regime might be more effective i.e. feeding every second day or feeding a base level of grain (0.8kg) and then pulsing an additional 1kg of grain every second day. This would reduce the period of low nutrition and so make the cycle between anabolic and catabolic states less severe. In the model used in this experiment, any stimulation of the lipid enzymes due to increased glucose availability (via increased feed intake promoting starch digestion in the small intestine) may have been negated by the catabolic events associated with the subsequent relatively low period of nutrition.

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Publications arising from work

Pethick, D.W., Phillips, N., Rowe, J.B. and McIntyre, B.L. (1995) The effect of different dietary grains on the expression of ATP citrate lyase in the adipose tissue of sheep. Proceedings of the Nutrition Society of Australia **19**,132

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Students associated with the work

Nathan Phillips

Honours degree in Biological Sciences, Murdoch University, 1994. Nathan played a key role in the project during both the glucose infusion and grain feeding studies.

Graham Gardner

Honours Degree in Agricultural Science, University of Western Australia. Graham's work on the effect of chromium supplementation on fat and glycogen metabolism represented work beyond the scope of this project but was done as a specific adjunct to the project.

Appendix I

Sheep Fat Assay Integration

Solutions

You need the following stock solutions prior to starting these assays:

- 15mM $MnCl_2$ (fridge)
- 0.15 M Tris-HCl, pH 7.4(fridge)
- 0.1 M $MgCl_2$ (fridge)
- 0.2 M K_3 Citrate (fridge)
- Chilled distilled water for Polytron
- Homogenising buffer stock: 300mM sucrose, 30mM Tris-HCl,1mM EDTA. pH 7.4 (fridge)
- Stock PMSF (7mg PMSF/ml Na_2SO_4-dehydrated propan-2-ol).
    - Propan-2-ol is prepared by shaking with a.few grams of
    - anhydrous Na_2SO_4 then filtering through Whatmans #1.paper (fridge)

- ACC premix buffer: 28.57 mM each of Tris,$MgCl_2$,and K_3 Citrate pH is 7.4
- ACC assay buffer: 40mM each of tris, $MgCl_2$, and K_3.Citrate;
- 0.4mM EDTA, pH 7.4 (fridge)
- 5M NaOH and 1M NaOH
- 6N HCl

Preweighed cofactors

The following cofactors need to be weighed out before the day of assay: (sufficient to assay up to 12 samples in duplicate)

- 26.4 mg CoA (freezer)
- 8.52 mg NADH (fridge)
- 22.72 mg ATP (fridge)
- 121 mg ATP (fridge)
- 9.44 mg NADP (fridge)
- 4.68 mg Na Malate (not maleate!!) (fridge)
- 3 x 30.7 mg reduced glutathione (fridge)
- 2 x 125 mg bovine serum albumin, fatty acid free (fridge)
- 4.84 mg Acetyl CoA (freezer)
- 31.5 mg $NaHCO_3$ (fridge or shelf)

Check that there is sufficient malate dehydrogenase (80 µl 10 000 U/1.2ml) and ß-mercaptoethanol.

Instrumentation

1) Booking Prior to the day of assay, book the Beckman Ultracentrifuge and 70.1 Ti head in either the Vet school or Biology. Book the counter in the Vet school for overnight. Either the Beckman DU-50 spectrophotometer in Biology, or, in emergency, the Vet school DU-8 can be booked.

2) Initial setup On the day of assay, switch on the ultracentrifuge and set the temperature to 4°C / maximum 30°C.

- Switch on the DU-50 and printer and allow the spectro to go through its warm up routine (under 5 minutes).When finished the DU-50 will print "test completed". Switch on temperature controller and set the dial to 36. Lift the return hose from the spectro and confirm that there is some flow of water (only about 1 ml per second)- if no flow then purge cell in theopposite direction of water flow with nitrogen gas - see Leon!). Later confirm that the real temperature of the bath is 37°C by checking the thermometer in the bottom. After warm up press [vis] key to switch on lamp.

Note that the lamp needs at least 15 minutes to settle down before taking any readings.

- Switch on the 37 °C waterbath in the fume hood.

Homogenisation of Sample

Reagents

Homogenisation buffer: Just prior to homogenisation, measure out 100 ml of Homogenisation buffer stock and add 30.7 mg reduced glutathione, and 300 µl PMSF in dehydrated propan-2-ol. This buffer must be used within an hour as both glutathione and PMSF rapidly go off in solution. Keep it in the fridge until you are ready to use it.

Equipment

- Screwdriver x2;
- Brown paper for the floor;
- Hammer;
- 2l of liquid N2 + dewars x2;
- N_2-chilled Macerator and piston x2;
- Plastic base x2;
- Large rubber mallet;
- One empty esky for liquid N_2;
- N_2-chilled medium-sized spatula (in esky) x2;
- N_2-chilled long test-tube for each sample;
- Beaker filled with ice for Polytron;
- Three ice filled eskys;
- Cotton and rubber gloves;
- Long N_2-chilled forceps x2;
- Chilled distilled water;
- Triplicate ice chilled test tubes for receiving infranatant;
- Whatman papers,5cm x 3cm;
- Ultra-centrifuge tubes (on ice);
- Plastic pasteur pipettes for balancing tubes;
- Cotton buds for cleaning rotor head;

Procedure

All work is to be carried out wearing double layer gloves - cotton under covered by latex outer gloves.

Transfer approximately 2g of fat tissue from liquid nitrogen to the chilled macerator (use the hammer if necessary). Install the piston and thump it hard 2-3 times with a **rubber mallet**. Remove the piston and transfer it to liquid nitrogen in esky, and then break up the pellet in the macerator with a pre-chilled screwdriver. Replace the piston and strike it again. Break up the pellet into a powder with the screwdriver and transfer 1.5 g accurately to a chilled, labelled test tube. Add 3 volumes of homogenisation buffer (4.5

ml) to the tube and store on ice for a few minutes to allow the pellet and tube to warm up. Knock it gently against the bench to disperse the pellet.

Chill the Polytron probe by immersing it into a measuring cylinder of chilled distilled water. Just before use wipe the probe dry with Kleenex. After placing the test-tube into an ice-filled beaker, disperse the fatty powder with 5 strokes of the polytron, power setting = "7". Immediately transfer the tubes to the ice bath. Rinse and dry the probe between homogenisations. After the last tube has been dispersed, clean the probe by 'homogenising' a 30% solution of Decon 90 and hot water. Rinse the probe in a further 5 changes of hot water, then one change of cold distilled water.

Centrifugation

Chill the ultracentrifuge tubes on ice. Transfer the homogenates into the tubes and balance the ultracentrifuge tubes to within +/- 0.01 gram. Insert balanced ultracentrifuge tubes into a prechilled ultracentrifuge 70.1Ti rotor. Install rotor into prechilled centrifuge and spin at 58 000 RPM for 20 minutes (~100 000 g h).

Set spin parameters according to the instructions accompanying the ultracentrifuge with particular note to the following:

rpm = 58 000
time = 20 min
temp = 4°C, max temp = 30°C
mode = time
vacuum = on

Parameters are entered by pressing either the up or down arrows and holding the keys under the appropriate displays.

YOU MUST SELECT VACUUM AND WAIT FOR THE RED VACUUM LIGHT TO EXTINGUISH BEFORE STARTING THE RUN.

Press Autostart, and wait for the centrifuge to attain full speed.

After the run has finished, release the vacuum, dry the motor head if necessary transfer the tubes to ice and remove the infranatant into chilled tubes.

Divide this fluid up into 3 sub lots: (680 µl) ATP Citrate Lyase, Malic Enzyme (680µl) and Acetyl CoA Carboxylase (450µl). **Keep cytosol on ice until just prior to assay.**

Setting up the DU-50 Spectrophotometer.

Ensure that the DU-50 is warmed up and that the blue program module labelled kinetics is installed in the memory socket. Follow the following sequence of keystrokes:

Keystroke	Action
[prog]	
[step]	until machine shows prog 6: kindata
[R/S]	
0 [enter]	edit the program
340 [enter]	selects the wavelength for the assay (340 nm)
6 [enter]	selects number of cells
0 [enter]	number of blanks in each run
1 [enter]	output results as a table
15 [enter]	sets interval time to 15 seconds
5 [enter]	sets total time of assay to 5 minutes
0 [enter]	don't plot results as a graph
0 [enter]	automatically span graph results (illogical I Know)
0 [enter]	select a negative slope for NADH disappearance
1 [enter]	returns user to a program at the end of the run to calculate linear regression of the data.
3.21543[enter]	outputs results as µmoles NADH consumed or NADPH generated. g/tissue/min
0 [enter]	select calibration for spectro

Insert dist. water

[R/S] calibrates spectro

ATP Citrate Lyase

Note: Enzyme is unstable and must be assayed before all others. **Keep cytosol on ice to minimise enzyme loss.**

Reagents (sufficient for 40 cuvette assay)

On the day of assay make up the following cofactors:

26.4mg CoA + 2ml H_2O

8.52mg NADH + 2ml H_2O

121 mg ATP + 380 µl 1M NaOH + 1.62 ml H_2O

Then make up the 'Stock solutions':

4ml	0.15M, pH 7.4 Tris-HCl
4ml	0.1M $MgCl_2$
4ml	0.2M K_3Citrate
1.2ml	CoA
2ml	NADH
28µl	ß-mercaptoethanol (in fume hood)
80µl	malate dehydrogenase (Sigma 10 000 U/1.2ml)(check activity)
18.66ml	distilled water

You will need to take the following items to run the assay:

- Stop watch
- Cuvettes
- loo paper
- 200 µl pipette and tips
- 1ml pipette and tips
- esky with ice and cytosol
- stock solutions, ATP (on ice)
- "golf club" cuvette mixers
- distilled water bottle
- beaker
- pencil

Prepare the following sets of cuvettes for each fat sample to be assayed:

	Volume of premix	**Volume of cytosol**	**Volume of water**	**Volume of ATP**
Blank 1	750 µl	200 µl	50 µl	-
Sample 1	750 µl	200 µl	-	50 µl
Sample 1	750 µl	200 µl	-	50 µl
Blank 2	750 µl	200 µl	50 µl	-
Sample 2	750 µl	200 µl	-	50 µl
Sample 2	750 µl	200 µl	-	50 µl

Wait until first set of samples is running before making up the next set (ATP citrate lyase goes off quickly so store on ice as close as possible preincubation)

Wait until 2nd set of samples is runnung before making up the next set...

Preincubate cuvettes for 10 minutes in spectrophotometer in order to bring them up to temperature and to remove endogenous substrate.

Start the assay by adding 50µl ATP to the sample cuvettes only. Mix and load cuvettes into the spectro and press [R/S] to start the kinetics program. The spectro will read the samples for 5 minutes. During this time start preparing the next set of cuvettes for warming up. When the run has finished, insert the new set of cuvettes and set your timer for 10 min. Then type in the numbers of the cells (1-6) in response to the spectros prompts to get the rate of NADH disappearance, as shown below:

#of cell [enter]
0 for blank [enter]
0 for time 1
5 for time 2
[enter] to use the default factor

Repeat this for all six cells.

At the end of calculating the rate for all cells, type 0 [enter] to quit the results program. Type [R/S] to go to the beginning of the kinetics program, 1 [enter] to start the program. Type [R/S] to start the assay after 10 min warm-up and addition of ATP.

Malic Enzyme

Reagents (This is sufficient for a 40 cuvette assay).

On the day of assay, make up the following cofactors:

NADP:	9.44 mg + 2ml H_2O
Na Malate:	4.68 mg + 2ml H_2O

Prepare the following premix:

Stock Tris-HCL	8ml
Stock $MnCl_2$	8ml
NADP	2ml
water	16ml

Set up the assay as shown on the table on the ATP citrate lyase assay.

Use the spectro program you set up for the DU-50 for ATP-Citrate Lyase.

Place 6 cuvettes in the spectro and preincubate for 10 minutes.

Start the reaction by quickly adding 50 µl Malate with the 200µl pipette to all cuvettes except the blanks, mix cuvettes then start the program [R/S], 1 to run the samples then [R/S] to start the main program.

At the end of the program type in the numbers of the cells (1-6) in response to the spectros prompts to get the rate of NADPH formation.

Follow the instructions for ATP citrate lyase.

Acetyl CoA Carboxylase Assay Protocol

Reagents

1) Stock isotope. 1mCi of $14NaHCO_3$ is made up to 5 ml with freshly-made 0.1M NaOH. This is stored sealed in the fume hood at room temperature.

2) Premix Solution. 11mM of Tris and 28.57mM each of $MgCl_2$,and K_3 Citrate, pH 7.4 is stored chilled in the fridge. On the day of assay, 30.7 mg glutathione and 125 mg BSA is added to 35 ml of this solution and prewarmed to 37°C for at least 10 minutes prior to preincubation. (final assay tube concentrations of GSH and BSA are 2mM and 2.5 mg/ml respectively).

3) Assay Mix. Make 25ml of assay buffer (in the fridge) up to 47.5ml with water and add 30.7 mg GSH, 125 mg BSA. 14.25 ml of this solution is removed, and to it are added 4.84 mg acetyl CoA, 22.72 mg ATP (which has been neutralised with 14.5µl 5M NaOH),and 31 mg $NaHCO_3$ premixed with 750 µl of 200µCi/ml $NaH14CO_3$. This is preincubated at least 10 minutes prior to addition to assay. Keep assay mix covered when not in use to avoid CO_2 exchange with the atmosphere. Add two 20µl aliquots of reaction mix straight into scintillant (ie without drying on the line) in order to determine specific activity of the reaction mix.

4) 6N HCl stop reagent.

Preincubation

Label and prechill your assay tubes.

Add 150µl of each sample to a clean test tube. Use 150µl of the homogenising buffer as a blank.

Start the preincubation by transferring tubes from the ice bucket to the 37°C waterbath and adding 350 µl of premix solution. Stagger the tubes at 10 second intervals. Mix carefully.

After 30 min, from the addition to the first tube, start main assay by adding 500 µl Assay mix to each tube. Stagger the tubes at 10 second intervals. Mix carefully

After 5 min, stop the reaction by adding 200µl HCl. Again, at 10 second intervals.

Transfer 400 µl to 2 layers of 5x 3cm bent Whatman #1 filter paper and air dry on line in fume hood. After 2h, roll up filters, place in vials and add 5ml scintillant (Packard optisafe).