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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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 (1) supplementation of Merino ewes with cholecalciferol during late pregnancy will increase the concentrations of vitamin D in the ewe and lamb at birth and (2) 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 1×10^6 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 1 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 with 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 that maternal supplementation with 1×10^6 IU cholecalciferol does not improve innate, passive or adaptive immune function in lambs.

Additional keywords: lamb survival, immunity.

Introduction

Lamb mortality represents a major production loss for the Australian sheep industry. On average, 30% of all lambs born will die before weaning, and ~80% of lamb deaths occur in the first 48–72 h 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 4.5–5.5 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 while active infections have been previously identified in lambs, primarily via postmortem 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 several 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, while 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 concentrations 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 concentrations 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. The present study, therefore, tested the hypotheses that (1) supplementation of Merino ewes with cholecalciferol during late pregnancy will increase the concentrations of vitamin D in the ewe and lamb at birth and (2) 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 (32°30′23″S, 116°59′31″E) between November 2013 and August 2014. Two hundred Merino ewes aged between 4 and 7 years 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, namely, control or vitamin D supplementation during late pregnancy. The ewes were shorn in November 2013 before 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, Sydney, NSW, Australia) that were inserted into the vagina of all ewes 14 days before artificial insemination and were withdrawn 48 h before artificial insemination. One day before artificial insemination, the ewes were weighed and body condition was 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 before 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 1–2 weeks and nutrition was managed accordingly, so as 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). Ewes were then allocated into three replicates for each of the two treatment groups, namely, 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 per treatment per 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, Sydney, NSW, Australia) at Days 113 and 141 of pregnancy. On each occasion, 1.0×10^6 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 ~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

Pretreatment blood samples were collected from a subgroup 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 0400 hours and 12 midnight each day, for ewe and lamb sampling to occur before the lamb suckled 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 that 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, whereas 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 min 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 min 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 were 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, Sydney, NSW, Australia). 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 analyses, 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 ~2-weeks post-birth. Four weeks post-birth (±1 week), 9 mL blood samples were collected from the subgroup 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 ($n = 69$) analyses. At marking, ~4 weeks post-birth, lambs were weighed and received their primary vaccinations (Glanvac® 6, Eryvac®, Gudair®, and Scabigard®; Zoetis). 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 2–3 weeks until weaning at ~14-weeks of age. At weaning, a 5 mL blood sample 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 concentrations of 25-hydroxyvitamin D 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 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. While 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 the present study.

Thus, the total 25-hydroxyvitamin D (25(OH)D) concentrations in the present 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 h for routine white blood cell differentials by using the Advia®120 automated hematology analyzer (Sequence 284; Siemens, Munich, Germany) in a high-output pathology laboratory (Clinical Pathology, Murdoch University Veterinary Hospital, Perth, WA, Australia). 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 on the basis of 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, Carlsbad, CA, USA) were resuspended at 1 mg/mL in phosphate-buffered solution (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 min at 37°C with 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, San Diego, CA, USA) for 15 min in the absence of light, for the identification of monocytes. Ice-cold PBS (100 µL) was added to each well before transfer of samples to FACS tubes containing 2 mL of ice-cold PBS. Tubes

were centrifuged at 134*g* for 5 min at 10°C, before aspirating the supernatant and washing the cells with 1 mL of ice-cold PBS. Cells were pelleted and red blood cells lysed with 1 mL BD FACS™ Lysing Solution (BD Biosciences, San Jose, CA, USA) for 15 min 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 of Stabilising Fixative (BD Biosciences). Samples were stored at 4°C in the dark before analysis by flow cytometry.

Stained cells were analysed with a FACSCanto™ II cell analyser (BD Biosciences), 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, USA) and checked weekly.

An inclusion gate for leukocytes was first identified on the basis of side- and forward-scatter properties and any debris was 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 monocyte subpopulations were established on the basis of 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 analyses were performed with applied compensation using Flowjo 10 software (TreeStar, Ashland, OR, 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® SheepandGoat IgG assay, IDBiotech, Issoire, France). The immunodiffusion precipitate rings were read using the

IDRing® Viewer S120 (IDBiotech) 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 100 µL (final volume) of 619 Lf/mL highly purified tetanus toxin from *Clostridium tetani* (tetanus toxoid for *in vitro* tests, Statens Serum Institut, Copenhagen, Denmark) diluted to 1 : 1200 by using 0.05 M bicarbonate buffer + 1% bovine serum albumin (BSA) to each well. Plates were incubated overnight (16 h) at 4°C, before washing with *Tris*-buffered solution (TBS, pH 7.2) + 0.05% tween (TBS Tween®-20 Buffer, Thermo Scientific, Melbourne, Vic., Australia). Plates were filled with TBS + 0.05% tween and incubated for 3 min at room temperature. The plates were then rinsed three times, refilled and incubated at room temperature for 3 min. The wash procedure was then repeated before emptying the plate. To prevent non-specific binding, plates were blocked by adding 100 µL of TBS + 1% BSA to each well, and incubating at 4°C for 1 h. Plates were washed before adding 100 µL of plasma diluted 1 : 20 by using TBS + 0.05% tween + 1% BSA to each well. Plates were incubated for 2 h 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™, Camarillo, CA, USA) diluted 1 : 6000 by using TBS + 0.05% tween + 1% BSA and plates were incubated for 2 h 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 plates at room temperature in the dark for 30 min. The reaction was terminated by adding 50 µL of 2 M sulfuric 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, Hercules, CA, USA).

Statistical analyses

All statistical analyses were performed using GENSTAT for Windows (15th edition; VSN International 2012, Hemel Hempstead, UK). For all analyses, interaction terms were included only if they were statistically significant ($P < 0.05$). Analysis also included, where appropriate, data from an additional 148 lambs that 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 the present 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 by 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 so as 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 were 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 $25(OH)D_2$) before 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 were 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 h and to weaning was assessed by fitting generalised 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. So as 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), before treatments (Day 111 pregnancy; 63.2 kg vs 62.5 kg), before 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), before treatments (Day 111 of pregnancy; 2.37 vs 2.38), before 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.

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 21.6 kg and 22.3 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 before 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 than in 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 with the controls ($P < 0.001$).

There was a significant ($P < 0.001$) 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 (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 than in that of the single-born lambs (110.3 vs 84.6 nmol/L; $P < 0.001$) and in the male lambs than in 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.

Using the method employed in the present study, the concentration of $25(OH)D_3$ in colostrum was detectable only in 10 samples (2–6 nmol/L), while the concentration of $25(OH)D_2$ was detectable only 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) and vitamin D-supplemented (5.6% and 28.7%, respectively) lambs.

The percentage of phagocytic monocytes in whole blood of control lambs did not differ significantly from that of the vitamin D-supplemented 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 phagocytic

polymorphonuclear leukocytes (PMNL) in the whole blood of control or vitamin D-supplemented 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 between control and vitamin D-supplemented (80.4 vs 82.3 mg/mL) ewes, and there was no significant effect of pregnancy status on colostrum 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 ($P < 0.001$) between consecutive time-points of analysis, but there was no significant interaction with treatment.

Single-born lambs had significantly ($P < 0.001$) higher plasma IgG concentrations at 1 week of age (18.3 mg/mL) than multiple-born lambs (15.6 mg/mL), but not at 4, 6 or 14 weeks of age. Male lambs had significantly ($P < 0.01$) higher plasma IgG concentrations than female lambs, but there was no interaction among plasma IgG concentrations, sex and the week of analysis. Gestational 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-supplemented lambs analysed 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 ($P < 0.001$) different, but there was no significant interaction with treatment. Plasma samples from single-born lambs (0.69 nm) had significantly ($P < 0.01$) higher absorbances than did those from multiple-born lambs at 1 week of age (0.57 nm), 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.

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 suggested that supplementing ewes twice with 1×10^6 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 the present 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. While the plasma half-life of cholecalciferol is only 4–6 h, cholecalciferol is stored in the adipose tissue, muscle and liver, and has a whole-body half-life of ~2 months (Jones 2008). Thus, the increase in the plasma 25(OH)D concentrations between lambing and 4 weeks post-lambing indicated 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 that ewe and lamb plasma 25(OH)D_3 concentrations were positively correlated at birth. The half-life of 25(OH)D is 2–3 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 persist only for up to 2–3 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. 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. While 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 concentrations 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 the present 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 than, and has an increased bioavailability compared with cholecalciferol, and, thus, supplementation with 25-hydroxyvitamin D_3 is recognised to be between 4.2 and 5 times more effective at improving the vitamin D status of an individual than supplementation with cholecalciferol (Cashman *et al*. 2012; Borel *et al*. 2015). Supplementing 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 than supplementation with cholecalciferol. Furthermore, it is unknown whether ewes and lambs in the present 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 with 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 *et al*. (1971), who observed that *S. aureus*was one of the major bacterial species infecting lambs and, furthermore, that this bacterium 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 concentrations 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 supplementation 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-supplemented 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-supplemented 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 the present 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 analysed for anti-tetanus-toxoid antibodies in the present study were similar to those reported by Adams *et al*. (1997) who analysed 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 4.5–5.5 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 h 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 h 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 1×10^6 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 concentrations 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 optimise immune function. Subsequent research could then determine which approach to vitamin D supplementation is most effective at safely boosting the immune competency of the lamb and whether this is associated with an improvement in lamb survival.

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Table 1. Mean weights, rectal temperatures and blood glucose concentrations at birth and survival at birth, to 72 h 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

Parameter	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 h (%)	89.8	95.1	0.276
Weaning (%)	69.9	80.8	0.186

Table 2. Mean plasma total 25-hydroxyvitamin D concentrations (nmol/L) of control and vitamin D-supplemented (Vit. D) ewes before 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

Parameter	Control	Vit. D	*P*-value
	Ewes (excluding C_3-epi-25(OH)D_3		
Pretreatment	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-25(OH)D_3		
Pretreatment	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-25(OH)D_3		
Birth	24.2	49.0	<0.001
4 weeks	21.9	25.6	0.168
	Lambs (including C_3-epi-25(OH)D_3		
Birth	69.2	134.8	<0.001
4 weeks	28.4	35.6	0.099

Fig. 1. Relationship between the total concentration of 25-hydroxyvitamin D (25(OH)D) in the plasma of control and vitamin D-supplemented ewes at lambing and their lambs at birth. The dashed lines represent the 95% confidence 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.

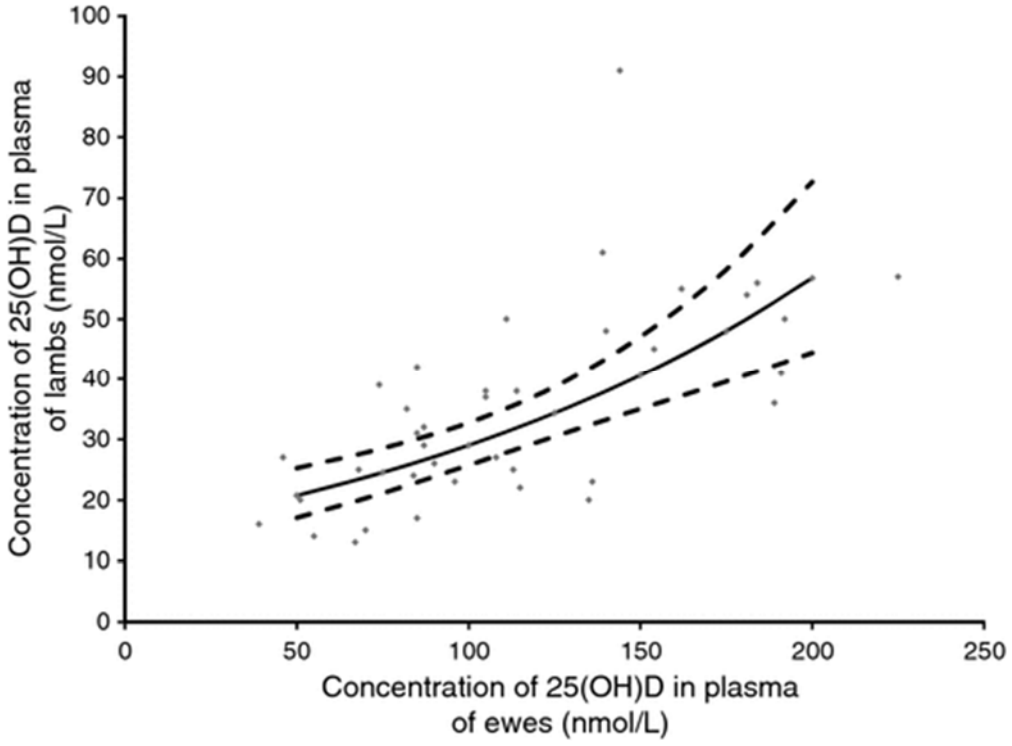

Fig. 2. Mean plasma total immunoglobulin-G concentrations (±least significant intervals) of control (black) and vitamin D (grey) lambs at birth and 1, 4, 6 and 14 weeks of age. The ewes were supplemented with cholecalciferol on Days 113 and 141 of pregnancy. Values are presented in the back-transformed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

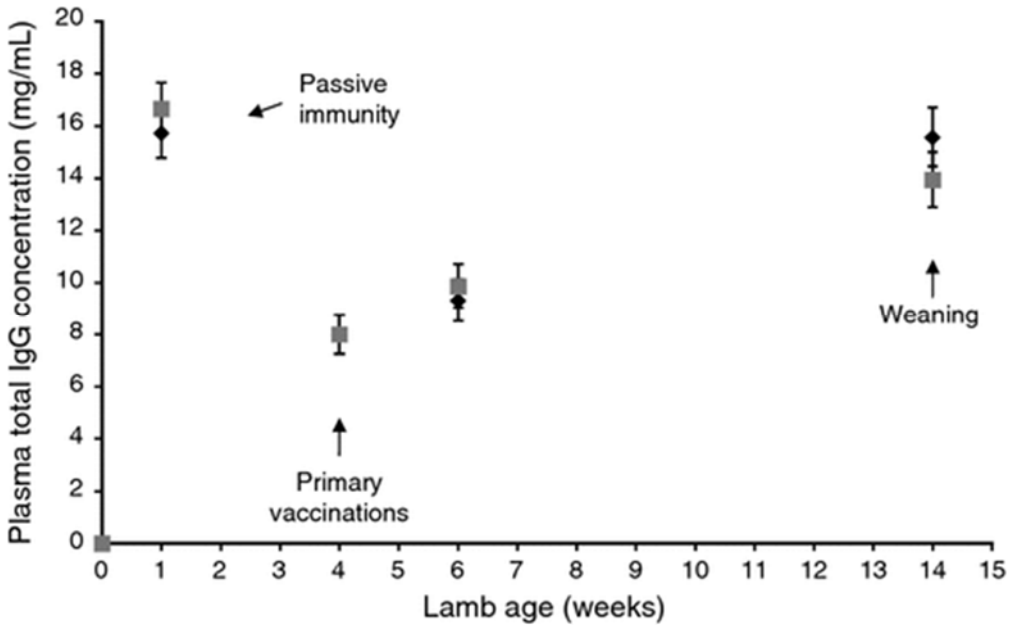

Fig. 3. Mean absorbances (±least significant intervals) of control (black) and vitamin D (grey) lamb plasma samples analysed for anti-tetanus-toxoid antibodies between birth and weaning. The ewes were supplemented with cholecalciferol on Days 113 and 141 of pregnancy. Values are presented in the back-transformed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