Australian Government
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AUSTRALIAN Pork™

Survey of the methyl donor status and the effectiveness of dietary methyl donor supplement in Australian pigs

Final Report
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Executive Summary

Homocysteine (HCY) is an intermediary product of the methionine cycle that provides methyl groups to DNA, protein and methylation reactions, thus influencing many cellular processes. Therefore, disruptions to the methyl donor status of the animal can have long reaching implications. Additionally, specific knowledge of the metabolic requirements for methyl donors may provide pathways for specific manipulation of tissue accretion. This experiment was conducted in two experiments to 1) conduct a screening of the plasma homocysteine (HCY) concentration in pigs at various growth stages from commercial piggeries in Australia; then 2) assess the ability of dietary supplementation of methyl group donating compounds to reduce HCY concentrations in a commercial system. Experiment 1 confirmed that the majority (71%) of the animals screened from Qld and South Australian sites had a HCY concentration above what is considered normal in pigs (determined to be < 20 μM); with a range in HCY concentration of 5-545 μM observed. Furthermore, the mean plasma HCY concentration (67 μM) was significantly higher than the normal threshold in the pigs sampled. This suggests that there is an imbalance in the methyl donor pathways in commercially raised pigs which is predicted to have negative effects on growth efficiency, health and body composition. Furthermore, there was a significant difference in HCY concentration due to piggery state, with concentrations greater in pigs sampled from S.A. (103 μM) compared to those sampled from Qld. (23 μM). Finally, HCY concentrations were greatest in weaners (129 μM) and lowest in sows (21 μM) with growers (46 μM) and finishers (56 μM) between these extremes. Thus, sows were the only group within the sampled animals that demonstrated a mean HCY concentration that was close to the accepted normal range.

The objective of Experiment 2 was to manipulate the HCY production by the provision of additional methyl donors in the diet via the supplementation of betaine (0.125 and 0.25 %), choline (0.15 and 0.3 %) and methionine (0.2 %). This experiment demonstrated that higher doses of betaine (0.25 %), choline (0.30 %) and methionine can successfully reduce plasma HCY concentrations, although not to below the accepted normal HCY concentration. The HCY concentrations were lower on day 84 (33 μM) compared to day 14 (44 μM), suggesting that either HCY production decreases as growth rates decline or that the continued consumption of the supplemented diets leads to a continual decline in HCY production as the requirement for methyl groups is provided by the diet. There was no difference in the production rates or carcase compositions between treatment groups, therefore it cannot be concluded that a reduction in HCY leads to improved production. As HCY concentrations remained above normal thresholds it may be that increased supplementation rates that lead to more drastic reductions in HCY are required to elicit a production effect. However, as over-supplementation (of betaine for example) may itself lead to production losses if additional energy is required for the animal

to excrete the excess concentration, the provision of increased supplements may not be beneficial in both efficiency and cost. Further studies in more controlled environments are recommended to further elucidate the basal HCY levels in pigs of different ages and to determine the efficacy of nutritional supplements for the manipulation of HCY. Additional measures such as repeated and more frequent blood samples of fasted animals as well as urine samples will further elucidate these basal responses in pigs. Further exploration of dietary supplements to reduce HCY production such as a wider range of doses, combinations of supplements and additional supplements such as folate is warranted.

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1. Background to Research

Methyl donors are involved in many metabolic processes such as hormone signalling, protein synthesis and cell growth. Therefore, abnormal methylation is associated with numerous negative consequences such as impaired embryonic development and embryonic death as well as diseases to most metabolically active tissues including the liver. Methylation is the addition of a methyl (CH_3) group via the methylation cycle whereby methionine is converted into homocysteine using methionine, betaine, choline or folate as sources of methyl groups (methyl donors) as demonstrated in Figure 1. If the source of methyl donors is low there will be an increase in remethylation of homocysteine (HCY), while excess methyl donors may be converted into cysteine for excretion. Thus, high HCY levels indicate an imbalance in the methionine cycle and have been demonstrated to occur in response to human illnesses such as cardiovascular disease, diabetes and other developmental diseases (Cronje 2008). While methyl donor compounds such as choline have been well studied to determine their optimum levels for production, less is understood about the metabolic effects of an imbalance (both deficiencies and excesses) to the methylation cycle.

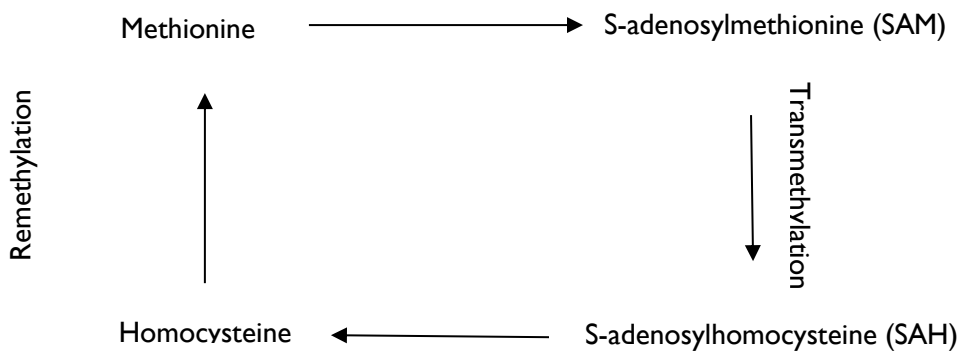

Figure 1. The methionine cycle (adapted from Cronje, 2008).

Reviews of the literature demonstrate that there is limited knowledge on the methyl donor requirements and therefore status of pig herds in Australia. However, numerous studies (including those conducted by the investigators on this application) highlight that the dietary supplementation of methyl donors can have production benefits in pigs. Establishing the methyl donor status of Australian commercial pigs will provide a valuable advancement towards the development of dietary manipulations to balance the methyl donor status and improve the health and production of pigs.

Human studies have demonstrated a positive health effect of reducing HCY levels via the supplementation of betaine, choline and vitamin B12, as increased HCY is a marker of metabolic

imbalance. While these supplements have been observed in pigs there has been no investigation into the health outcomes of these supplements (while growth rate and efficiency are regularly observed) in relation to methylation pathways. The development of dietary supplementation methods that allow specific manipulation of the homocysteine balance, provide a unique opportunity to improve production efficiency in pigs in a cost effective and easy to administer method.

2. Objectives of the Research Project

1. To establish the methyl donor status of an Australian commercial pig herd and provide a prediction of the total daily use of methyl donors of an Australian commercial pig herd
2. To confirm that supplementation of dietary methyl donors can improve production and efficiency in pigs
3. To measure the physiological and metabolic responses to a range of doses of dietary betaine (as a methyl donor) and observe the effects on the methyl donor status of the animal

3. Introductory Technical Information

Methyl donors have multiple key functions in animals such as increasing metabolite absorption, alterations to gene expression and methylation reactions that include hormonal signals, cell growth and protein synthesis. For example. disruptions to the methyl donor balance of an animal is involved in inflammatory diseases such as fatty liver (Cronje 2016). The key methyl donors involved in the methionine cycle are choline, betaine and folate. Methionine, an essential amino acid for pigs, is either supplied in the diet through methyl donors including betaine and choline or is produced from HCY. The production of methionine from non-dietary sources occurs mainly in the liver or the kidney by converting HCY via the actions of methionine synthase or betaine homocysteine methyltransferase (BHMT). For example, creatine is a major consumer of methyl groups and is formed in the liver and then exported to muscle tissue (for storage) making creatine an important determinant of muscle mass. Therefore, there are large stores of betaine found in liver and kidney tissues. If the supply of methionine is low then the methylation of HCY will be increased and conversely excess supplies of methionine is converted into cysteine via HCY. Thus, HCY is a key control point for the methionine cycle and high HCY concentrations suggest an imbalance in this pathway. These transmethylation pathways are regulated by feedback mechanisms and therefore dietary sources of methyl groups or methyl group donors are important.

Currently, the methyl donor status of the Australian pig herd is unknown and the value of providing additional methyl donor substrates has not been evaluated. However, given the long ranging implications of a methyl group deficiency it is logical to assume that an undersupply of methyl donors will have negative effects on animal health and production. Additionally, specific knowledge of the metabolic requirements for methyl donors may provide pathways for specific manipulation of tissue accretion. For example, manipulation of the betaine pathway may influence the accretion of muscle and fat tissues (Lipinski *et al.* 2012). The methylation cycle involves many steps and the main methyl donor of the cell is S-adenosylmethionine (SAM), which is involved in many cellular reactions such as DNA methylation, antioxidant reactions, neurotransmitter pathways and creatinine pathways

As betaine can act as a methyl donor during its conversion into glycine it can provide substrates for re-methylation into methionine and thus replace a portion an animal's methionine requirement and providing additional substrates for protein synthesis (Matthews *et al.* 2001a; Matthews *et al.* 2001b). Additionally, betaine can enhance carnitine concentrations in liver and muscle tissues; and as carnitine is required for fatty acid transport through mitochondrial membranes betaine supplementation may improve fatty acid oxidation thereby reducing carcase fat (Eklund *et al.* 2005). As betaine is a direct donor of methyl groups (unlike choline that requires a two-step enzymatic process to be converted

into betaine), betaine is a cheaper and considered a more effective methyl donor than choline and methionine, although the transfer of methyl groups donated by betaine into the cytoplasm is a complex and energy consuming process (Lipinski *et al.* 2012). For example, it is estimated that the supply of methyl donors provided by anhydrous betaine (97 %) is equivalent to 1.25 kg of DL-methionine or 1.65 kg of choline chloride (Kidd *et al.* 1997). High HCY levels are indicative of an imbalance in the methionine cycle and have been demonstrated in pigs and increased HCY levels can increase the production of reactive oxygen species (ROS) as it can impair the actions of ROS scavengers such as glutathione (GSH) or the enzymes that catalyse these reactions such as glutathione peroxidase (GPx). GSH is particularly important in gut tissues and exposure of the gut to ROS will increase the conversion of methionine to cysteine which is required for the synthesis of GSH. In addition, excess concentrations of HCY are oxidated to for ROS which can lead to damage to tissues and the formation of gut ulcers (for example). The liver is the major site of methionine and cysteine metabolism, although the gut is a major consumer of dietary methionine which is highlighted by the improved gut health in pigs fed betaine.

Human studies have demonstrated a positive health effect of reducing HCY levels via the supplementation of betaine, and while these supplements have been observed in pigs there has been no investigation into the health outcomes of these supplements (while growth rate and efficiency are regularly observed) (Cronje 2008). The development of dietary supplementation methods that allow specific manipulation of homocysteine balance provide a unique opportunity to improve production efficiency in pigs in a cost effective and easy to administer method.

4. Research Methodology

Experiment one

Blood samples from a subset of 30 female animals each from lactating sow, sucker, grower and finisher groups (n = 120 per piggery) were obtained via jugular venepuncture from two commercial piggeries each in Queensland (Tonga Park (TP) and Westbrook (W)) and South Australia (Wasleys (WA) and Shea Oak (SO)). Plasma samples were obtained via jugular venepuncture and stored at -20 °C then analysed for plasma creatinine, betaine and HCY concentrations. Feed samples from each of the piggeries and growth stages were obtained (ingredients are listed in Tables 1 and 2) and samples were analysed for amino acid content.

Table 1. Queensland commercial piggery diets

	Lactating sow mash	Grower 1 pellet	Grower 2 pellet	Finisher pellet
	Ingredient (% of diet)			
Barley	22.6	0	0	11.5
Sorghum	0	8.7	44.0	26.0
Wheat	42.2	61.8	20.0	20.0
Millrun	7.0	0	0	11.7
Mixed grading hammered	0	0	5	7.0
Chick peas	2.0	4.3	5	3.0
Dried distillers grain sorghum	0	0	2.3	7.0
Canola meal	8.0	13.0	7.6	4.8
Soybean meal	5.0	4.1	10.0	2.1
Blood meal	1.2	2.6	0	0
Meat meal	3.0	0	0	0
Fish meal	2.0	0	0	0
Levucell	0.01	0	0	0
Tallow	3.5	2.7	1.3	1
Salmate oil	0.5	0	0	0
Molasses	1.0	0	0	0
Dextrose	0.5	0	0	0
Limestone (fine)	0.7	1.2	1.1	1.2
Di-calcium phosphate	0	0.7	0.9	0.8
Salt	0.2	0.2	0.2	0.2
Choline chloride (60%)	0	0.01	0.025	0.04
Betaine	0.2	0	0	0
DL methionine	0.03	0.05	0.06	0.04
Lysine (HCl)	0.3	0.4	0.4	0.4
L-threonine	0.065	0.038	0.045	0.095
L-tryptophan	0.005	0	0	0
Natuphos	0.015	0.015	0.015	0.015
Premix	0.2	0.2	0.2	0.2

Table 2. South Australia farm diets.

	Lactating sow	Grower	Finisher 1	Finisher 2
	Ingredient (% of diet)			
Barley	0	0	0	17.5
Sorghum	0	20.0	40.3	50.0
Wheat	61.9	57.2	39.1	10.0
Millrun	14.9	0	0	7.0
Canola meal	8.5	10.0	10.0	9.3
Soybean meal	0	3.1	3.7	0
Blood meal	1.9	2.5	2.8	1.6
Meat meal	5.3	4.7	0	0
Tallow	5.3	0	0	0
Blended Oil	0	1.3	1.0	0.5
Limestone (fine)	0.55	0.3	1.1	1.3
Di-calcium phosphate	0	0	1.1	0.8
Salt	0.2	0.2	0.2	0.2
Choline chloride	0.05	0.04	0.1	0.0
MHA Calcium	0	0.06	0.06	0.04
Lysine (HCL)	0.3	0.4	0.4	0.4
L-threonine	0.03	0.03	0.02	0.02
L-tryptophan	0	0.005	0	0
Hi-Phos	0	0.008	0.008	0.008
Nemtonite	0	0	0	1.1
Deodorase	0	0.1	0.1	0.1
Luctarom (sweet apple flavour)	0.03	0	0	0
Luvucell	0.01	0	0	0
Salmate	0.4	0	0	0
Betaine (liquid)	0.6	0	0	0
Premix	0.2	0.2	0.2	0.2

Amino acid analysis

Amino acid analysis was performed by the Australian Proteome Analysis Facility (Sydney, NSW). Briefly, samples were homogenized by grinding, weighed then underwent liquid hydrolysis in 6*M* HCl at 110 °C for 24 h. During this procedure asparagine is hydrolysed to aspartic acid and glutamine to glutamic acid and therefore the reported amount of these two acids is the sum of their respective components. After hydrolysis all amino acids except cysteine were labelled (Waters AccQTag Ultra chemistry) and analysed using a Waters Acquity UPLC with detection at 260 nm (UV). Cysteine analysis was performed using performic acid oxidation followed by 24 h gas phase hydrolysis using 6*M* HCl at 110 °C. All samples were run in duplicate and reported as mg/g.

Plasma creatinine, betaine and dimethylglycine analysis

Plasma betaine, creatinine and dimethylglycine (DMG) were measured as per the methods of (Laryea *et al.* 1998). Briefly, samples were diluted 50:50 in 100 mmol/L KH_2PO_4 then mixed with 900 µL derivitization reagent then heated to 80 °C for 60 min. Samples were then cooled to room

temperature and 15 µL of the supernatant injected directly into the HPLC (Waters Associates) using a Supelcosil LC-SCX, 5 m^2, 25 cm x 4.6 cm column (Supelco Inc.). Sample elution was isocratic over 10 min at a flow rate of 0.5 mL/min and measured at 254 nm at 25 ◦C.

Plasma HCY analysis

Analysis of HCY was conducted via liquid chromatography/ mass spectrometry (LCMS) using a Shimadzu application note C92. Plasma samples were extracted for LCMS using DTT, 0.2% HCOOH-CH_3-CN prior to analysis. Briefly, 1 µL of sample and standard was injected at a flow rate of 0.45 mL/min and analysis performed over a 5 min period.

Statistical analysis

Statistical analysis was performed using the analysis of variance or REML functions in GenStat 18th edition. The main factors in experiment 1 were piggery location or state (as described in text), pig age (weaner, grower, finisher or sow) and interactions between the two factors; while the random factor was pig ID. For experiment 2 the main factors were diet and sample day and any interactions between these factors were explored; with the random effects of pen and replicate included in the model. Responses were considered significant when $P \leq 0.05$ and a trend when $P \geq 0.05$ and ≤ 0.10.

Experiment two

A feeding experiment was conducted at a commercial piggery (Rivalea) to 1) confirm that supplementation of dietary methyl donors can improve production and efficiency in pigs and 2) to measure some metabolic and physiological responses to supplementation of methyl donors. 672 female finisher pigs (large white landrace, 70 d of age, 26.2 ± 0.69 kg) were sourced from the Rivalea herd and divided into 6 groups (14 pigs per group, 8 pens per treatment) for 12 weeks. Treatment groups were control, choline (0.15% of feed), choline (0.30% of feed), betaine (0.125% of feed), betaine (0.25% of feed) and DL-methionine (0.20 % of feed). Pen weights were collected every two weeks. Blood samples were collected via jugular venepuncture into lithium heparin tubes (BD) from a subset of 3 pigs per pen (approx. 21% of the population, 24 pigs per treatment per sample time) every two weeks. After the 12-week treatment period animals were slaughtered commercially and slaughter data collected, including HSCW, P2 fat depth and loin depth.

Plasma samples were analysed for betaine and creatinine samples as described previously. Plasma samples from days 14 and 84 (first and last sample days) were analysed for HCY concentrations using the commercial HCY kit (Crystal Chem #80494) as per kit instructions.

Table 3. Treatment diet ingredients fed to growing and finishing female pigs in experiment 2.

	Growers						Finishers					
	Control	Choline 0.15	Choline 0.30	Betaine 0.125	Betaine 0.25	Methionine	Control	Choline 0.15	Choline 0.30	Betaine 0.125	Betaine 0.25	Methionine
Wheat	76.89	76.89	76.89	76.89	76.89	76.89	58.53	58.48				
Barley							28.33	28.33	28.33	28.33	28.33	28.33
Canola meal 37%	15.00	15.00	15.00	15.00	15.00	15.00	7.00	7.00	7.00	7.00	7.00	7.00
Meatmeal 56%	2.00	2.00	2.00	2.00	2.00	2.00	1.33	1.33	1.33	1.33	1.33	1.33
Blood meal	1.00	1.00	1.00	1.00	1.00	1.00						
Water	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Tallow-mixer	1.33	1.33	1.33	1.33	1.33	1.33	0.83	0.83	0.83	0.83	0.83	0.83
Limestone	1.67	1.67	1.67	1.67	1.67	1.67	1.33	1.33	1.33	1.33	1.33	1.33
Dicalciumphos	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
DL-Methionine	0.04	0.04	0.04	0.04	0.04	0.24	0.04	0.04	0.04	0.04	0.04	0.04
Choline Chloride 60%		0.25	0.50					0.15	0.30			
Betaine				0.13	0.25					0.13	0.25	
Lysine	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Threonine	0.15	0.15	0.15	0.15	0.15	0.15	0.18	0.18	0.18	0.18	0.18	0.18
Fysal SP dry micro	0.30	0.30	0.30	0.30	0.30	0.30	0.01	0.01	0.01	0.01	0.01	0.01
Copper proteinate micro 24%	0.04	0.04	0.04	0.04	0.04	0.04	0.05	0.05	0.05	0.05	0.05	0.05
KeyQuindox micro	0.03	0.03	0.03	0.03	0.03	0.03						
Salt	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Rovabio	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Vitamin blend A	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
Salinomycin 120 micro	0.05	0.05	0.05	0.05	0.05	0.05	0.00	0.00	0.00	0.00	0.00	0.00
Mineral ruminant micro	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
Mineral monogastric micro	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.40	0.40	0.40	0.40	0.40
Vitamin blend B micro	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03

Table 4. Treatment diet contents fed to growing and finishing female pigs in experiment 2.

	Growers						Finishers					
	Control	Choline 0.15	Choline 0.30	Betaine 0.125	Betaine 0.25	Methionine	Control	Choline 0.15	Choline 0.30	Betaine 0.125	Betaine 0.25	Methionine
DE Pig	13.92	13.88	13.84	13.92	13.93	13.92	13.50	13.48	13.46	13.51	13.52	13.50
Protein	17.77	17.71	17.67	17.76	17.75	17.92	14.26	14.23	14.21	14.25	14.25	14.42
Fat	3.05	3.05	3.04	3.05	3.05	3.05	2.39	2.38	2.38	2.38	2.38	2.38
Calcium	0.95	0.95	0.95	0.95	0.95	0.95	0.91	0.91	0.91	0.91	0.91	0.91
Total Phos.	0.57	0.57	0.57	0.57	0.57	0.57	0.49	0.49	0.49	0.49	0.49	0.49
Available Phos.	0.47	0.47	0.47	0.47	0.47	0.47	0.42	0.42	0.42	0.42	0.42	0.42
Lysine	1.11	1.10	1.10	1.10	1.10	1.10	0.89	0.89	0.89	0.89	0.89	0.89
Methionine	0.33	0.33	0.33	0.36	0.38	0.53	0.27	0.27	0.26	0.29	0.31	0.46
M+C	0.70	0.70	0.70	0.70	0.70	0.89	0.56	0.56	0.56	0.56	0.56	0.76
ALY/DE	0.07	0.07	0.07	0.07	0.07	0.07	0.06	0.06	0.06	0.06	0.06	0.06
Met/Lys	0.30	0.30	0.30	0.32	0.35	0.48	0.30	0.30	0.30	0.33	0.36	0.52
M+C/Lys	0.63	0.63	0.63	0.63	0.63	0.81	0.63	0.63	0.63	0.63	0.63	0.85
Thr/Lys	0.67	0.67	0.67	0.67	0.67	0.67	0.70	0.70	0.70	0.70	0.70	0.70
Iso/Lys	0.53	0.53	0.53	0.53	0.53	0.53	0.54	0.54	0.54	0.54	0.54	0.54
Try/Lys	0.20	0.20	0.20	0.20	0.20	0.20	0.19	0.19	0.19	0.19	0.19	0.19
Val/Lys	0.74	0.74	0.74	0.74	0.74	0.74	0.70	0.70	0.70	0.70	0.70	0.70
Choline	1690.66	3176.63	4655.67	1688.12	1686.02	1686.86	1292.07	2188.23	3082.17	1290.01	1288.40	1289.04

5. Results

Optimisation of HCY measurement in porcine plasma

As described previously in various methods for HCY detection in porcine plasma were assessed. It was determined that the use of LCMS was the most sensitive method of detection and this was utilised for the sample analysis of experiment 1 samples (appendix 1). However, due to limited availability of the required equipment the HCY analysis in experiment 2 was conducted using a commercial kit assay.

Experiment 1:

Amino Acid concentration of diets

The amino acid (AA) content of the standard diets fed at each piggery are presented in Table 5. While there were numerical differences in the total AA concentration due to individual piggery and growth stage, these differences were not significant ($P > 0.14$). Furthermore, the noted numerical differences in key AA concentrations of methionine ($P > 0.11$), lysine ($P > 0.17$) and cysteine ($P > 0.24$) were not significantly different due to individual piggery or stage of growth. However, when the data was combined to assess the AA concentrations by state there was no difference in total AA concentrations while dietary cysteine concentration was greater in SA compared to Qld diets (3.47 vs. 3.10 mg/g ± 0.123 respectively, $P = 0.016$).

Table 5. Amino acid (mg/g) composition of commercial piggery diets

Diet	W Grower	TP Grower	SO & Wa Grower	W Finisher	TP finisher	SO & Wa Finisher	W Sow	TP Sow	Wa Gilt Lactation	Wa Sow	SO sow
Lysine	10.8	11.5	12.2	8.2	8.5	8.1	8.4	10.0	10.4	9.1	9.1
Methionine	1.8	2.6	2.4	1.4	1.8	1.6	2.0	2.1	2.6	2.4	2.1
Cysteine	3.2	3.2	3.8	2.7	3.3	2.9	2.9	3.3	4.0	3.7	3.6
Glycine	9.4	12.0	10.8	5.2	6.7	5.6	9.2	9.0	10.1	8.5	8.9
Histidine	4.6	6.2	5.3	3.5	3.9	3.0	4.1	4.7	5.1	4.3	4.4
Serine	7.5	9.9	9.2	5.4	7.1	5.4	7.3	7.6	8.5	7.9	7.9
Arginine	9.0	11.8	11.1	6.0	8.1	6.4	8.6	9.0	10.9	10.5	9.9
Aspartic acid	12.4	17.3	15.5	8.4	11.1	7.8	11.0	12.3	14.0	12.8	12.4
Glutamic acid	34.2	43.8	42.2	24.7	33.4	31.4	37.4	38.1	44.3	42.4	41.3
Threonine	6.4	8.0	7.6	4.6	5.8	5.0	5.6	6.4	7.5	6.5	6.5
Alanine	8.8	10.9	9.3	7.5	9.2	5.0	8.2	7.8	8.7	7.3	7.6
Proline	12.4	15.6	14.4	9.2	11.6	11.2	13.3	13.5	15.1	13.7	13.8
Tyrosine	2.5	3.6	3.1	2.1	3.1	2.0	2.9	2.6	3.5	3.2	2.8
Valine	9.1	11.7	10.8	7.1	8.5	6.6	8.6	9.2	10.2	8.8	9.0
Isoleucine	6.3	7.4	7.4	4.7	6.4	4.8	5.5	5.8	7.2	6.7	6.5
Leucine	13.5	17.0	14.8	11.7	14.5	8.6	12.5	12.6	13.8	12.1	12.3
Phenylalanine	7.9	10.5	9.3	6.1	7.5	5.8	7.5	8.1	8.9	8.0	7.9
Total	156.8	199.8	185.3	116.0	147.2	118.3	152.0	159.0	180.6	164.3	162.4

*W – Westbrook, TP – Tonga Park, SO – Shea Oak, Wa - Wasleys

Plasma metabolite concentrations

Plasma HCY concentrations ranged from 5 to 545 µM, with the median value of 29 µM and mean of 67 µM observed. Of the samples analysed, 71% were classified as high HCY (above 20µM). The age of the pigs influenced plasma HCY concentrations such that concentrations were greatest in weaners (129 µM) and lowest in sows (21 µM) with growers (46 µM) and finishers (56 µM) between these extremes (± 9.1 µM, $P < 0.001$; Figure 2). Furthermore, there was a significant difference in HCY concentration between piggery states such that concentrations were greater in pigs sampled from SA compared to those sampled from Qld. (103 vs. 23 ± 7.6 µM, $P < 0.001$; Figure 3). As demonstrated by Table 6, there was a significant relationship between piggery state and pig age such that the concentration was the greatest for weaner pigs from SA and lowest for sows from Qld.

Plasma creatinine concentrations varied between piggery sites and were significantly greater in pigs sampled from TP (481 µmol) and W (491 µmol) compared to SO (317 µmol) and Wa (314 µmol, ± 41.8; $P < 0.001$). When comparing samples by state the plasma creatinine concentration was greater in Qld compared to SA sites (479 vs. 316 µmol, ± 28.7 respectively; $P < 0.001$). Plasma creatinine concentrations also varied due to stage of growth of the pig such that concentrations were lowest in weaners (300 µmol) and greatest in lactating sows (525 µmol) with growers (376 µmol) and finishers (402 µmol) between these values (± 42.0, $P < 0.001$). As demonstrated in Figure 4, there was no interaction between age and piggery state ($P = 0.125$).

Plasma betaine concentrations varied between piggery sites and were significantly greater in pigs sampled from TP (729 µmol) and W (673 µmol) compared to SO (595 µmol) and Wa (520 µmol, ± 50.4; $P = 0.004$). When comparing samples by state the plasma betaine concentration was greater in Qld. compared to SA sites (704 vs. 556 µmol, ±36.2; $P < 0.001$). Plasma betaine concentrations also varied due to stage of growth of the pig such that concentrations were lowest in weaners (282 µmol) and greatest in lactating sows (957 µmol) with growers (690 µmol) and finishers (590 µmol) between these values (± 52.4, $P <0.001$). As demonstrated in Figure 5, there was also an interaction between age and piggery state following the same trends as the individual factors ($P = 0.001$).

Table 6. Plasma HCY (µM) concentrations measured from a subset of animals from commercial piggeries in Queensland (Qld) and South Australia (SA).

	Plasma HCY (µM) concentrations					*P* - Values		
	Weaner	Grower	Finisher	Sow	SED	State	Age	State x Age
Qld	30.4	20.8	21.3	17.8	13.15	<0.001	<0.001	<0.001
SA	227.5	71.5	90.3	23.9				

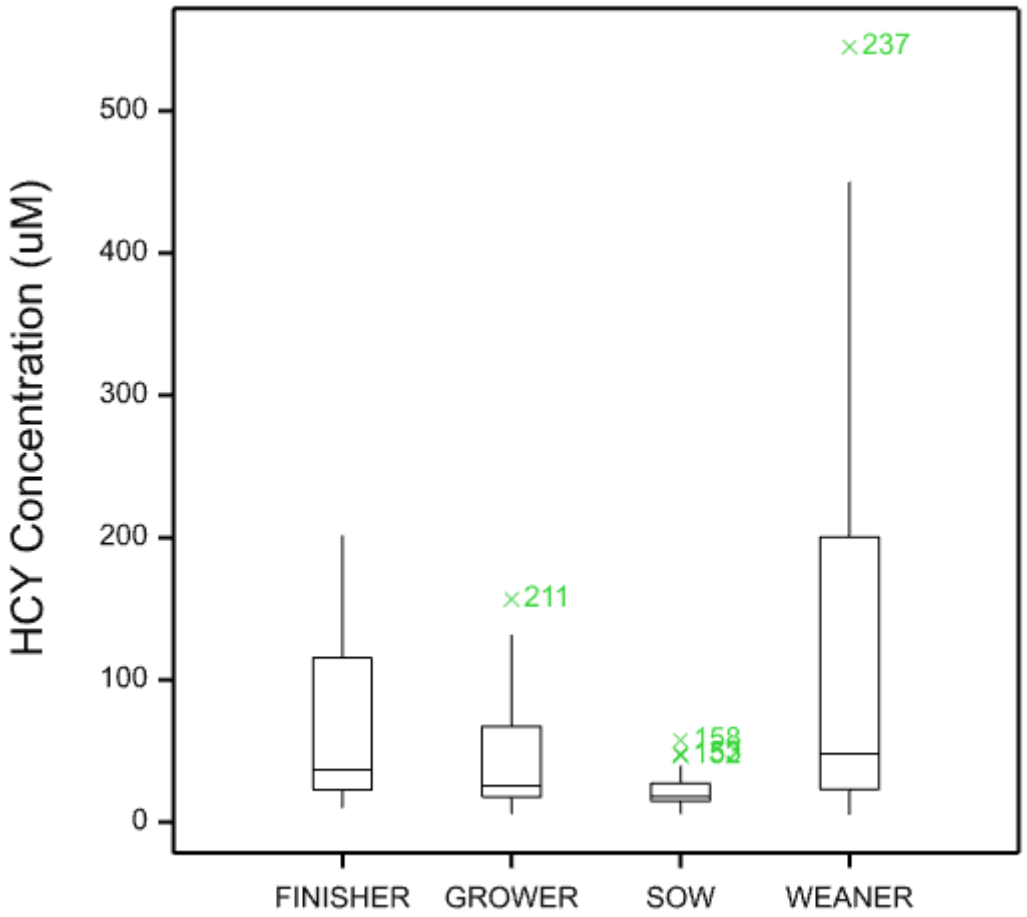

Figure 2. Boxplot demonstrating the range of plasma HCY concentration in plasma samples sourced from commercial piggeries in Australia.

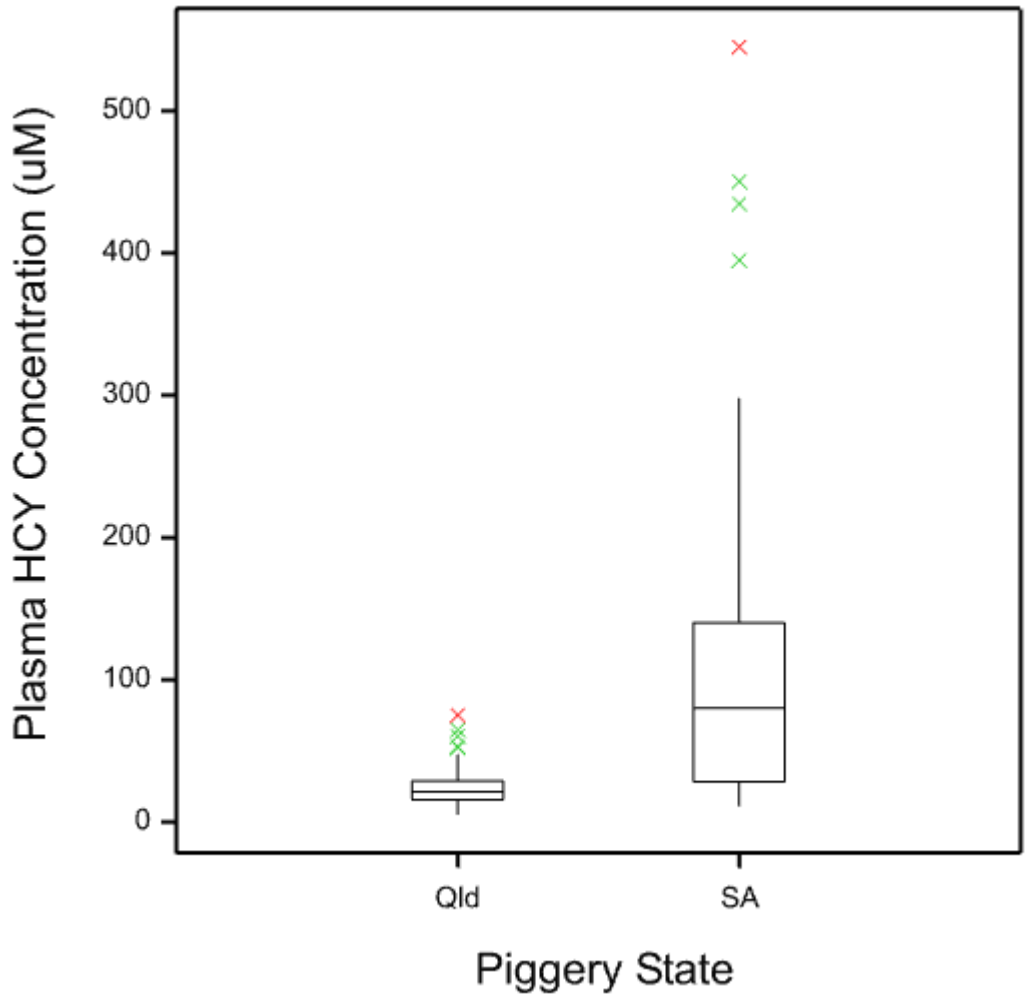

Figure 3. Boxplot demonstrating the range of plasma HCY concentration by state in plasma samples sourced from commercial piggeries in Queensland and South Australia.

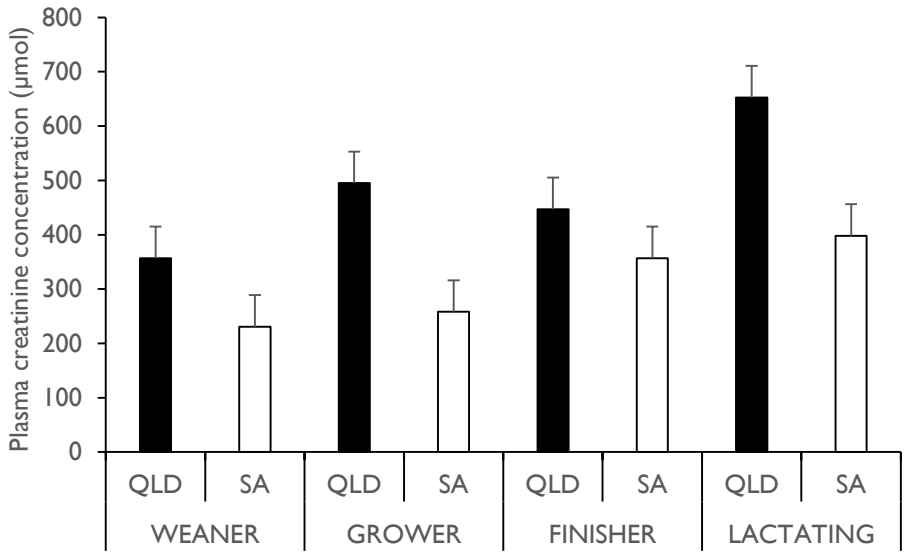

Figure 4. Plasma creatinine concentration in pigs of different stages of growth from commercial piggeries in Queensland and South Australia (P = 0.125).

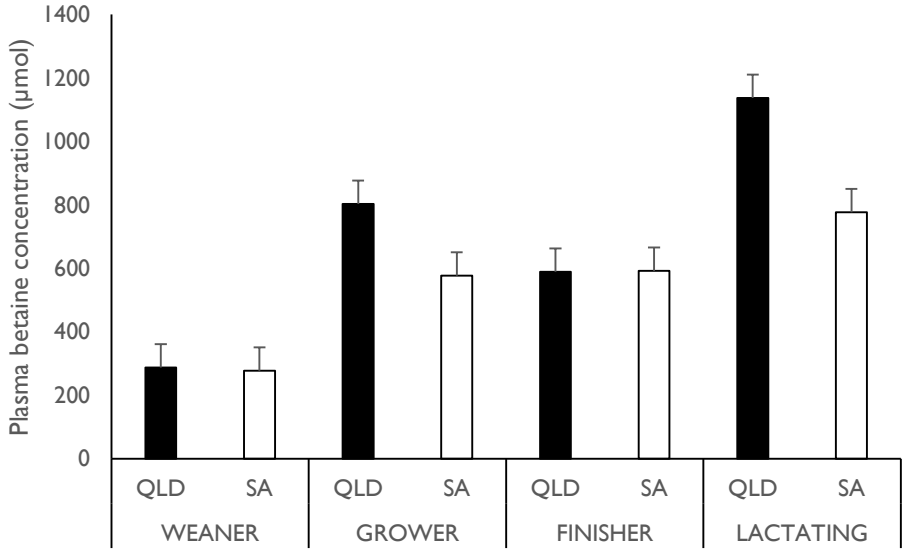

Figure 5. Plasma betaine concentration in pigs of different stages of growth from four commercial piggeries in Australia (P = 0.001).

Experiment 2

Plasma metabolite concentrations

Plasma HCY concentrations were greater on day 14 compared to day 84 of treatment (44.1 vs. 33.3 μM ± 4.13, $P = 0.010$). Plasma HCY concentrations were greatest in pigs fed betaine at 0.125% and lowest in pigs fed betaine at 0.25% (Figure 6, $P = 0.012$), although there was no interaction between day and diet (Table 7, $P = 0.575$). Compared to those fed the control diet, pigs fed choline at 0.3%, betaine at 0.25% and methionine had lower plasma HCY concentrations; and while betaine at 0.125% and choline at 0.15% numerically increased HCY concentrations this response was different from pigs fed control diets.

Plasma creatinine concentration was greatest on day 28 of treatment (1024 μmol) and lowest on day 84 (225 μmol) with days 14 (421 μmol), 42 (348 μmol) and 70 (627 μmol) intermediate (± 64.4, $P < 0.001$). There was no effect of diet on plasma creatinine concentration ($P = 0.710$) and there was no interaction between the two factors (Table 8; $P = 0.954$).

Plasma betaine concentration followed the same pattern of creatinine and was greatest on day 28 of treatment (1155 μmol) and lowest on day 84 (659 μmol) with days 14 (687 μmol), 42 (735 μmol) and 70 (764 μmol) intermediate (± 107.3; $P < 0.001$). There tended to be an effect of diet on plasma betaine concentration such that pigs fed control (683 μmol) and methionine (645 μmol) diets had lower betaine concentrations than pigs fed betaine at 0.125% (829 μmol) or 0.25% (887 μmol); pigs fed choline at 0.15% (951 μmol) and 0.3% (805 μmol; ± 117.2, $P = 0.055$). There was no interaction between the two factors (Table 8; $P = 0.838$).

Plasma DMG was only present in a subset of the samples measured, as demonstrated in Table 8 where there is missing data for some combinations of factors. However, there was a significant difference in plasma DMG concentration due to sample day such that concentrations were greater on day 14 of treatment (1105 μmol) compared to days 28 (270 μmol), 42 (66 μmol) and 70 (60 μmol, ± 63.1, $P < 0.001$) while there was insufficient data to determine the mean concentration on day 84. There was also a significant effect of diet on plasma DMG concentrations such that concentration was greatest in pigs fed choline at 0.3% (286 μmol), betaine at 0.25% (259 μmol) and methionine (216 μmol) compared to control (88 μmol), betaine at 0.125% (143 μmol) and choline at 0.15% (139 μmol, ± 143.8; $P = 0.029$). As described in Table 8; there was no effect of an interaction between diet and sample day on plasma DMG concentrations.

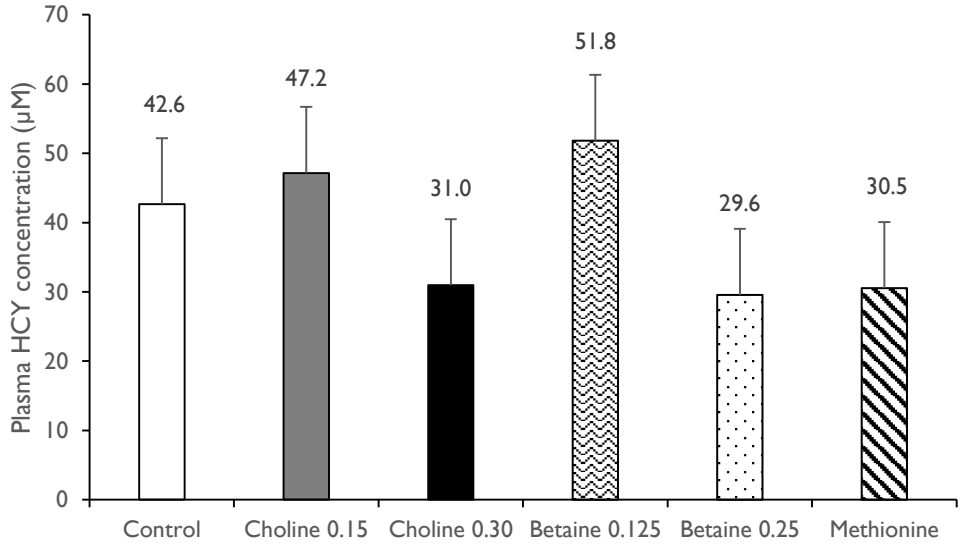

Figure 6. Mean plasma HCY concentration in female finishing pigs fed diets supplemented with either choline at 0.15 and 0.3% of the diet, betaine at 0.125 or 0.25% of the diet or methionine at 0.2% of the diet (P = 0.012).

Table 7. Plasma HCY (µM) concentration in female finishing pigs fed diets supplemented with either choline at 0.15 and 0.3% of the diet, betaine at 0.125 or 0.25% of the diet or methionine at 0.2% of the diet for 84 days.

	Day of treatment			*P*-values		
	14	84	SED	Diet	Day	Diet x Day
Control	56.8	28.5	12.24	0.012	0.010	0.575
Betaine 0.125	52.1	51.5				
Betaine 0.25	29.4	29.7				
Choline 0.15	54.7	39.7				
Choline 0.3	33.0	29.0				
Methionine	38.8	22.3				

Table 8. Plasma betaine, creatinine and dimethyl-glycine concentrations in female finishing pigs fed diets supplemented with either choline at 0.15 and 0.3% of the diet, betaine at 0.125 or 0.25% of the diet or methionine at 0.2% of the diet for 84 days.

		Diet							*P*-values		
	Day of treatment	Control	Betaine 0.125	Betaine 0.25	Choline 0.15	Choline 0.3	methionine	SED	Day	Diet	Day x Diet
Creatinine (µmol)	14	470	445	420	411	342	440	151.7	<0.001	0.71	0.954
	28	1115	1003	998	894	1169	963				
	42	356	324	415	421	260	311				
	70	544	649	684	620	654	611				
	84	214	198	269	213	223	234				
Betaine (µmol)	14	614	781	665	735	731	595	262.0	<0.001	0.055	0.838
	28	900	1113	1205	1523	1429	761				
	42	564	761	791	1127	646	524				
	70	728	785	949	763	643	715				
	84	610	704	825	609	574	631				
DMG (µmol)	14	767	1259	916	1144	*	1235	134.5	<0.001	0.029	0.198
	28	403	296	295	221	315	244				
	42	145	*	81	*	135	44				
	70	78	100	79	36	93	109				
	84	*	*	*	*	*	*				

Animal growth and carcase composition measures

There was no effect of dietary supplementation on intake, growth and carcase measures in finishing pigs (Tables 9 and 10). Unfortunately, an outbreak of disease occurred on trial days 42-56 leading to increased illness and death and decreased intake and hence ADG of the pigs across this period.

Table 9. Average pen intake and growth responses to supplemental diets during the finisher period.

		Treatment days					
		0-14	14-28	28-42	42-56	56-70	70-84
Intake (kg)	Control	300.0	354.5	397.4	442.9	468.4	518.1
	Choline 0.15	300.0	349.2	400.1	423.5	473.7	522.7
	Choline 0.3	297.7	354.4	398.8	430.9	477.5	513.7
	Betaine 0.125	290.7	355.5	389.2	385.5	431.1	487.5
	Betaine 0.25	307.8	362.9	391.1	416.2	438.0	514.2
	Methionine 0.2	298.2	357.3	402.6	404.7	439.4	506.9
Weight (kg)	Control	528.2	693.3	846.2	960.2	1108.2	1237.9
	Choline 0.15	529.8	681.8	840.9	964.8	1149.3	1322.8
	Choline 0.3	529.0	691.6	849.8	963.8	1133.2	1273.2
	Betaine 0.125	514.0	685.4	831.1	880.0	1047.3	1184.6
	Betaine 0.25	529.8	690.2	817.0	909.8	1082.4	1245.0
	Methionine 0.2	518.4	685.3	823.1	909.3	1067.1	1231.1
Ave. gain (kg)	Control	11.5	12.1	12.4	12.1	14.0	14.0
	Choline 0.15	11.2	12.3	12.8	11.2	14.1	13.2
	Choline 0.3	11.6	12.1	12.4	12.2	13.7	12.8
	Betaine 0.125	11.4	12.4	12.5	9.0	14.5	13.4
	Betaine 0.25	11.7	12.3	12.8	10.5	13.8	13.9
	Methionine 0.2	11.4	12.1	12.2	11.5	13.5	13.5
Adj. FCR	Control	1.87	2.09	2.34	2.72	2.67	2.98
	Choline 0.15	1.87	2.01	2.33	2.93	2.58	3.03
	Choline 0.3	1.84	2.11	2.37	2.67	2.71	3.20
	Betaine 0.125	1.85	2.06	2.35	3.70	2.46	3.06
	Betaine 0.25	1.88	2.11	2.30	3.19	2.57	2.99
	Methionine 0.2	1.87	2.14	2.42	2.85	2.67	3.09

Table 10. Growth and carcase responses to supplemented diets in growing pigs.

	Control	Betaine 0.125	Betaine 0.25	Choline 0.15	Choline 0.3	Methionine 0.2	SED	*P*-value
ADI (kg/day)	2.21	2.15	2.20	2.19	2.19	2.19	0.043	0.758
ADG (kg/day)	0.91	0.87	0.89	0.89	0.89	0.88	0.016	0.385
FCR	2.44	2.46	2.46	2.45	2.46	2.47	0.027	0.788
Ave ROG	0.74	0.70	0.75	0.81	0.77	0.74	0.054	0.415
HSCW (kg)	81.9	79.5	81.0	81.2	80.9	80.4	1.15	0.440
P2 (mm)	12.0	11.6	12.2	11.9	11.6	11.7	0.38	0.648
Loin depth (mm)	58.2	57.6	57.5	57.3	57.1	58.7	1.05	0.694
Dressing %	80.1	80.0	80.0	80.2	80.2	80.0	0.51	0.997

6. Discussion

The initial screening of plasma HCY concentrations in commercial piggeries conducted in experiment 1 indicated that according to the previously described threshold of 20 µM over 70% of the commercial pig herd were classified as having 'high' or 'abnormal' HCY concentrations. Furthermore, the average (67 µM) HCY concentration of the screened animals was also well above the threshold below which is considered normal. The highest HCY concentration was observed in the weaner group of animals, with only the sow group demonstrating an average HCY concentration almost equal to the 20 µM threshold (at 21 µM). In humans, minor increases above normal HCY concentrations are suggested to be associated with vascular diseases (Ueland and Refsum 1989). This suggests that the extreme HCY concentrations observed in experiment 1 may indicate a significant imbalance in commercial pigs, although as summarised by Cronje (2008) pigs appear to have a consistently higher HCY concentration compared to humans and may be tolerant of these higher levels.

In addition, pigs sampled from SA piggeries had greater HCY concentrations than those sampled from Qld. As demonstrated in Table 5, the AA concentration of the diets provided to the pigs sampled in experiment 1 did not vary in total AA concentration or individual concentrations of the key AA's methionine and lysine, although there was a greater concentration of cysteine in SA diets compared to Qld. Cysteine is an AA that can be irreversibly synthesised from methionine and in adults cysteine is not considered to be an essential AA for this reason. Thus, the supply of dietary cysteine can reduce the catabolism of, and thus spare, methionine (Shoveller *et al.* 2003). However, in growing animals cysteine synthesis may be limited and therefore is considered to be essential in the diet. As cysteine can spare methionine it is imperative that the requirements for this AA are understood in order to optimise diets; and as such it has been suggested that 40-70% of methionine requirements can be spared by cysteine in growing pigs. As the pigs sampled from SA had higher HCY concentrations (that were considered to be abnormal), the reduced supply of dietary cysteine may be contributing to the disruption to the methyl donor pathway.

Creatine is an amino acid derivative formed in the liver that is utilized as an energy source for muscle tissue during times of short and intense activity. Creatinine is formed in muscle as a product of irreversible non-enzymatic dehydration and loss of phosphate from creatine. As the loss of creatine is proportional to muscle mass, circulating creatinine can be a marker for muscle wastage. Furthermore, creatine resynthesis is required to maintain muscle mass and is responsible for 33% of the demand for methyl groups in growing piglets (Brosnan *et al.* 2007). Male pigs fed a high fat diet demonstrated a decreased plasma creatine and creatinine concentration compared to those fed a low fat diet, likely driven by reduced activities of glycolysis and glycogenolysis (Jégou *et al.* 2016). In the present

experiment plasma creatinine concentrations increased with increasing age of the pigs, which is logical as the animals slow their muscle anabolic processes. Further, plasma creatinine concentrations were greater in lactating sows which again is logical as lactation is an energetically demanding process that likely requires muscle catabolism to provide energy substrates for milk synthesis. Muscle production requires a high percentage of the methyl group supply, which is supported by the findings of experiment 1 whereby weaner pigs had the highest HCY concentrations indicating that these animals are methyl group deficient and may benefit from additional dietary supplementation of methyl donor compounds. This is further highlighted by the diets provided to the weaner pigs in experiment 1 which did not contain any supplementary betaine. As elevated HCY concentrations influence biochemical pathways such as lipid, glucose and insulin metabolisms as well as aspects of the immune system (Sharma *et al.* 2006) the effects of high HCY concentrations as vast. Thus, in growing weaner pigs the consequences of a HCY imbalance are likely to include impaired growth and changes to body composition, although these negative responses were not observed by the data obtained in experiment 2.

Plasma HCY concentrations can be reduced by betaine supplementation in humans (Craig 2004); while betaine supplementation upregulates the concentration of betaine-homocysteine methyltransferase (BHMT) which is suggested to play a role in betaines ability to decrease HCY concentrations (Ahn *et al.* 2016). Increased BHMT concentrations are noted when pigs were supplemented with betaine and choline; although the greatest increase was noted when cysteine was also supplemented (Emmert *et al.* 1998). As heat exposure will decrease BHMT concentrations, animals exposed to heat may have a limited ability to re-methylate HCY to methionine under heat conditions. Thus, environmental temperature may influence plasma HCY concentrations, although this is not supported by the responses presented in experiment 1 where pigs sampled from Qld (which is expected to be warmer with mean maximum temperature in 2016 of 27.3 °C in Brisbane and 21.8 °C in Adelaide (Australian Bureau of Meteorology) demonstrated lower HCY concentrations compared to those sampled in SA.

Unfortunately, an outbreak of disease occurred on days 42-56 leading to increased illness and death and decreased intake and hence ADG of the pigs. All treatment groups were affected by this and while blood samples were not obtained for this fortnight, the experiment was continued and sampling continued the following fortnight. This may have contributed to the lack of significant growth and production responses noted throughout the trial as demonstrated in Tables 9 and 10. This is in contrast to previous experiments that have demonstrated an effect of betaine supplementation on growth and carcase characteristics in pigs (Matthews *et al.* 1998; Matthews *et al.* 2001a; Matthews *et al.* 2001b; Lawrence *et al.* 2002). While there were no differences in production data in experiment 2, a reduction in plasma HCY concentrations was demonstrated in pigs supplemented with choline at 0.3

%, betaine at 0.25 % or methionine at 0.2 % of the diet. While this supports previous findings that methionine, betaine and choline can act as methyl donors and decrease the concentration of circulating HCY, it was expected that the lower doses of betaine (0.125 %) and choline (0.15 %) would also have beneficial effects. For example, a meta-analysis of available data demonstrated that betaine supplementation decreases backfat thickness and increases dressing % in pigs; although the changes to backfat thickness appear to be influenced by betaine dose (Sales 2011). Higher levels of betaine supplementation may decrease efficiency of betaine usage due to the energetic costs of betaine excretion (Eklund *et al.* 2005; DiGiacomo *et al.* 2016). However, experiment 2 did not demonstrate any effects of dietary supplementation on production or carcase outcomes which does not support previous findings that betaine can act as a carcase modifier. Similarly, no variation in production was shown between pig's supplemented betaine at 0.125 vs 0.5 % of feed (Wray-Cahen *et al.* 2004), while in another experiment betaine supplemented at 0.5 % of feed was the most effective increaser of lean gain in pigs compared to 0.125 and 0.25 % (Fernandez-Figares *et al.* 2002). A linear decrease in carcase fat has been demonstrated in correlation to increased betaine levels, although this only occurred in male feed restricted pigs (Fernandez-Figares *et al.* 2002). As betaine metabolism results in HCY being methylated to methionine (Lawrence *et al.* 2002), high supplementation rates of betaine may be beneficial for the reduction of HCY, although the associated effects upon production would need to be considered.

Betaine was present in all samples analysed, while plasma betaine concentrations were greater in Qld piggeries, and these pigs also had a lower plasma HCY concentration compared to SA sites. Both Qld and SA sites included supplementary betaine in their sow diets, with SA including a higher amount in their sow diets. As betaine can be metabolised from other nutrients such as choline this increased plasma betaine may be driven by variations in specific nutrients between states. This is supported by the dietary inclusion levels of choline which were greater in Qld finisher diets and further supported by an increased plasma betaine concentration in growing and finishing pigs compared to weaners.

Plasma DMG was continually unable to be observed in plasma samples, which supports recent findings from our group (unpublished data) in dairy cattle whereby DMG was undetected in the majority of plasma samples however, when the cows were heat stressed urinary betaine excretion more than doubled (1152 vs 2829 umol/L) and DMG excretion dropped 1.5 fold (3931 vs 2525 umol/L). Thus, there is betaine and DMG activity occurring in animals that is not able to be detected by plasma sample analysis. Urinary excretion of DMG increases in response to the metabolism of betaine, and thus can be a useful marker of betaine and HCY metabolism (Schwab *et al.* 2006).

In conclusion, the results from experiment 1 and 2 indicate that plasma HCY concentrations are elevated in the majority of the commercial pig population in Australia. While there were no effects of dietary supplementation on production variables it is clear that the supplementation of methyl donors such as betaine, methionine and choline fed at appropriate doses can improve (reduce) plasma HCY concentrations. While the results from the present experiment cannot make conclusive statements regarding the potential for a reduction in HCY concentrations to improve health and production, the published information in human subjects suggest that high HCY concentrations are eliciting negative effects. However, due to the shorter productive life of growing pigs these negative responses may not manifest in commercial piggeries.

7. Implications & Recommendations

The results from the experiments presented here support the suggestion from Cronje (2016) that there is an imbalance in the HCY concentrations of commercial pigs in Australia. This experiment confirmed that over 70% of the pigs sampled from multiple piggeries and in multiple growth stages were considered to have abnormally high HCY concentrations, which was most prominently noted in weaner pigs. This was associated with a lower plasma creatinine in weaner pigs which is logical as these pigs are in the anabolic stages of growth, which further highlights that there is a specific and significant requirement for methyl groups to support anabolic processes at this time. Experiment 2 demonstrated that dietary supplementation of methyl donors can successfully reduce plasma HCY concentrations, although not to levels considered to be normal. This positive finding highlights that further exploration of the manipulation of HCY production via dietary supplementation are warranted. Specifically, more controlled experiments are merited to more closely determine the growth and production effects of HCY manipulation via dietary supplementation in order to avoid the potential risks associated with commercial studies that include illness and disease outbreaks as shown in experiment 2. Further, controlled experiments would allow for the collection of additional and more frequent blood samples combined with the collection of urine which will be useful for the measurement of betaine and DMG excretion. As the samples in experiment 1 were obtained as a one off ad hoc sample it is not possible to conclude that the sampled animals were consistently high in HCY throughout the entire day (although all samples were collected at the same time of day); although the majority were at the time of sampling. Repeated sampling of fasted animals would provide a clearer insight into these measures. Controlled experiments can then be followed by commercial experiments to confirm the production effects of HCY manipulation.

8. Technical Summary

The major technical finding from this study was that the measurement of HCY in porcine plasma is complicated and expensive due to the higher concentrations noted in porcine plasma. While commercial kits designed for human subjects are available the detection range of these kits limited their use in this general screening project and our results using the LCMS determined that samples from the pigs were both below and above the limit of detection of the kits. The high installation and running costs of LCMS equipment make this method prohibitive for large scale research projects; while the limited range of kit assays also limits their use. Further development of methods to measure HCY in porcine plasma in a cost-effective manner are warranted.

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10. Appendices

Appendix 1. Plasma HCY measurement optimisation.

Optimisation of HCY measurement in porcine plasma

Initially various methods for the measurement of HCY in porcine plasma were explored to establish the most cost effective and reliable measure of this metabolite in plasma. The following HCY analytical methods were considered in this project. The selection criteria was that the assay had to have the required sensitivity, cost effective, low capital equipment and technical requirements.

Commercial colorimetric kit based plate assay

These are available from suppliers such as CrystalChem (Assay Matrix, Ivanhoe, Vic, catalog #80494). These kits have the advantage of being routine colorimetric assays, only requiring a 96-well visible plate reader. However; the assays are cost prohibitive (at approximately $20 AUD per sample) for the throughput required for experiment 1. Cell Biolabs also offer an ELISA based competitive immunoassay, although as this lacked sensitivity with a limit of quantitation (LOQ) of 10 μmol/L this was deemed unsuitable for the experiments described in this project.

Cycling assay

Based on the method of Tan *et al.* (2000) this fluometric based assay has a desirable LOQ of 1 μmol/L. This assay works on the principal of the single step conversion of homocysteine to α-ketobutyrate by homocysteine α,γ lyase (HCYase), NH_4 and H_2S. In the second step the H_2S is reacted with *N,N*-Dibutyl phenylene diamine (DPBDA) for indirect detection of homocysteine. The disadvantage of this assay is that HCYase is not commercially available and would need custom synthesis if a recombinant protein which would be costly in terms of both time and funding.

7-Fluorobenzo-2-oxa-1,3-diazole-4-sulfonic acid ammonium salt (SBD-F)

Halogenobenzofurazans such as SBD-F off the advantage of selectively reacting with thiol compounds and do not fluoresce themselves. This provides a highly sensitive detection mechanism (<1 μmol/L) that can be used for the simultaneous detection of cysteine, cysteine-glycine and glutathione using a HPLC coupled with a fluorescence detector (Frick *et al.* 2003; Ferin *et al.* 2012). These methods are widely published and the sample preparation is straightforward and comparatively inexpensive. The difficulty with this method is with the shift in analytical tools it is more difficult to access fluorescence HPLC than a mass spectrometer (MS), which offers superior sensitivity (but at a greater cost). Purchasing of a fluorescence detector was cost prohibitive for this project but the investigators on this project are actively investigating options to fund a fluorescence detector by other funding sources for future projects.

Derivatisation and ultraviolet HPLC detection

Derivatisation is the process whereby the target molecule is reacted with a chromogen to form a stable derivative that has enhanced detection properties (such as SBD-F). Three UV absorbing derivatives were evaluated, 2-chloro-1-methylquinolinium tetrafluoroborate (CMQT) (Bald *et al.* 2004; Glowacki and Bald 2009), 2-chloro-1-methylpyridinium iodide (CMPI) (Bald *et al.* 2000) and *p*-bromophenacyl bromide (Peng *et al.* 2012). The advantage of this methodology is the near ubiquitous nature of UV-HPLC in research institutions and relative low cost of this analysis. Although these publications indicated a LOQ down to 0.5 μmol/L for CQMT and CMPI these derivatives absorbed poorly in the UV spectrum with absorbance in the mAU range, meaning that analysis could be easily compromised by interfering proteins in the sample matrix. Given that we only had single 300-500 μL of plasma sample it was decided not to compromise these samples for this analysis. Homocysteine Phenacyl-esters formed after reaction with *p*-bromophenacyl bromide absorb at 263 nm and are prone to spectral interference and we were unable to locate any peaks in the physiological range. Homocysteine itself absorbs UV poorly and is not suitable for direct detection in biological concentrations.

Additionally, proprietary products such as Waters AccQ-Tag, which is a stable derivative that can be quantified by both UV and fluorescence, were evaluated. This method would require additional investment in new analytical columns and has higher costs than other methods evaluated.

Detection by liquid chromatography/ mass spectrometry (LCMS)

Analysis of homocysteine by LCMS is routine and there are numerous easy to follow technical notes available by various manufacturers. Due in part to the narrower bore of modern UPLC columns (Ultra Performance Liquid Chromatography), LCMS has faster run times- from 10-60 min by HPLC to 2-3 min, greatly increasing sample throughput. Further advantages of LCMS are the determination by molecular weight. This removes variability in derivatisation efficiency and allows the improved use of internal standards. LCMS was evaluated with an initial run of 13 samples using Shimadzu application note C92.