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Enhancing immune competency to improve lamb and weaner survival

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Abstract

Lamb birth weight is the major determinant of lamb survival to weaning. Lamb survival rarely exceeds 90% for singles, 75% for multiples and 60% for triplets which suggests that factors independent of birthweight must also influence lamb survival. Innate and adaptive immune deficiencies in neonatal mammals are known to predispose them to infections and increase the risk of death, however the role of immune competency in the survival of lambs is poorly understood. This project investigated the impacts of supplementation with vitamin D, vitamin E and selenium or sulphur amino acids during late pregnancy on the immune system of the ewe and lamb(s) and the subsequent impact on lamb survival. Maternal supplementation with Vitamin E plus selenium or cholecalciferol were effective at increasing the concentration of α-tocopherol and selenium or 25(OH)D concentrations in both the ewe and the lamb at parturition, but supplementation with methionine did not elevate total glutathione in the ewe or lambs. Whilst there was some evidence that the supplements improved lamb survival there was no evidence that this change in survival resulted from the supplements boosting the lamb's innate, passive or adaptive immune responses.

Executive Summary

On average, 30% of all lambs born will die prior to weaning, and approximately 80% of lamb deaths occur in the first 48 to 72 hours of life. Improving lamb survival is recognised as the highest priority to improve reproductive efficiency of the Australian sheep flock. Lamb birthweight is the greatest contributor to lamb survival, and significant steps have been made with both Merino and maternal ewes to improve ewe nutrition during pregnancy, optimise lamb birth weights and improve lamb survival. However, even at the optimal birthweight of 4.5 kg to 5.5 kg, lamb survival to weaning rarely exceeds 90% for singles, 75% for multiples and 60% for triplets, suggesting that factors independent of birthweight must also influence lamb survival. Functional innate and adaptive immune deficiencies in neonatal mammals are known to predispose them to infections, and the associated inflammation may cause tissue damage and/or dysfunction, and death, particularly in the perinatal period. However, the role of immune competency in the survival of lambs is poorly understood.

This project has successfully completed a large field experiment to better understand the effects of maternal supplementation with Vitamin D, Vitamin E plus selenium and methionine in late gestation on enhancing immune competency to improve lamb survival. Furthermore this project investigated the influence of the lamb's genetic potential for fleece weight on immune function and survival. Two hundred and thirty four pregnant Merino ewes that had been artificially inseminated using semen from four sires were allocated to three replicates of each treatment on day 111 of pregnancy. Ewes and lambs were intensively monitored and blood, colostrum and milk samples were collected between allocation of ewes to treatments and weaning. Lamb immune function was assessed by analysing the functional capacity of phagocytes, and the plasma IgG and anti-tetanus-toxoid antibody concentrations between birth and weaning. There were no significant effects of treatments on lamb birth weights, growth rates or survival to weaning, however survival to weaning was approximately 10% higher for all treatments than the Control (80% *vs.* 70%) and also differed by up to 16% between sire groups. Increasing the genetic potential for fleece weight significantly decreased the survival of lambs to 72 hours of life.

Maternal supplementation with Vitamin E plus selenium or cholecalciferol were effective at increasing the concentration of α-tocopherol and Se or 25(OH)D concentrations in both the ewe and the lamb at parturition, whereas maternal supplementation with methionine did not elevate total glutathione in the ewe or lambs. Supplementation reduced the proportion of ewes that were deficient in Vitamin E (<0.7 mg/L) from 61% to 9% and deficient to marginal (<0.1 mg/L) in selenium from 87% to 46%, but the increases in the concentration of α-tocopherol (0.08 *vs.* 0.14 mg/L;

P<0.001) and selenium (0.05 *vs* 0.08 mg/L; P<0.01) in lambs at birth were not as large as expected. Prior to suckling it is likely that all lambs were still deficient in Vitamin E and 40% of lambs were deficient or marginal in selenium even when ewes were supplemented. Maternal supplementation with cholecalciferol increased the plasma 25(OH)D concentrations of both ewes (137 *vs* 79 nmol/L; P < 0.001) and lambs (49 vs 24 nmol/L; P < 0.001) at birth compared to the Controls, but it is unknown whether ewes and lambs were still in a state of Vitamin D sufficiency. The estimated efficiency of placental transfer was 0.09 to 0.15 for α-tocopherol, 0.4 to 0.46 for selenium and 0.24 for 25(OH)D. Supplementation boosted the concentration of α-tocopherol and selenium in colostrum but there was negligible 25(OH)D in colostrum or milk.

There was no consistent nor large effects of increasing α-tocopherol and selenium, 25(OH)D or methionine concentrations in the ewe or lamb on the phagocytic capacity of monocytes or polymorphonuclear leukocytes, the concentration of IgG in the colostrum or plasma of lambs, or the vaccine-specific antibody response against tetanus-toxoid. Nor was there any consistent effect of nutritional treatments on the concentration of inflammatory cytokines. Lambs born with a higher percentage of phagocytic PMNL had significantly higher survival to weaning, and while the percentage of phagocytic PMNL was related to sire it was not manipulated by the nutritional interventions. Sterile swabs that were taken from ewes and their lambs at birth indicated complex patterns of microbial diversity but there were insufficient deaths during the first 72 hours to identify any clear associations between nutritional interventions, microbial diversity and the likelihood of mortality. Whilst there is some evidence that the supplements provided during late pregnancy improved lamb survival there was no evidence that this potential change in survival resulted from the supplements boosting the lamb's innate, passive or adaptive immune responses.

This project identified that there may be scope to improve lamb survival by providing specific nutrients to autumn lambing ewes during late pregnancy, regardless of the mechanism. The potential for the supplements used in this study to increase the survival of lambs born in autumn requires further validation at paddock scale across a range of environmental conditions. It is likely that the lambing environment that was necessary to enable the intense sampling of ewes and lambs to quantify nutrient status and immune responses to the supplements inadvertently limited the lamb's exposure to environmental or pathogenic stressors and hence reduced the impacts of the nutritional treatments on both immune function and lamb survival.

Project background

Lamb mortality represents a major production loss for the Australian sheep industry. On average, 30% of all lambs born will die prior to weaning, and approximately 80% of lamb deaths occur in the first 48 to 72 hours of life (Miller *et al.* 2010; Oldham *et al.* 2011; Hawken *et al.* 2012; Hinch and Brien 2014; Paganoni *et al.* 2014). Lamb birthweight is the greatest contributor to lamb survival and is strongly influenced by ewe nutrition during pregnancy (Oldham *et al.* 2011; Paganoni *et al.* 2014). However, even at the optimal birthweight of 4.5 kg to 5.5 kg (Oldham *et al.* 2011; Hinch and Brien 2014), lamb survival to weaning rarely exceeds 90% for singles, 75% for multiples and 60% for triplets (Paganoni *et al.* 2014), suggesting that factors independent of birthweight must also influence lamb survival. Functional innate and adaptive immune deficiencies in neonatal mammals are known to predispose them to infections, and the associated inflammation may cause tissue damage and/or dysfunction, and death, particularly in the perinatal period (Firth *et al.* 2005; Futata *et al.* 2012). However, the role of immune competency in the survival of lambs is poorly understood. The incidence of infection directly causing death in lambs during the prenatal and neonatal periods ranges from 0.2% to 30% (Hughes *et al.* 1971; Dennis 1974; Dwyer 2008; Rad *et al.* 2011), and whilst active infections have been previously identified in lambs, primarily via post-mortem examination, the ability of lambs to mount immune responses in early life have not been widely investigated.

Neonatal lambs are reliant on passive transfer of antibodies from the ewe's colostrum for protection in early life, and failure of passive transfer of immunity is well recognised as a cause of postnatal infection and death in lambs during the first week of life (Gokce and Erdogan 2009; Gokce *et al.* 2014). Immunoglobulin-G (IgG) is the predominant immunoglobulin found in ewe colostrum and measurement of serum IgG concentration in neonatal lambs can be used as an indicator of immune status (Bernadina *et al.* 1991; Hashemi *et al.* 2008). Passively acquired IgG molecules have a number of functions that promote neonatal innate immune responses including activation of complement, prevention of microorganism attachment to mucosal membranes and the opsonisation of bacteria for phagocytosis and killing (Parkin and Cohen 2001). Phagocytosis is an essential innate immune defence whereby phagocytes identify, engulf and destroy invading pathogens through an array of cellular mechanisms (Tosi 2005). Phagocytosis is also essential in linking innate to adaptive immune responses by promoting the induction of T cell responses and hence humoral immunity (Prosser *et al.* 2013). Impaired phagocytosis in the newborn can lead to inadequate control of pathogen replication, resulting in higher microbial loads and greater induction of pathogen-driven inflammatory responses in affected tissues and organs (Garvy 2004). Hence, poor adaptive immune competency in ewes, and poor passive immunity in the lamb, along with immature phagocyte function in newborns, may be associated with adverse infection outcomes in lambs, and therefore could contribute to lamb mortality.

Prior to commencing this project, a workshop involving representatives from MLA, UWA, Murdoch University and DPI VIC confirmed a short list of nutritional interventions that could be applied to enhance immune competency of ewes and lambs. This included Vitamin D, Vitamins E and selenium and sulphur amino acids:

(1) Vitamin D has been recognised to have several roles in the regulation of immune function and can enhance innate antimicrobial immune responses, whilst dampening excessive adaptive responses and inflammation to maintain self-tolerance and prevent auto-immunity (Lang *et al.* 2013). During pregnancy Vitamin D is thought to enhance innate anti-microbial and anti-inflammatory responses within the placenta and reproductive tissues, which may protect the fetus from intrauterine infection and subsequent inflammation (Grayson and Hewison 2011). Maternal transfer of Vitamin D to the lamb may occur *in utero*, or to a lesser degree postnatally via intake of milk (Lapillonne 2010). Thus any Vitamin D deficiency in pregnant ewes could have negative impacts on the immune system of the ewe and/or lamb. Increasing the Vitamin D levels in neonatal lambs at birth may confer a greater ability to control infection and/or limit infection-driven inflammation, and thus may contribute to improved lamb survival.

(2) Vitamin E is the major lipid soluble anti-oxidant in mammalian tissues and there is evidence that Vitamin E influences the reproductive performance of both the ram and ewe (Liu *et al.* 2014). Plasma Vitamin E levels are reduced when there is no green feed and also drops by 50% during the period from a few weeks prior to lambing until 20-30 days postpartum. Vitamin E supplementation in late pregnancy has been reported to improve lamb birth weights (Yaprak *et al.* 2004; Capper *et al.* 2005), lamb vigour (Knott *et al*. 1983; Williamson *et al.* 2008), lamb survival (Thomas *et al.* 1995; Kott *et al.* 1998; Ali *et al.* 2004; Yaprak *et al.* 2004) and lamb growth to weaning (Kott *et al.* 1998; Ali *et al.* 2004), however the responses vary between studies. The impacts of Vitamin E are undoubtedly related to enhanced immune function of the ewe and lamb, but could also be due to a combination of improved immune competence, improved activation of brown adipose tissue and thermogenesis of the lamb and a neonate more capable of dealing with stressors. Regardless of the mechanism, further work is required to determine the optimum supplementation level of Vitamin E during different stages of pregnancy and the conditions that will lead to more

predictable responses to Vitamin E supplementation. The responses to Vitamin E supplementation are also likely to be related to interactions with selenium. The selenium concentrations in pastures and supplements are often below daily recommended requirements.

(3) Sulphur amino acids are critical nutrients for immune competency. Methionine (Met) is required for the synthesis of polyamines which facilitate rapid division of cells throughout the body, including those in the activated immune system (Grimble 2002 Methionine is also converted to cysteine (Cys) in the body which is the limiting amino acid for the synthesis of glutathione (GSH). Both GSH and Cys are small molecular antioxidants, and essential to stabilise the redox status of the body and reduce the development of inflammation in tissues. Dietary supplementation of SAA has been reported to improve immune competency in farm animals. Supplementation of rumen protected Met to dairy cows from 21 days before calving until 30 days after calving increased the voluntary feed intake of cows and milk protein concentration and milk yield. The supplement was also associated with an elevation in blood neutrophil phagocytosis (Osorio *et al*. 2013) and reduced introleukin-6, a pre-inflammatory cytokine (Osorio *et al.* 2014). These changes are indicative of improved immune function in the cows. Supplementing super-nutritional levels of SAA to broilers resulted in significant dose-related increases in total antibody and IgG, suggesting more SAA are required for optimal antibody response (Tsiagbe et al. 1987). An abomasal infusion of 2 g/d Cys to sheep challenged by intestinal parasites tended to increase the peripheral eosinophils and globular leukocytes in the abomasum and the anti-ovalbumin IgG response (Miller *et al*. 2000).

Previous studies have also suggested immunity may also be a heritable trait. For example, genetic variation in the ability to acquire and absorb immunoglobulins in colostrum in calves has been estimated (Norman *et al.*, 1981, Berggren-Thomas *et al.*, 1987), suggesting a genetic link with passive immunity. Furthermore, Berggren-Thomas (1987) reported the antibody response against foreign antigens in ewes may have genetic components, suggesting adaptive immune responses are also influenced by genes. Therefore, despite the heritability of lamb survival being very low, sire and dam genetics may influence immune function which may influence survival of the lamb.

Project Objectives

- To determine if supplementation with vitamin D, vitamin E and selenium or sulphur amino acids, during late pregnancy boosts innate immune defences and improves vaccine-specific adaptive immunity in the first year of life.

- To determine if maternal supplementation alters the micro biome of the newborn and reduces exposure to potentially pathogenic microorganisms.

- To determine if high producing sheep have impaired maternal and lamb innate immune defences and vaccine-specific adaptive immunity, and if they are more responsive to maternal supplementation.

- To determine if maternal supplementation reduces the prevalence of pre- and post-weaning lamb mortality.

Results and Discussion

The final report is comprised of papers that have been or will be submitted for publication and are includeds below.

Appendix 1: A Lockwood, A Currie, S Hancock, S Broomfield, S Liu, V Scanlan, G Kearney, AN Thompson (2015). Supplementation of Merino ewes with cholecalciferol in late pregnancy improves the Vitamin D status of ewes and lambs at birth but is not correlated with an improvement in immune function in lambs. *Animal Production Systems* (in press)

Appendix 2: S Sterndale, S Broomfield, A Currie, S Hancock, G Kearney, J Lei, A Lockwood, S Liu, V Scanlan, G Smith and AN Thompson (2016). Supplementation of Vitamin E plus selenium in Merino ewes increases alpha-tocopherol and selenium concentrations of the lamb but does not improve their immune function. *Animal Production Systems* (to be submitted)

Appendix 3: S Liu, J, Lei, S Hancock, V Scanlan, S Broomfield, A Currie and AN Thompson (2016). Lamb survival, glutathione redox state and immune function of lambs from periparturient Merino ewes supplemented with rumen protected methionine. *Animal* (to be submitted)

Appendix 4: A Lockwood, A Currie, S Hancock, S Broomfield, V Scanlan, G Kearney and A Thompson (2016). A higher genetic potential for fleece weight decreases innate immune function and survival to 72 hours of age in Merino lambs. *Animal* (submitted)

Further results that have not been published are presented in Appendix 5.

Appendix 1

Supplementation of Merino ewes with cholecalciferol in late pregnancy improves the Vitamin D status of ewes and lambs at birth but is not correlated with an improvement in immune function in lambs

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Short title: Supplementing ewes with cholecalciferol does not improve lamb immunity

Abstract. Functional deficiencies of the immune system are known to predispose human and animal neonates to death. Thus immune competency may be a significant factor influencing the mortality of lambs. Vitamin D has been recognised to improve immune function and is transferred across the placenta. This study tested the hypotheses that (i) Supplementation of Merino ewes with cholecalciferol during late pregnancy will increase the levels of Vitamin D in the ewe and lamb at birth and (ii) Supplementation of Merino ewes with cholecalciferol during late pregnancy is correlated with an increase in innate phagocytic and adaptive antibody immune responses in the lamb. Merino ewes (*n* = 53) were injected intramuscularly with 1 x 10^6 IU cholecalciferol at days 113 and 141 of pregnancy. A Control group (*n* = 58) consisted of ewes receiving no additional nutritional treatments. The Vitamin D status of ewes and lambs was assessed up until one-month post-lambing. Lamb immune function was assessed by analysing the functional capacity of phagocytes, and the plasma IgG and anti-tetanus-toxoid antibody concentrations between birth and weaning. Maternal supplementation with cholecalciferol increased the plasma 25(OH)D concentrations of both ewes (137 vs 79 nmol/L; $P < 0.001$) and lambs (49 vs 24 nmol/L; $P < 0.001$) at birth compared to the Controls. Supplementation with cholecalciferol had no significant effect on the phagocytic capacity of monocytes or polymorphonuclear leukocytes, the concentration of IgG in the colostrum or plasma of lambs, or the vaccine-specific antibody response against tetanus-toxoid. Overall the results support our first hypothesis, but suggest maternal supplementation with 1 x 10^6 IU cholecalciferol does not improve innate, passive or adaptive immune function in lambs.

Introduction

Lamb mortality represents a major production loss for the Australian sheep industry. On average, 30% of all lambs born will die prior to weaning, and approximately 80% of lamb deaths occur in the first 48 to 72 hours of life (Miller *et al.* 2010; Oldham *et al.* 2011; Hawken *et al.* 2012; Hinch and Brien 2014; Paganoni *et al.* 2014). Lamb birthweight is the greatest contributor to lamb survival and is strongly influenced by ewe nutrition during pregnancy (Oldham *et al.* 2011; Paganoni *et al.* 2014). However, even at the optimal birthweight of 4.5 kg to 5.5 kg (Oldham *et al.* 2011; Hinch and Brien 2014), lamb survival to weaning rarely exceeds 90% for singles, 75% for multiples and 60% for triplets (Paganoni *et al.* 2014), suggesting that factors independent of birthweight must also influence lamb survival. Functional innate and adaptive immune deficiencies in neonatal mammals are known to predispose them to infections, and the associated inflammation may cause tissue damage and/or dysfunction, and death, particularly in the perinatal period (Firth *et al.* 2005; Futata *et al.* 2012). However, the role of immune competency in the survival of lambs is poorly understood. The incidence of infection directly causing death in lambs during the prenatal and neonatal periods

ranges from 0.2% to 30% (Hughes *et al.* 1971; Dennis 1974; Dwyer 2008; Rad *et al.* 2011), and whilst active infections have been previously identified in lambs, primarily via post-mortem examination, the abilities of lambs to mount immune responses in early life have not been widely investigated.

Neonatal lambs are reliant on passive transfer of antibodies from the ewe's colostrum for protection in early life, and failure of passive transfer of immunity is well recognised as a cause of postnatal infection and death in lambs during the first week of life (Gokce and Erdogan 2009; Gokce *et al.* 2014). Immunoglobulin-G (IgG) is the predominant immunoglobulin found in ewe colostrum and measurement of serum IgG concentration in neonatal lambs can be used as an indicator of immune status (Bernadina *et al.* 1991; Hashemi *et al.* 2008). Passively acquired IgG molecules have a number of functions that promote neonatal innate immune responses including activation of complement, prevention of microorganism attachment to mucosal membranes and the opsonisation of bacteria for phagocytosis and killing (Parkin and Cohen 2001). Phagocytosis is an essential innate immune defence whereby phagocytes identify, engulf and destroy invading pathogens through an array of cellular mechanisms (Tosi 2005). Phagocytosis is also essential in linking innate to adaptive immune responses by promoting the induction of T cell responses and hence humoral immunity (Prosser *et al.* 2013). Impaired phagocytosis in the newborn can lead to inadequate control of pathogen replication, resulting in higher microbial loads and greater induction of pathogen-driven inflammatory responses in affected tissues and organs (Garvy 2004). Hence, poor adaptive immune competency in ewes, and poor passive immunity in the lamb, along with immature phagocyte function in newborns, may be associated with adverse infection outcomes in lambs, and therefore could contribute to lamb mortality.

Vitamin D has been recognised to have several roles in the regulation of immune function and can enhance innate antimicrobial immune responses, whilst dampening excessive adaptive responses and inflammation to maintain self-tolerance and prevent auto-immunity (Lang *et al.* 2013). During pregnancy Vitamin D is thought to enhance innate anti-microbial and anti-inflammatory responses within the placenta and reproductive tissues, which may protect the fetus from intrauterine infection and subsequent inflammation (Grayson and Hewison 2011). Maternal transfer of Vitamin D to the lamb may occur *in utero*, or to a lesser degree postnatally via intake of milk (Lapillonne 2010). Thus any Vitamin D deficiency in pregnant ewes could have negative impacts on the immune system of the ewe and/or lamb. Increasing the Vitamin D levels in neonatal lambs at birth may confer a greater ability to control infection and/or limit infection-driven inflammation, and thus may contribute to improved lamb survival. Supplementation of ewes with Vitamin D during pregnancy could provide an effective means to increase the levels of Vitamin D in the ewe and her lamb/s *in utero*, thereby improving innate and/or adaptive immunity in the ewe, and innate and/or passive immunity in the lamb. This study therefore tested the hypotheses that; (i) Supplementation of Merino ewes with cholecalciferol during late pregnancy will increase the levels of Vitamin D in the ewe and lamb at birth and (ii) Supplementation of Merino ewes with cholecalciferol during late pregnancy is correlated with an increase in innate phagocytic and adaptive antibody immune responses in the lamb.

Materials and methods

All procedures described were performed according to the guidelines of the Australian Code of Practice for the Use of Animals for Scientific Purposes 2013 and received approval from the Murdoch University Animal Ethics Committee.

Research site, animals and experimental design

The research was performed at the University of Western Australia Future Farm near Pingelly in Western Australia (S 32° 30' 23" – E 116° 59' 31") between November 2013 and August 2014. Two hundred Merino ewes aged between four and seven years old were sourced from the 'Maternal

Efficiency Flock' (Rosales Nieto *et al.* 2013). All ewes had full pedigree records. Ewes were artificially inseminated with semen from four Merino sires and those identified to be pregnant were allocated into three replicates of each of the two treatment groups; Control or Vitamin D supplementation during late pregnancy. The ewes were shorn in November 2013 prior to artificial insemination. Lambing occurred during late April/early May 2014 and lambs were weaned in early August 2014.

Animal management and treatments

Ewes were managed to achieve a body condition score greater than three for joining. Oestrus was synchronised in ewes via Controlled Internal Drug Releases (Eazi-Breed™ CIDR® Sheep and Goat Device, Zoetis Australia) which were inserted into the vagina of all ewes 14 days prior to artificial insemination and were withdrawn 48 hours prior to artificial insemination. One day prior to artificial insemination the ewes were weighed and body condition scored (Jefferies 1961). Ewes were artificially inseminated laproscopically in November 2013. Semen from the four sires was allocated to the ewes according to their age and liveweight and condition score prior to insemination. The ewes were managed as a single flock and grazed the same paddocks until day 113 of pregnancy.

Ewes were pregnancy scanned via trans-abdominal ultrasonography on day 55 of pregnancy to identify single- and twin-bearing ewes (Fowler and Wilkins 1984). After confirmation of pregnancy status, ewes were weighed and condition scored every 1- to 2-weeks and nutrition was managed accordingly in order to achieve a body condition score of 2.5 at lambing. The ewes grazed paddocks with very low levels of dry annual pastures and on average were supplemented with 700 g/day of supplementary feed (52% lupins (13.9 MJ ME/kg DM and 31.2% CP), 31% oats (12.8 MJ ME/kg DM and 12.2% CP) and 17% oaten chaff (9.3 MJ ME/kg DM and 7.6% CP)) between pregnancy scanning and lambing.

On day 111 of pregnancy, ewes were administered a clostridial 6 in 1 booster vaccine (Glanvac® 6, Zoetis Australia). Ewes were then allocated into three replicates for each of the two treatment groups; Control or Vitamin D supplementation, according to ewe age, sire of the lamb, pregnancy status, and liveweight and condition score at insemination (n = 17-20 ewes/treatment/replicate). The Control group (n = 58) consisted of ewes receiving no additional nutritional treatments. Ewes supplemented with Vitamin D (n = 53) received an injection of cholecalciferol (Vitamin D_3; Bova Compounding, NSW) at days 113 and 141 of pregnancy. On each occasion, 1.0 x 10^6 IU cholecalciferol, in oil, was injected intramuscularly into the hind-limb. Each replicate of the two treatments was combined after the first injection of cholecalciferol and the three flocks of 37 Control and Vitamin D ewes grazed separate pre-lambing plots until day 141 of pregnancy. At this time the ewes within each replicate were reallocated to a lambing plot according to treatment group, pregnancy status and lamb sire into two smaller groups, so there were six groups of 18 – 19 Control and Vitamin D ewes for lambing. Ewes remained in these six lambing plots until approximately 1-week post-lambing, at which point all ewes and their lambs were joined together and run as a single flock until weaning.

Animal measurements and sample collection

Pre-treatment blood samples were collected from a sub-group of Vitamin D and Control ewes (n = 40) on day 111 of pregnancy for subsequent analysis of Vitamin D concentrations. At lambing, between days 143 and 153 of pregnancy, ewes were intensively monitored between 4 a.m. and 12 midnight each day in order for ewe and lamb sampling to occur prior to the lamb suckling following birth. All blood samples were collected via jugular venipuncture into heparinised vacutainer tubes. Following the initial collection of blood samples from lambs, only those lambs which were bled at birth were bled at subsequent time points in the study. All blood samples collected for Vitamin D analysis were transported on ice while those collected for immunological analysis were stored at

room temperature following collection and for transport. Following transport, samples collected for Vitamin D analysis were centrifuged for 15 minutes at 2278 g to isolate plasma which was then stored at -20˚C. For immunological analysis, whole blood was isolated from blood samples collected from lambs at birth. The remainder of the sample, and all other lamb blood samples for immunological analysis were centrifuged for 10 minutes at 952 g to isolate plasma which was then stored at -80˚C.

At birth, all lambs were weighed, and their dam, sex and birth type was recorded (*n* = 154). Two 5 mL blood samples were collected from all single-born lambs and from the first lamb born only for lambs born in litters, provided they had not already suckled (*n* = 91), and the blood glucose concentration of the sample was measured immediately using a glucometer (Accu-Chek® Go, Roche Diagnostics). Rectal temperatures were taken from most lambs using a digital thermometer. At least 30 mL of colostrum and a 9 mL blood sample were collected from all ewes for immunological and Vitamin D analysis respectively. Colostrum was temporarily stored at 4˚C before being aliquoted and stored at -80˚C for subsequent immunological analysis.

One-week post-birth (± 3 days), 5 mL blood samples were collected from the lambs (*n* = 91) for immunological analysis. Lambs were weighed approximately 2-weeks post-birth. Four-weeks post-birth (± 1-week) 9 mL blood samples were collected from the sub-group of Control and Vitamin D-supplemented ewes (*n* = 40) for Vitamin D analysis, and two 5 mL blood samples were collected from the lambs for Vitamin D (*n* = 40) and immunological (*n* = 69) analysis. At marking, approximately 4-weeks post-birth, lambs were weighed and received their primary vaccinations (Glanvac® 6, Eryvac®, Gudair®, and Scabigard®; Zoetis, Australia). Two-weeks following administration of the primary vaccines (week-6), lambs were weighed and 5 mL blood samples were collected from the lambs for immunological analysis (*n* = 69 Lambs were then weighed every 2- to 3-weeks until weaning at approximately 14-weeks of age. At weaning a 5 mL blood samples was also collected for immunological analysis (*n* = 65) and the lambs were administered clostridial booster vaccines (Glanvac® 6, Zoetis Australia).

Vitamin D concentration assay

Plasma samples collected from ewes at day 111 of pregnancy, before treatments commenced (*n* = 40), and from both ewes and lambs at lambing (*n* = 80) and 4-weeks post-birth (*n* = 80) were analysed for 25-hydroxyvitamin D concentrations using liquid chromatography tandem mass spectrometry (LC-MS/MS) with electrospray ionisation in positive mode (ESI+). Colostrum samples (*n* = 40) collected from ewes at lambing and milk samples (*n* = 40) collected from ewes 4-weeks post-lambing were also analysed for 25-hydroxyvitamin D concentrations. Briefly, the internal standards d_6-25-hydroxyvitamin D_2 and d_6-25-hydroxyvitamin D_3 were added into samples and then the sample was extracted using acetonitrile to release Vitamin D from Vitamin D-binding protein and precipitate proteins. Extracts were then chromatographed in order to further isolate Vitamin D from potentially interfering substances and to separate the different forms of Vitamin D (25-hydroxyvitamin D_2 ($25(OH)D_2$), 25-hydroxyvitamin D_3 ($25(OH)D_3$) and C-3 epimer of 25-hydroxyvitamin D_3 (C3-epi-$25(OH)D_3$)). The various forms of Vitamin D were quantified in a mass spectrometer (Waters Micromass Quattro Premier XE) with selected ion mode. Whilst C3-epi-$25(OH)D_3$ is not currently known to have any functionally active roles in the body and is not widely included in the measure of Vitamin D status, a significant amount of C3-epi-$25(OH)D_3$ was detected in the plasma samples in this study. Thus the total 25-hydroxyvitamin D (25(OH)D) concentrations in this paper are presented as the total of $25(OH)D_3$ and $25(OH)D_2$, either excluding or including C3-epi-$25(OH)D_3$.

Whole blood differential cell counts

Whole blood samples collected from lambs at birth and 4-weeks of age were analysed within 24 hours for routine white blood cell differentials using the Advia® 120 automated Hematology

Analyzer (sequence 284) in a high output pathology laboratory (Clinical Pathology, Murdoch University Veterinary Hospital). Briefly, the Advia machine adds peroxidase reagents to the samples to achieve erythrocyte lysis and fixation and staining of white blood cells. Monocytes and neutrophils are identified based on light scatter and absorption data generated through analysis of samples in a peroxidase flow cytometry channel. Monocyte and neutrophil cell counts are displayed as a percentage of white blood cells. Manual counts by blood smear microscopy were conducted where necessary, such as in the presence of blood clots or to confirm Advia data.

Whole blood phagocytosis uptake assay

Whole blood samples collected from lambs at birth and 4-weeks of age were analysed for monocyte and polymorphonuclear leukocyte phagocytic capacities using flow cytometry. pHrodo™ Red Staphylococcus aureus bioparticles® (Life technologies, U.S.A) were resuspended at 1 mg/mL in PBS. Resuspended bioparticles were vortexed and sonicated to remove aggregates before adding 40 µL to wells of a 96-well polypropylene plate. HEPES buffered RMPI media supplemented with 5% heat inactivated fetal calf serum was then added. Whole blood (25 µL) was added to the wells in duplicate such that each blood sample was analysed with and without pHrodo™ bioparticles. Plates were incubated for 60 minutes at 37˚C/ 5% CO_2 before transferring to ice. All samples were then stained with Brilliant Violet 421™ anti-human CD14 antibody (cross-reactive with sheep CD14), clone M5E2 (BioLegend) for 15 minutes in the absence of light for the identification of monocytes. Ice-cold PBS (100 µL) was added to each well prior to transfer of samples to FACS tubes containing 2 mL ice-cold PBS. Tubes were centrifuged at 134 g for 5 minutes at 10˚C before aspirating the supernatant and washing the cells with 1 mL ice-cold PBS. Cells were pelleted and red blood cells lysed with 1 mL BD FACS™ Lysing Solution (BD Biosciences) for 15 minutes at room temperature in the dark. Cells were then washed with 1 mL PBS before centrifuging and aspirating the supernatant. Cells were fixed by the addition of 200 µL Stabilizing Fixative (BD Biosciences). Samples were stored at 4˚C in the dark prior to analysis by flow cytometry.

Stained cells were analysed with a FACSCanto™ II cell analyser (BD Biosciences, USA) alongside single stained compensation control beads (BD) for AlexaFluor405-anti-CD14 and pHrodo labelled cells. Detection of AF405 fluorescence was using a 530/30 nm band-pass filter while pHrodo fluorescence was detected through a 670 nm long-pass filter. Equivalent fluorescence intensities for the instrument across the study were established using SPHERO Rainbow calibration beads (SpheroTech Inc., Lake Forest, IL) and checked weekly.

An inclusion gate for leukocytes were first identified based on side and forward scatter properties and any debris excluded. Singlet cells were identified from this inclusion gate by gating on side-scatter height and width. Phagocytes (high side scatter) and lymphocytes (low side scatter) were then identified, and the neutrophil and monocytes sub-populations established based on the absence or presence of CD14 staining. Histograms of pHrodo fluorescence were to determine the percentage of phagocytic neutrophils and monocytes and the median fluorescence intensity of positive cells recorded. Untreated whole blood samples served to determine the cut-off for pHrodo fluorescence and >5000 cells were collected from each sample. All flow cytometry analysis was performed with applied compensation using Flowjo 10 software (TreeStar, USA).

Total IgG assay

Ewe colostrum samples, and plasma isolated from blood samples collected from lambs at 1-, 4-, 6- and 14-weeks of age were analysed for total IgG concentrations using a single radial immunodiffusion assay for the detection of IgG, according to the manufacturer's guidelines (IDRing® Sheep&Goat IgG assay, IDBiotech, France). The immunodiffusion precipitate rings were read using the IDRing® Viewer S120 (IDBiotech, France) and the total IgG concentration of each sample was

calculated against a known standard (supplied) using the calculation spreadsheet provided by IDBiotech.

Anti-tetanus-toxoid antibody assay

The antibody assay described by (Adams et al. 1997) was optimised for use with ovine plasma samples. High-binding 96-well ELISA plates were coated with tetanus-toxoid by adding 100µL (final volume) of 619 Lf/mL highly purified tetanus toxin from Clostridium tetani (Tetanus-toxoid for in vitro tests, Statens Serum Institut) diluted 1:1200 using 0.05 M bicarbonate buffer + 1% Bovine Serum Albumin (BSA) to each well. Plates were incubated overnight (16 hrs) at 4˚C before washing with Tris-buffered solution (TBS; pH 7.2) + 0.05% tween (TBS Tween®-20 Buffer, Thermo Scientific). Plates were filled with TBS + 0.05% tween and incubated for 3 minutes at room temperature. The plate was then rinsed three times, refilled and incubated at room temperature for 3 minutes. The wash procedure was then repeated before emptying the plate. To prevent non-specific binding, plates were blocked by adding 100µL of TBS + 1% BSA to each well and incubating at 4˚C for 1 hour. Plates were washed before adding 100 µL of plasma diluted 1:20 using TBS + 0.05% tween + 1% BSA to each well. Plates were incubated for 2 hours at 37˚C in humid conditions, to allow antibody-antigen binding. Following incubation, plates were washed to remove unbound antibody. Antibody binding was detected with 100 µL of developing antibody (HRP-Rabbit Anti-Sheep IgG, invitrogen™) diluted 1:6000 using TBS + 0.05% - tween + 1% BSA and plates were incubated for 2 hours at 37˚C in humid conditions. Plates were washed before adding 100 µL of TMB substrate (1-Step™ Ultra TMB-ELISA, Thermo Scientific) to each well and incubating the plate in the dark for 30 minutes at room temperature. The reaction was terminated by adding 50 µL of 2M sulphuric acid to each well. The absorbance of each sample was determined by reading the plate at 450 nm in a spectrophotometer (iMark™ Microplate Reader, Bio-Rad).

Statistical analyses

All statistical analyses were performed using GENSTAT (VSN International 2012). For all analyses, interaction terms were only included if they were statistically significant ($P < 0.05$). Analysis also included, where appropriate, data from an additional 148 lambs which were concurrently studied to examine the effects of supplementation of the ewe with Vitamin E or methionine during late pregnancy on similar parameters to those that are reported in this paper. Where significant treatment effects were observed, the results presented include only data from Vitamin D and Control lambs. Where no treatment effects were observed, results presented for effects of gestational length, birthweight, birth type, sex and sire of the lamb include lambs from both Control and Vitamin D treatments and the additional lambs concurrently studied.

Liveweights of ewes during pregnancy were corrected for weight of the conceptus using the formula from the Grazfeed model (Freer *et al.* 1997), where day 0 of pregnancy was the day of artificial insemination. Ewe liveweights corrected for the weight of the conceptus and ewe body condition scores were assessed using Restricted Maximum Likelihood (REML) with a heterogenous power model in which the correlation between observations from the same animal decays as the time delay between the observations increases along with allowing the variances at each time to be different in order to model repeated measurements over time. For analysis of ewe liveweights and condition scores, treatment, pregnancy status (single- or multiple-bearing), date and interactions thereof were fitted as fixed effects and pre-lambing plot, stud from which the ewe was sourced (ewe source), sire of the ewe and ewe birth year were fitted as random effects. For pregnancy status of the ewe, multiple-bearing refers to both twin- and triple-bearing ewes. Lamb liveweights were also assessed by REML with treatment, sire of the lamb, birth type (single- or multiple-born) and sex fitted as fixed effects and pre-lambing plot, lambing plot (nested with pre-lambing plot), dam source, dam identification, sire of the dam and dam birth year fitted as random effects. For birth type of the lamb, multiple-born refers to both twin- and triple-born lambs.

Ewe and lamb plasma Vitamin D metabolite concentrations (total 25(OH)D excluding or including 3-epi-25(OH)D_3, 25(OH)D_3, 3-epi-25(OH)D_3 and 25(OH)D_2) were assessed using REML. Where appropriate the data was log-transformed. For analyses of data from ewes, treatment was fitted as a fixed effect and pre-lambing plot, ewe source, sire of the ewe and ewe birth year were fitted as random effects. In addition, for analyses of ewe data at lambing and 4-weeks after lambing, the respective Vitamin D metabolite concentration (total 25(OH)D excluding or including the C3-epimer, 25(OH)D_3, 3-epi-25(OH)D_3 or 25(OH)D_2) prior to treatment was fitted as a covariate. For analyses of lamb data, treatment, sire of the lamb, birth type and sex and interactions thereof, where appropriate, were fitted as fixed effects, and pre-lambing plot, lambing plot (nested within pre-lambing plot), dam source, sire of the dam and dam birth year were fitted as random effects. Analysis of the relationship between ewe total 25(OH)D at lambing and lamb total 25(OH)D at birth was assessed using REML with treatment, sire of the lamb, birth type and sex and interactions thereof, where appropriate, fitted as fixed effects, and pre-lambing plot, lambing plot (nested within pre-lambing plot), dam source, sire of the dam, dam birth year and birth date of lamb were fitted as random effects. For this model, fixed terms were removed if not significant ($P \geq 0.05$).

Lamb immune function parameters of monocyte and neutrophil cell counts and monocyte and polymorphonuclear leukocyte (PMNL) phagocytic capacities were assessed using REML. For analyses at 4-weeks of age, the respective cell count or phagocytic capacity at birth was fitted as a covariate. Plasma IgG concentrations and anti-tetanus-toxoid antibody absorbances were modelled over time using REML with a power model in which the correlation between observations from the same animal decays as the time delay between observations increases. Where necessary, the aforementioned data was angular- or log-transformed. For these analyses, treatment, sire of the lamb, gestational length, birthweight, birth type and sex and interactions thereof, where appropriate, were fitted as fixed effects and pre-lambing plot and lambing plot (nested within pre-lambing plot), dam source, dam identification, sire of the dam, dam birth year and laboratory batch information, where appropriate, were fitted as random effects.

Rectal temperatures and blood glucose concentrations of the lambs at birth were assessed using REML. Due to very strong leverage, five lambs that had rectal temperatures of <30˚C and subsequently died were removed from the analysis. Blood glucose concentrations were log-transformed. Treatment, sire of the lamb, birthweight, birth type, sex and interactions thereof, where appropriate, were fitted as fixed effects and pre-lambing plot, lambing plot (nested within pre-lambing plot), dam source, dam identification, sire of the dam and dam birth year were fitted as random effects.

Lamb survival at birth, to 72 hours and to weaning was assessed by fitting Generalized Linear Mixed models. The approach used a logit-transformation and binomial distribution. Using additive models, logits were predicted as a function of the variables fitted as fixed and random effects. Treatment, birthweight, birthweight^2, birth type, sex and sire of the lamb were fitted as fixed effects and lamb date of birth, pre-lambing plot, lambing plot (nested within pre-lambing plot), dam source, dam identification, sire of the dam and dam birth year were fitted as random effects. In order to assess the effects of the monocyte and neutrophil cell counts and monocyte and PMNL phagocytic capacities at birth on the survival of lambs to weaning, the respective immune measure was fitted as a covariate in the survival to weaning analyses.

Results

Ewe liveweights and body condition scores

There was no significant difference in the mean liveweights of Control and Vitamin D ewes at artificial insemination (64.2 kg vs 63.8 kg), prior to treatments (day 111 pregnancy; 63.2 kg vs 62.5

kg), prior to lambing (day 141 pregnancy; 64.7 kg vs 65.0 kg), at lamb marking (57.9 kg vs 57.4 kg) or at lamb weaning (55.9 kg vs 55.5 kg). The mean body condition scores of Control and Vitamin D ewes also did not differ significantly at insemination (3.39 vs 3.38), prior to treatments (day 111 pregnancy; 2.37 vs 2.38), prior to lambing (day 141 of pregnancy; 2.59 vs 2.64), at lamb marking (2.61 vs 2.66) or at lamb weaning (2.77 vs 2.76).

Gestational length, birthweight and lamb survival

The mean gestational length of Control and Vitamin D ewes (148.5 vs 148.8 days) did not differ significantly and there were no significant differences in the gestational lengths of single- or multiple-bearing ewes (150.1 vs 149.0 days). There was no significant difference between Control and Vitamin D lambs for birthweight, rectal temperature or blood glucose concentration at birth (Table 1). Male lambs were heavier than female lambs (4.62 vs 4.35 kg; $P < 0.01$), and single-born lambs were heavier than multiple-born lambs at birth (4.99 vs 3.97 kg; $P < 0.001$). Birthweight had no significant effect on the blood glucose concentrations of lambs, however it had a significant positive effect on the rectal temperatures of lambs at birth (+0.5 ± 0.13˚C/kg birthweight; $P < 0.001$). There was no significant effect of birth type or sex of the lamb on rectal temperature or blood glucose concentration at birth.

Table 1. Mean weights, rectal temperatures and blood glucose concentrations at birth and survival at birth, to 72 hours and to weaning for lambs born to Control and Vitamin-D supplemented (Vit. D) ewes. The ewes were supplemented with cholecalciferol on days 113 and 141 of pregnancy. Values are presented in the back-transformed state where appropriate.

	Control	Vit. D	P value
Birthweight (kg)	4.20	4.26	0.243
Rectal temperature (˚C)	37.7	38.0	0.730
Blood glucose (mmol/L)	3.40	3.49	0.794
Survival			
Birth (%)	94.3	98.9	0.193
72 hours (%)	89.8	95.1	0.276
Weaning (%)	69.9	80.8	0.186

Maternal supplementation with cholecalciferol had no significant effect on the survival of lambs (Table 1), or the growth of lambs to weaning. The survival of single-born lambs to weaning was significantly greater than that of multiple-born lambs (88.3% vs 68.5% ; $P < 0.001$). Mean liveweights of Vitamin D and Control lambs at marking (week-4) were 11.8 kg and 12.3 kg respectively, and at weaning (week-14) were 22.3 kg and 21.6 kg respectively. Single-born lambs were consistently heavier than multiple-born lambs between birth and weaning ($P < 0.001$). There was no significant difference in the survival or growth of male and female lambs to weaning.

Vitamin D concentrations in plasma, colostrum and milk

The total 25(OH)D concentrations in plasma of Control and Vitamin D ewes did not differ significantly prior to supplementation (Table 2). Plasma total 25(OH)D concentrations, both including and excluding C3-epi-25(OH)D_3, were significantly higher in Vitamin D ewes compared to Control ewes at lambing and 4-weeks after lambing (Table 2).

In comparison to the Control lambs, the total 25(OH)D concentrations in plasma of lambs born to Vitamin D-supplemented ewes were 65.6 nmol/L and 24.8 nmol/L higher at birth, with and without the inclusion of C3-epi-25(OH)D_3, respectively. Both with or without the inclusion of C3-epi-25(OH)D_3, the plasma total 25(OH)D concentrations of Vitamin D and Control lambs did not differ significantly at 4-weeks of age (Table 2). Increased plasma total 25(OH)D concentrations in Vitamin D-supplemented ewes at lambing and 4-weeks after lambing, and in their lambs at birth, were associated with a significant increase in the concentration of 25(OH)D_3 and not 25(OH)D_2 compared to the Controls ($P < 0.001$).

Table 2. Mean plasma total 25-hydroxyvitamin D concentrations (nmol/L) of Control and Vitamin D-supplemented (Vit. D) ewes prior to supplementation (day 111 pregnancy), at lambing and 4-weeks after lambing, and of their lambs at birth and 4-weeks of age. The ewes were supplemented with cholecalciferol on days 113 and 141 of pregnancy. Values are presented in the back-transformed state where appropriate.

	Control	Vit. D	P value
Ewes (excluding C_3-epi-OH-D3)			
Pre-treatment	66.3	72.5	0.259
Lambing	78.6	137.0	< 0.001
4 weeks	73.0	157.0	< 0.001
Ewes (including C_3-epi-OH-D3)			
Pre-treatment	98.5	106.2	0.354
Lambing	105.7	198.9	< 0.001
4 weeks	89.5	222.5	< 0.001
Lambs (excluding C_3-epi-OH-D3)			
Birth	24.2	49.0	< 0.001
4 weeks	21.9	25.6	0.168
Lambs (including C_3-epi-OH-D3)			
Birth	69.2	134.8	< 0.001
4 weeks	28.4	35.6	0.099

There was a significant positive correlation between the total 25(OH)D (excluding C3-epi-25(OH)D_3) concentrations in the plasma of ewes at lambing and their lambs at birth ($P < 0.001$; Fig. 1). With the exclusion of C3-epi-25(OH)D_3, birth type and sex had no significant effect on the total concentrations

of 25(OH)D in the plasma of lambs at birth, however with the inclusion of C3-epi-25(OH)D_3, the total 25(OH)D concentrations were significantly higher in the plasma of multiple-born lambs compared to the single-born lambs (110.3 vs 84.6 nmol/L; $P < 0.001$) and in the male lambs compared to the female lambs (106.3 vs 87.8 nmol/L; $P < 0.001$). At 4-weeks of age, birth type and sex had no significant effect on the total concentrations of 25(OH)D in the plasma of lambs.

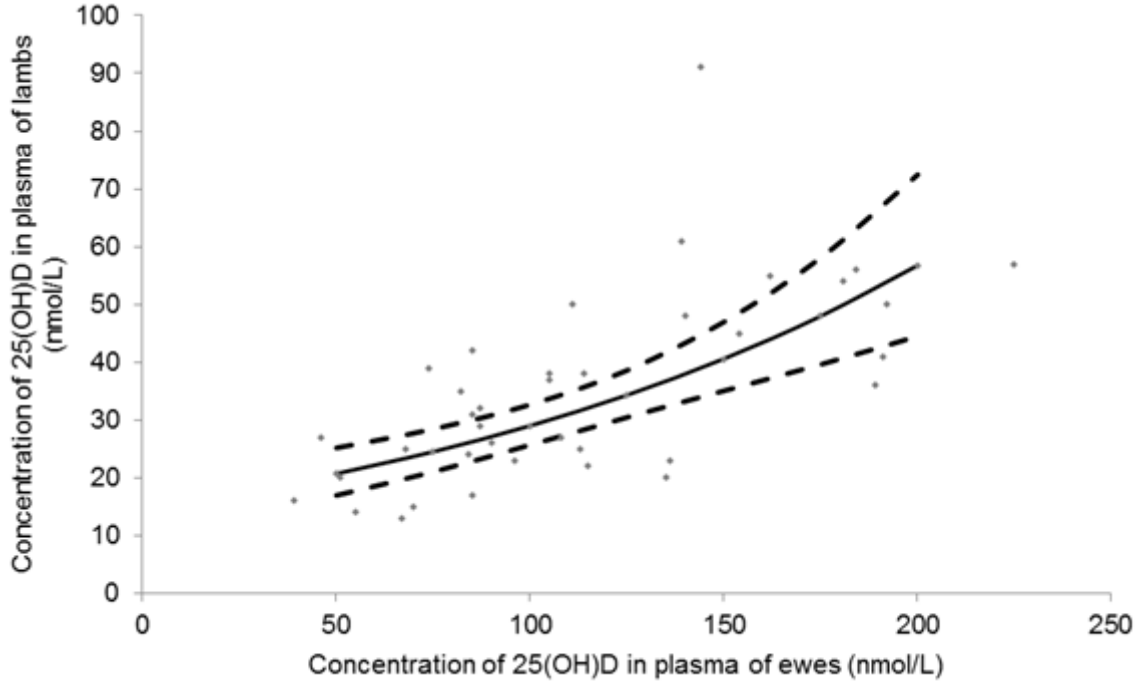

Fig. 1. Relationship between the total concentration of 25-hydroxyvitamin D (25(OH)D) in plasma of Control and Vitamin D-supplemented ewes at lambing and their lambs at birth (black solid line). The dashed lines represent the 95% confidence intervals (summary of data intervals). The fitted line and confidence intervals are presented in the back-transformed state. The dots represent the raw data for the plasma 25(OH)D concentrations of an individual ewe and her lamb.

Using the method employed in this study, the concentration of 25(OH)D_3 in colostrum was only detectable in 10 samples (2 – 6 nmol/L) whilst the concentration of 25(OH)D_2 was only detectable in five samples (1 – 10 nmol/L). The concentrations of 25(OH)D_3 and 25(OH)D_2 in milk samples collected 4-weeks after lambing were all below the level of detection.

Leukocyte counts and phagocytic capacities

There were no significant differences between lambs from Control and Vitamin D-supplemented ewes in the percentages of circulating monocytes (2.9% vs 3.0%) or neutrophils (55.3 vs 47.0%) in whole blood at birth. At 4-weeks of age, there were no significant differences in the percentages of monocytes or neutrophils in the whole blood of Control (5.3 and 29.4% respectively) or Vitamin D (5.6 and 28.7% respectively) lambs.

The percentage of phagocytic monocytes in whole blood of Control lambs did not differ significantly to that of the Vitamin D lambs at birth (84.2% vs 85.0%) or 4-weeks of age (98.6% vs 98.2%). There were also no significant differences in the percentages of polymorphonuclear leukocytes (PMNL) in the whole blood of Control or Vitamin D lambs at birth (60.0% vs 60.2%) or 4-weeks of age (91.2% vs 92.8%).

Gestational length, birthweight, birth type or sex of the lamb had no significant effect on the percentages of circulating monocytes or neutrophils or the percentages of phagocytic monocytes or PMNL at birth or 4-weeks of age.

IgG concentrations of colostrum and lamb plasma

There was no significant difference in the concentration of IgG in the colostrum of Control and Vitamin D-supplemented ewes (80.4 vs 82.3 mg/mL), and there was no significant effect of pregnancy status on colosrum IgG concentration. Lambs were born agammaglobulinemic (0 mg IgG/mL) and plasma IgG concentrations of Control and Vitamin D lambs did not differ at 1-, 4-, 6- or 14-weeks of age (Fig. 2). The plasma IgG concentrations of lambs differed significantly between consecutive time-points of analysis ($P < 0.001$), but there was no significant interaction with treatment.

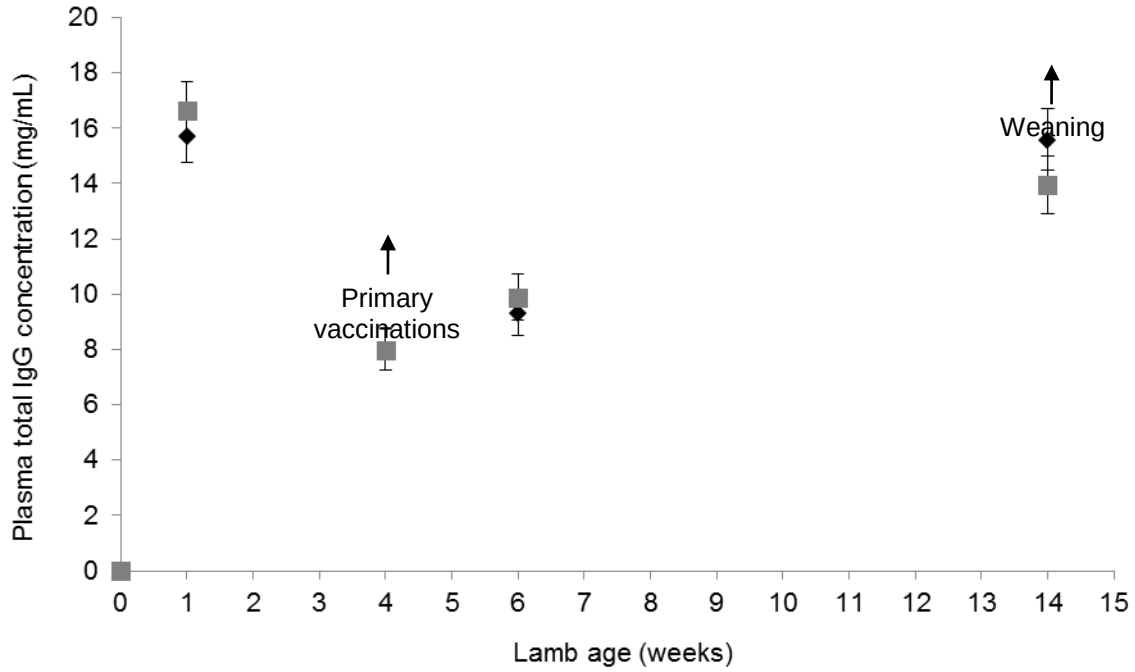

Figure. 2. Mean plasma total immunoglobulin-G concentrations (± least significant intervals) of Control (black) and Vitamin D (grey) lambs at birth and 1-, 4-, 6- and 14-weeks of age. The ewes were supplemented with cholecalciferol on days 113 and 141 of pregnancy. Values are presented in the back-transformed state.

Single-born lambs had significantly higher plasma IgG concentrations at 1-week of age (18.3 mg/mL) compared to multiple-born lambs (15.6 mg/mL; $P < 0.001$) but not at 4-, 6- or 14-weeks of age. Male lambs had significantly higher plasma IgG concentrations than female lambs ($P < 0.01$), but there was no interaction between plasma IgG concentrations, sex and the week of analysis. Gestation length and birthweight had no significant effect on the plasma IgG concentrations of lambs.

Anti-tetanus-toxoid antibody concentrations

The absorbances of plasma samples of Control and Vitamin D lambs analyzed for anti-tetanus-toxoid antibodies did not differ at 1-, 4-, 6- or 14-weeks of age (Fig. 3). Between consecutive time-points of analysis the absorbances of lamb plasma samples were significantly different ($P < 0.001$), but there was no significant interaction with treatment. Plasma samples from single-born lambs (0.69 nm) had significantly higher absorbances than multiple-born lambs at 1-week of age (0.57 nm; $P < 0.01$), but

not at 4-, 6- or 14-weeks of age. There was no significant effect of gestational length, birthweight or sex on the absorbances of plasma samples at 1-, 4-, 6- or 14-weeks.

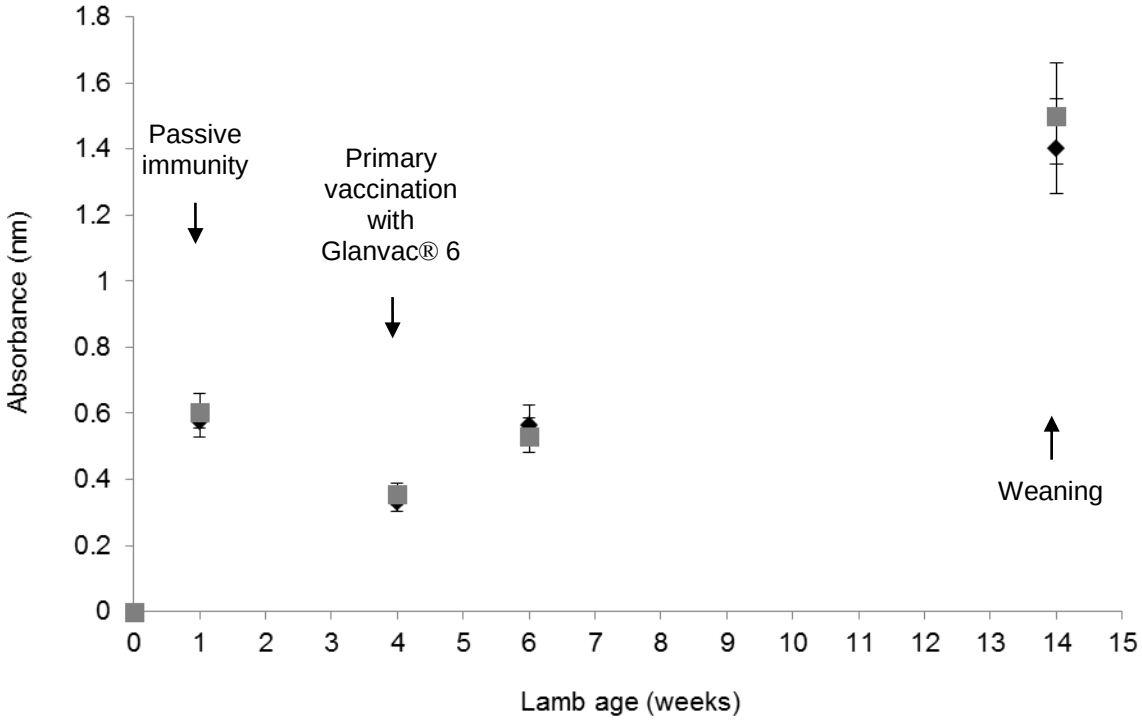

Figure 3. Mean absorbances (± least significant intervals) of Control (black) and Vitamin D (grey) lamb plasma samples analysed for anti-tetanus-toxoid antibodies between birth and weaning. The ewes were supplemented with cholecalciferol on days 113 and 141 of pregnancy. Values are presented in the back-transformed state

Discussion

Maternal supplementation with cholecalciferol in late pregnancy increased the plasma concentrations of 25(OH)D, excluding C3-epi-25(OH)D_3, in supplemented ewes by 74%, at lambing and this doubled the plasma 25(OH)D concentrations in their lambs at birth, supporting our first hypothesis. Supplementation of ewes with cholecalciferol in late pregnancy was not correlated with an increase in the percentage of phagocytic monocytes or PMNLs in lambs at birth or 4-weeks of age, or in the plasma concentrations of IgG or anti-tetanus-toxoid antibody of lambs between birth and weaning, thus our second hypothesis was rejected. Overall, our findings suggest that supplementing ewes twice with 1 x 10^6 IU cholecalciferol during late pregnancy is not effective at boosting the immune competency of lambs during the neonatal period and therefore is unlikely to reduce lamb mortality.

The plasma 25(OH)D_3 concentrations of ewes and lambs in this study are similar to those reported by Smith *et al.* (1987). In response to supplementation, the plasma 25(OH)D concentrations of the ewes continued to increase during the first month following lambing, whereas the plasma 25(OH)D concentrations of lambs born both to supplemented and Control ewes had declined by 4-weeks of age. Whilst the plasma half-life of cholecalciferol is only 4 to 6 hours, cholecalciferol is stored in the adipose tissue, muscle and liver, and has a whole-body half-life of approximately 2 months (Jones 2008). Thus the increase in the plasma 25(OH)D concentrations between lambing and 4-weeks post-lambing indicates that release of stored cholecalciferol continued to increase the plasma 25(OH)D concentrations of ewes for at least 2 months following the initial supplementation. 25-hydroxyvitamin D, which is the precursor for biologically active Vitamin D (1,25-dihydroxyvitamin D), is the only Vitamin D metabolite known to readily cross the placenta of humans and rodents (Lapillonne 2010; Roth 2011; Thorne-Lyman and Fawzi 2012; Kovacs 2013), and consistent with our findings, Smith *et al.* (1987) found ewe and lamb plasma 25(OH)D_3 concentrations were positively correlated at birth. The half-life of 25(OH)D is 2- to 3-weeks (Jones 2008; Holick 2009; Gezmish and Black 2013), and therefore 25(OH)D in the plasma of lambs at birth, acquired via placental transfer, would only persist for up to 2- to 3-weeks after birth. The decline in plasma 25(OH)D concentrations during the first 4-weeks of life in lambs born to Vitamin D-supplemented ewes also suggests that ewe colostrum and milk were a poor source of Vitamin D for the lambs. This is consistent with the analytic results of 25(OH)D concentrations in colostrum and milk samples, which were below 10 nmol/L at lambing and 4-weeks after lambing, respectively. Very low concentrations of Vitamin D metabolites are also found in human and bovine milk, which is understood to be due to the very low concentrations of Vitamin D-binding protein in the milk (Hollis *et al.* 1981); (Kovacs 2013). We therefore conclude that large intramuscular doses of cholecalciferol in late pregnancy could not significantly increase the concentrations of Vitamin D in the plasma of lambs during early post-natal life due to a rapid decline in 25(OH)D acquired via placental transfer from the ewe, along with a very low rate of transport of maternal Vitamin D into the milk and thus to the offspring via suckling.

The minor improvement in the Vitamin D status of lambs following supplementation of ewes with cholecalciferol in late pregnancy may also be associated with the efficacy of the route of supplementation and the Vitamin D metabolite used to supplement the ewes. Whilst intravenous or large oral doses of cholecalciferol in humans generate a more rapid response in plasma 25(OH)D concentrations, large intramuscular or subcutaneous doses of cholecalciferol in oil are able to provide a sustained source of 25(OH)D to increase plasma levels over time (Whyte *et al.* 1979). However, peak plasma 25(OH)D concentrations may not be reached until up to 2 months post-supplementation when cholecalciferol is administered via intramuscular or subcutaneous injection, as seen in this study, which may be associated with a reduced bioavailability of Vitamin D when administered via these routes (Whyte *et al.* 1979; Vieth 1999). 25-hydroxyvitamin D_3 is known to be more potent and has an increased bioavailability compared to cholecalciferol, and thus supplementation with 25-hydroxyvitamin D_3 is recognized to be between 4.2 and 5 times more effective at improving the Vitamin D status of an individual compared to supplementation with cholecalciferol (Cashman *et al.* 2012; Borel *et al.* 2013). Supplementating ewes with 25(OH)D_3 during late pregnancy may therefore be more effective at boosting the Vitamin D status of ewes and fetal lambs compared to supplementation with cholecalciferol. Furthermore, it is unknown whether ewes and lambs in this study were in a state of Vitamin D sufficiency, hence the plasma 25(OH)D concentration thresholds for Vitamin D deficiency, sufficiency and toxicity in young and adult sheep need to be defined. Further research is also required to determine the biological significance of C3-epi-25(OH)D_3 and whether it should be included when reporting the Vitamin D status of the body.

The ability of lamb monocytes and PMNLs to engulf *S. aureus* bioparticles was not influenced by maternal supplementation with cholecalciferol during late pregnancy. In contrast, Abu-Amer and Bar-Shavit (1993) showed that the phagocytic capacity of macrophages was poorer in Vitamin D-

deficient mice compared to Vitamin D-replete mice. Furthermore, nutritional supplementation of weanling pigs with cholecalciferol or 25(OH)D significantly increased the phagocytic capacity of blood monocytes and granulocytes (Konowalchuk *et al.* 2013). The percentages of phagocytic monocytes and PMNLs in the whole blood of lambs at birth were similar to those seen in the cord blood (Hallwirth *et al.* 2002) and peripheral blood (Prosser *et al.* 2013) of human infants, and as expected the ability of the lamb monocytes and PMNLs to engulf the bacterial bioparticles was higher at 4-weeks of age than at birth, reflecting maturation of the lamb's immune system (Menge *et al.* 1998). The use of *S. aureus* bioparticles to assess phagocytic uptake of bacteria in lambs appears to be an appropriate bacterial model based on the findings of Dennis (1974) and Hughes (1971), who observed *S. aureus* was one of the major bacterial species infecting lambs and furthermore that this bacteria was associated with pneumonia, polyarthritis and peritonitis, which are recognised to be common causes of infection-related death in lambs (Mellor and Stafford 2004). However, the interactions between the plasma Vitamin D levels of the lambs and the antimicrobial killing and inflammatory responses of lamb monocytes and PMNLs, including the production of reactive oxygen species, anti-microbial peptides and cytokines, should be investigated.

Supplementation of ewes with cholecalciferol did not affect the concentrations of IgG in the colostrum or in the plasma of lambs at 1-week of age or following administration of primary vaccines. This indicates that supplementaion did not influence the production of maternal antibodies in the colostrum or the passive immune status of lambs, and also had no effect on the adaptive antibody immune responses in the lambs. The colostral IgG concentrations of Control and Vitamin D ewes at lambing were within the range of 64.2 – 99.4 mg/mL reported by previous studies (Gilbert *et al.* 1988; al-Sabbagh *et al.* 1995; Loste *et al.* 2008; Swanson *et al.* 2008). The plasma concentrations of IgG in Control and Vitamin D lambs at 1- and 4-weeks of age were slightly lower than those reported in other breeds of sheep (Yılmaz *et al.* 2011; Gokce *et al.* 2014). However, the optimal plasma concentrations of IgG in lambs in early life have not been reported and therefore it is difficult to establish whether the lambs in this study had an adequate passive immune status.

Vaccination clearly boosted vaccine-specific anti-tetanus-toxoid antibody titers in lambs, but we did not observe any effect of maternal supplementation with cholecalciferol on the antibody immune response to tetanus-toxoid. The absorbance values of the lamb plasma samples analyzed for anti-tetanus-toxoid antibodies in the present study were similar to those reported by Adams *et al* (1997) who analyzed anti-tetanus-toxoid antibody titers in adult ewes following administration of booster vaccines, however to the best of our knowledge, our study is the first to measure the vaccine-specific antibody response to tetanus-toxoid in neonatal lambs. Although supplementation did not appear to influence the vaccine-specific response to tetanus-toxoid in the lambs, it is possible that vaccine-specific antibody immune responses of the lambs to other vaccine antigens were affected by maternal supplementation with cholecalciferol.

Maternal supplementation with cholecalciferol had no significant effect on the overall metabolic status of lambs at birth or on the growth or survival of lambs to weaning. Furthermore, the birthweights of the lambs were close to the range of 4.5 to 5.5 kg at which maximum survival occurs, and the rectal temperatures and blood glucose concentrations at birth were similar to those reported previously, therefore indicating that the lambs were not at an increased risk of death in the first 72 hours of life (Stafford *et al.* 2007; Miller *et al.* 2010; Chniter *et al.* 2013). On average, only 4.6% of lambs born alive died within the first 72 hours of life in the present study, which is considerably lower than the expected mortality of 20 – 30% (Oldham *et al.* 2011; Hawken *et al.* 2012; Hinch and Brien 2014). We therefore expect that the limited number of lamb deaths during this high risk period was due to the high level of intervention associated with intensive sampling at lambing.

Overall our novel findings showed that supplementation of ewes with two large intramuscular doses of 1 x 10^6 IU cholecalciferol in late pregnancy is unsuccessful in boosting the immune competency of the lamb during early life. Colostrum and milk are poor sources of Vitamin D and therefore young lambs may require direct supplementation in the early neonatal period in order to increase plasma Vitamin D levels sufficiently high to generate a response in the immune system. Further research is therefore needed to determine the requirements for Vitamin D in young lambs and additionally what levels of Vitamin D are required to optimize immune function. Subsequent research could then determine what approach to Vitamin D-supplementation is most effective at safely boosting the immune competency of the lamb and if this is associated with an improvement in lamb survival.

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Appendix 2

Supplementation of autumn lambing Merino ewes with vitamin E plus selenium increases alpha-tocopherol and selenium concentrations of the lamb but does not improve their immune function or survival

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Short title: Supplementing ewes with Vitamin E and selenium does not improve lamb immunity

Abstract

Vitamin E and selenium have been reported to improve immune function and lamb survival. Ewes lambing in autumn in Western Australia are at risk of being deficient in Vitamin E and selenium at lambing thus predisposing their lambs to deficiencies and increasing the likelihood of infection and disease resulting in peri-natal mortality. This study tested the hypothesis that supplementation with Vitamin E plus selenium in late gestation will increase the levels of vitamin E and selenium in the ewe and lamb and this will boost the lamb's innate and adaptive immune responses and improve their survival. Pregnant Merino ewes were divided into a Control group (*n* = 58) which received no supplement or a group supplemented with Vitamin E plus selenium (n = 55). On day 111, 125 and 140 of pregnancy ewes in the Vitamin E plus selenium group were given 4 g dl-α-tocopherol acetate orally, and on day 111 they were also given 60 mg of selenium as barium selenite by subcutaneous injection. The concentrations of α-tocopherol and selenium were measured in ewes and/or lambs from day 111 of pregnancy to weaning. Immune function of the lamb was assessed by analyzing the phagocytic capacities of monocytes and polymorphonuclear leukocytes and plasma IgG concentrations between birth and weaning. Maternal supplementation with Vitamin E plus selenium increased the concentration of α-tocopherol in plasma (0.67 *vs*. 1.13 mg/L; P<0.001) and selenium in whole blood (0.12 *vs*. 0.07 mg/L; P<0.01) of the ewes at lambing compared to Controls. Supplementation also increased the concentration of α-tocopherol (0.08 *vs.* 0.14 mg/L; P<0.001) and selenium (0.05 *vs* 0.08 mg/L; P<0.01) in lambs at birth compared to controls. Our initial hypothesis was therefore supported, however prior to suckling it is likely that all lambs were still deficient in Vitamin E and 40% of lambs were deficient or marginal in selenium even when ewes were supplemented. There was no significant effect of supplementation on the innate phagocytic or adaptive antibody immune responses of the lambs, so this part of our hypothesis was rejected. The survival of lambs to weaning from ewes supplemented with Vitamin E plus selenium was 11% higher than the survival of control lambs, but this effect was not statistically significant and there was no evidence that this change in survival resulted from the supplements boosting the lamb's innate or adaptive immune responses.

1.1 Introduction

The mortality of lambs from birth to weaning can exceed 50%, especially in multiple born Merino lambs (Behrendt *et al*. 2011; Hinch and Brien 2014; Paganoni *et al*. 2014), and improving survival is recognised as the highest priority to improve reproductive efficiency of the Australian sheep flock (Young *et al.* 2014). Most lambs die within the first 72 hours of life, and the causes of death include dystocia, starvation, mismothering and exposure and infection and disease (Brown *et al.* 2014; Refshauge *et al.* 2015). In Western Australia many ewes lamb during autumn/early winter (Curtis and Croker 2005) and these ewes typically lose significant live weight and condition score during late gestation as nutrient supply from dry pastures and crop stubbles seldom meet energy requirements for maintenance and foetal growth (Kelly 1992). Poor nutrition during late pregnancy can result in low lamb birth weights which increases the risk of lamb mortality (Oldham *et al*. 2011; Paganoni *et al*. 2014). The adverse effects of poor paddock feed on ewe live weight and condition score can be offset by providing supplementary feeds but high rates of lamb mortality may still occur and this may be related to deficiencies in specific nutrients.

Several studies have reported that ewes lambing in autumn in Western Australia are at risk of being deficient in vitamin E and selenium, due to the increased requirements of vitamin E and selenium during gestation and their low concentrations in dry pasture, crop stubbles and grain supplements (Gardner and Gorman 1963; Kumagai and White 1995; White *et al.* 1997; White and Rewell 2007). Vitamin E and selenium function synergistically as antioxidants that can counteract oxidative stress occurring in the peri partum period (Gitto *et al.* 2001; Celi *et al.* 2010; Casamassima *et al.* 2012). A deficiency in vitamin E and selenium can also compromise immune responses (Meydani and Beharka 1996; Rooke *et al.* 2004) which may increase the likelihood of infection and disease in the new born lambs resulting in peri-natal mortality. Selenium more readily crosses the placenta than vitamin E and Liu *et al*. (2014) proposed that immune responses are more closely related to the concentration of selenium than vitamin E. The innate and adaptive immune response of a neonate is naïve at birth and the lamb is reliant on passive transfer of antibodies, predominantly immunoglobulin G (IgG), from colostrum immediately after birth. Sufficient antibody concentrations in the neonate significantly boost phagocytosis which links the innate immune response to the adaptive immune response. Phagocytosis involves the recognition, binding, engulfment and ultimate destruction of antibody- and/or complement-opsonised viruses, bacteria and fungi. The ability for phagocytes to clear a challenge with exogenous bacteria is a good indicator of the lamb's combined immune defences.

In this paper we hypothesize that supplementation of Vitamin E and selenium to autumn lambing ewes during late pregnancy increases the levels of vitamin E and selenium in the ewe and lamb, and that the increased levels of vitamin E and selenium in the lambs will improve their innate and adaptive immune responses and hence survival to weaning.

1.2 Materials and Methods:

This experiment was approved by Murdoch University's Animal Ethics Committee in accordance with the guidelines of the Australian Code of Practice for the Use of Animals for Scientific purposes.

Animals and experimental design

Two hundred Merino ewes aged between 4 and 7 years old were sourced from the Maternal Efficiency Flock (Rosales Nieto *et al.* 2013) located at the UWA Research farm near Pingelly in Western Australia (S 32° 30' 23" – E 116° 59' 31"). Ewes were artificially inseminated in November 2013 and 120 pregnant ewes were allocated into three replicates of each of two treatment groups; Control or Vitamin E plus selenium supplementation during late pregnancy. Lambing occurred during late April/early May 2014 and lambs were weaned in August 2014.

Animal management and treatments

The ewes were managed to achieve a condition score of 3.3 prior to artificial insemination. Controlled Internal Drug Releases (Eazi-Breed™ CIDR® Sheep and Goat Device, Zoetis Australia) were inserted into the vagina of all ewes 14 days prior to artificial insemination to synchronise oestrus. Two days prior to artificial insemination they were removed and the ewes were injected with 1.6 ml of Pregnant Mare Serum Gonadotropin (PMSG; Pregnecol, Bayer). Ewes were artificially inseminated in November 2013 with semen from four sires which was allocated to the ewes according to their age and live weight and condition score measured on the previous day.

After artificial insemination, the ewes were managed as a single flock until day 111 of pregnancy. They were pregnancy scanned on day 55 of pregnancy to identify single- and twin-bearing ewes (Fowler and Wilkins 1984) and then weighed and condition scored every one to two weeks and supplementary fed to achieve a condition score of 2.5 at lambing. The live weights of ewes during pregnancy were corrected for weight of the conceptus using the formula from the Grazfeed model (Freer *et al.* 1997), where day 0 of pregnancy was the day of artificial insemination. The ewes grazed dry annual pasture during mid and late pregnancy (6.8 MJ ME/kg, 9.8% CP, 4.3 mg/kg Vitamin E, 0.019 mg/kg selenium) and green vegetative pastures from early lactation (128.2 mg/kg Vitamin E, 0.027 mg/kg selenium) to weaning. Between pregnancy scanning and lambing they were offered 700 g/day of supplement consisting of 52% lupins (13.9 MJ ME/kg, 31% CP, 9.1 mg/kg Vitamin E, 0.038 mg/kg selenium), 31% oats (12.8 MJ ME/kg, 12.2% CP, 6.8 mg/kg Vitamin E, 0.012 mg/kg selenium) and 17% oaten chaff (9.3 MJ ME/kg, 7.6% CP, 4.3 mg/kg Vitamin E, 0.018 mg/kg selenium).

On day 111 of gestation, the ewes were administered a clostridial 6 in 1 booster vaccine (Glanvac® 6, Zoetis Australia) and then allocated into three replicates of each of the two treatment groups in the basis of ewe age, sire of the lamb, pregnancy status, and live weight and condition score at insemination (n = 17-20 ewes/treatment/replicate). Ewes in the treatment group received an oral dose of 4 g dl-α-tocopherol acetate (Advanced Liquid E, 7mL, Advanced feeds PTY LTD) and were subcutaneously injected with 1.2 mL of Bayer Selovin (The Farm Co. WA, active constituent barium selenate; 1 mg selenium per kg live weight). On day 124 and 140 of gestation, a second and third oral dose of vitamin E (4 g per sheep) was also administrated. The control group received no intervention during gestation.

Each replicate of the two treatments was combined after the first injection of α-tocopherol acetate and the three flocks of control and vitamin E plus selenium ewes grazed separate pre-lambing plots until day 140 of pregnancy. At this time the ewes within each replicate were reallocated into smaller lambing plots according to treatment group, pregnancy status and sire of lamb so there were six groups of 17 – 20 control and vitamin E plus selenium ewes for lambing. Ewes remained in the lambing plots until approximately 1-week post-lambing at which point all ewes and their lambs were joined together and run as a single flock and weighed every 1 to 4 weeks until weaning. The lambs were marked (castrated and tailed) and vaccinated with Glanvac® 6 (Zoetis Australia), Eryvac® (Zoetis Australia), Gudair® (Zoetis Australia) and Scabigard® (Zoetis, Australia) at 4-weeks of age (± 1 week) and administered a Glanvac® 6 booster vaccine at weaning at 14 weeks of age (± 1 week).

Animal measurements and blood sampling

Lambing commenced on day 143 of gestation and ceased on day 153 of gestation. The ewes were intensively monitored during lambing between the hours of 4 am and midnight. Immediately after parturition and before suckling the lambs were weighed, ear tagged and birth type and gender identified. The rectal temperature was also recorded using a digital thermometer. Two 5 ml blood samples were then collected from the lambs via jugular venipuncture into heparinised vacutainers

and blood glucose concentrations were measured using a glucometer (Accu-Chek Go, Roche Diagnostics). Additional blood samples were collected from lambs at one week of age (± 3 days; n = 92), at marking (n = 73), 2-weeks following their vaccination at marking (n = X) and at weaning (n = Y). These samples were used to measure nutrient status and immune function. Blood samples that were collected for analysis nutrient status were placed on ice whilst those collected for analysis of immune function were kept at room temperature.

A single 9 ml blood sample was also collected from ewes via jugular venipuncture into heparinised vacutainer tubes on day 111 (pre-treatment; n = 114), at lambing (n = 92) and at lamb marking (n = 84) and weaning (n = X) for further analysis of α-tocopherol and selenium concentrations. A 30 ml colostrum sample was collected pre-suckling (n = 82) and placed on ice before being aliquoted and stored at -80˚C for subsequent immunological analysis. Milk samples were collected at lamb marking (n = 81) from a sub set 40 Control and 40 treatment ewes. Blood samples were centrifuged at 2278 g for 15 min and plasma was removed and stored at -20°C for analysis for nutrient analysis. The blood samples used for immune function analysis were centrifuged at 952 g for 10 minutes and at 134 g for the phagocytosis assay. Plasma was then aliquoted and stored at -80 C.

Vitamin E and selenium concentration in plasma and colostrum

Plasma samples collected from ewes on day 111 of gestation and ewes and lambs at lambing, marking and weaning were analysed for α-tocopherol concentrations using high performance liquid chromatography (HPLC) as described by McMurray and Blanchflower (1979). Colostrum samples collected at lambing and milk samples collected at 4 weeks post lambing were also analysed using HPLC. Briefly 1 mL of sample was deproteinized with 1mL 1% pyrogallol in ethanol. Five ml hexane was added, vortexed and then 4 ml of hexane layer was transferred into another tube and evaporated under nitrogen. The hexane extraction was repeated once. The dryness was reconstituted in 0.5 ml methanol (0.1% butylated hydroxytoluene), and the reconstitute was applied to an Agilent HPLC system (1100) equipped with a Zorbax SB-C18 column (3 mm x 150 mm, 3.5 µm) (Agilent) with a methanol mobile phase. α-tocopherol was quantified using fluorescence detection (ex. 296 nm and em. 330 nm). Selenium concentration in whole blood was estimated from the relationships derived between selenium concentration and glutathione peroxidase (GSHpx) activity in erythrocytes, as described by Lui *et al.* (2015a).

Vitamin E and selenium concentration in feedstuffs and soils

Pasture samples on paddocks used for grazing the ewes were harvested on two occasions during late pregnancy and lactation. Each sample was collected from 8-10 sites per paddock and pooled. All pasture samples were freeze-dried and then stored at -20˚C. Samples of oaten chaff, oats and lupin seeds were collected five times during late pregnancy, pooled and dried at 65˚C. All feedstuff samples were ground to pass 1 mm sieve and used for analyses of vitamin E and selenium concentrations and dry matter at 105˚C. Soil samples were also collected from paddocks grazed by the ewes during the experimental period. Samples were collected from five spots in each paddock and pooled. All samples were dried at 65˚C, ground and then used for analysis of selenium content.

The α-tocopherol concentrations in feedstuff samples were also analysed using a HPLC procedure as described by (McMurray *et al.* 1980). Briefly, 1 g of feed was homogenized in 10 mL 6% pyrogallol by ultraturrex. Then 1 ml 60% KOH (w/v) in water was added, the tube was sealed, and tubes heated at 70°C for 30 min. The procedures of hexane extraction, reconstitutions, and instrumental analysis were the same as described for the analysis of plasma samples. Selenium concentrations in feedstuffs and soils were determined using Agilent VGA-76 hydride generator. One gram of feedstuff and soil samples was wet digested using nitric and perchloric acids under a temperature program which ensures residual nitric acid is removed from the digest. Selenium was then converted to the Se(IV) state using hydrochloric acid in preparation for measurement by

hydride generation and atomic absorption (Varian VGA-77 and Varian AA280FS respectively, Agilent Technologies Inc.). Selenium concentration in the soil ranged from 0.02 mg/kg DM to 0.15 mg/kg DM, with an average of 0.058 mg/kg DM (SD0.043).

Analysis of immune function

Whole blood deferential cells counts, monocyte and polymorphnuclear leukocyte phagocytic capacities, total immunoglobulin-G concentrations and anti-tetanus toxoid antibodies were measured as described in detail by Lockwood e*t al.* (2015). Briefly, whole blood differential cell counts were analysed using Advia® 120 automated Hematology Analyzer (sequence 284) in a high output pathology laboratory (Clinical Pathology, Murdoch University Veterinary Hospital). The abilities of monocytes and polymorphnuclear leukocytes to phagocytose bacterial bioparticles were assessed using a flow cytometry method. Plasma total IgG concentrations of lambs were assessed using a single ImmunoDiffusion assay (IDRing® Sheep&Goat IgG assay, IDBiotech, France). Anti-tetanus toxoid antibodies in lamb plasma samples were assessed by optimising the enzyme-linked immunosorbent assay described by Adams *et al.* (1997) for use with ovine plasma samples.

Statistical analysis

All statistical analyses were performed using GENSTAT (VSN International 2012). For all analyses, interaction terms were only included if they were statistically significant ($P < 0.05$). Analysis also included, where appropriate, data from an additional 148 lambs which were concurrently studied to examine the effects of supplementation of the ewe with Vitamin D (Lookwood *et al.* 2015) or methionine (Liu *et al.* 2015b) during late pregnancy on similar parameters to those that are reported in this paper. Where significant treatment effects were observed, the results presented include only data from Vitamin E plus selenium and Control lambs. Where no treatment effects were observed, results presented for effects of gestational length, birth weight, birth type, and sex of the lamb include lambs from both Control and Vitamin E plus selenium treatments and the additional lambs concurrently studied.

Lamb and ewe data was analysed using restricted maximum likelihood method (REML) with treatment, sex, birth type, sire of lamb, any covariate of interest and interactions, where appropriate, fitted as fixed effects. Pre-lambing and lambing paddocks (nested with pre-lambing paddock), ewe source, ewe identification, sire of ewe, dam birth year and laboratory batch information, where appropriate, fitted as random effects. Liveweights of ewes during pregnancy were corrected for weight of the conceptus using the formula from the Grazfeed model (Freer *et al.* 1997), where day 0 of pregnancy was the day of artificial insemination. Lamb survival was analysed by fitting General linear mixed models. The approach used a logit-transformation and binomial distribution. Using additive models, logits were predicted as a function of the variables fitted as fixed and random effects. Treatment, birthweight, birthweight^2, birth type, sex, sire of the lamb and variates of interest were fitted as fixed effects and lamb date of birth, pre-lambing plot, lambing plot (nested within pre-lambing plot), dam source, sire of the dam and dam birth year were fitted as random effects. For birth type of the lamb, multiple-born refers to both twin- and triple-born lambs. Some immunity data and nutritional status values were not normally distributed and had to be transformed (natural log) for analyses. A full description of this analysis is provided by Lookwood *et al*. (2015). Analysis of the relationship between ewe α-tocopherol and selenium at lambing and lamb α-tocopherol and selenium at birth was assessed using REML with treatment, sire of the lamb, birth type and sex and interactions thereof, where appropriate, fitted as fixed effects, and pre-lambing plot, lambing plot (nested within pre-lambing plot), dam source, sire of the dam, dam birth year and birth date of lamb were fitted as random effects. For this model, fixed terms were removed if not significant ($P \geq 0.05$).

Results

Ewe live weight and condition score

There were no significant differences in the average maternal live weight of Control and Vitamin E plus selenium treated ewes prior to treatments (63.2 *vs* 62.8 kg), prior to lambing (day 141 of gestation 64.7 *vs* 65.1 kg), at lamb marking (58.0 *vs* 57.5 kg) or at weaning (56.0 *vs* 55.6 kg). Similarly, the average condition score of Control and Vitamin E plus Selenium ewes did not differ significantly prior to treatments (2.4 *vs* 2.4), prior to lambing (day 141 of gestation (2.6 *vs* 2.6), at lamb marking (2.6 *vs* 2.7) or at weaning (2.8 *vs* 2.8). There was a significant effect ($P<0.01$) of birth type on live weight and condition score, and a significant birth type x time interaction ($P<0.01$), mostly because ewes that subsequently had twins were heavier and fatter between artificial insemination and the start of the treatment period.

Gestation length and lamb characteristics

There was no significant difference in the average gestation length between Control and Vitamin E plus selenium ewes (148.5 *vs* 149.2 days) or between single- or multiple-bearing ewes (150.1 *vs* 149.0 days). There was no significant effect of maternal supplementation on lamb weight, blood glucose or rectal temperature at birth, or live weight of lambs at marking or weaning (Table 1). Survival to weaning was also not significantly different, despite the average survival of lambs from supplemented ewes being 81% compared to 70% for those from Control ewes. Single born lambs were significantly heavier than twins at birth (5.0 vs 4.0 kg; $P<0.001$), at marking (13.4 *vs* 10.5 kg; $P<0.001$) and weaning (23.9 *vs* 19.5 kg; $P <0.001$). Male lambs were heavier than female lambs at birth (4.6 *vs* 4.3 kg; $P < 0.01$) but not at marking or weaning.

Table 1. Average blood glucose concentration and rectal temperature of the lamb at birth, lamb weights from birth to weaning and survival to weaning from ewes in the control group or ewes that were supplemented with Vitamin E and selenium (Vit E + Se). Data is presented in the back-transformed state where applicable.

	Control	Vit E + Se	P value
Blood glucose (mmol/L)	3.40	3.29	0.794
Rectal temperature (°C)	37.7	37.7	0.730
Birth Weight (kg)	4.20	4.45	0.243
Marking (kg)	12.3	12.1	0.508
Weaning (kg)	22.2	21.9	0.648
Survival at day 0 (%)	94	98	0.193
Survival to day 3 (%)	90	95	0.276
Survival to weaning (%)	70	81	0.186

Vitamin E status in the ewe and lamb

There was no significant difference in the concentration of α-tocopherol between Control and Vitamin E plus selenium ewes prior to the treatment period (Table 2). During late pregnancy the concentration of α-tocopherol was maintained for ewes supplemented with Vitamin E and selenium but decreased by about 50% for Control ewes. At lambing the concentration of α-tocopherol was significantly higher for Vitamin E plus selenium ewes compared to Controls, and supplementation reduced the proportion of ewes that were deficient in Vitamin E (<0.7 mg/L) from 61% to 9%. At marking the concentration of α-tocopherol was still significantly higher in the supplemented ewes ($P<0.001$) than the Control ewes, however there was no difference at weaning. There was a significant difference in the concentration of α-tocopherol in colostrum from Control and Vitamin E plus selenium ewes (4.1 *vs* 10.6 mg/L; $P<0.001$) however there was no significant difference in the

concentration of α-tocopherol in milk at marking (1.0 *vs* 1.1 mg/L). There were also no significant differences in the concentration of α-tocopherol in plasma, colostrum or milk between single and twin bearing ewes.

Table 2. Average concentration of α-tocopherol and selenium (mg/L) in plasma of ewes at day 111 (pre-treatment) of gestation and of ewes and lambs from lambing until weaning (week 14). The ewes were supplemented with Vitamin E plus selenium on days 111, and then again with Vitamin E on day 124 and 140 of gestation. Values are presented in the back transformed state where appropriate.

		α-tocopherol concentrations (mg/L)			Selenium concentrations (mg/L)		
		Control	Vit E + Se	P value	Control	Vit E + Se	P value
Ewe	Pre treatment	1.29	1.28	n.s.	0.10	0.10	n.s.
	Lambing	0.67	1.13	<0.001	0.06	0.13	<0.001
	Marking	1.51	1.67	<0.001	0.05	0.12	<0.001
	Weaning	1.95	1.86	n.s.	0.04	0.12	<0.001
Lambs	Birth	0.08	0.14	<0.001	0.05	0.08	<0.001
	Marking	0.96	1.20	<0.01	0.04	0.08	<0.001
	Weaning	1.02	1.10	n.s.	0.04	0.06	<0.001

Lambs born to ewes supplemented with Vitamin E plus selenium had significantly higher α-tocopherol concentrations at lambing compared to the control lambs (Table 2; P<0.001), however all lambs were still Vitamin E deficient at birth (<0.7 mg/L). The concentration of α-tocopherol increased 10-fold during the 4-week period to marking and remained significantly higher in Vitamin E plus selenium lambs that Control lambs at marking (P<0.01), however this difference was not significant at weaning. There was no significant effect of sex on the concentration α-tocopherol between birth and weaning, but the concentration α-tocopherol was higher for twin-born than single-born lambs at marking (1.27 *vs* 1.10; P = 0.05).

There was a positive non-linear correlation between the concentration of α-tocopherol in plasma from ewes and their lamb at birth (Fig. 1). When the maternal concentration of α-tocopherol was less than 1.0 ml/L, a unit change of α-tocopherol concentration in the ewe was associated with a 0.09 unit change in the concentration of α-tocopherol in the lamb, whereas when the maternal concentration of α-tocopherol was greater than 1.0 ml/L a unit change of α-tocopherol concentration in the ewe was associated with a 0.15 unit change in α-tocopherol concentration in the lamb.

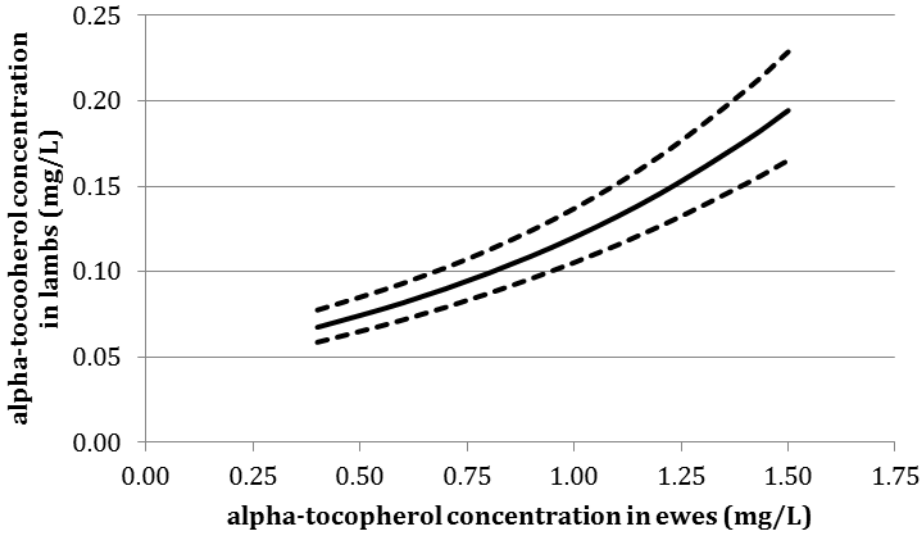

Figure 1. The relationship between the concentration of α-tocopherol in plasma from ewes and their lambs immediately after at birth and prior to suckling. The dashed lines represent the 95% confidence intervals.

Estimated Selenium concentration in whole blood in the ewe and lamb

There were no significant differences in estimated concentration of selenium between Control and Vitamin E plus selenium ewes prior to the treatment period (Table 2). During late pregnancy the concentration of selenium in blood increased slightly for ewes supplemented with Vitamin E and selenium but decreased by about 40% for Control ewes. At lambing, marking and weaning the concentration of selenium in blood was significantly higher for ewes supplemented with Vitamin E plus selenium compared to Controls. Supplementation reduced the proportion of ewes that were deficient to marginal (<0.1 mg/L) in selenium from 87% to 46%. There were no significant differences in the concentration of Se in blood between single and twin bearing ewes.

Maternal supplementation with Vitamin E and selenium in late gestation increased the concentration of selenium in the blood of lambs at lambing, marking and weaning by 50 to 100% compared to lambs from Control ewes (Table 2; P<0.001). The blood selenium concentration was marginally higher in ewe lambs than male lambs at birth (0.06 *vs* 0.05 mg/L; P<0.05), but there were no other consistent effects of birth type or sex of lamb on the concentration of selenium in blood up until weaning. There was a positive correlation between selenium concentrations in the ewes and lamb at birth, and this relationship differed significantly (P<0.001) for female and male lambs. A one unit change in the selenium concentration in the ewes resulted in a 0.41 and 0.46 unit change in the selenium concentration in their female and male lambs, respectively.

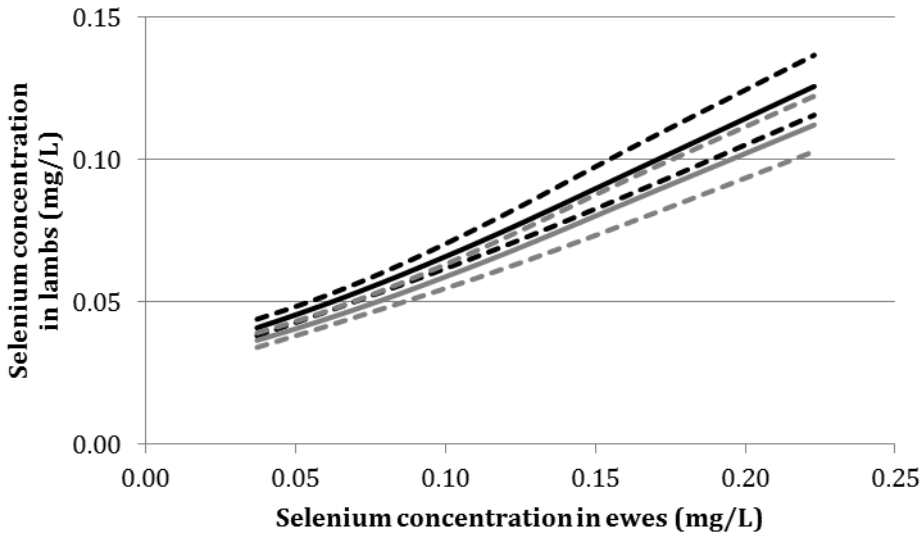

Figure 3. The relationship between the concentration of selenium in blood from ewes and their male (grey) and female (black) lambs immediately after at birth and prior to suckling. The dashed lines represent the 95% confidence intervals.

Innate immune response

The total number of white blood cells in whole blood of Control and Vitamin E plus Selenium lambs did not differ significantly at birth (3.75 x 10^9/L *vs* 3.71 x 10^9/L) or one month of age (6.6 x 10^9/L *vs* 6.7 x 10^9/L). There was also no significant difference in the relative proportion, total number or phagocytic activity of the monocytes or neutrophils in the Control or Vitamin E plus selenium lambs at birth or one month of age. As a result, the total number of phagocytic monocytes in whole blood of Control lambs did not differ to that of the Vitamin E plus selenium lambs at birth (0.12 x 10^9/L *vs* 0.10 x 10^9/L) or one month of age (0.35 x 10^9/L *vs* 0.40 x 10^9/L), and the total number of phagocytic neutrophils did not differ at birth (1.1 x 10^9/L *vs* 0.98 x 10^9/L) or one month of age (1.8 x 10^9/L *vs* 1.5 x 10^9/L). Across all lambs, on no occasion was there a significant relationship between the actual concentrations of α-tocopherol or selenium in the lambs at birth or one month of age on their innate immune responses.

Adaptive immune response

There was no significant difference in the concentration of IgG in colostrum of control or supplemented ewes (80.4 *vs* 72.2 mg/mL). There was no significant difference in the total number of lymphocytes in Control or Vitamin E plus selenium lambs at birth (2.1 x 10^9/L *vs* 1.7 x 10^9/L) or one month of age (2.0 x 10^9/L *vs* 1.6 x 10^9/L). The total concentrations of IgG in the plasma of lambs born to control or supplemented ewes did not differ significantly at 1-week (25.7 vs 26.9 mg/mL), 4-weeks (18.0 *vs* 18.5 mg/mL), 6-weeks (19.3 *vs* 19.3 mg/mL) or 14-weeks (25.6 *vs* 24.7 mg/mL) of age. There was a significant difference in plasma IgG concentrations between consecutive time points of analyses ($P<0.001$), but there was no significant interaction with treatment. There was no significant effect of gestation length or birth weight on plasma IgG concentrations of lambs, and the only effect of birth type and sex of lambs was that plasma IgG concentrations were higher in single-born lambs at one week of age and in males compared to females.

Anti-tetanus-toxoid antibody concentrations

There were no significant differences in the absorbances of plasma samples from control or Vitamin E plus selenium lambs at 1-week (0.58 *vs* 0.58 nm), 4-weeks (0.33 *vs* 0.37 nm), 6-weeks (0.56 *vs* 0.49 nm) or 14-weeks (1.40 *vs* 1.57 nm) of age. On no occasion was there a significant relationship between the concentrations of α-tocopherol or selenium in the lambs on their adaptive immune

response. Absorbances of plasma samples differed significantly between consecutive time points of analysis ($P<0.001$), however there was no significant interaction with treatment. There was no significant effect of gestation length, birth weight or sex on the absorbances of lamb plasma samples. At one week of age, absorbances of plasma samples from single-born lambs were significantly higher than those from multiple-born lambs.

Discussion

Maternal supplementation with Vitamin E plus selenium during late gestation increased the concentrations of plasma α-tocopherol in plasma and selenium in whole blood in the ewe and her lambs at parturition, thus supporting our first hypothesis. However, supplementation of ewes with Vitamin E plus selenium during late gestation did not influence the production of maternal antibodies in the colostrum and the actual concentrations of α-tocopherol and selenium in the lambs was not correlated to the total number of phagocytic monocytes or neutrophils at birth prior to suckling or at marking, or the concentrations of IgG in plasma or anti-tetanus-toxoid antibody between birth and weaning. Therefore these results suggest that immune competency was not enhanced in the lamb by supplementing ewes with Vitamin E plus selenium during late gestation. The survival of lambs to weaning from ewes supplemented with Vitamin E and selenium was 11% higher than the survival of control lambs, but this effect was not statistically significant and there was no evidence that the changes in survival resulted directly from the supplements boosting the lamb's innate or adaptive immune responses. Our second hypothesis was therefore rejected.

The concentrations of α-tocopherol and selenium decreased by 40 to 50% during late pregnancy when no Vitamin E and selenium supplements were provided, which was consistent with Hatfield *et al.* (2000), Rock *et al.* (2001) and Hall *et al*. (2012). The depletion in α-tocopherol and selenium during late pregnancy, which were equivalent to 0.016 mg/L and 0.001 mg/L per day respectively, was due to low intakes of Vitamin E and selenium in the diets and increased demand from rapid growth of the fetus and production of colostrum which has a high concentration of α-tocopherol and selenium (Njeru *et al*. 1994; Rock *et al*. 2001; Stewart *et al*. 2013). The autumn lambing ewes in our study grazed dry annual pasture and were supplemented with oaten chaff, oats and lupins during their entire pregnancy. During late pregnancy they gained about 1.5 kg or 50 g/day maternal live weight and this aligned closely with a gain of 0.2 in condition score. The estimated concentration of Vitamin E and selenium in their base diet during this period was 5.9 and 0.023 mg per kg of dry matter and the estimated total intake was about 8.50 and 0.033 mg per day, respectively. These estimates of the intake of Vitamin E and selenium are significantly below the recommended requirements for pregnant sheep (Agricultural Research Council 1980; Freer *et al.* 2007). Consequently, at lambing more than 60% of the ewes that were not supplemented had plasma α-tocopherol concentrations lower than 0.7 mg/L, which is considered the critical threshold for deficiency (White and Rewell 2007), and almost 90% of ewes had blood selenium concentrations less that 0.1 mg/L which is considered to be deficient to marginal (Counotte and Hartmans 1989; Barceloux 1999; Lee 1999). The concentrations of α-tocopherol and selenium at birth were lower than that reported by most studies, and these results demonstrate that both Vitamin E and selenium deficiency was highly prevalent in these ewes and maybe a more widespread problem for ewes lambing in late autumn onto dry pasture prior to the break of season. Inconsistencies in the literature concerning production and immune responses to supplementation with Vitamin E and selenium can often be attributed to the base diets of the un-supplemented ewes and the ewes themselves not being deficient in Vitamin E or selenium (Kumugai and White 1995; Daniels *et al*. 2000; Gabryszuk and Klewiec 2002; Rooke *et al*. 2004; Anugu *et al.* 2013), but this was not the case in the current study.

Maternal supplementation of ewes with Vitamin E plus selenium during late pregnancy increased the concentrations of both α-tocopherol in plasma and selenium in whole blood by about 70% at lambing compared to ewes that were not supplemented. However, 9% of supplemented ewes were still deficient in Vitamin E and 46% were still deficient to marginal in selenium at birth. The maximum concentrations of α-tocopherol in plasma in supplemented ewes were significantly lower than that achieved in numerous other studies (Kumagai and White 1995, Daniels *et al*. 2000; Rooke *et al*. 2004; Capper *et al*. 2005; Anugu *et al.* 2013), despite using higher levels of Vitamin E in the supplement than some studies and using dl-α-tocopherol acetate which is considered to have the highest bioavailability (Hatfield *et al.* 2000). The lower concentrations of α-tocopherol in plasma of supplemented ewes in the current study may reflect that ewes actually gained condition score and hence fat during late pregnancy enabling greater storage of Vitamin E. The ewes in the Kumagai and White (1995) study lost 5 to 6 kg of live weight during late pregnancy excluding the weight of the conceptus, and whilst Daniels *et al*. (2000) and Rooke *et al*. (2004) did not report the changes in live weight during pregnancy the diets they offered were designed to result in mobilization of fat reserves. This proposition is consistent with the findings that animals are less susceptible to nutritional myopathy caused by Vitamin E deficiency when nutrition is poorer and more susceptible when they are well fed with a diet containing low levels of Vitamin E (Allen *et al*. 1986). The concentrations of selenium in supplemented ewes were also lower than expected in comparison to other studies (Rock *et al.* 2001; Stewart *et al.* 2013). This may be attributed to the lower doses of selenium administered in the current study, that only a single does was given almost 6-weeks prior to lambing, plus we used inorganic forms of selenium whereas organic selenium if more effective at increasing the concentration of selenium in the blood (Stewart *et al*. 2013). It is clear that supplementation was effective at increasing α-tocopherol and selenium levels in the ewe, however the responses were generally less than other studies due to lower concentration of Vitamin E and selenium in the base diet, the levels, form and time the Vitamin E and selenium supplements were provided and that the ewes were in positive energy balance during late pregnancy.

Prior to suckling the concentration of plasma α-tocopherol and selenium in whole blood was higher in lambs born to ewes supplemented with Vitamin E plus selenium compared to control lambs, indicating that both α-tocopherol and selenium were able to cross the placenta. However, the concentrations of α-tocopherol and selenium in the lambs was generally lower than other studies (Kumagai and White 1995; Rock *et al.* 2001; Stewart *et al.* 2012, 2013) and suggest that all lambs were still deficient in Vitamin E and 40% of lambs were deficient to marginal in selenium even when ewes were supplemented. The lower concentrations of α-tocopherol and selenium in the lambs in the current study are indicative of the lower concentrations achieved in the supplemented ewes for reasons outlined previously plus lower efficiency of placental transfer compared to other studies, especially for selenium. The efficiency of maternal transfer varied from 0.08 to 0.15 for α-tocopherol depending on the maternal concentration and from 0.40 to 0.46 for selenium depending on sex of lamb. The upper level of placental transfer of α-tocopherol closely aligns with Kumagai and White (1995) who reported plasma concentrations in perinatal foetuses that were about 15% of those in their mothers. It is likely that the lower efficiency of placental transfer of selenium in our study compared to others (Rock *et al*. 2011; Stewart *et al.* 2012, 2013) partially reflected the lower selenium concentrations in the ewes as there appears to be a positive correlation between the selenium concentration in the ewe and transfer efficiency when selenium is provided as selenite (Stewart *et al.* 2012). The ewes were also supplemented with inorganic rather than organic selenium, and inorganic selenium is transferred across the placenta up to 50% less effectively than organic selenium (Rock *et al*. 2001; Stewart *et al*. 2012). It would seem that multiple injections of inorganic selenium which is cheaper and more practical could have improved the selenium concentration in the lambs above the threshold concentration considered to be marginal, but there is minimal scope to improve neonatal α-tocopherol status in plasma by maternal supplementation. It has been proposed however that plasma concentrations may not be a reliable indicator of

neonatal Vitamin E status, and Capper *et al.* (1995) reported significant increases in the α-tocopherol concentrations in brain tissue and muscle of neonates from supplemented ewes in the absence of changes concentrations in plasma. The absence of nutritional myopathy in new born lambs in the current study would support this contention, although clinical signs of nutritional myopothy could also have been avoided by rapid intake of colostrum which had a much higher α-tocopherol concentration.

Maternal supplementation with Vitamin E plus selenium in late gestation did not influence the total number or ability of monocytes or polymorphonuclear leukocytes to engulf *S. aureus* bioparticles in whole blood from their lambs at birth or marking. Furthermore, on no occasion was there a significant effect of supplementation with Vitamin E plus selenium on the concentration of IgG in colostrum, the total number of lymphocytes or total concentrations of IgG in the plasma of lambs or their tetanus toxoid antibody response. It is very clear that in this study both the innate and adaptive immune responses in the lamb were not influenced by Vitamin E plus selenium supplementation of their mothers during late pregnancy, despite evidence across species that improving Vitamin E and selenium status can enhance these responses (Finch and Turner 1996; Meydani and Beharka 1996; Rooke *et al.* 2004). Liu *et al.* (2014) reviewed the effects of Vitamin E and selenium supplementation on the responses of the immune system and concluded that dietary selenium was more critical than Vitamin E, however it is not clear from the literature what concentration of α-tocopherol in the plasma and selenium in the blood are required to elicit the immune responses. In a study investigating the effect of dietary vitamin E in rats, the requirement for optimal lymphocyte proliferation was 3-13 times that considered to avoid a deficiency (Meydani and Beharka 1996). Hall *et al.* (2013) also found that the killing capacity of neutrophils was greater when selenium supplements were fed to ewes to increase the concentration of selenium in the blood to about 300 ng/ml, however, even then the response to selenium was only evident in sheep infected with footrot and was not significant in healthy sheep. Previous studies have reported conflicting results on the effects of vitamin E and/or selenium on IgG concentrations found in both colostrum and lamb plasma (Gentry *et al.* 1992; Daniels *et al.* 2000; Rock *et al.* 2001). As there was no direct relationship between the actual concentrations of α-tocopherol or selenium in the lambs and their innate or adaptive immune response, it appears that the average and range in concentration of α-tocopherol and selenium between treatment groups and individuals, and/or their level of exposure to environmental or pathogenic stress, may have been too low to elicit measurable immune responses.

The changes in α-tocopherol and selenium status of the ewe and their lamb during lactation can be attributed largely to variations in the Vitamin E concentrations in the diet. Significant rainfall occurred (30 mm over 5 days) at the very end of lambing and subsequent consumption of green pasture high in Vitamin E (128 mg α-tocopherol/kg) rapidly elevated α-tocopherol concentration in plasma especially in Control ewes. The concentration of α-tocopherol in plasma of lambs also increased by 10-fold between birth and marking at 4-weeks of age following consumption of colostrum and milk. The α-tocopherol in plasma was even higher for lambs from ewes supplemented with Vitamin E plus selenium as the concentration of α-tocopherol in the colostrum from supplemented ewes was 2.5 fold greater than that from Control ewes. The higher concentration α-tocopherol in plasma from twin-born than single-born lambs at marking suggests that the increase in α-tocopherol between birth and 4-weeks was also due to consumption of green pasture by the lambs, as it is well-known that twin lambs start consuming pasture at a younger age than single lambs. Unlike Vitamin E, there was also a deficiency of selenium in the green pasture so the concentration of selenium in the blood of ewes either remained constant or declined during lactation due to higher demands for selenium associated with milk production. The selenium status for the lambs declined during lactation probably due to the increasing demand of selenium required to support growth, which has been previously reported by White *et al.* (1997) and White and Revell

(2007), and two-thirds of lambs were considered deficient in selenium and the balance marginal in selenium at weaning even when ewes were supplemented. It is clear that low concentrations of selenium in soil and then in feedstuffs in Western Australia could lead to a high risk of selenium deficiency in ewes and lambs even when supplements are provided.

The vitamin E plus selenium treatment had no significant effect on the overall metabolic status of lambs at birth or on the growth of lambs to weaning. There was an 11% difference in the lamb survival to weaning between the vitamin E plus selenium and control group, but this was not significant. The design of this experiment and the high intensity of measurements necessary immediately post-lambing to collect the samples required to quantify the response in nutrient status and immune function may have reduced the capacity of the experiment to detect treatment effects on lamb survival. The care of the animals immediately post lambing was greater than under normal farm conditions, and this is reflected in the low deaths rates during the first 72 hrs. In the current study about 30% of total deaths to weaning occurred during the first 72 hours, whereas normally about 70-80% of total deaths occur during this time (Oldham *et al.* 2011; Hinch and Brien 2014). Literature concerning the effects of Vitamin E plus selenium supplementation on lambs survival is variable, but Knott *et al.* (1983) and Thomas *et al*. (1995) using larger numbers of ewes reported increases in survival of 8 to 20% and the effects were more apparent when adverse conditions increased the stress on the new born. Monoz *et al*. (2008) reported that supplementation of selenium deficient ewes during early and mid-pregnancy improved survival significantly between 24 hours and weeks of age but a difference of 7% at weaning was not significant. The potential for Vitamin E and selenium supplementation to increase the survival of lambs born in autumn required further validation at paddock scale across a range of environmental conditions.

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Appendix 3

Lamb survival, glutathione redox state and immune function of neonates and lambs from periparturient Merino ewes supplemented with rumen protected methionine

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Abstract

In Merino sheep there is a high demand for the sulphur amino acids (SAA), methionine (Met) and cysteine (Cys), for wool and body growth. SAA also play a role in the management of oxidative stress through the conversion of reduced glutathione (GSH) to oxidised glutathione (GSSG) and are required for immune function. Ewes undergo oxidative stress during pregnancy and a combination of oxidative stress and suppressed immune function may play a role in increasing the risk of lamb mortality. For these reasons it was hypothesised that dietary SAA supplementation to Merino ewes during the late stages of pregnancy will increase the supply of SAA to the maternal body and improve foetal growth and immune competency of ewes and lambs. A total of 120 grazing, pregnant Merino ewes were used in this study, 60 of these were supplemented with 6.3 g/d rumen protected Met (Met-Plus, n = 60) from day 111 of pregnancy until 5 days after lambing there were compared with 60 non-supplemented control ewes. Lambs from Met supplemented ewes tended to be slightly heavier than control lambs ($P = 0.10$) and ewes had slightly higher concentrations of total GSH in plasma at lambing ($P < 0.06$). No other differences in GSH, GSSG of the GSH redox state (GSSG:GSH) were observed. So, despite the observation that GSH redox state was elevated at the end of pregnancy and that ewes were undergoing increased oxidate stress ($P < 0.05$), Met supplementation did not reduce oxidative stress indicating a lack of Met did not contribute significantly to the GSH redox state. The total IgG concentration in colostrum and in plasma of one-week old lambs was also not different between the control and Met groups ($P > 0.05$), but was higher ($P < 0.05$) in lambs aged 4 and 6 weeks. White blood cell counts, the percentage of lymphocytes, monocytes and neutrophils, and the capacity of phagocytosis of monocytes and neutrophils in the neonates and one-month old lambs were not changed by Met supplementation. It is uncertain whether Met supply from both the diet and the supplementation met the high demand of Met for supporting both foetal growth and immune system. A trend towards higher lamb birth weights in the Met supplemented group requires further investigation as this may influence survival at birth and weaning.

Key words: methionine, lamb mortality, redox state, immunity, pregnancy, sheep

Introduction

Poor lamb survival in Australian sheep flocks has been recently reviewed and identified as a major source of reproductive inefficiency (Brien *et al.* 2014). Others have also reported higher lamb mortality rates from Merino ewes than for crossbred ewes in six out eight locations in the Sheep CRC Information Nucleus program (Geenty *et al.* 2014). While Merino ewes have been identified as high risk during the transition period (3 weeks before to several weeks after parturition), all ewes are under physiological stress and have been shown to have a lowered immune response and increased susceptibility to disease at this time (Anugu *et al.* 2013; Fthenakis *et al.* 2015). This susceptibility is further increased in twin bearing ewes compared to single bearing ewes (Caroprese

et al. 2006), and similar changes have been reported in dairy cattle (Mallard *et al.* 1998; Spears and Weiss 2008; Esposito G *et al.*, 2014).

The sulphur containing amino acids (SAA) are critical nutrients to immune competency. Methionine (Met) is required for the synthesis of polyamines which facilitate rapid division of cells throughout the body, including those in the activated immune system (Grimble 2002). Methionine is also converted to cysteine (Cys) in the body. This is the limiting amino acid for the synthesis of glutathione (GSH). Both GSH and Cys are small molecular antioxidants, and essential to stabilize the redox status of the body and reduce the development of inflammation in tissues. Dietary supplementation of SAA has been reported to improve immune competency in farm animals. Supplementation of rumen protected Met to dairy cows from 21 days before calving until 30 days after calving increased the voluntary feed intake of cows and milk protein concentration and milk yield. The supplement was also associated with an elevation in blood neutrophil phagocytosis (Osorio *et al*. 2013) and reduced introleukin-6. Introleukin-6 is a pre-inflammatory cytokine (Osorio *et al.* 2014). These changes are indicative of improved immune function in the cows. Soder and Holder (1999) reported that dietary supplementation of 30 g/d rumen protected Met increased proliferative ability of peripheral blood T lymphocytes in lactating cows, but did not influence mononuclear cell compositions in either blood or milk. Supplementing super-nutritional levels of SAA to broilers resulted in significant dose-related increases in total antibody and IgG, suggesting more SAA are required for optimal antibody response (Tsiagbe et al. 1987). An abomasal infusion of 2 g/d Cys to sheep challenged by intestinal parasites tended to increase the peripheral eosinophils and globular leukocytes in the abomasum and the anti-ovalbumin IgG response (Miller *et al*. 2000).

This interaction between SAA and immune function is of special interest for sheep production and for Merinos in particular. Wool proteins contain a high concentration of SAA (>10%; Reis, 1979). This is 2-3 times higher than the concentration in rumen microbial proteins (the major source of protein for ruminants) and similarly higher than in other body proteins (see (Liu and Masters, 2003)). Hence, sheep growing 10 g/d wool deposit approximately 1 g SAA into wool protein, equivalent to the SAA deposited in about 200 g/d weight gain (assuming the gained weight gain contains 15% protein and the protein contains 3.1% SAA). By breeding sheep for high wool production, we have also bred a sheep with a high demand for SAA, a demand that cannot be adequately met from rumen microbial protein. For this reason, SAA are the primary limiting nutrients for wool growth in growing Merino sheep. This has been described for sheep housed internally and fed roughage diets (Reis, 1967) and in sheep grazing dry pastures (Mata *et al.* 1995). Whether SAA are the limiting amino acids for pregnant sheep is less certain (Stewart *et al.*, 1993; Williams *et al.*, 1988).

This high demand for SAA may be part of the reason for observed differences in lamb survival between wool and meat breeds of sheep and may contribute to the suppressed immune function in all breeds in late pregnancy and early lactation. It is also possible that the competition for SAA at this time may impact on foetal growth and milk production also contributory factors to lamb survival. We hypothesized, therefore, that dietary SAA supplementation to Merino ewes during the late stages of pregnancy will increase the supply of SAA to the maternal body and improve foetal growth and immune competency of ewes and lambs. The hypothesis was tested in with Merino ewes under grazing conditions. Ewes supplemented with rumen protected Met were compared with non-supplemented control. The GSH redox state in ewes and lambs was also monitored to examine the changes of the oxidative state of ewes during the transition period, and the effect of Met supplementation. This study was part of a larger experiment that also evaluated the role of vitamin D (Lockwood *et al.* 2015) or vitamin E and selenium (Sterndale *et al*. 2015) on immune competency and lamb survival.

Materials and Methods

The use of the animals and the experimental procedures of this experiment were performed according to the guidelines of the Australian Code of Practice for the Use of Animals for Scientific Purposes 2013 and approval by the Animal Ethics Committees of both the University of Western Australia and Murdoch University.

Animals and management

The experiment was completed at the University of Western Australia's farm "Ridgefield" at West Pingelly, Western Australia (S 32° 30' 23" – E 116° 59' 31"). A total of 400 Merino ewes aged from 4 to 7 years old were initially used. They were grazed as a single flock from the beginning of the experiment in November 2013 until the start of the nutrient interventions in early March 2014. During this period, ewes were weighed and condition scored at fortnight intervals. Liveweights of ewes during pregnancy were corrected for weight of the conceptus using the formula from the Grazfeed model (Freer *et al.* 1997), where day 0 of pregnancy was the day of artificial insemination. Oaten chaff, oats and lupin seeds were supplemented during the late stage of pregnancy and lactation period (from February to July 2014). The quantity of the feeds offered was calculated to meet the energy requirements for pregnant ewes (CSIRO 2007) for a target body condition score of 2.5 at lambing.

Ewes were randomly allocated for artificially insemination with semen from one of four Merino sires in November 2013, and were pregnancy scanned via trans-abdominal ultrasonography on day 55 of pregnancy. A total of about 240 ewes were diagnosed as pregnant. Non-pregnant ewes were removed from the experiment. Lambing occurred in late April 2014. The details of the artificial insemination, pregnancy scanning and lambing management have been described previously (Lockwood *et al*. 2015).

Experimental design and nutrient intervention

On day 111 of pregnancy, ewes were divided into two groups of 60 balanced for liveweight, sire of lamb and pregnancy status. These groups were allocated to the following nutritional treatments: 1) Control: no supplementation; and 2) Methionine supplementation (Met): a rumen protected Met product, Met Plus (Niso Shoji Co, Japan) was mixed with oats and supplied to ewes at 2-3 day intervals from day 111 of pregnancy until day 10 post lambing. The quantity of Met Plus offered was 6.3 g/d per sheep. According Niso Shoji Co, this product contains 63% Met with an estimated rumen by-pass rate of 75%, thus 6.3 g product was expected to deliver 3 g/d Met into the small intestine.

Sampling procedures

Blood samples, each approximately 10 mL, were taken from the jugular veins of all ewes on day 113 of pregnancy (referred to as pre-treatment) and at lambing, and from 44 ewes (22 ewes from each group) at one month (marking) and three months (weaning) post-lambing, respectively. Blood samples, each of approximately 5-7 mL, were collected from all neonates (the first born in case of twins or triplet litters) before suckling colostrum, and from about 44 lambs (22 from each group) at one month and three months of age. The 44 ewes selected for sampling were balanced, as much as possible, for treatments (targeted about 20 sheep for each treatment), replicate, gender of their lambs, birth type and gestational length. Some lambs and a few ewes died after birth, so the number of samples at each sampling time varied slightly.

Blood samples were collected into heparinised vacutainer tubes. One tube was placed on ice and kept for analysis of white blood cell differentials and phagocytosis. An aliquot of 0.3 mL blood was added into 2.7 mL trichloroacetic acid-EDTA solution, vortexed thoroughly and placed on ice. The sample was then centrifuged for 15 min at 4000×g at 4°C for deproteinization. The supernatant was

transferred into an Eppendorf tube for measurement of the GSH redox state within two days of sampling. The rest of blood samples were immediately centrifuged at 2278 ×g for 15 min to harvest plasma. The plasma samples were stored at -20˚C until analysis of Met concentration and total IgG. Colostrum samples were collected from lambing ewes and stored at -20˚C for analysis of the total IgG.

Chemical analysis

Met analysis: Plasma Met concentration was determined using a colorimetric method as originally described for an automated analyser (Collins et al. 1978). Briefly, 0.535 mL of plasma sample was mixed with 0.3 mL of formaldehyde and Sorensen buffer (ratio 1 : 7.6) solution and left standing at room temperature for 10 min. Then 0.2 mL of ice cooled 30% trichloroacetic acid was added, and the solution was centrifuged at 17,000×g for 5 min at 4˚C. An aliquot of 180 µL of clean supernatant was taken and mixed with 45 µL of the colour reagent (a solution of chloroplatinic acid (1 mg/mL), potassium iodide (1M), and 8M HCl in ratio of 5.67 : 1 : 104) on a microplate. After 5 min of incubation, the colour intensity was recorded in a PolarStar Omega Multi-mode microplate reader (BMG Labtech, Offenburg, Germany). A calibration curve was generated using a series of Met standard solutions ranging from 10 µM/L to 75 µM/L.

GSH analysis: Reduced GSH and the total GSH (reduced GSH plus oxidized GSH) in whole blood were determined. Total GSH assay determined using Sigma-Aldrich GSH Assay kit (Cat No CS0260) following the manufacturer's instructions. GSH concentration in blood was determined using the method originally as described by Sedlak and Lindsay (1968) and modified for an auto-analyzer (Mata et al., 1995). For GSH, an aliquot of the supernatant, as described above, was reacted with a solution of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in 0.4 M phosphate buffer (pH 8.4). A calibration curve was generated using a series of five GSH standard solutions ranging from 0.05 mM/L to 0.1 mM/L. This calibration curve was used to calculate GSH concentration in the blood samples. To analyse total GSH, an aliquot of the supernatant was firstly reacted with β-nicotinamide adenine dinucleotide phosphate and GSH reductase to reduce GSSG to GSH in 0.4M phosphate buffer, followed with the reaction with DTNB to measure the total GSH. The reactions were all carried out on microplate, and the colour intensity was recorded in a PolarStar Omega Multi-mode microplate reader (BMG Labtech, Offenburg, Germany). The total GSH concentration was calculated against the GSH calibration curve. Oxidized GSH, ie GSSG, concentration (mM/L) was then calculated as the difference between the total GSH and GSH, divided by 2 (1 GSSG is formed from 2 GSH).

Immune assays

Whole blood deferential cells counts, monocyte and polymorphnuclear leukocyte phagocytic capacities, total immunoglobulin-G concentrations and anti-tetanus toxoid antibodies were measured as described in detail by Lockwood e*t al.* (2015). Briefly, whole blood differential cell counts were analysed using Advia® 120 automated Hematology Analyzer (sequence 284) in a high output pathology laboratory (Clinical Pathology, Murdoch University Veterinary Hospital). The abilities of monocytes and polymorphnuclear leukocytes to phagocytose bacterial bioparticles were assessed using a flow cytometry method. Plasma total IgG concentrations of lambs were assessed using a single ImmunoDiffusion assay (IDRing® Sheep&Goat IgG assay, IDBiotech, France). Anti-tetanus toxoid antibodies in lamb plasma samples were assessed by optimising the enzyme-linked immunosorbent assay described by Adams *et al.* (1997) for use with ovine plasma samples.

Statistical analysis

The differences between the two groups in all the measures were analysed using a REML procedure of GenStat (Version 16, VSN International). In a Linear Mixed model of REML, fixed factors included nutrient treatment, birth type (single or twin), sire (for lambs only) and their interactions. Since the

live weight of ewes and the GSH redox status were repeated measures at four time points (pre-treatment, lambing, one month and three months post lambing), time was included as another factor, and the measure at pre-treatment was used as a covariate. The random model included replicate and individual. The means and standard error of the differences (SED) from output of REML analyses are presented, unless specified, in this paper. In all analyses P values < 0.05 were declared to be statistically significant.

Results

Productive performances of ewes and lambs

The live weights of ewes immediately before Met supplementation (i.e., pre-treatment), one week before lambing, and 1 month post lambing did not show any significant difference (P > 0.05) between the Met supplemented group and the Control. Lambs from the Met supplemented ewes tended to be heavier (10%) at birth than lambs from the Control ewes (P = 0.10). There were no significant differences in the live weight of lambs when the two groups were compared at one month of age (P > 0.05). Lamb survival rate was 4% higher at birth and 9% higher at weaning in Met supplementation group, but these differences were not statistically different (P > 0.05).

Table 1. Live weights of ewes and lambs and lamb survival. The ewes were supplemented with methionine between days 1113 of pregnancy and day 10 of lactation. Values are presented in the back transformed state where appropriate.

	Control	Met	SED	P values
Ewes				
Pre-treatment (n=120)	63.2	63.3		
1 week pre lambing (n=120)	64.7	62.2	1.20	0.46
1 month post lambing (n=110)	58.0	58.5		
Lambs				
Birth weight (n=150; kg)	4.2	4.6	0.16	0.10
Live weight at 1 month age (n=12; kg)	11.9	11.5	0.37	0.40
Lamb survival at birth*	94	98		0.19
Lamb survival at weaning*	70	79		0.32

* Lamb survival rate was calculated from all lambing ewes for those two groups.

Plasma Met concentration in ewes and lambs

The concentration of Met in the plasma of ewes fed rumen protected Met was higher than control ewes at lambing (25%) and one month post lambing (11%), however, these differences were not significant (P > 0.05). The plasma Met concentration in the neonate of Met supplementation group was higher by 21% as compared with the mean of control group, but as with the ewes the difference did not reach statistical significance (P > 0.05). At one month of age, there were no significant differences in plasma Met concentration between the two groups of lambs.

Table 2. Methionine concentrations (µM/L) in plasma of ewes and lambs. The ewes were supplemented with methionine between days 113 of pregnancy and day 10 of lactation. Values are presented in the back transformed state where appropriate.

	Control	Met	SED	P values
Ewes				
Lambing day (n=41)	17.0	21.2	2.47	0.20
1 month post lambing (n=41)	19.1	21.2	2.54	0.45
Lambs				
Neonatal (n=43)	31.4	38.0	4.16	0.18
1 month old (n=43)	28.0	29.3	1.63	0.69

Glutathione redox state in whole blood of ewes, neonates and lambs

The GSH redox status in whole blood, including the concentrations of reduced glutathione (GSH), oxidized glutathione (GSSG), and total glutathione (t-GSH), and the GSSG : GSH ratio, are presented in Table 3. There were no significant differences in any of the measures when the two groups of ewes were compared before the nutrient intervention ($P > 0.05$), however, GSSG in the control group was slightly higher than in the Met supplemented group ($P<0.06$). On lambing day, ewes supplemented with Met had a slightly higher total GSH (t-GSH) concentration compared with the control group ($P < 0.06$), there were no differences in GSH, GSSG and the GSSG:GSH ratios when the two groups of ewes were compared. Three months after lambing (i.e., at weaning), there were no differences in the GSH redox status between the two groups of ewes ($P > 0.05$). There were also no significant differences between the groups in blood GSH, GSSG, total GSH, and the GSSG:GSH ratio in lambs at one-month and three months of age ($P > 0.05$; Table 3). The GSH redox state was not measured in the neonate.

The GSSG : GSH ratio changed substantially from pregnancy (pre-treatment) to lactation (lambing and one month post lambing) (Table 3, Fig. 1, $P < 0.05$), but there was no interaction between the nutrient treatment and time ($P > 0.05$). On average the GSSG : GSH ratio for ewes before the nutrient intervention was 0.21, increased to 0.41 on lambing day, and then declined to 0.32 one month post lambing and 0.13 at weaning (Fig. 1). The average of the GSSG:GSH ratio across two groups of lambs was 0.40 at one month of age and 0.17 at three months of age respectively (Table 1).

Table 3 (next page). Reduced glutathione (GSH, mM/L), oxidized GSH (GSSG, mM/L), total GSH (t-GSH, mM/L) and ratio of GSSG to GSH in the whole blood of ewes and lambs. The ewes were supplemented with methionine between days 113 of pregnancy and day 10 of lactation. Values are presented in the back transformed state where appropriate.

		Control	Met	SED	P values
Ewes					
Pre-treatment (n=116)	GSH	0.38	0.41	0.030	0.36
	t-GSH	0.52	0.52	0.035	0.88
	GSSG	0.07	0.06	0.006	0.06
	GSSG:GSH	0.23	0.19	0.041	0.36
Lambing day (n=43)	GSH	0.34	0.41	0.053	0.30
	t-GSH	0.66	0.69	0.027	0.06
	GSSG	0.14	0.14	0.012	0.61
	GSSG:GSH	0.43	0.38	0.039	0.86
1 month post lambing (n=43)	GSH	0.55	0.40	0.035	0.26
	t-GSH	0.82	0.84	0.037	0.67
	GSSG	0.14	0.16	0.018	0.83
	GSSG:GSH	0.31	0.33	0.044	0.14
3 months post lambing	GSH	0.45	0.43	0.069	0.58
(n=43)	t-GSH	0.56	0.53	0.088	0.85
	GSSG	0.06	0.05	0.015	0.74
	GSSG:GSH	0.13	0.12	0.030	0.45
Lambs					
1 month old (n=43)	GSH	0.45	0.43	0.053	0.98
	t-GSH	0.77	0.74	0.069	0.79
	GSSG	0.15	0.16	0.029	0.64
	GSSG:GSH	0.40	0.41	0.070	0.69
3 months old (weaning, n=41)	GSH	0.70	0.63	0.091	0.20
	t-GSH	0.73	1.00	0.203	0.10
	GSSG	0.09	0.11	0.022	0.11
	GSSG:GSH	0.14	0.20	0.037	0.32

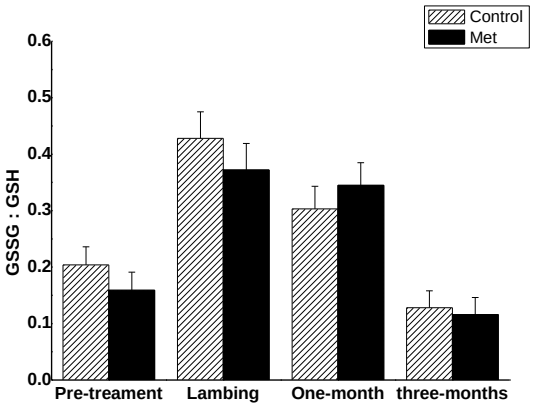

Figure 1 The glutathione redox states (GSSG : GSH) of Merino ewes from day 113 of pregnancy until weaning. The ewes were not supplemented or supplemented with methionine between days 113 of pregnancy and day 10 of lactation. Values are presented in the back transformed state where appropriate. * indicates significant different (P < 0.05) as compared with pre-treatment.

Immune status of neonates and one-month old lambs

The immune status of neonates and one-month old lambs is shown in Table 4. Of all the measures of immune status only lymphocyte % was slightly higher (P = 0.07) in the Met supplementation group compared with the control neonates. In one-month old lambs, phagocytic monocytes MFI tended to be higher (P = 0.07) and the plasma total IgG concentration was significantly higher (P < 0.05) in Met group as compared with those in the control, so was the IgG concentration in 6 weeks old lambs (P < 0.05).

Table 4. Immune measurements of lambs in response to dietary supplementation of rumen protected methionine to pregnant ewes. The ewes were not supplemented or supplemented with methionine between days 113 of pregnancy and day 10 of lactation. Values are presented in the back transformed state where appropriate.

	Control	Met	SED	P values
Total IgG in colostrum (mg/L)	80.4	82.4	1.08	0.63
Neonates				
White blood cells ($\times 10^9$/L)	3.8	3.5	1.09	0.69
Lymphocyte %	41.1	49.1	2.43	0.075
Monocyte %	2.9	2.9	0.93	0.82
Neutrophils %	54.9	48.5	3.95	0.11
Phagocytic monocytes %	83.7	89.4	3.63	0.68
Phagocytic monocytes MFI*	597	588	38.1	0.64
Phagocytic neutrophils %	57.1	61.2	5.21	0.88
Phagocytic neutrophils MFI	424	438	41.5	0.26
Plasma total IgG (mg/mL), one week old	23.4	24.8	1.05	0.72
Lambs, 1 month old				
White blood cells ($\times 10^9$/L)	6.8	6.9	0.45	0.36
Lymphocyte %	58.2	54.2	2.75	0.46
Monocyte %	5.3	5.7	0.52	0.72
Neutrophils %	30.8	34.4	2.84	0.20
Phagocytic monocytes %	98.7	98.3	1.30	0.96
Phagocytic monocytes MFI	632	664	33.8	0.07
Phagocytic neutrophils %	91.3	91.2	1.81	0.50
Phagocytic neutrophils MFI	772	807	41.2	0.23
Plasma total IgG (mg/L)	8.0	10.4	0.44	<0.05
Plasma total IgG (mg/L), 6 weeks old	9.1	10.5	0.46	<0.05

*MFI: Median pHrodo fluorescence intensity of phagocytic cells, as described by Prosser *et al.* (2013).

Discussion

It was hypothesized that feeding rumen protected methionine to pregnant Merino ewes in the late stages of pregnancy would increase the supply of SAA to the maternal body and improve foetal growth and immune competency in ewes and lambs. The results indicate some of the hypothesized changes were observed but overall supplements with Met did not result in large changes in growth, oxidative state or immune function. The weight of lambs born to Met supplemented ewes tended to be higher (0.45 kg) than lambs from Control ewes ($P < 0.1$). While this difference failed to reach a significance levels of $P < 0.05$, an increase of 0.45 kg would expect to be associated with an improvement in lamb survival (Kelly and Lindsay, 1987). Also Geenty *et al*. (2014) has reported a linear relationship between lamb survival rate at weaning and birth weight when birth weight ranged from 2 kg up to 6 kg. An increase in birth weight by 0.45 kg would be expected to increase lamb survival rate to weaning by 6%. The lamb survival rate for Met group in the current

experiment was 9% higher than Control lambs, though not statistically significant, this is in line with the relationship between lamb survival and birth weight as report by Geenty *et al* (2014). The lack of a large production responses in the Met supplemented ewes is not entirely unexpected as others has suggested that nutritional and hormonal factors other than Met influence production close to the time of parturition (Stewart *et al.* 1993; Williams *et al.* 1988).

The GSH redox state, as indicated by the GSSG:GSH ratio, increased in both treatments between day 113 of pregnancy and lambing. This result indicates that the ewes were under increased oxidative stress during the transition period. The higher GSSG:GSH ratio indicated that more reactive oxygen species (ROS) were generated during this period, leading to oxidation of GSH. The increase of ROS in ewes during the transition period was firstly reported by Rizzo *et al*. (2008) where ROS concentration in serum, as determined with a photometric analytic system, increases by 20% - 30% at birth until 36 hours after birth, as compared with 3 days before birth. The author explained parturition is an acute inflammatory process during which the uterus and the cervix undergo a series of deep structural and functional reorganizations including tissue damage (Rizzo *et al*. 2008). The acute inflammation process is accompanied with generation of large quantities of cytokines, leading to an increase of ROS production and, if ROS is in excess, to a challenged defence capacity and oxidative stress (Rizzo *et al*. 2008). The oxidative stress can lead to a delay in foetal growth, pregnancy interruption and stillbirths, as reviewed by Mutinati *et al.* (2013). Oxidative stress in dairy cows during the transition period has also been frequently reported, for example by Castillo *et al*. (2005) and Konvicna *et al*. (2015). In their experiments, plasma malondialdehyde (MDA) concentration (an indicator of peroxidation of fatty acid) increased sharply and the plasma total antioxidant capacity dropped during this period (Castillo *et al.* 2005; Konvicna *et al*. (2015). In the current experiment, the trend towards higher Met concentrations in plasma and higher concentrations of total GSH in Met supplemented ewes at lambing time is consistent with the feeding of a Met supplement. Met is not an antioxidant, but can be converted to Cys in the body and this is used for GSH synthesis. The conversion of Met to Cys in sheep is usually less than 10% of its metabolic pool (see Liu and Masters 2003), so Met supplementation may not make a substantial change in Cys and then in GSH. The conversion of GSH to GSSG is part of the antioxidation process and higher utilisation of GSH would have resulted in higher concentrations of GSSG – this was not observed. So, while the increase in GSSG:GSH indicates higher oxidative stress, a limited supply of GSH is not the major cause of the elevated ratio, and other oxidants could play an important role. Lambs at one month old also had a higher GSSG : GSH ratio compared with those that at three months old. GSH redox state was not measured in neonates. As with ewes, treatment with Met did not influence the ratio and so a lack of Met and GSH are unlikely to contribute to any elevation is oxidative stress in the young sheep.

Consistent with the lack of treatment effect on GSH redox state was a similar lack of a consistent treatment effect on immune competency. Met supplements to ewes did not result in any changes in total IgG concentrations in colostrum or in plasma of one-week old lambs although the IgG concentration in one-month old lambs from Met supplemented ewes was higher than in lambs from control ewes. There were no treatment differences in white blood cell count or in the capacity of phagocytic neutrophils and monocytes in both the neonates and one-month old lambs. The IgG concentrations in serum of the lambs were in the normal range (9.2 mg/mL) and the leukocytes counts were also in line with the normal physiological values (4-12 $\times 10^9$) (Pastiret *et al.* 1998). The immune system competes for nutrients, amino acids and energy with the rest of the body, thus is suppressed over the transition period to support foetal growth and milk production (Esposito *et al*. 2014; Kehrli Jr 2015). For example, this high demand for amino acids is demonstrated by the high Met concentration in the pregnant uterus of sheep and cattle - over 12 times higher than in plasma (see Wiltbank *et al*. 2015). Despite this high level of competition for nutrients within the body, this study provides no evidence that Met supplementation during late pregnancy could change the immune competency in newborns and young lambs, as measured as serum IgG concentration,

leukocyte counts and phagocytic activity of the granulose. It is uncertain that if Met supply from both the diet and the supplementation had met the high demand of Met for supporting both foetal growth and immune system. This warrants further research,

This experiment showed that increasing Met supply through a dietary supplement of rumen protected methionine to ewes during late pregnancy resulted in no significant changes in the growth of ewes, the foetus or lambs. Lambs born to ewes supplemented with Met were slightly heavier than lambs from control ewes, the validity of this observation and potential to influence survival of lambs at birth and weaning requires investigation with larger numbers of sheep. All ewes were subject to increased oxidative stress during the transition period; this was not influenced by Met supplements suggesting Met supplementation does not contribute substantially to this oxidative stress. Similar results were observed with the lambs. Supplements with Met also were not consistently associated with the measured differences in immune function in newborns and young lambs.

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Appendix 4

A higher genetic potential for fleece weight decreases innate immune function and survival to 72 hours of age in Merino lambs

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Short title: Genetics for fleece weight influence innate immunity and survival in lambs

Abstract
Lambs with a higher genetic potential for fleece weight can be at greater risk of death which may be caused by poor immune function. Therefore, we tested the hypothesis that immune function and lamb survival is poorer in lambs bred for increased fleece weight. Merino ewes (*n* = 400) were artificially inseminated with semen from four sires which had different ASBVs for clean fleece weight at yearling age. Lamb immune function between birth and weaning was assessed by analysing the numbers of monocytes and neutrophils in whole blood, the functional capacity of phagocytes, and the plasma IgG and anti-tetanus-toxoid antibody concentrations. The genetic potential for fleece weight significantly decreased the proportion of phagocytic PMNL in lambs at birth and the survival of lambs to 72 hours of life, but there was no effect on other innate or adaptive immune measures or the survival of lambs to weaning. In conclusion, selecting sheep for increased fleece weight will reduce some innate immune functions and the survival of lambs in the early neonatal period.

Keywords: fleece weight, immunity, survival, lamb

Introduction

Improving the survival of lambs is a priority for the Australian wool industry in order to reverse the decline of the national Merino sheep population. Additionally, Merino ewes have poorer maternal ability compared to other sheep breeds (Hinch and Brien, 2014). The sire of the lamb and sire of the dam can also influence lamb survival despite lamb survival having a low heritability of between 0.02 and 0.05 (Safari *et al.*, 2005, Hatcher *et al.*, 2010). Previous studies have suggested immunity may also be a heritable trait. For example, genetic variation in the ability to acquire and absorb immunoglobulins in colostrum in calves has been estimated (Norman *et al.*, 1981, Berggren-Thomas *et al.*, 1987), suggesting a genetic link with passive immunity. Furthermore, Berggren-Thomas (1987) reported the antibody response against foreign antigens in ewes may have genetic components, suggesting adaptive immune responses are also influenced by genes. Therefore, despite the heritability of lamb survival being very low, sire and dam genetics may influence immune function which may influence survival of the lamb.

Increasing selection for fleece weight in Merino sheep relative to body weight decreases lifetime reproductive performance (Greeff, 2005). In addition, fleece weight has negative genetic correlations with reproductive traits including fertility and the number of lambs weaned (Cloete *et al.*, 2004, Safari *et al.*, 2007). This may be because of higher metabolic requirements of the wool follicles and/or skin for nutrients and/or reduced metabolic reserves which may decrease the nutrition, health and bonding-behaviour of the ewe and/or lamb (Adams *et al.*, 2006). Immune

responses against gastrointestinal nematode infections in sheep are associated with metabolic costs which can decrease wool growth (Sykes and Coop, 2001, Greer, 2008). Furthermore, high wool-producing sheep are more susceptible to gastrointestinal nematode infections (Williamson *et al.*, 1995, Miller *et al.*, 2000, Khusro *et al.*, 2004). Hence, competition between the wool follicle and immune system for essential nutrients may decrease the immune function of lambs bred for greater fleece weight thus predisposing them to infection, subsequent inflammation and death (Sykes and Coop, 2001, Liu *et al.*, 2005, Greer, 2008). Therefore, we tested the hypothesis that immune function and lamb survival is poorer in lambs bred for increased fleece weight.

Materials and methods

All procedures were performed according to the guidelines of the Australian Code of Practice for the Use of Animals for Scientific Purposes 2013 and received approval from the Murdoch University Animal Ethics Committee.

Experimental design

Research was performed at the University of Western Australia Future Farm near Pingelly in Western Australia between November 2013 and August 2014. Four-hundred Merino ewes aged between 4 and 7 years old were artificially inseminated with semen from four sires with different Australian Sheep Breeding Values (ASBVs) for Clean Fleece Weight at yearling age (YCFW; Table 1). All ewes had full pedigree records. The mean ASBV for the sire of the dam for YCFW was 8.9 ± 0.54. Ewes were pregnancy scanned via trans-abdominal ultrasonography on day 55 of pregnancy to identify single- and twin-bearing ewes (*n* = 219) (Fowler and Wilkins, 1984). These pregnant ewes were managed as a single flock and grazed the same paddocks following artificial insemination until day 111 of pregnancy, when they were divided into four treatment groups; Control (*n* = 58), or supplementation with Vitamin D (*n* = 53), Vitamin E and selenium (*n* = 55), or Methionine (*n* = 52). Details of supplementation and the effects of these nutritional treatments on lamb immune function and survival have been reported by Lockwood *et al.* (2015), Sterndale *et al* (2015) and Liu *et al* (2015). This paper focuses on the effects of the genetic potential of the lamb for fleece weight on immune function and survival to weaning for all lambs, as there were no significant interactions between treatment and the genetic potential of the lamb for fleece weight on immune function or lamb survival.

Table 1. Sire Australian Sheep Breeding Values for birthweight (BWT) and liveweight (YWT), eye muscle depth (YEMD), fat (YFAT), clean fleece weight (YCFW), fibre diameter (YFD) and worm egg count (YWEC) at yearling age (Sheep Genetics 2014)

Sire Identification	BWT (kg)	YWT (kg)	YEMD (mm)	YFAT (mm)	YCFW (%)	YFD (µm)	YWEC (%)
L1	0.27	10.2	2.5	1.6	3.7	0.3	-38
L2	0.31	11.6	1.1	0.08	5.8	0.1	-16
H1	-0.16	7.9	1.5	0.5	24.7	0.4	-21
H2	0.16	9.3	1.2	0.4	36.2	0.7	7
Averages[1]	0.03	4.2	0.6	0.2	10.9	-1.1	-11

[1] Merinoselect Averages are the average ASBVs of all Australian Merino sheep recorded by Sheep Genetics as at 21 September 2014.

Animal management and measurements

Ewes were weighed and condition scored, as per the 1 to 5 scale described by Russel (1984), every 1 to 2 weeks between pregnancy scanning and lambing. The ewes grazed paddocks with very low levels of dry annual pastures and were fed on average 700 g/day of mixed grain and chaff between pregnancy scanning and lambing in order to achieve a body condition score of 2.5 at lambing. This feed mix consisted of 52% lupins (13.9 MJ ME/kg DM and 31.2% CP), 31% oats (12.8 MJ ME/kg DM and 12.2% CP) and 17% oaten chaff (9.3 MJ ME/kg DM and 7.6% CP). On day 111 of pregnancy ewes were injected with a clostridial 6 in 1 booster vaccine (Glanvac® 6, Zoetis Australia) and then allocated into treatment groups to commence nutritional treatments (Lockwood *et al.*, 2015) Sterndale *et al* 2015, Liu *et al* 2015). Control, Vitamin D and Vitamin E and selenium ewes were also allocated into one of three replicates (*n* = 54-56 ewes/replicate). The ewes supplemented with methionine were also allocated into three replicates (*n* = 17-18 ewes/replicate) but were grazed separately to the other treatment groups as methionine was provided as an oral supplement. On Day 141 of pregnancy, the three replicates of Control, Vitamin D, and Vitamin E and selenium ewes were reallocated into six smaller groups of 26-29 ewes for lambing, whilst the ewes supplemented with methionine remained in three groups.

At birth all lambs were weighed and their dam, sex and birth type recorded (*n* = 302). Immediately after birth before suckling a 5 mL blood sample was collected from all single-born lambs and from only the first lamb born for lambs born in litters (*n* = 174). The blood glucose concentration of the sample was measured immediately using a glucometer (Accu-Chek® Go, Roche Diagnostics). Rectal temperatures were taken from lambs using a digital thermometer (*n* = 266).

Following lambing, all ewes and lambs were managed as a single flock until weaning. Ewes and lambs were weighed and ewes were condition scored every 2 to 3 weeks between lambing and weaning. At marking, approximately 4-weeks after birth, lambs were vaccinated (Glanvac® 6, Eryvac®, Gudair®, and Scabigard®; Zoetis, Australia). A 5 mL blood sample was also collected at 1-, 4-, 6- and 14-weeks of age from lambs which had blood samples at birth.

Blood samples collected from the lambs were analysed for whole blood differential cell counts (relative and absolute), monocyte and polymorphonuclear leukocyte (PMNL) phagocytic capacities, and plasma immunoglobulin-G and anti-tetanus toxoid antibodies as described by Lockwood *et al.* (2015). The total numbers of phagocytic monocytes and PMNLs (cells/L) were calculated from the product of the respective absolute cell count and the proportion of phagocytic cells determined by flow cytometry.

Statistical analyses

All statistical analyses were performed using GENSTAT (VSN International 2012). The statistical analyses have been described by Lockwood *et al.* (2015). Briefly, restricted maximum likelihood was used to assess the effects of the measures of lamb immune function, rectal temperature at birth, blood glucose concentration at birth and lamb liveweights on lamb survival. For these analyses, treatment, sire of the lamb, gestational length, birthweight, birth type, sex, and sire of the lamb and sire of the dam ASBVs for YCFW and YWT, were fitted as fixed effects. Lamb survival at birth, 72 hours and weaning were assessed by fitting generalised linear mixed models. The total and percentage of monocytes, phagocytic monocytes, neutrophils and phagocytic PMNLs in whole blood were each fitted separately as covariates in the survival to weaning analyses. Data was angular- or log-transformed, where appropriate, and means are presented in the back-transformed state.

Results

Lamb characteristics and survival

Birthweights and rectal temperatures of the lambs were not affected by sire of the lamb (Table 2). At birth, lambs sired by L2 had significantly lower blood glucose concentrations than lambs from other sires (Table 2). Lambs sired by L1 tended to have higher survival rates to 72 hours of age and to weaning than lambs sired by H2 (Table 2). Sire of the lamb ASBV for YCFW significantly decreased the survival of lambs to 72 hours of age when YWT was not included in the model (P=0.031). However, there was no effect of sire of the lamb or sire of the dam ASBV for YCFW on the survival of lambs to weaning with or without YWT in the model. There was no significant effect of sire of the lamb on the growth of lambs to weaning and there was no significant effect of sire of the lamb or sire of the dam ASBV for YCFW on lamb birthweight.

Table 2. Mean weights, rectal temperatures and blood glucose concentrations at birth and survival at birth, to 72 hours and to weaning for lambs sired by L1, L2, H1 and H2. Values are presented in the back-transformed state where applicable.

	L1	L2	H1	H2	P value
Birthweight (kg)	4.39	4.55	4.47	4.61	0.541
Rectal temperature (˚C)	37.8	37.4	37.3	37.5	0.427
Blood glucose (mmol/L)	3.64[1]	2.72[2]	3.53[1]	3.46[1]	<0.001
Survival (%)					
Birth	98.7	98.4	99.2	96.3	0.462
72 hours	98.0	95.8	91.5	87.7	0.052
Weaning	87.9	82.9	74.9	72.3	0.079

[1, 2] Means with different superscripts differ significantly

Monocyte counts and phagocytic capacities

There was no significant difference in the total number or proportion of monocytes at birth or at 4-weeks of age in the whole blood of lambs sired by L1, L2, H1 or H2 (Table 3). There was also no significant difference in the total number or proportion of phagocytic monocytes at birth or at 4-weeks of age in the whole blood of lambs sired by L1, L2, H1 or H2. There was no significant effect of sire of the lamb or sire of the dam ASBV for YCFW on the total number or percentage of monocytes or phagocytic monocytes in whole blood of lambs at birth or at 4-weeks of age.

Table 3. Mean total number and percentage of monocytes, neutrophils, phagocytic monocytes and phagocytic polymorphonuclear leukocytes (PMNL) in whole blood at birth and 4-weeks of age in lambs sired by L1, L2, H1 and H2. Values are presented in the back-transformed state where applicable.

	L1	L2	H1	H2	P value
Total monocytes (10^8/L)					
Birth	1.00	1.20	1.40	1.10	0.342
Week 4	4.10	3.60	4.10	4.20	0.735
Total monocytes (%)					
Birth	2.4	3.2	3.2	2.9	0.305
Week 4	6.0	5.3	6.0	6.0	0.863
Total phagocytic monocytes (10^8/L)					
Birth	0.80	0.90	1.20	1.10	0.249
Week 4	4.10	3.40	4.10	4.00	0.592
Total phagocytic monocytes (%)					
Birth	86.9	85.6	88.5	84.3	0.633
Week 4	98.0	98.3	98.1	97.7	0.684
Total neutrophils (10^9/L)					
Birth	1.50^1	1.47^1	2.25^2	1.64^1	0.003
Week 4	1.81	1.73	2.34	2.04	0.121
Total neutrophils (%)					
Birth	43.2^1	44.7^1	56.4^2	48.5^1	0.004
Week 4	27.5	29.2	34.5	31.2	0.155
Total phagocytic PMNL (10^9/L)					
Birth	1.01^1	0.89^1	1.40^2	0.89^1	0.016
Week 4	1.60	1.56	2.07	1.85	0.127
Phagocytic PMNL (%)					
Birth	65.6	58.9	63.1	54.7	0.073
Week 4	89.9	92.3	93.2	91.7	0.090

[1, 2] Means with different superscripts differ significantly

Neutrophil counts and PMNL phagocytic capacities

At birth, lambs sired by H1 had had a greater absolute number and percentage of neutrophils in whole blood compared to lambs sired by H2, L1 and L2 (Table 3). However, at 4-weeks of age there were no significant differences in the total number or percentage of neutrophils in the whole blood of lambs born to different sires (Table 3). There was no significant effect of sire of the lamb or sire of the dam ASBV for YCFW on the total number or percentage of neutrophils in whole blood of lambs at birth or at 4-weeks of age.

At birth, lambs sired by H1 had a greater number of phagocytic PMNL than lambs born to H2, L1 or L2, but there was no significant difference in the percentage of phagocytic PMNL in whole blood (Table 3). There was no significant difference in the total number or percentage of phagocytic PMNLs in the whole blood of lambs born to different sires at 4-weeks of age. There were no significant effects of sire of the lamb or sire of the dam ASBV for YCFW on the total number of phagocytic PMNLs in whole blood of lambs at birth or at 4-weeks of age. Sire of the lamb ASBV for YCFW significantly decreased the percentage of phagocytic PMNL in whole blood of lambs at birth ($P < 0.01$), but not at 4-weeks of age. There was no significant effect of sire of the dam ASBV for YCFW on the percentage of phagocytic PMNL in whole blood of lambs at birth or 4-weeks of age.

IgG concentrations of colostrum and lamb plasma

The plasma IgG concentrations of lambs sired by L1, L2, H1 and H2 did not differ significantly at 1-week (16.7, 18.5, 16.0 and 16.0 mg/mL), 4-weeks (8.3, 9.4, 8.0 and 8.2 mg/mL), 6-weeks (9.3, 10.2, 9.6 and 10.3 mg/mL) or 14-weeks of age (13.7, 14.6, 15.8 and 15.1 mg/mL). There were no significant effects of sire of the lamb or sire of the dam ASBV for YCFW on lamb plasma IgG concentrations.

Anti-tetanus-toxoid antibody concentrations

The absorbance of plasma samples from lambs sired by L1, L2, H1 and H2 did not differ significantly at 1-week (0.63, 0.65, 0.59 and 0.63 nm), 4-weeks (0.36, 0.39, 0.34 and 0.35 nm), 6-weeks (0.53, 0.55, 0.54 and 0.59 nm) or 14-weeks of age (1.42, 1.45, 1.67 and 1.29 nm) . There was no significant effect of sire of the lamb or sire of the dam ASBV for YCFW on the absorbance of lamb plasma samples.

Immunity and lamb survival

Lambs born with a lower percentage of phagocytic PMNL had significantly lower survival to weaning ($P<0.01$; Figure 1). However, there was no significant effect of the total number of phagocytic PMNL in whole blood of lambs at birth on lamb survival. There was also no significant effect of the total number or percentage of phagocytic monocytes in whole blood of lambs at birth on lamb survival. There was no significant effect of the total number or percentage of monocytes or neutrophils in whole blood of lambs at birth on the survival of lambs to weaning.

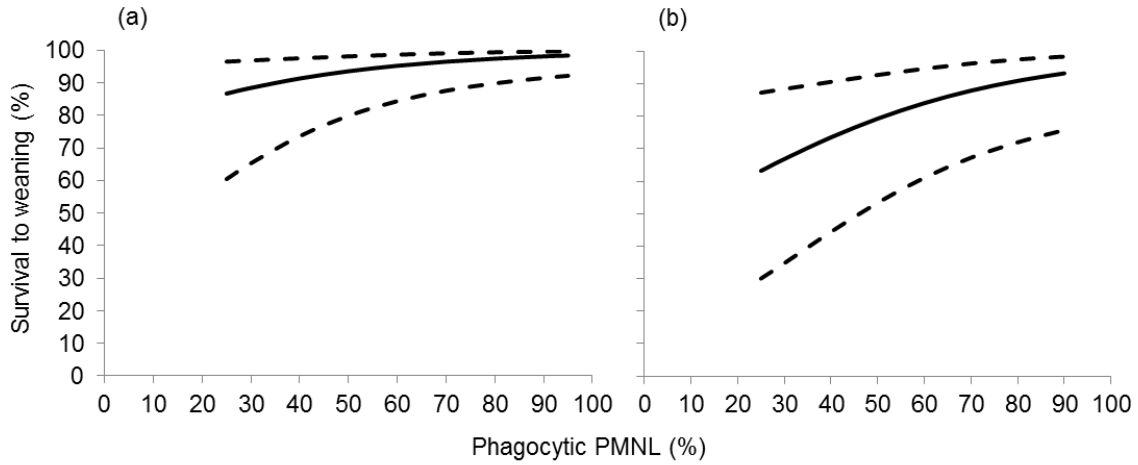

Figure 1. Relationship between the percentage of phagocytic polymorphonuclear leukocytes (PMNL) in whole blood at birth and survival to weaning in (a) single-born and (b) twin-born lambs at the mean birthweight of 4.93 kg in single-born lambs and 4.12 kg in twin-born lambs. The dashed lines represent the 95% confidence intervals. Data is presented in the back-transformed state.

Discussion

We found no significant effects of selecting for high fleece weight on the numbers of monocytes or neutrophils in whole blood, the phagocytic capacities of monocytes, the passive transfer of immunity following birth or the adaptive antibody immune responses against tetanus-toxoid between birth and weaning. Lambs born from sires with high breeding values for fleece weight had a significantly lower proportion of phagocytic PMNL in whole blood at birth and poorer survival to 72 hours of life, but these effects were not significant at weaning. Therefore, our hypothesis that selecting Merino sheep for greater fleece weight will decrease immune function and lamb survival is partially accepted.

Lambs born with a lower percentage of phagocytic PMNL had poorer survival to weaning. Phagocytosis of bacteria by neutrophils, the primary PMNL, is an important mechanism for the rapid removal of foreign bacteria by the innate immune system and also results in the activation of humoral immunity to ultimately control bacterial infections within the body (Paape *et al.*, 2002, Prosser *et al.*, 2013). Neonatal neutrophils have functional deficiencies compared to adult cells which limits their ability to control infecting microbes and thus predisposes neonates to overwhelming infections and death (Carroll *et al.*, 1999, Basha *et al.*, 2014). Thus lambs born with a lower percentage of phagocytic PMNL would have a poorer ability to control bacterial infections within the body, predisposing them to sepsis, and decreasing survival.

Sire of the lamb ASBV for YCFW significantly decreased the percentage of phagocytic PMNL in whole blood of lambs at birth. Therefore, whilst we observed no effect of the sire ASBV for YCFW on the number of phagocytic PMNL, our results suggest that the genetic potential for fleece weight could decrease the proportion of lamb PMNL which are able phagocytose bacteria in the early neonatal period. However, lambs born to sires with high breeding values for fleece weight did not have a lower percentage of phagocytic PMNL compared to lambs born to sires with a lower genetic potential for fleece weight. Therefore, the effect of the sire ASBV for YCFW on the proportion of phagocytic PMNL in the lambs must have been confounded by other genetic, animal or environmental factors which are beyond the scope of this study.

At birth, lambs sired by H1 had a significantly higher absolute number and proportion of neutrophils in their blood compared to lambs from other sires. Consequently, lambs sired by H1 had a greater

absolute number of PMNL in whole blood which were able to phagocytose *S.aureus* bioparticles, although the proportions of phagocytic neutrophils in lambs sired by H1 was not different to that from lambs from other sires. As there was no significant effect of sire of the lamb ASBV for YCFW on the absolute number or proportion of neutrophils or the absolute number of phagocytic PMNL, the greater number of neutrophils and phagocytic PMNL at birth in lambs sired by H1 cannot be explained by the sire's breeding value for YCFW. Nevertheless, this study provides evidence that sire can influence the immune function of the lamb. Further research is required to investigate whether sire effects on lamb immune function are driven by genetic and/or physiological factors.

Overall there were no significant effects of breeding value for fleece weight or sire of the lamb on the metabolic status of lambs at birth or on the growth of lambs to weaning. However, lambs sired by L1 tended to have higher survival rates to 72 hours of age and to weaning compared to lambs sired by H2, which represent the sires with the lowest and highest ASBVs for CFW respectively. Furthermore, a greater genetic potential for YCFW in the sire resulted in poorer survival of lambs to 72 hours of life. Surprisingly, there was no effect of the sire of the lamb ASBV for CFW on lamb survival to weaning, despite lambs born to H2 tending to have poorer survival. Therefore, our results indicate that the genetic potential for fleece weight may influence the survival of lambs in the early neonatal period. This could be associated with the lambs having a lower proportion of phagocytic PMNL at birth and therefore a poorer ability to control bacterial infections following birth.

To our knowledge, the effect of the genetic potential for fleece weight on the immune function of the lamb has not been previously studied. Whilst it appears unlikely that differences in adaptive immune responses can explain the lower survival of lambs bred for greater fleece weight (Adams *et al.*, 2006, Safari *et al.*, 2007, Brien *et al.*, 2014), it is possible that a poorer ability of lamb PMNL to phagocytose exogenous bacteria could be associated with poorer survival of lambs in the early neonatal period. Therefore, further investigation into the relationship between the genetic potential of the lamb for fleece weight, innate immune function and the risk of death due to sepsis in the first week of life is needed. Further research to investigate alternative immunological, physiological and/or genetic factors which may predispose lambs bred for more wool to specific causes of death prior to weaning would also be beneficial. This would improve strategies to increase the survival of Merino lambs by understanding the physiological changes in animals when they are bred for more wool.

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Appendix 5

Overall lamb mortality characteristics and associations with infectious agents

Lamb mortality to weaning was >20% and slightly higher in Male lambs (Table 1). Twin and multiple births accounted for 75% of all deaths. Lamb survival to weaning was positively associated with birth weight with surviving lambs ~0.5 kg heavier at birth than those that died before weaning (4.5±0.9 vs 3.9±1.2 kg; $p<0.0001$).

Table 1: Lamb mortality to the start of weaning

	Males	Females
Total born	162	140
Total died by start of weaning	42	32
% mortality	26	23

Forty percent of lamb deaths occurred in the first 72h after birth, with 19% of lambs stillborn (Table 2)

Table 2: Timing and proportions of early lamb deaths

Total lambs dead:	74		
Died at (%):	0 day:	14	19%
	1 - 3 days:	16	21%
	4 - 60 days:	44	60%

Observations and PM data were available for 14 of 30 lambs that died in the first 72 h (Table 3).

Table 3: Causes of death based on PM data and observations (0-3 days)

Cause	Number of deaths	% of examined deaths
Stillborn:	6	43
Born live:	8	57
Abnormality	3	21
Mismothering	1	7
Predation	1	7
Exposure	1	7
Dystocia	1	7
Unknown	1	7

All stillborn lambs were male and 4 of the 6 were in the Control group with the remaining 2 in the Vitamin D group.

Observations were available for 12 of the 44 lambs that died between Day 3 and the start or weaning. Of these, eight (66%) were associated with exposure, three (25%) were culled due to the presence of white muscle disease, and one lamb was found without signs of standing.

A case/control design was used to compare microbial colonization in lambs which died <72h (cases) with matched controls that survived past weaning, to investigate associations with infectious agents. Cases and controls were matched for birth date, gestation length, sire, dam, treatment group and birth type. Swabs where available from 21 of the 30 cases (Table 2). Three cases, with identified non-infectious causes of death (Table 3) were excluded. The remaining cases were classified as stillborn (n = 7; 1 case was classified as born dead with no PM) or born live but died (n = 10) and analysed as separate groups.

A total of 160 swabs from the 38 selected animals were processed for 16S bacterial rRNA sequencing. Sufficient microbial DNA for analysis was available from 107 swabs, the remaining swabs had levels of DNA below detectable limits or of insufficient quality for sequencing (Table 4).

Table 4: Number and proportion of bacterial swabs with sufficient bacterial load for DNA analysis

	Nose	Rectal	Ear	Placental	Vaginal
Still born (n =7)	6 (86%)	5 (71%)	6 (100%)	1 (50)	3 (100)
Born Live (n = 10)	7 (70%)	8 (89%)	7 (70%)	0 (0)	4 (80)
Controls (n = 20)	18 (90%)	15 (75%)	11 (55%)	6 (55)	10 (83)

As expected, the majority of external sites (nose, ear, rectal and vaginal) had swabs positive for bacterial DNA, while the placental swabs had the lowest detectable frequency. Nose and ear swabs immediately at birth provide a potential measure of exposure to vaginal microorganisms (acquired during birth), as well as a sample site for any intraamniotic exposures. Consistent with this, stillborn lambs had the highest frequency of detectable bacterial DNA in their nose, ear and dam vaginal swabs.

Heat-map analysis of all DNA positive swabs revealed that the majority of variation in sequence data was attributed to bacterial sequences within the following 17 identifiable familes: Corynebacteriaceae, Chitinophagaceae, Planococcaceae, Staphylococcaceae, Streptococcaceae, Clostridiaceae, Lachnospiraceae, Clostridiales; Ruminococcaceae, Sphingomonadaceae, Oxalobacteraceae, Helicobacteraceae, Enterobacteriaceae, Pasteurellaceae, Moraxellaceae, Pseudomonadaceae, Mycoplasmataceae. Major differences were also detected in an, as yet uncharacterised bacterial family, listed as Bacteria; other.

Due to the small sample size for some sample groups and the large number of parameters detected, descriptive comparison were made based on the mean frequencies in each lamb group (Figure 4).

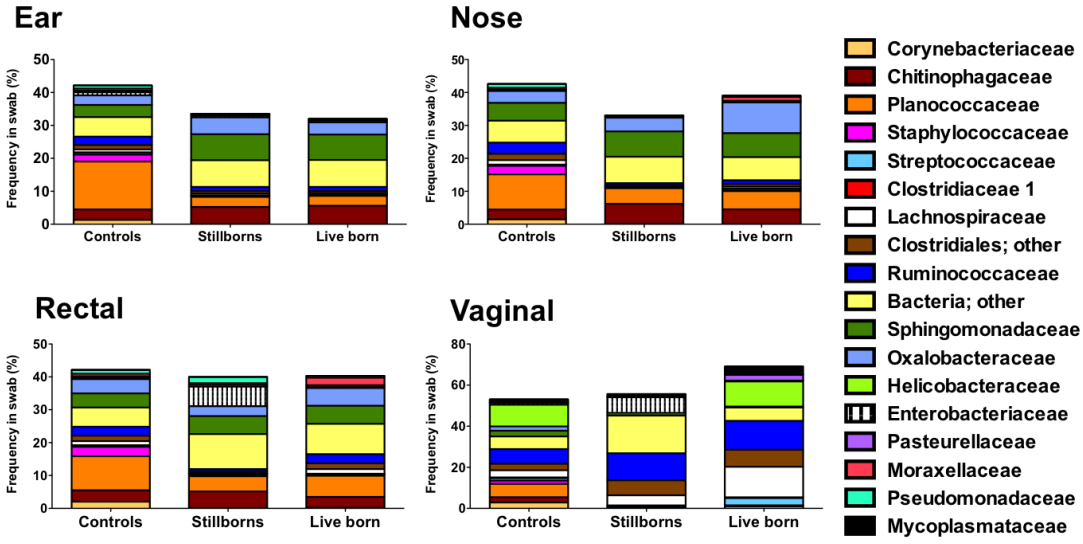

Figure 1: Relative mean distributions of major bacterial families identified in swabs from cases and controls (n = as per Table 4).

Overall, bacterial diversity was highest in control lambs with the main 18 bacterial families contributing to >40% of detectable bacterial sequences. The remaining sequences were spread across 236 recognizable bacterial organisation taxonomic units (OTU) at a family level. The overall patterns of microbial sampling across the 3 lamb groups were very similar, with the following notable exceptions:

1) Planococcaceae, attributed to the genus Planomicrobium, were more abundant (at least 2-fold) in the control lambs at all sites, and were the most abundant organism in these animals (comprising 28% of all microbes in some animals). The function of these microbes in sheep is not known, but their relative abundance and detection in at least one ruminant study (Omoniyi et. al. 2013), suggests that they may be commensal organisms. Notably, Planomicrobium were also readily detectable in vaginal swabs of controls, but absent from either of the two case groups. This suggests that colonization with such organisms may be assisted transvaginally. Future studies should identify these organisms to the species level and determine their potential role in ruminant digestion.

2) Rectal and dam vaginal swabs of stillborn lambs had higher proportions of Enterobacteriaceae than control and live born lambs (5.9% in stillborns versus <0.5% abundance in controls and live born lambs). This was a significant difference (p=0.0245) by one-way ANOVA of log-normalised data, before correction for multiple comparisons. The majority of these sequences were mapped to the genera of *Escherichia*/*Shigella*. An analysis of stillbirths/abortions in US lambs by Kirkwood et.al (1993), found that *Salmonella sp.*, *Escherichia coli* and *Enterobacter sp.* contributed to 9.5% of all infectious deaths. Enterobacteriaceae were also overrepresented in vaginal swabs of dams that had stillborn lambs, suggesting that vaginal carriage of these organisms may be associated with stillbirths. Future studies should determine which of these Enterobacteriaceae species dominate the vaginal site during pregnancy and assess any association in their bacterial load with lamb deaths at birth.

Analysis of blood pathology data for live born cases and controls

Pathology and phagocytic data was available from only 3 of the live born lamb cases and 10 of the lamb controls. No blood measurements were available from stillborn cases.

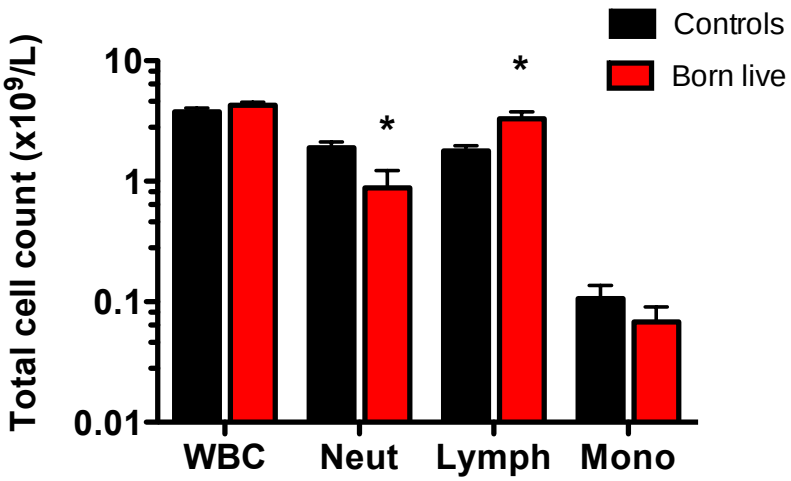

Figure 2: Total and differential white blood cell counts at birth from control lambs and cases born live.

Even though group sizes were small, significant differences in WBC proportions were evident between cases and controls. Live born cases had slightly higher WBC counts than controls (4.3±0.4 x10^9/L vs 3.8±0.9 x10^9/L) although this was not significant. This difference was comprised of significantly higher lymphocyte counts in cases compared to controls (3.3±0.8 x10^9/L vs 1.8±0.6 x10^9/L; p=0.014, Mann-Whitney test), but countered by a significantly lower neutrophil count in the cases (0.9±0.6 x10^9/L vs 1.9±0.7 x10^9/L; p=0.028). Monocyte counts were not significantly different between cases and controls. There were no significant differences in the proportions of either neutrophils or monocytes that were capable of bacterial phagocytosis, although due to the reduced numbers of neutrophils in the blood of cases, the total number of phagocytic neutrophils was significantly lower (0.33±0.2 x10^9/L vs 1.1±0.6 x10^9/L; p=0.014).

Communications

Scientific communications include at least four refereed papers (Appendix 1 to 4). There has been limited industry communication due to the nature of the project outputs. One feature article has been produced for the MLA feedback magazine. There has also been one mediate article (The Land, July 2015), a UWA Agricultural Instititute newsletter article and two honours presentations at Murdoch University.

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