# Factors influencing Barbervax® immunity and effects on wellbeing and production in Merino ewes and lambs

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# SUMMARY

Barbervax® is a vaccine released in 2014 for use in sheep of all ages to protect them against *Haemonchus contortus* infection. The overall aims of this thesis were to learn more about the course of action of the vaccine in a commercial environment, simplifying its use on-farm and conducting preliminary observations on performance and production in vaccinated ewes and lambs. The work completed in this thesis is novel, with no previous publication found dealing with the specific hypotheses under test.

Chapter 1 consists of a review of the relevant literature including that on the cost of gastrointestinal nematodes to industry, the pathophysiological effects of *H. contortus* infection in sheep and methods of control. Also reviewed are the development of the immune response to haemonchus infection and the history of development of vaccines against gastrointestinal nematodes, ultimately leading to the production of Barbervax®.

Chapter 2 contains the results of an experiment in two phases, each testing a different hypothesis. The first hypothesis was that the duration of vaccinal protection in Merino hogget ewes following a full vaccination course in years 1 and 2 of life would be longer than the claimed 6 weeks. The second hypothesis was that natural exposure to *H. contortus* infection would boost the Barbervax immune response following a pre-lambing vaccination. A total of 60 age-matched ewes, running together in a larger mob, were split into three treatment groups to test the hypotheses and WEC and ELISA sampled at frequent intervals. Results revealed that neither hypotheses could be supported by the data.

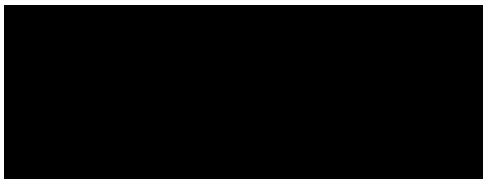
In Chapter 3 I tested the hypothesis that the second 'priming' vaccination in lambs could be removed by reducing the marking to weaning interval to 6 weeks and doubling the vaccine dose at marking and/or weaning. The progeny from the group of ewes referred to in Chapter were split into five treatment groups, each with a different vaccine protocol. The results confirmed that the second priming vaccination could be removed with a 6-week marking to weaning interval and provision of a double dose of Barbervax® at either marking or weaning.

Finally, in Chapter 4 I report on the effects of the different Barbervax® treatments applied in the previous chapters on ewe and lamb production and performance. Performance measures included; greasy fleece weight, fibre diameter, ewe body condition score and ewe and lamb bodyweights at routine husbandry time points, and transfer of maternal Barbervax® antibody to their progeny prior to weaning. There was clear evidence of maternal transfer of Barbervax® antibody to progeny, but were no negative or positive effects on ewe and lamb performance.

## CERTIFICATE

I certify that the work in this thesis is original and has not been previously submitted for any other award, degree or qualification.

I certify that any help in preparing this thesis are entirely my own effort, except where otherwise acknowledged.



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# List of Abbreviations

| Acronym | Definition |
|---|---|
| AAD | Amino-acetonitrile derivatives |
| AH | Aluminium hydroxide |
| BOM | Bureau of Meteorology |
| BZ | Benzimidazoles |
| BV | Barbervax® |
| c.i. | Confidence interval |
| CP | Crude protein |
| CSIRO | Commonwealth Scientific and Industrial Research Organisation |
| DAFWA | Department of Agriculture and Food Western Australia |
| CRC | Controlled Release Capsule |
| CWG | Clean wool growth |
| DDA | dimethyldioctadecylammonium |
| DP | Digestable protein |
| ELISA | Enzyme-linked Immunoasorbant assay |
| epg | eggs per gram |
| ES | Excretory/secretory |
| FD | Fibre diameter |
| FECRT | Faecal egg count reduction test |
| g | Grams |
| GFW | Greasy fleece weight |
| GIN | Gastrointestinal nematode |
| HC | *Haemonchus contortus* |
| hd | head |
| hrs | hours |
| Ig | Immunoglobulin |
| IL | Interleukin |
| IFA | Incomplete Freunds Adjuvant |

| | |
|---|---|
| IPM | Integrated Pest Management |
| kg | Kilogram |
| L | Litres |
| LWG | Liveweight gain |
| MddW | Marking double dose + weaning vaccinated lamb (Chapter 3) |
| MddWdd | Marking double dose + weaning double dose vaccinated lamb (Chapter 3) |
| ME | Metabolisable energy |
| mg | miligrams |
| mL | Mililitres |
| ML | Macocyclic Lactones |
| mm | Milimetres |
| MWdd | Marking + Weaning double dose (Chapter 3) |
| N/kt | Newtons per kilotex |
| NR | Non-responder |
| NSW | New South Wales |
| OP | Organophosphate |
| PBST | Phosphate buffered saline with Tween20 |
| PCV | Packed cell volume |
| PI | Protective index |
| p.i. | Post infection |
| PPR | Peri-parturient rise |
| RBC | Red blood cells |
| R | Responder |
| s.d. | Standard deviation |
| s.e. | Standard error |
| TNTT | Tris  NaCL Tween Buffer |
| µL | Microlitres |
| µm | Micrometres |
| UNE | University of New England |

| | |
|---|---|
| UNVACC | Unvaccinated ewe (Chapter 2) |
| UV | Unvaccinated lamb (Chapter 3) |
| VACC | Vaccinated ewe (Chapter 2) |
| VACCMOX | Vaccinated and Moxidectin treated ewe (Chapter 2) |
| VFI | Voluntary feed intake |
| VP | Vaccinated as per protocol lamb (Chapter 3) |
| WEC | Worm egg count |

# List of Publications (Conference Abstracts)

Broomfield, M.A., Walkden-Brown, S.W., Doyle, E.K., Kahn, L.P., Smith, W.D., (2016) Optimising Barbervax use and prediction of well-being of Merinos, *Sheep CRC Postgraduate Annual Conference*, Sydney, pp. 26.

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Broomfield, MA, Walkden-Brown, SW, Doyle, EK, Kahn, LP, Smith, WD, (2017) Strategies to optimise management of pre-weaning Barbervax® vaccination in Merino lambs, *26th International Conference of the World Association for the Advancement of Veterinary Parasitology*, Kuala Lumpur, Malaysia, pp. 218.

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# CHAPTER 1

# Review of the Literature

## 1.1. Cost of gastrointestinal nematodes (GIN) to the Australian Sheep Industry

Gastrointestinal nematodes (GIN) are the most economically damaging disease to the Australian sheep industry, costing AUD$435 M annually, with the majority of this ($342 M) due to production loss (Lane et al. 2015). The production costs and effects of GIN vary depending on location in Australia and the dominant nematode species. In the Northern Tablelands and summer rainfall areas, *Haemonchus contortus* (Barber's pole) is the dominant species and is estimated to cost the industry ~$29 M annually in the high rainfall areas (Kelly 2010). During *H. contortus* dominated infections, every 1% increase in mortality rate increases the cost/ewe by $1.24 (Kelly 2010).

Under typical management practices parasitism costs producers $11.09/ewe/year and can decrease to $5.80/ewe when integrated parasite management strategies are implemented (Kelly 2010). The cost/head however is dependent on meat and wool prices, with a 25% price increase increasing the costs per ewe under an IPM system by $4.18.

The costs associated with GIN are rising due to the increase in drench resistance and low adoption of alternative control methods. Production losses due to drench resistance include up to 10% reduction in wool growth and additional death rates up to 7%, equaling ~$2.45/hd (Besier *et al.* 1996). Lane *et al.* (2015) state that if the severity of parasite burdens is reduced and use of effective control methods is adopted, the industry would gain $164 M annually.

## 1.2. Pathophysiology of Haemonchosis

### 1.2.1. Anaemia

Due to the voracious blood-feeding behavior of *H. contortus* the principal pathophysiological effect of haemonchosis is anaemia (Dunn 1978; Besier *et al.* 2016). Both acute and chronic cases are common, with each worm capable of causing blood loss of 50 µl per day, causing a sheep with a burden of 5000 *H. contortus* to potentially lose upwards of 250 mL per day (Urquhart *et al.* 1996). Blood loss will be even greater with larger infections (Dargie and Allonby 1975; Le Jambre 1995).

The degree of anaemia is also largely dependent upon the animal's ability to replace the blood loss (Dargie and Allonby 1975).

Acute haemonchosis occurs in three stages. The first stage, during the pre-patent period, is recognizable approximately 2-3 weeks post infection by a rapid decrease in packed cell volume (PCV) (Dargie and Allonby 1975; Urquhart *et al.* 1996). The second stage of the disease occurs at the early post-patent period (Nielsen 1976), where maximum blood loss is observed and PCV plateaus at a low level due to the stabilisation of the haemopoietic system and increase of erythropoiesis. In sheep 14 days post an artificial infection with 10,000 *H. contortus* L3, early stages of anaemia and an enhanced haemopoietic response occurred, although not large enough to compensate for blood loss. The hosts protein and iron reserves continue to decrease due to loss of iron and protein into the gastrointestinal tract and increasing inappetance (Urquhart *et al.* 1996). This leading to severe iron deficiency and the final stage of acute haemonchosis (Dargie and Allonby 1975), where exhaustion of iron and protein reserves occurs with a further PCV decrease (Dargie and Allonby 1975; Urquhart *et al.* 1996).

Anaemia, oedema, lethargy and death can be seen within 4-6 weeks of infection with worm burdens between 2000 and 20,000 worms per sheep and worm egg counts (WEC) above 50,000 epg (Urquhart *et al.* 1996; Besier *et al.* 2016). On post-mortem examination of animals with acute haemonchosis, a pale carcass, ascites, submandibular oedema and watery blood may be observed (Craig 2009; Besier *et al.* 2016). Chronic haemonchosis develops over a longer period of time, and usually due to dryer weather causing an intermittent or low level of larval intake (Besier *et al.* 2016). Due to the slower development of chronic haemonchosis, signs include weight loss and weakness due to anaemia.

### 1.2.2. Reduced feed intake and reduced weight gain

Reductions in live weight gain (LWG) can result from large infections over an extended period of time, although reductions in LWG are not as severe for the abomasal and intestinal scour worms such as *Teladorsagia circumcincta* and *Trichostrongylus colubriformis* infections (Wilson *et al.* 1969; Hoste *et al.* 2016). In *T. colubriformis* infections reductions in LWG of up to 30% can occur, although effects are not observed till 4 weeks post challenge (Sykes 1987). In comparison, 5-month old Merino lambs infected thrice weekly with 200 *H. contortus* supplemented with urea (3%) consumed 9-24% more chaff and gained 13 g/day more than un-supplemented infected lambs (Knox and Steel 1999). An increase in protein supply has a positive effect on voluntary feed intake (VFI) and LWG during *H. contortus* burdens. Lambs infected with 200 L3/kg

bodyweight thrice weekly for 10 weeks, gained 16% more body weight per day when fed a high protein diet (173 g/kg Crude Protein (CP)), than when fed the basal diet of 98 g/kg CP (Wallace *et al.* 1996).

Lambs infected with between 200 and 6000 *H. contortus* larvae via bolus dose or trickle infected showed greater weight loss of between 3.4 kg and 6.4 kg over 12-16 weeks than control animals (Abbott *et al.* 1988; Kelkele *et al.* 2012). An artificial infection of 11,000 *H. contortus* L3 caused an average reduction of 1.29 kg in Merino lambs over 9 weeks (Albers *et al.* 1989). These demonstrate the importance of adequate feeding for controlling infections to prevent reductions in weight loss and VFI.

### 1.2.3. Hypoalbuminaemia

Acute cases of hypoalbuminaemia and hypoproteinaemia are more pronounced in animals on low-protein diets and result in sub-mandibular oedema or “bottle jaw”, accompanied by severe anaemia and weight-loss. Studies using differing protein diets and levels of *H. contortus* infection have found the effects on serum protein and albumin levels varies between sheep breeds (ie. Dorset, Scottish Black-face and Hampshire Downs), reflecting differences in resistance to the parasite (Abbott *et al.* 1985; Abbott *et al.* 1988; Wallace *et al.* 1995; Wallace *et al.* 1996).

In 4 month old Finn Dorset or Dorset Horn lambs infected weekly with 350 *H. contortus* L3/kg serum protein and albumin were recorded weekly for 2 and 10 weeks pre and post infection. Serum protein and albumin decreased 16 g/L and 10 g/L in the two breeds respectively when fed a high protein diet (169 g CP/kg) (Abbott *et al.* 1986). In comparison, lambs fed a low protein diet (88 g CP/kg) were impacted more-so by infection with decreases in protein and albumin of 20 g/l and 15 g/L respectively (Abbott *et al.* 1986). Scottish Blackface and Hampshire Down breeds had smaller decreases in serum protein and albumin concentrations of between 2 and 10g/L when fed similar protein diets (98–173 g CP/kg) (Wallace *et al.* 1995; Wallace *et al.* 1996). During the 10 weeks of infection, serum measures were made three times per week, with a greater decrease in protein and albumin concentrations of the aforementioned experiments, the longer the infection was present.

### 1.2.4. Production Losses – Wool and Fibre

The effects of wool growth and fibre diameter in animals affected by haemonchosis are not seen immediately, but rather over a period of time. A bolus dose of 11,000 *H. contortus* infection under field conditions reduced clean wool growth (CWG) and fibre diameter (FD) over a 4 to 9-week

period post-infection associated with declining haematocrit. For every decrease of 0.01 hematocrit a proportional 0.004 µm and 0.007 kg reduction in fibre diameter and CWG were observed respectively (Albers *et al.* 1990). Under the same field conditions a lag phase of 3 weeks existed between administration of infection and reductions in wool growth (11 to 96 g) and FD (0.39 to 0.79 µm) (Albers *et al.* 1989).

Weaner crossbred lambs infected with 750 *H. contortus* larvae twice weekly were given diets varying in CP content. Lambs fed a diet containing 22% CP had an average clean fleece weight of 2.35 kg compared to 1.9 kg in lambs fed a 10% CP diet (Datta *et al.* 1999). Markedly higher values for yield (5.7% extra), FD (2.9 µm extra), staple length (10.8 mm) and staple strength (6.9 N/kt) were also observed in weaners fed the diet containing 22% CP, demonstrating the important role of protein supply in modulating the production effects of *H. contortus* infection.

## 1.3. Control Methods for *H. contortus*

### 1.3.1. Anthelmintics

Anthelmintic compounds have long been used to control gastrointestinal nematodes in sheep initially with comparatively toxic chemicals including arsenic, copper sulphate, nicotine sulphate, carbon tetrachloride and later phenothiazine. The era of safe, highly effective broad-spectrum anthelmintics commenced with the release of thiabendazole in the early 1960s. The predominant method of control of *H. contortus* is the use of anthelmintic treatments, with the main anthelmintic classes including:

- Benzimidazoles (BZs) eg. Thiabendazole, Albendazole
- Levamisole/Morantel group eg. Levamisole
- Organophosphates (OPs) eg. Napthalaphos
- Macrocyclic lactones (MLs) eg. Ivermectin, Avermectin, Moxidectin
- Newer broad-spectrum classes eg.
    - Amino-acetonitrile derivatives (AADs) Eg. Monepantel
    - Derquantel, supplied mixed with avermectin
- Narrow spectrum classes with specific activity against haematophagous nematodes
    - Salicylanilides eg. Closantel

Thiabendazole demonstrated efficacy (defined as 95% reduction in worm burden or egg count) against 10 different gastrointestinal nematodes in the 1960s (Brown *et al.* 1961). When sheep received 50 mg/kg adult parasites were successfully killed, inhibiting further egg and larval development The 1980s saw the introduction of MLs to the Australian market as a further broad-spectrum option with longer duration of action, however resistance to MLs has been reported on ~85% of Australian properties undertaking FECRT tests between 2003 and 2005, particularly against *H. contortus* (Besier 2006; Besier 2007).

Development of anthelmintic resistance is placing pressure on chemical control using anthelmintics, but this has been relieved somewhat with the recent commercial release of two new active ingredients, monepantel and a derquantel (with the currently used active abamectin). In sheep infected with 3000 *T. colubriformis* and 6000 *H. contortus* L3 with BZ, ML and levamisole resistance, monepantel had a 99.9% and 99.98% efficacy respectively (Kaminsky *et al.* 2011). For the same resistant strains efficacy of the derquantel/abamectin combination to *H. contortus* was 18.3% (Kaminsky *et al.* 2011).

In a recent field trial in northern NSW, yearling wethers naturally infected with *H. contortus* and *T. colubriformis* were treated with 5 different anthelmintics to determine their efficacy (Sales and Love 2016). Wethers treated with a combination anthelmintic (abamectin, albendazole, levamisole, closantel) attained >95% reduction in WEC, whilst wethers treated with derquantel/abamectin, monepantel or moxidectin showed reductions of 93%, 31% and 30% respectively (Sales and Love 2016). Wethers treated with abamectin showed a WEC increase of 22%. Larval differentiation revealed post-treatment 100% of the survivors were *H. contortus*. In a second follow-up FECRT the same wethers treated with either moxidectin or abamectin were used. These wethers were then given a monepantel treatment which reduced WECs of the moxidectin and abamectin treated wethers by 51% and 29% respectively (Sales and Love 2016). This demonstrated that treatment using monepantel and moxidectin can provide moderate control to severely abamectin resistant *H. contortus*. strains. However, the efficacy of both new generation chemicals, monepantel and derquantel was below the 95% efficacy threshold indicating emerging and significant resistance respectively further requiring the need for new and effective control measures.

### 1.3.2. Nutrition

Nematode infections are most pronounced in younger animals and peri-parturient ewes, however increasing the protein supply lowers worm burden, WEC and improves the immune response.

The metabolisable energy (ME) requirements of a single-bearing ewe triples between day 1 of gestation (6.8 MJ/d) and 3 weeks post-partum (19.4 MJ/d), with digestible protein (DP) requirements showing a similar increase (30-75 g/d to 160 g/d) in Merino and Merino crosses (Freer *et al.* 1997). Once lactation begins the requirements for DP increases at a marked rate compared to the needs of ME.

Supplementation with 250 g cottonseed meal 6 weeks before and after parturition in 2 to 6-year-old Merino ewes exposed to natural infection and a bolus dose (3000 *T. colubriformis* and 1000 *H. contortus* L3) reduced the extent of the PPR post-partum when MP pressure is greatest. WEC's were reduced in supplemented groups by 66% coinciding with a reduction in maternal bodyweight (-134 g/d), all be it less than the weight loss of the un-supplemented ewes (-168 g/d) (Kahn *et al.* 2003b). No effect on bodyweight or reduction of WEC was observed during the pre-partum period, however the provision of increased MP prior to and during parturition and lactation, increased birth weight (100-300 g heavier) and weight gain (600-700 g extra) of lambs by 125 days post-birth (Kahn *et al.* 2003b). Interestingly, in this study the provision of supplemental MP resulted in greater WEC reduction in single-bearing than twin-bearing ewes in contrast to the findings of Donaldson *et al.* (1998) who found that an increased MP supply caused a significantly greater reduction in WEC in twin bearing ewes.

In weaner lambs, susceptibility to infection is marked until an immune response is mounted, and the provision of protein supplement assists in this process. Weaned and un-weaned lambs fed 80 g cottonseed meal during a trickle *H. contortus* infection (300 L3 twice weekly), revealed that supplemented lambs had peak WEC at day 50, in contrast to un-supplemented lambs which peaked a further 25 days later (Shaw *et al.* 1995). The ability for lambs to amount an earlier protective response was also shown by Roberts and Adams (1990) in Merino wethers given a *H. contortus* 500 L3 trickle infection, provided 400 g or 600 g of mixed protein diet. Weaners fed the high protein intake diet had a 76-98% reduction in WEC and an extra 2.7 kg in LWG throughout the experiment (Roberts and Adams 1990).

### 1.3.3. Breeding – Genetic Selection

Selectively bred sheep for resistance or resilience is becoming an important element to nematode control allowing a reduced reliance on anthelmintics. Animals selected for resistance have lower worm burdens, a lower nematode establishment rate and lower worm fecundity (Woolaston and Eady 1995). The heritability of WEC appears to fluctuate with age and breed so estimates range between 0.08 to 0.42 with a commonly accepted heritability estimate of 0.2-0.25 (Eady *et al.* 1996;

Greeff and Karlsson 1997, 1998; Pollott *et al.* 2004). Despite the moderate heritability of WEC current selective breeding regimes are quite widely adopted and producing gradual improvements.

Whilst reduced WEC is a good marker for selection of more resistant animals within breeds or flocks, major breed differences in resistance are present. During *H. contortus* infections, Red Masai were the most resistant breed and showed signs of 'self-cure' phenomenon, followed by Blackhead Persian, Merino, Dorper, Corriedale and Hampshire (Preston and Allonby 1978, 1979). Reductions in WEC's of 69-70% in Merinos genetically selected for increased resistance have been reported by Woolaston *et al.* (1997); Eady *et al.* (2003) although contrasted with 35% reductions in response to protein supplementation and 12-28% in response to strategic drenching. While selective breeding successfully reduces WEC there are challenges in simultaneously breeding for resistance and maintaining production, with genetically selected sheep producing 9% and 10% less clean wool and greasy wool respectively, than randomly bred Merinos (Eady *et al.* 2003). On the other hand the use of nutrition to improve worm control was associated with an increase of 17% in clean fleece weight and an increase in fibre diameter of 1.1 µm (Eady *et al.* 2003).

The repeatability of WEC as a selection measure has been shown to be up to 0.91, with other selection methods such as PCV and eosinophils not as repeatable. In Merino sheep selected for increased or decreased *H. contortus* resistance based on WEC, WEC's were lowest and eosinophils the highest in the resistant line although the differences in eosinophils were not significant (Woolaston *et al.* 1996). However, in *T. colubriformis* infections both WEC and eosinophil counts were heritable and associated with resistance. It was concluded that eosinophils can't be used as a predictor for resistance and have no advantage over WEC measurements. This was due to lower and more variable heritability estimates for eosinophils and a non-significant and negative correlation between eosinophil and WEC (Woolaston *et al.* 1996). Furthermore, WEC is significantly correlated with worm burden and a good indication for infection (Cabaret *et al.* 1998). In a resistant line of sheep heritability of WEC and PCV were 0.29 and 0.21, with the genetic correlation between the two being 0.87, suggesting PCV could be a determinant of resistance to GIN (Woolaston and Piper 1996). The correlation was weaker in the susceptible line (0.76) and studies have shown greater correlations of WEC's to total worm burden, so WEC remains the preferred technique for determining resistance (Woolaston and Piper 1996).

### 1.3.4. Grazing Management

Reducing pasture contamination can be accomplished by a variety of methods including grazing with a non-host species or by removing the host from the paddock for an extended period of time, allowing interruption of the parasitic life-cycle.

In sheep-only systems, intensive rotational grazing systems are highly effective at reducing exposure to and worm burdens, particularly for weaners and peri-parturient ewes (Besier and Love 2003). Despite only minimal effect on WEC, during a 4 year experiment using Merinos, a rotational grazing system was preferred to a continuous grazing system. This was due to rotational grazing not favouring the persistence of nematode re-infections due to regular animal movement through different paddocks (Roe *et al.* 1959). In this study the full epidemiological benefit of the rotational grazing system may not have been observed because under favourable conditions infective larvae may survive longer than 3 weeks (Gordon 1948) which was the grazing interval in this study (weekly rotations between 4 paddocks). The week-long grazing period would also have allowed development of egg to L3 (minimum time requirement of 3-4 days) ensuring infection from current contamination during each grazing period. Use of an intensive rotational grazing system with longer periods of paddock rest and (8-14 days grazing with 60-140 days rest) found significant benefits of reduced reinfections and WEC's within the grazing cycle, with the lowest mean WEC and drench treatment frequency of 444 epg and 3.1 treatments/year respectively. In farms utilising improved nutrition and typical management practices WEC's of 1374 epg and 1122 epg were observed respectively with anthelmintic treatments/year of 4.7 and 4.3 respectively, a 60% difference in WEC's and increased susceptibility to haemonchosis (Walkden-Brown *et al.* 2013). During artificial *H. contortus* infections reductions in WEC of 87-94% have been observed apart of intensive rotation during Spring and Summer, however these animals were more susceptible to worms due to lack of exposure and maintenance of immunity (Colvin *et al.* 2012). The interruption of the lifecycle during rotation reduces the prevalence of infective larvae on pastures, although this is dependent upon weather conditions, duration of grazing period and length of paddock rest.

Mixed species grazing of cattle and sheep simultaneously or grazing cattle prior to sheep into also assists in the interruption of the lifecycle (Sayers and Sweeney 2005; Bailey *et al.* 2009b). A study by Southcott and Barger (1975) on the Northern Tablelands used paddocks naturally infected by sheep for 11 months before replacing with cattle for 6, 12 and 24 weeks, with effectiveness evaluated by replacing the cattle with tracer lambs. Significant reductions in *H. contortus* of 97.2%, 92.6% and 99.9% were observed at 6, 12 or 24 weeks respectively (Southcott and Barger 1975). The most common technique is combining effective anthelmintic treatment and

paddock rotation. By allowing pastures to have a longer 'rest-period' of 1 or 2 months followed by a shorter grazing period of recently drenched weaners Niven *et al.* (2002) found that such grazing reduced WEC and pasture contamination by 50%, animals grew 254 g more clean wool and animal bodyweights were heavier by an extra 3.2 kg.

### 1.3.5. Integrated Parasite Management (IPM)

The main aims of IPM include enhanced nematode control whilst slowing development of chemical resistance and simultaneously increasing flock productivity. Methods that contribute to IPM include timely and correct use of anthelmintic treatment, grazing management, improved nutrition and the use of selective breeding, although it has been noted that the complexity of integrating these different management practices are reasons for its slow adoption (Kahn and Woodgate 2012). Following the release of a highly successful anthelmintic Closantel in the early 1980's, a strategic control program called 'WormKill' was released with a strong focus on anthelmintic control strategies. The program was highly successful but over reliance on chemical control resulted in the widespread development of closantel resistance and deterioration of the program (Kahn and Woodgate 2012).

The advantages of IPM have been documented by Scrivener *et al.* (2006) and Kelly *et al.* (2010). Lower WEC's were achieved in animals managed according to IPM with an average of 68% reduction in WEC's the entire year, and fewer tactical anthelmintic treatments required, 3.5 vs 4.5 treatments/yr in TYP. In areas where *H. contortus* is the dominant summer species, high mortality rates are the main impact of *H. contortus* infection, making the benefit of implementing a successful IPM strategy greater than that of other parasite control options. In all studies, little difference was detected in live-weight and fleece weight between IPM-treated and typically managed sheep.

## 1.4. The immune response to *H. contortus* infection

An innate immune response is displayed during first exposure to worms and involves the first non-specific lines of defence such as increased mucus production in the GI tract and the inflammatory response (McRae *et al.* 2015). The innate immune response is not key to GIN protection (McRae *et al.* 2015). Mucus covering the abomasum and intestinal tract assists in the stimulation of inflammation and provides a protective layer against GIN (McRae *et al.* 2015). It is the development of acquired immunity and the adaptive immune response that plays the greater role in developing resistance to GIN burdens, with the rate of development of immunity dependent upon the size of the worm burden (Dobson *et al.* 1990). Adams and Beh (1981) found in Merinos,

exposed to primary *H. contortus* infection, increases in total antibody titre were observed 5 weeks post infection, however reductions in WEC's were not observed until 5 weeks post reinfection. In comparison, Ingham *et al.* (2008) showed an enhanced protective effect earlier in the second infection, with observable reductions in WEC from the onset of the patent period at day 21.

### 1.4.1 Immune response to infection

***Immunoglobulins (Ig)***

The adaptive immune response to GIN is predominantly a Th2 response resulting in an effective humoral or antibody-mediated response. The effector cells are B-lymphocytes which form into antibody-secreting plasma cells and can down-regulate responses and activities of T-lymphocytes. During nematode infection a number of Ig isotypes are produced including; $IgG_1$, $IgG_2$, IgA, IgM and IgE (Kooyman *et al.* 1997b).

Increases in plasma IgG occurs in animals selected for increased resistance to *H. contortus* and aids in the hosts defence (Gill *et al.* 1992; Doyle 2007). Gill *et al.* (1992) found significant levels of $IgG_1$ locally in the abomasum, clearly indicating its role in host defence. IgA is produced both in the plasma and locally at mucosal surfaces of the abomasum and intestine (McRae *et al.* 2015). Increased levels of IgA have been associated with suppressing worm growth and controlling fecundity (Strain and Stear 2001). Provision of a high protein diet (90-173 g MP/kg DM) increased the IgA response significantly, however regardless of protein quantity, IgA levels were significantly correlated with worm length (Strain and Stear 2001; Doyle 2007).

The dominant immunoglobulin during *H. contortus* infection, IgG, is positively correlated with immunity against infection (Gill *et al.* 1993a; Watson *et al.* 1994). The lack of acquired immunity in lambs until >6months of age (Manton *et al.* 1962), is due to low circulating $IgG_1$ and IgA directed against GIN (Duncan *et al.* 1978; Kassai *et al.* 1990). In yearling Merinos, significant increases in $IgG_1$, IgG2 and IgA directed against GIN occur 7 d post *H. contortus* infection with these levels 20-40 times greater than the controls at 21 d pi. Whilst control Ig levels remained unchanged, IgA, IgM and $IgG_1$ and $IgG_2$ peaked 21 and 28 days pi respectively, with IgA the most frequent immunoglobulin (66-84%) followed by $IgG_1$ ( 8-30%) (Gill *et al.* 1992). IgA appears to be associated with protection against *H. contortus* during primary infection (Amarante *et al.* 2005), with IgA and IgG shown to have a greater increase during secondary infection (Charley-Poulain *et al.* 1984).

During *H. contortus* infection a minimal circulating IgE response occurs, with the induction of an IgE response 2-4 weeks pi. and a negative association between worm egg count and IgE serum

levels (Kooyman *et al.* 1997b). IgE is locally produced in the lymph nodes (Balic *et al.* 2000) and bound to mast cells causes degranulation

Vervelde *et al.* (2001) found no significant or prolonged increase in IgE levels following infection with *H. contortus*. However, during *T. colubriformis* and *T. circumcincta* infections higher serum and lymph concentrations of IgE have been observed peaking 8-27 days pi, with IgE increasing more rapidly in sheep previously exposed (Huntley *et al.* 1998; Shaw *et al.* 1998). This suggests that the IgE response is species dependent and is of lesser importance during *H. contortus* infection.

***Lymphocytes***

T-helper 2 (Th2) cells play a key role in driving the Th2 response resulting in immunity to GIN infection and the increase in specific antibodies directed against GIN.

Genetically resistant sheep are able to prevent larval establishment and have a strong Th2 immune response resulting in greater increases in serum $IgG_1$ and IgE compared to non-resistant counterparts (Gill *et al.* 2000; Alba-Hurtado and Munoz-Guzman 2013). Watson *et al.* (1994) observed with adult Merino 3-6 years old had a significantly higher number of CD4+ and CD8+ cells than lambs 4-8 months old in blood, lymph and skin. Interestingly, breed affects the Th2 immune response to natural *H. contortus* infections, with Gulf Coast Native lambs developing greater increases in IgE, eosinophil and leukocyte counts than Suffolk lambs (Shakya *et al.* 2009). Comparison of these aforementioned studies, shows that Merinos, despite a marked response to primary and secondary infection, have Ig and lymphocyte responses that are not as high as for some other breeds. This may be expected as the Merino breed is one of the least resistant to infection.

The T-lymphocyte phenotype, CD4+ (helper T-cell) increases in the abomasal wall and peripheral blood system during *H. contortus* infection (Alba-Hurtado and Munoz-Guzman 2013). A rapid increase in CD4+ cells in the lymph nodes is observed 3 d pi. By 5 d pi. a 2-fold increase in the lymph node weight, increases in eosinophils and CD4+ cells in the abomasum has been observed (Balic *et al.* 2000, 2002). In genetically resistant Merinos the injection of Anti-CD4+ cells, caused the depletion of both CD4+ and CD8+. Depletion of CD4+, but not CD8+, abolished resistance to infection of 6-month-old lambs against *H. contortus* infection and reduced tissue eosinophils (Gill *et al.* 1993b). Furthermore, animals vaccinated with *H. contortus* gut antigen and anti-CD4+ cells, showed a depression in immunity with increases in WEC at 21 dpi (Karanu *et al.* 1997).

### *Eosinophils*

The primary function of eosinophils is by binding to invading parasite antigens and creating acute inflammation by releasing enzymes at the site of infection. Eosinophils have been shown to congregate and develop around the attaching L3 6 hours post artificial infection with *H. contortus,* causing larvae injury and degeneration 24 hours pi (Rainburd *et al.* 1998; Balic *et al.* 2006). A continued increase in eosinophils *in vitro* showed that immobilization of *H. contortus* occurred in the presence of IL5, suggesting that eosinophils can induce a killing effect. However, studies conducted prior to this using *Trichostrongylus vitrinus* and *T. circumcincta* do not agree with increases in the number of circulating eosinophils in primary and secondary infections (Huntley *et al.* 1995). These authors concluded that eosinophils don't have a direct killing effect in the *Trichostrongylus* species, while the role of eosinophil's during *H. contortus* infection can't be discounted.

## 1.4.2. Factors affecting *H.contortus* immunity

### *Age*

Lambs display a degree of immunity to gastrointestinal nematode infection from 6-12 months of age (Stear *et al.* 1999), with some lambs under 6 months of age failing to demonstrate an effective response. This hypo-responsiveness is primarily due to a delay in the development of acquired immunity, rather than lack of exposure or suppressive effects of maternal antibodies (Colditz *et al.* 1996). Immunologically, younger lambs (<8 months) have significantly fewer CD4+ and CD8+ lymphocytes compared to animals 3+ years (Watson *et al.* 1994; Colditz *et al.* 1996; Alba-Hurtado and Munoz-Guzman 2013). Stress to lambs resulting from weaning, contributes to a delay in the development of the immune response to *H. contortus* and *T. colubriformis* infection (Watson and Gill 1991). Lambs weaned at 3 months of age have an increased ability to mount an immune response, when compared to lambs weaned at 8 weeks of age. The early weaned lambs were 6 kg lighter, significantly increased worm egg counts and reduced *H. contortus* antibody responses. Provision of a supplemented protein diet (28% CP) to lambs 4-6months old assisted in enhancing immunity with WEC significantly suppressed and further increases in WEC prevented (Shaw *et al.* 1995).

### *Sex*

An artificial *H. contortus* infection given at or after puberty shows that males are more susceptible to infection than females (Luffau *et al.* 1981). Adams (1989) also found that 5-month-old wethers

are more susceptible to a large artificial *H. contortus* infection than ewes the same age. Differences between male and female are likely due to differing hormones, anatomy, and circulating antibodies and immunoglobulin classes (Barger 1993), however Woolaston *et al.* (1990) and Albers *et al.* (1987) found that sex of pre-pubertal lambs had no effect when lambs are selected for resistance to *H. contortus*. When the sexes are grazed together, rams have been shown to acquire a larger *H. contortus* burden, although the effects (both production and worm establishment) are more severe to the reproductive female particularly pre and post-lambing. It has been speculated that the increased susceptibility of males is accounted for by the higher protein requirements of males, due to a higher protein demand for larger weight gain (Shaw *et al.* 1995).

***Peri-Parturient Rise (PPR) in WEC***

The PPR has been defined as the 'temporary loss of acquired immunity to nematode parasites at the time of parturition and lactation' (Barger 1993), and corresponds with an increase in WEC. Selecting Merinos for resistance to *H. contortus* significantly reduced PPR (Woolaston 1992). During lactation, WEC increased for a period of 4 weeks and then decreased in resistant ewes, whilst WEC continued to increase in susceptible ewes (Woolaston 1992). During *T. colubriformis* infection a relaxation in immunity of pregnant ewes occurred with decreased levels of total plasma antibody titre, eosinophils and IgG1, followed by a marked increase in WEC's (Beasley *et al.* 2010). A rapid recovery of the immune response and decrease in WEC's was observed in ewes with lambs weaned 2 days post lambing, compared ewes suckled for a further 6 weeks post lambing (Beasley *et al.* 2010).

Provision of a MP supplement during pre-partum and lactation reduces WEC in resistant and susceptible ewes. The most notable reductions in WEC were exhibited in the supplemented susceptible ewes during pre-partum and lactation, as well as increases in maternal weight (Kahn *et al.* 2003a).

## 1.5. Overview of history of research and development of vaccines against GIN

Vaccines are proven effective if the target antigens produce an effective immune response in the host (Newton and Munn 1999). During the 1960's the first successful vaccine against a nematode parasite was produced against *Dictyocaulus viviparous* (Bovine lungworm) using radiation attenuated larvae (Jarrett and Sharp 1963; Smith and Zarlenga 2006). This is still available today in some parts of Europe as HuskVac®. This method was extended to *H. contortus* and was

successful under experimental conditions in mature sheep, however the response was inadequate under field conditions, particularly in young lambs (Smith and Angus 1980; Smith 1999; Smith and Zarlenga 2006). Below is a review of the work that went into the discovery and development of attenuated vaccines first against *T. colubriformis,* followed by *H. contortus*.

First attempts at a vaccine against GIN was for *T. colubriformis* using irradiated larvae in the 1970s. Three oral vaccinations containing 20,000 irradiated *T. colubriformis* larvae were given a fortnight apart to 9-10-month-old Merino ewes resulting in protective indexes (the percentage in WEC reduction of treated animals relative to untreated controls) between 97 and 99% (Gregg and Dineen 1978). Due to the large quantity of larvae required and associated cost to invoke protection, it is unrealistic to use this method across large flock numbers. It was apparent from these positive results using larval vaccines that immune responses in sheep to protect against nematode infection could be successful. A later study used Merino lambs that were vaccinated twice with 80,000 irradiated *T. colubriformis* larvae, and then challenged with 30,000 L3. Reductions in WEC of >82% occurred (Wagland *et al.* 1984), although as previously, a large quantity of larvae was required to induce a protective response.

Priming lambs to induce an immune response has also been used to improve protection in young animals in which immunity is not yet established. One-month-old Merino lambs were trickle infected with 6,000 *T. colubrifomis* L3/week and exhibited a 45-91% reduction in WEC for 7 weeks post challenge, but this effect gradually decreased over time (McClure *et al.* 1998; Emery *et al.* 1999). Trickle infection of *T. colubriformis* causes a greater increase in $IgG_1$, IgA and IgE antibody titres to soluble L3 *T. colubriformis* antigens compared to lambs infected with a bolus dose (McClure *et al.* 1998).

It has been found that the ability of vaccinated lambs to mount a vaccinal protective response was unaffected by maternal vaccinal antibodies when suckling. Ewes grazed naturally *T. colubriformis* pastures and were housed prior to lambing, and lambs born were either colostrum-deprived for the first 72 hours or remained with their mothers. Lambs were vaccinated at 3 months of age with irradiated *T. colubriformis* larvae and challenged at 5-months of age. No significant differences in WEC's of 3-month-old Merino lambs either colostrum fed or deprived 48 hrs post birth were observed (Dineen *et al.* 1978).

Due to variation in protective index obtained, a concept of animals as 'non-responders' and 'responders' to vaccination emerged, as indicated by the individual WEC response following vaccination. Separation of vaccinated ram and ewe lambs into 'responders' and 'non-responders'

was shown to cause more consistent reduction values. Of 'responders' to *T colubriformis* irradiated larval vaccination following primary or secondary infections, reductions in WEC ranged between 66-99% in three experiments (Dineen *et al.* 1978; Dineen and Windon 1980; Windon *et al.* 1980). Following on from this, it was found that of the animals that were classified for 'responding', they would be more likely to produce progeny which 'responded'. Rams selected for responsiveness to irradiated larval vaccination, produced progeny more responsive to vaccination, with mean reduction in WEC's of 66% and steady increase in eosinophils during infection (Dineen and Windon 1980; Dawkins *et al.* 1989). Furthermore, in Merino lambs selectively bred for responsiveness, approximately 70% of 'responders' contained the lymphocyte antigens SY1 and SY2, however further attempts to investigate use of this antigen to induce protection have not occurred (Outteridge *et al.* 1985). Ultimately, the cost of vaccination using irradiated larvae is too great and inefficient for effective commercial vaccine development.

Following the relative success in inducing protection using irradiated larvae vaccines, use of an excretory/secretory (ES) antigen from *T. colubriformis* was investigated. It was found that the inability of lambs pre-weaning to mount an acquired immune response, can be improved by vaccination using a recombinant *T. colubriformis* 17 kDa ES antigen. In lambs 2-6 months old vaccination reduced WEC by 50%-65% and a marked increase in jejunal IgA and mucosal IgA and IgE response occurred (Emery *et al.* 1999; McClure 2009).

### 1.5.1 Vaccine adjuvants

The choice of adjuvant is important for increased stimulation and maintaining the Th1 or Th2 immune response. Five adjuvants found to be useful during early vaccination trial work for both *H. contortus* and *T. colubriformis* were Alhydrogel, Aluminium hydroxide (AH), dimethyl-dioctadecyl ammonium (DDA), Freunds and QuilA. Using an excretory/secretory (ES) *H. contortus* antigen with Alhydrogel adjuvant, vaccination produced an 89% reduction in worm burden, and maintained high immunoglobulin levels (IgG and IgA) for approximately 8 weeks before titres began to decrease (Vervelde *et al.* 2001; Vervelde *et al.* 2003). In comparison DDA adjuvant produced a 42% reduction in worm burden (Vervelde *et al.* 2003) and appears only capable of inducing an immune response in adult sheep (Schallig and van Leeuwen 1997; Vervelde *et al.* 2001). Intradermal immunisation with purified *H. contortus* surface antigen and QuilA did not cause a significant protective response in 5-month old lambs. However, use of AH adjuvant and the same antigen caused reductions of ~64% in worm burden, and near a two-fold increase in the anti-vaccine antibody response. Despite this, the duration of this response was shorter lasting ~3-4 weeks (Jacobs *et al.* 1999). Incomplete Freunds adjuvant (IFA), as used in

early vaccine trial work, elevates the Th2 immune response, however provides only a short duration of action and a minor antibody response to immunization requiring more frequent vaccinations (InvivoGen 2016). QuilA induces a strong CD+ lymphocyte response and potentiates the response to mucosal antigens, as well as activating both cell- and antibody- mediated immune responses. QuilA induces a stronger and more prolonged immune response lasting for ~6weeks (Newton and Munn 1999).

### 1.5.2. Development of the Barbervax® vaccine

Simultaneously to this vaccine work using *T. colubriformis,* similar work was occurring using *H. contortus,* all working towards finding an effective vaccine, both easy and repeatable to reproduce and induce repeatable protection.

***Identification of H. contortus antigens***

In the early vaccination work, X-irradiated (larvae exposed to X-rays) *H. contortus* larvae were used to reduce WEC (Urquhart *et al.* 1966). Two vaccinations of 10,000 X-irradiated *H. contortus* larvae in 7-month-old sheep reduced WEC by 100% and reduced adult worm numbers by 93% (Smith and Christie 1978). Reductions in worm numbers occurred simultaneously with increases in serum IgG antibodies post vaccination and significant increases in abomasal mucosal IgA (Smith and Christie 1978).

Excretory or secretory antigens (ES) produced by *H. contortus* are recognized by the host's immune system following an infection and classified as 'natural antigens' (Smith and Zarlenga 2006). Infection with *H. contortus* produces protective responses to two particular ES antigens, 24 and 15 kDa proteins (Schallig *et al.* 1994). Vaccinations using 24 and 15 kDa antigens, in 8-month-old Texel sheep followed by challenge with 20,000 *H. contortus* L3, resulted in WEC and worm burden reductions of 99.9% and 97.6% respectively. Interestingly, unlike previous vaccination trials, no increased IgG response was observed (Schallig and van Leeuwen 1997). However, immunisation of lambs 3 months of age proved ineffective, most likely as a result of the lack of maturity of their adaptive immune system (Stear *et al.* 1999). Further research using the 24 and 15kDa protein antigens showed no significant reduction in worm burden in vaccinated 3 month old lambs compared with an 82% reduction in worm burden in 9-month old lambs along with marked increase in antibody response 1-week post vaccination (Kooyman *et al.* 2000; Vervelde *et al.* 2001). Further attempts to reproduce this protection with 15 and 24kDa antigens in 3 and 9-month old lambs have been unsuccessful (Vervelde *et al.* 2002). Reductions in this repeat experiment in 9-month old lambs between 46-49% and 55-65% in WEC and worm burden

respectively were observed, although no protection was observed in 3-month old lambs. The authors suggested that batch differences and ability to produce identical batches of refolded recombinant ES proteins was the reason for variation in the protection.

***Hidden antigens***

Following the success of a hidden antigen-based vaccine to the cattle tick *Rhipicepahlus* (*Boophilus) microplus (*Willadsen et al. 1989; Cobon et al. 1995), further vaccine work following on from irradiated larvae and ES antigens, has focused on hidden gut antigens. A 'hidden antigen' is one which is not recognized by the host during infection. The host is not exposed to the surface proteins from the epithelial cells lining the gut of the nematode making these ideal candidate hidden antigens (Knox *et al.* 2003). Vaccination with a 'hidden antigen' stimulates the production of circulating antibodies directed against these antigens. Following ingestion of host blood by *H. contortus,* the antibodies in the blood bind to the proteins on the microvilli surface of the worm gut epithelial cells and interfere with their function. Two 'hidden antigens' that proved to be successful in damaging the parasite were H11 and H-gal-GP. Whilst these antigens don't cause immediate nematode death, they damage gut function and impair the growth and function of the parasite. Starvation and eventual death of the nematodes reduces worm egg output and pasture contamination (Moredun Research Institute 2017).

The degree of vaccinal immunity provided from hidden antigens is highly correlated with antibody titre (Munn *et al.* 1997; Smith *et al.* 1999) and is able to be passively transferred by serum (Smith 1993) or by colostrum (Andrews *et al.* 1995). A disadvantage of the 'hidden antigen' is the immune system requires frequent booster vaccinations in order to maintain a high level of immunity since natural challenge does not reinforce immunity. As noted above the two hidden antigens which have shown to have repeat success for inducing protective immunity are H11 and H-gal-GP, two gut surface proteins of *H. contortus.*

H11 is a glycoprotein complex with amino-peptidase activity and vaccines based on it have induced marked protection in a variety of sheep ages and breeds (Newton and Munn 1999) particularly young lambs vaccinated at 7 and 9 weeks of age (Tavernor *et al.* 1992). This additional protective immunity does not affect the host's innate or acquired immune response to natural infection (Smith and Smith 1993). Pregnant 17-month-old grazing ewes were immunized with a highly enriched fraction of H11. These ewes, following challenge with 10,000 *H. contortus* L3 during the final trimester of pregnancy, showed a 89-99% reduction in WEC and 90% increase in titres in vaccinated ewes (Andrews *et al.* 1995). Furthermore, lambs reared from these vaccinated

ewes had higher H11 titres and a 51% reduction in WEC at 2-3 weeks of age, compared to lambs reared from control ewes (Andrews *et al.* 1995). Older 8 month old lambs showed greater WEC reduction (79-99.8%) and worm burden (54-95%) when vaccinated with H11(Munn *et al.* 1997).

H-gal-GP, a galactose containing glycoprotein from *H. contortus,* is also obtained from the microvilli surface of adult worms using lectins specifically targeting galactose complexes (Smith *et al.* 1994; Newlands *et al.* 2013). H-gal-GP is a stable complex which is important because, when dissociated, the complex loses its protective capacity (Knox *et al.* 2003). Lambs 3-5 months old given an artificial challenge and immunized with H-gal-GP, showed a 93-98% reduction in WEC and a 53-75% reduction in worm burden, relative to controls (Smith *et al.* 1994; Smith and Smith 1996).

Combining both H11 and H-gal-GP provided even greater success in protection against *H. contortus.* Both H11 and H-gal-GP antigens were used in a combination vaccine trial in South Africa using 12-18-month-old Dorpers under natural infection. Reductions in WEC of >82% occurred post vaccination. Following a rain event a high *H. contortus* challenge occurred, with a follow-up vaccination boosting and re-instating effective immunity (Smith *et al.* 2001b). A second trial using the same combination vaccine in Louisiana USA, found vaccination to reduce mean worm egg output by >65% in 2-year-old Suffolk ewes. The flock consisted of resistant and susceptible animals, which were determined based on their WEC prior to vaccination (Kabagambe *et al.* 2000). In a subsequent study, a combination of 100 µg each of the H11 and H-gal-GP antigens, suspended in QuilA adjuvant was administered in 3-4-month-old Merino weaners to prime immunity. Four vaccinations were administered from at 5-6 weekly intervals (Le Jambre *et al.* 2008). WEC was reduced between 65 and 96% in vaccinates which also maintained normal PCV values (28-32%), compared to controls which became increasingly anaemic, due to a simultaneously increasing *H. contortus* infection. Following each vaccination, vaccine specific-antibody-titres increased by 92% compared to controls and total immunoglobulin and $IgG_1$ titres by approximately 80% (Le Jambre *et al.* 2008). This shows that exposure of the host to these 'hidden antigens' induces a strong antibody-mediated immune response. Once a vaccinal antibody response developed a highly significant negative correlation ($P<0.01$) was found between total antibody titres and WEC prior to and following the second, third and fourth vaccination. Interestingly, vaccination of naive peri-parturient and lactating Bergamacia ewes with 5 µg or 50 µg H11 and H-gal-GP antigen stimulated a significant antibody response but no significant reductions in WEC or differences in PCV occurred (Basetto *et al.* 2013, 2014). This lack of response in vaccinated ewes is likely a result of two possibilities. Firstly, their titres were

some 10-fold lower than their vaccinated lambs and may have been too low for a WEC response to be observed. Secondly, ewe treatments were grazed separately and pasture contamination levels prior to experimentation were variable (Basetto *et al.* 2014). Interestingly, lambs which received 5 µg had a greater reduction (72%) in *H. contortus* egg counts compared to lambs which received 50 µg (57% reduction in WEC) (Basetto *et al.* 2014). It is unclear why the lower volume of antigen vaccine had a greater protective response in lambs, and no reduction was observed in the ewes despite greater antigen quantity also applied. The authors also suggested the lack of a response in the ewes is likely an effect of pregnant/lactating ewes lacking the physiological resources to mount an immune response to parasite challenge (Valderrabano and Uriarte 2003; Valderrabano *et al.* 2006), and in Australian field trials vaccine naïve peri-parturient ewes, vaccination has also shown to be ineffective (Smith 2014b).

Vaccination using H11 and H-gal-GP has also proven effective in other species, other than ovine (Fitzpatrick *et al.* 2013). Male 4-month-old Holstein calves, given 3 vaccine doses at 3-weekly intervals, artificially infected with *H. contortus* or *H. placei*, showed reductions in WEC of 99% and 97% respectively (Basetto *et al.* 2011). Chamoix (*Rupicapra rupicapra)* at 2-5-months of age had worm burden reductions between 47-89%, although an antibody response was slow to develop and no correlation between WEC or worm burden and antibody titre was observed (Meier *et al.* 2016). In 6-month old Saanen and Anglo-Nubian goats a slow antibody response was also observed, and smaller WEC reductions of 69.8% and 57.4% respectively for each breed (de Matos *et al.* 2017). Interestingly, controls had antibodies to both antigens suggesting two possibilities for the positive reaction during the ELISA process. Firstly, the antigenic complexes somehow came in contact with the host, or secondly, unknown epitopes recognized by the host are causing a humoral response (de Matos *et al.* 2017). Attempts to register the product in Boer and Boer/feral cross gwoats was unsuccessful, due to variable results in WEC reductions of 17%, 44% and 73% (Smith 2016b). Despite these variable reductions in WEC, there appeared to be no indication of delayed or a shortened antibody response in the goats, with titres in the same order as that found in lambs following the second vaccination. It may be possible that goats require a greater quantity of antigen to achieve effective protection (Meier *et al.* 2016; de Matos *et al.* 2017). A trial using Boer goats in South Africa found no differences in antibody titre or WEC of animals given 5 µg or 50 µg of antigen (Smith 2016b). The reasons to the inconsistent results in goat studies and between goats and sheep are unresolved (Smith 2016b).

***Barbervax® registration trials and current label instructions***

Following some 20 years of work by Moredun Research Institute, Scotland, in collaboration with the Department of Food and Agriculture, Western Australia, registration of Barbervax® as a commercial vaccine for control of *Haemonchus contortus* in Australia was completed in 2014.

The continued success of the vaccine using hidden antigens H11 and H-gal-GP in sheep initiated production of the vaccine for early Barbervax® field trials in Western Australia and 5 on-farm trials in the Northern Tablelands of NSW during 2011/2012. Vaccinating grazing weaners caused WEC reductions over 5 months of between 71-87% relative to unvaccinated controls running in the same paddock (Besier *et al.* 2012b, 2012a; Besier *et al.* 2013; Smith unpublished-a). Unvaccinated lambs required more than three times the number of anthelmintic treatments than vaccinates, and ~3% of lambs were classified as 'non-responders'. The simultaneous grazing treatments led to further field trials, with treatments in separate paddocks to gain further information of the epidemiological effects of reduced WEC (Smith 2014a).

Trial work with *H. contortus* confirmed the notion that animals can be classified as 'responders' or not, although the expected epidemiological effect from a vaccine could be expected to protect 'non-responders' by reducing larval uptake. Non-responders were classified as those whose WEC fell above the lower 95% confidence interval of the respective control group. No attempt was made to classify animals as 'non-responders' based on antibody response throughout the registration trials. It was also not defined in the trials how many times animals had to 'non-respond' to be classified as a 'non-responder' throughout the experiments, particularly when multiple WEC samples were obtained.

The vaccination frequency, as determined by a dose response trial at Mt Barker, Western Australia in 2010/11 and a further 5 trials over the 2011/12 season (Smith *et al.* 2013; Smith unpublished-a), formed the on-label vaccination schedule for lambs (Moredun Research Institute 2017). This was to give the first vaccination at marking with intervals of 3-4 weeks between the first 3 vaccinations then every 6 weeks for a further 3 vaccinations. The first 3 vaccinations allow for 'priming' of the lambs and initial development of immunity with the remaining 3 booster vaccinations providing sustained protection for the remainder of the season. Depending on levels of the infective larvae on pasture the sixth vaccination may or may not be required.

Lambs are not fully 'primed' until after the third vaccination, based on the antibody titre at which a reduction in WEC occurs. Trials during 2011/12 conducted near Armidale, NSW used two selected lines of super-fine wool Merino lambs, selected for either super-fine wool and resistance

or susceptibility to *H. contortus*. Lambs were 3-8 weeks old at the beginning of the experiments with controls and vaccinates grazed together. Average reduction of 84.1% in WEC of all vaccinated lambs occurred across all trials (excluding 7 animals classified as 'non-responders') and 60% more animals in the control groups requiring a salvage treatment (Smith unpublished-a). Two further trials conducted by CSIRO and Invetus (formerly Veterinary Health Research), followed the same vaccine regime during the 2012/13 summer season using Merino lambs, although the treatments were grazed separately. Mean WEC reductions of the vaccinates across all plots were highly significant at CSIRO and Invetus, with a 91% and 71% reduction respectively (Knox *et al.* 2013; Smith 2014a). The lower reduction in WEC's of the latter location was thought to be due to higher green pasture availability and genetic variation of lambs, which unlike the former property were not from a genetically defined flock (Smith 2014a). The epidemiological benefit of grazing vaccinates and controls separately was observed in lambs at CSIRO. Grazing the vaccinated and unvaccinated treatments together in 2011/12 caused a lower protective index, than lambs grazed separately in 2012/13. The same results were not observed at the Invetus site, possibly due to lusher pasture and local climatic conditions. Barbervax® antibody titres of the vaccinated lambs followed the same pattern at both locations, with titres ranging from 8,000 to 40,000 post vaccination, followed by a slow decrease prior to another booster vaccination (Smith 2014a).

Trials using vaccinated lambs from the 2011/12 season as yearlings the following season (2012/13) provided data showing Barbervax® controlled *H. contortus* burdens in adult sheep. Four vaccinations 6 weeks apart, provided adequate protection for the summer season (Smith 2014a). The on-label Barbervax® schedule for hoggets vaccinated the previous year is that the first vaccination should be given November/December but can be given earlier depending on the wetness of spring. Thereafter, booster vaccinations are given at 6-weekly intervals for the duration of the *H. contortus* worm season, commonly a total of 4-5 vaccinations. Three on-farm trials running 60 yearlings (30 vaccinated, 30 controls) as a single mob, found reductions in WEC's of vaccinated sheep ranged from 63.7% to 82.2%, with >34% more control animals needing salvage drenches compared to the vaccinated. Whilst reductions in WEC appeared lower than observed in the lambs, it is likely this is due to the development of acquired immunity to nematode infection reducing the additional protective effect of Barbervax®. Antibody titres of the yearlings, vaccinated the year prior, were over 2000 units higher than controls at the beginning of the experiment some 8-9 months post their last vaccination. The estimated half-life of circulating IgG is 14 days so this suggests that the vaccine antigens are not entirely 'hidden' and receive antigenic stimulation over the past 8+ months (Smith 2014a). As per the situation in lambs, provision of a booster vaccination

caused a significant increase in specific antibody titres matched with a decrease in WEC with titres then declining and WEC increasing until the next vaccination.

The vaccine is also effective in pre-lambing and lactating Merino ewes. It was assumed that a similar booster regime as that given to ewe hoggets would provide adequate protection and trial work based on this gave rise to the current label instructions for breeding ewes previously vaccinated. These are given their first vaccination 1-2 weeks pre-lambing with this vaccination concurrently with an effective anthelmintic treatment before being moved to a low-risk worm paddock. A further booster (V2) is then given at lamb marking, and thereafter boosters are given at 6-week intervals as protection is required during the summer season (Moredun Research Institute 2017). For breeding ewes not previously vaccinated (ie. as lambs or hoggets) the on-label instructions are for 3 booster vaccinations to be given at 8-9 weeks, 4-5 weeks and 1-2 weeks prior to the commencement of lambing, V4 at marking and thereafter vaccinations every 6 weeks whilst protection is required.

Three trials were conducted on pregnant and lactating ewes, with two of these trials also using previously vaccinated ewes (as lambs and hoggets) resulting in the above vaccination instructions. All ewes in the different vaccination treatments grazed together for the duration of the trials, ensuring animals were subjected to equal natural challenge. On-farm trials conducted throughout the New England during a high-risk summer season, used ewes exposed to the vaccine as lambs and yearlings, or ewes naive to the vaccine. In ewes naive to the vaccine 2 pre-lambing vaccinations were administered (6-8 weeks and 2 weeks prior to lambing), and at 6 weekly intervals thereafter. Ewes previously exposed followed the same vaccination schedule although omitting the first pre-lambing vaccination. Reductions of WEC in ewes previously vaccinated during lactation (lambing to weaning) ranged between 55 to 71% and 55 to 73% in the two trials respectively. In comparison, ewes vaccinated for the first time saw a lesser reduction in WEC throughout lactation of 18-50% and 21-61% respectively, presumed to be due to a lower titre (Smith 2014b). Whilst a two to three fold difference in titres was observed between naïve and previously-vaccinated ewes, the pattern of their antibody titres was almost identical, increasing post vaccination, before beginning to decline prior to the next vaccination. More ewes in the control groups required salvage drench throughout the experiments compared to ewes previously vaccinated (26-86% higher). The greatest protection and increase in the titres of previously vaccinated ewes occurred post booster vaccinations (Smith 2014b). Throughout all registration trials, no differences bodyweights between treatment groups occurred at any time point, however

differences in other production values such as body condition score, fleece values and lamb survival were not measured.

As a result of these registration trials, the commercial release of a *H. contortus* vaccine was achieved successful and the Barbervax® vaccine is now widely used throughout *Haemonchus* endemic areas (Besier and Smith 2015). Whilst nematode vaccines may not achieve the sterile immunity of some viral vaccines, nor attain the efficacy levels of effective anthelmintics, modelling studies have shown vaccine efficacy of >65% will deliver desirable benefits to control haemonchosis (Dobson *et al.* 2011; Dobson *et al.* 2013). An efficacy of >65% results in a 4.5% mortality rate in vaccinates, compared to weaners drenched 4 times/year and a 27.7% mortality rate (Smith 2015).

***Barbervax® production***

Following the success of the registration trials, production of Barbervax® was increased. Production occurs at the Department of Agriculture and Food (DAFWA) laboratory in Albany, Western Australia. Lambs aged 5-10 months old are maintained at a feedlot near Mt Barker and artificially infected with *H. contortus* L3. Once infection is established, lambs are sold to a commercial abattoir where the carcasses are sent into the meat market and abomasum's (Ab's) retained and transported to DAFWA. The adult worms are harvested from the abomasum and stored at -70$^{o}$C, until required for the antigen purification process. After the worms are thawed and homogenized in a saline buffer, the remaining homogenate is centrifuged and the pellet extracted with a non-ionic detergent to solubilize the integral membrane proteins. Detergent soluble extract is then put through a column of ConA lectin, where the glycosylated fraction of the membrane proteins binds to the column. 5 µg of combined H11 and H-gal-GP antigen is then added to 1 mg of QuilA saponin adjuvant to make 1 mL Barbervax®, and dispensed into 100, 250 or 500 mL pillow packs (Smith 2016a). Once dispensed into pillow packs, the vaccine can be stored unopened for a year and a half at 2-8$^{o}$C. Vaccinations are then given subcutaneously to the neck of sheep at a dose of 1 mL/sheep irrespective of the animal's bodyweight at a cost of $0.50 USD. The vaccine has no expected slaughter interval (ESI) or with-holding period (WHP), and can be given simultaneously with other vaccines (eg. ScabiGuard), oral and topical treatments.

## 1.6. Conclusion

Internal parasites, particularly *H. contortus* are a persistent, costly and challenging problem for the Australian sheep industry. Heavy reliance on anthelmintics and uncontrolled use resulted in

the development of anthelmintic resistance, and few commercially available anthelmintics retain widespread efficacy >95%. Slow adoption of alternative and combined control methods and negative impact of parasitism on production, has caused demand for new successful control products. The development of vaccination approaches faced particular problems in the delay in maturation of the sheep's immune response until >6 months of age and decline in immune responsiveness in the peri-parturient ewe. Barbervax® is providing farmers a new control method that overcomes these issues to some extent, can be used in all classes of sheep, and due to the 'hidden antigen' nature is unlikely to interfere with the animal's ability to mount additional responses to antigens encountered during natural infection. However, whilst this new product is proving successful, adoption by some farmers is reduced due to the need for frequent vaccinations and associated musters, which is timely, costly and poses welfare risks for pre-weaned lambs. During the lambs 'priming' stages, all vaccinations except the second vaccination, coincide with a routine husbandry practice and associated muster. The second lamb vaccination requires an additional and perhaps unnecessary muster increasing the risk of mis-mothering with lambs still at foot of ewes. Exploring the potential for removing the requirement for V2 between lamb marking and weaning is therefore an important practical consideration for the further uptake of Barbervax®. Furthermore, while Barbervax® is known to provide at least 6 weeks immunity following a full booster course in year 2 as hoggets, the full length of protective immunity has not been investigated and neither has the question of whether immunity is stimulated via natural infection between vaccinations. In this thesis, I investigate these issues and also determine whether Barbervax® vaccination has any positive and/or negative effects on the productive performance of ewes/lambs. To the best of my knowledge this work is the first of its kind in regard to furthering research and knowledge of the Barbervax® vaccine.

# CHAPTER 2

# Response to Barbervax® vaccination in Merino ewe hoggets

## 1. Introduction

Internal parasites cost the Australian sheep industry $436 M annually in production losses and control costs (Lane *et al.* 2015) and anthelmintic resistance to BZ and LV classes of anthelmintic has been observed on over 90% and 80% of Australian properties respectively (Hucker *et al.* 1999; Besier and Love 2003; Love 2011). The first ever commercial gastrointestinal nematode vaccine, Barbervax® directed against *H. contortus*, was released in 2014 as a non-chemical option for use in lambs, weaners, hoggets and peri-parturient ewes. Amongst sheep producers subjected to high *H. contortus* challenge, particularly the Northern Tableland's of northern New South Wales, the vaccine has been widely adopted.

Early research into vaccination to protect against *H. contortus* infection included experiments in which vaccination with irradiated *H. contortus* larvae and excretory/secretory (15/24 kDa) antigens caused reductions in worm burdens of 82-99.9% and simultaneous increase in serum IgG antibodies in Merino lambs aged 7-9 months old (Smith and Christie 1978; Schallig *et al.* 1997; Kooyman *et al.* 2000). However immunization of 3 month old lambs and further attempts to reproduce protection with ES antigens proved unsuccessful (Vervelde *et al.* 2001; Vervelde *et al.* 2002). Subsequently two 'hidden antigens' which induced significant and repeatable reductions in worm burdens were identified, H11 and H-gal-GP. These 'hidden antigens' are gut-derived *H. contortus* antigens to which the host is not exposed during natural infection. Vaccination with them stimulates the production of circulating IgG antibodies which are ingested by *H. contortus* while feeding. The antibody then binds to the target antigens in the nematode gut, inhibiting blood-meal digestion, and causing weakness and death of the parasite, reducing worm egg output and pasture contamination (Smith *et al.* 1994; Knox *et al.* 2003). Vaccination with these two proteins has shown repeatable reductions in WEC's of 51-99% in sheep aged 3-months to 2-years, and it is these two antigens that are used in the Barbervax® formulation (Smith *et al.* 1994; Andrews *et al.* 1995; Smith and Smith 1996; Kabagambe *et al.* 2000; Smith *et al.* 2001b; Le Jambre *et al.* 2008; Smith unpublished-a).

Barbervax® usage recommendations are that animals are first vaccinated as lambs with 6 vaccinations at 3-4 weekly intervals in the first year or life, followed by a further 4 vaccinations at 6-week intervals during the *H. contortus* risk period as hoggets in the second year of life. The short duration of protection and resultant high frequency of administration is a significant drawback of vaccines based on hidden antigens. The 6-week vaccination interval in hoggets is based on persistence of immunity for at least 6 weeks during Barbervax® registration trials (Smith 2014a). This provides around 24 weeks of protection and it is of practical interest whether the duration of immunity after the final booster is prolonged beyond 6 weeks, extending the total protection period somewhat. Separately, later registration trials found that hoggets given booster vaccinations 8-9-months after the last vaccination exhibited elevated antibody titres and reduced WEC (Smith 2014b). This may represent an anamnestic response due to persistent memory cells or an indication that the vaccine antigens may not be entirely 'hidden' and immunity continues to be stimulated by natural infection. In light of the above, two hypotheses were tested in separate phases of a field experiment as follows:

1. The duration of vaccinal protection in hoggets following a full course of vaccination in year 2 will persist longer than 6 weeks; and
2. Although based on a 'hidden antigen' the response to booster Barbervax® vaccination prior to first lambing is enhanced by continual natural challenge. Suppression of natural challenge will therefore reduce the booster response.

A final aim was to determine effects (if any) of Barbervax® treatment on ewe performance as determined by scanned litter size, pregnancy status, greasy fleece weight (GFW) and fibre diameter (FD).

# 2. Materials and Methods

## 2.1. Experimental site

The experiment was conducted at TA Fields 'Congi Station' near Woolbrook, NSW (latitude: 30.91$^{o}$S, longitude: 151.29$^{o}$E, altitude: approx. 975 m) with the experiment split into two phases. Phase 1 was from April to July 2016 (weeks 4 to 16 after the 4$^{th}$ hogget booster vaccination) and phase 2 conducted from the beginning of August 2016 to pre-lambing at the beginning of September 2016 (week 21 to 25). During and following these periods, data was collected on ewes and lambs with lamb performance data presented in the following chapters. The region experiences summer dominant rainfall coinciding with increased *H. contortus* burdens and haemonchosis outbreaks. Ewes grazed native pastures for the duration of the experiments,

rotating between 3 paddocks of 50, 63 and 71 ha subjected to natural parasite challenge, predominantly *H. contortus* [96-100% (Table 1)]. Ewes were fed whole barley grain at introductory rates of 100 g/ewe 3-times per week which was increased to 1 kg/ewe 3-times per week for the duration of the first experiment due to dry weather and poor feed availability. A group of 200 experimental ewe hoggets was drafted at random from a larger mob on the property and remained together as a single mob, separated to other mobs on the property for the duration of the experiment. Of this mob 60 ewes selected at random were used in this experiment continuing to run in the mob of 200 ewes throughout. Ewes were managed by farm personnel according to regular farm husbandry practices. Weather and pasture measurements were made throughout the experiment.

## 2.2 Experimental design ethics approval and application of treatments.

The experiment used 60, 2014 born, fine-wool Merino ewe hoggets and was approved by the University of New England's Animal Ethics Committee (Approval number: AEC16-048). All animals (vaccinated and controls) were given a full Barbervax® vaccination course totalling 6 vaccinations as lambs in 2014-15 (the previous year). Day 0 was the day of the 4$^{th}$ booster vaccination as hoggets on 4$^{th}$ December 2016. Ewes were joined with rams from 4$^{th}$ April 2016 to 26$^{h}$ May 2016 (week 3 to 8), and shearing and the start of lambing were on the 21$^{st}$ July 2016 (week 19) and 11$^{th}$ September 2016 (week 26) respectively. The approach taken to testing the first hypothesis was to compare WECs and antibody levels of ewes receiving the full Barbervax® course as lambs and hoggets (VACC) with those of ewes which only received the full Barbervax® course as lambs and one booster as hoggets (UNVACC), for a 16-week period following the final hogget booster vaccination. To test the second hypothesis a third "worm-free" group was included in which worm infection was suppressed for a total of 125 days using long acting Moxidectin injections. On day 21 ewes were randomly split into the three treatment groups [n=20, Unvaccinated controls (UNVACC), Barbervax® treated (VACC) and Barbervax® + Moxidectin LA treatment (VACCMOX)] and had treatments applied as follows.

The two vaccinated treatments (VACC and VACCMOX) received 1 mL Barbervax® vaccinations on 4$^{th}$ December 2015 (week -14), 8$^{th}$ January (week -9), 5$^{th}$ February (week -5) and 11$^{th}$ March 2016 (week 0), prior to the beginning of phase 1, and a pre-lambing vaccination on 4$^{h}$ August 2016 (week 21) (Batch: 11/1 Expiry: February 2017; Wormvax Australia Pty Ltd. Western Australia). The VACCMOX treatment group received 2 injections of Moxidectin LA (Virbac Cydectin LA injection for sheep) at weeks 3 and 8 subcutaneously in the neck at a dose rate of 1

mL/20 kg (dosed to the heaviest animal). Moxidectin has a label claim to protect against re-infection with *H. contortus* up to 4 months so it could be expected that the LA Moxi ewes had an 18 week "worm-free" period between their final booster vaccination and the pre-lambing Barbervax® booster vaccination. Unvaccinated controls were given an initial vaccination as hoggets on 4th December 2016 (week -14), but were given no further booster vaccinations providing a 4-month period vaccination-free prior to the start of the experiment.

Animals were administered a single dose of Barbervax® [1 mL (containing 5 µg antigen) regardless of bodyweight] injected subcutaneously behind the ear. Unvaccinated control ewes were administered 1 mL saline solution at the same site. All ewes including the controls were given anthelmintic treatments [TriGuard® (Abamectin 1.0 g/L, Oxfendazole 22.7 g/L and Levamisole 33.9 g/L)] at weeks 4 and 21 in response to rising worm egg counts and a strategic pre-lambing drench at week 25 [Q-Drench® (Abamectin1.0 g/L, Albendazole 25.0 g/L, Closantel 37.5 g/L, Levamisole hydrochloride 40.0 g/L)]. One individual from the VACC group was given an extra precautionary anthelmintic treatment due to a high and increasing WEC and excluded from the statistical analysis. No animal deaths were recorded during the experiment and the timeline for the experiment can be seen in Figure 1.

## 2.3. Measurements, sampling and methods

### 2.3.1. Measurements and sampling

#### *2.3.1.1. Blood samples*

Blood samples were collected by jugular venipuncture into 1 x 5 mL serum separator (SST) Vacutainer® tubes. For phase 1, blood samples were collected at monthly intervals for a total of four months after the final 4th booster vaccination (day 28, 56, 84, 111) (Figure 1). For phase 2, samples were collected at weekly intervals for a total of five weeks (days 146, 154, 160, 168, 175) following the pre-lambing booster vaccination (Figure 1). Samples were centrifuged on the day of collection at 2763 x gravity at 4°C for 15 minutes using a Beckham Coulter Allegra X-15R centrifuge. Serum was stored at -20°C until required for analysis.

#### *2.3.1.2. Faecal samples*

Individual faecal samples were collected per rectum using fresh disposable gloves between sheep. For phase 1, faecal samples were collected at monthly intervals for a total of four months post the final 4th booster vaccination (week 4 to 16), and no samples were obtained for phase 2 (Figure 1).

## 2.3.2. Performance Measures

### *2.3.2.1. Bodyweight and body condition score (BCS)*

Individual ewe bodyweights were weighed using Ruddweigh electronic scales pre- and post-joining and at shearing (Figure 1). BCS was measured by the principal author at all time points. BCS of each individual ewe was measured at these times by the author using the Lifetime Wool condition score guide (LifetimeWool 2011). The BCS was assessed by palpating over the dorsal, medial and lateral surface of the backbone and short ribs and a score from 1 to 5 given on the basis of palpated fat cover and muscle depth.

### *2.3.2.2. Pregnancy and Fleece scanning*

Individual ewes were pregnancy scanned at week 18 (Figure 1). Ultrasound scanning for fetal number was undertaken by a contracted business. The foetal number was recorded as 'empty' (0), 'single' (1) or 'twin' (2). Ewes were shorn at week 19, and the unskirted fleece without the belly weighed. Fleeces were then skirted and a mid-side sample of the fleece taken for laser scanning to determine mean fibre diameter, fibre curvature + SD and total fibres >30.5 μm (AWTA 2017a, 2017b). This was completed by a contracted business (LaserWool).

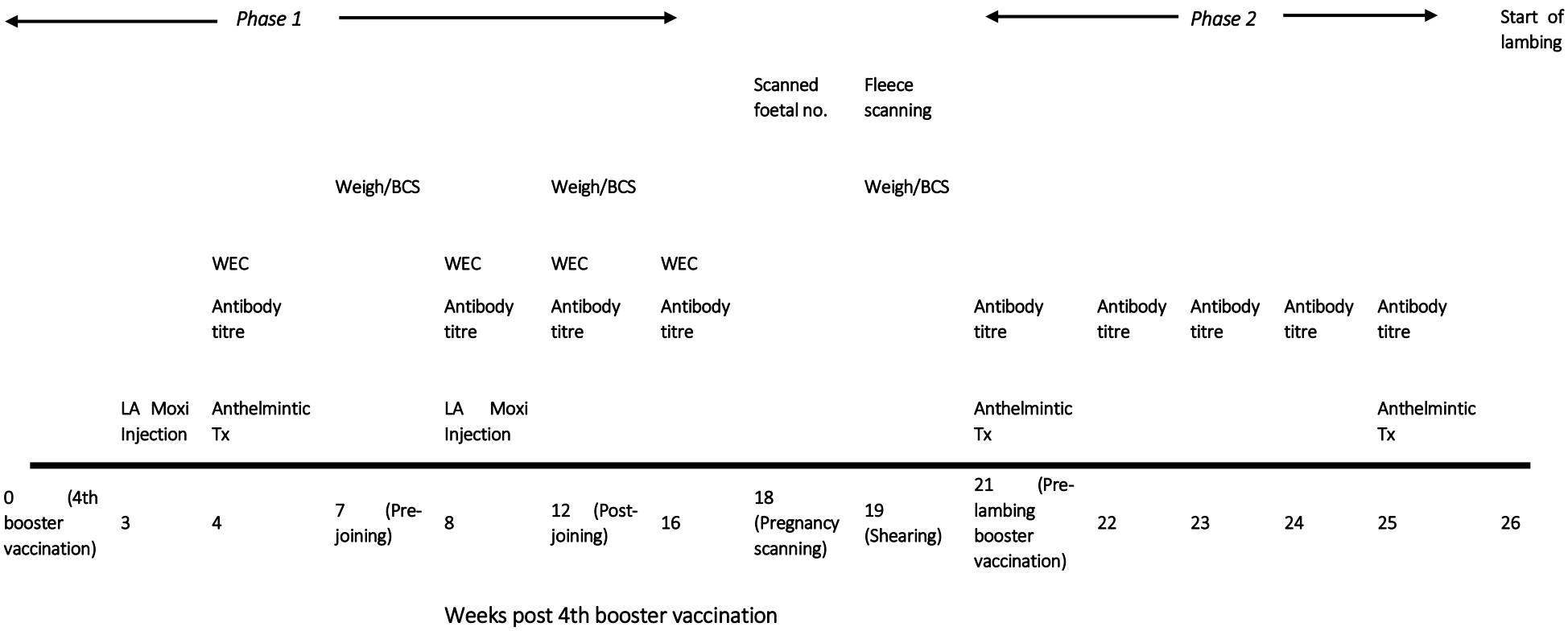


**Figure 1.** Timeline of treatments and measurements for the two phases in the experiment (not to scale)

### 2.3.3. Laboratory methods

#### *2.3.3.1. Enzyme Linked Immuno-absorbent Assay (ELISA) – Barbervax® antigens*

Serum samples were tested for the presence of IgG antibodies directed against Barbervax® antigens using a Barbervax® antibody specific ELISA method provided by Moredun Research Institute. Microtitre plates (Costar EIA/RIA High-Binding 96 well, USA) were coated with 50 µl/well using a sodium carbonate buffer (pH 9.6) mixed with stock vaccine antigen to concentration 1 µg/mL and incubated at 4 $^{o}$C for 24hours. Plates were washed three times with PBST (PBS + 0.05% Tween 20) and 100 µl/well of blocking solution added (10% w/v Karikare+ in TNTT) and incubated at 4$^{o}$C for a further 24 hours. Plates were washed thrice with PBST. Each plate had a layout of 7 samples in a serial doubling dilution from 1:100 to 1:204,800 triplicate negative controls and the remaining 15 wells left blank. The serial doubling dilutions were first completed in TNTT on low-binding plates (96 well ThermoScientific, Denmark). 50 µl of each sample was transferred from the low-binding to the high-binding plate from smallest to largest dilution avoiding cross-contamination and incubated for 1 hour at room temperature. High-binding plates were washed another 3 times with PBST and 50 µl/well of secondary antibody mouse monoclonal anti goat/sheep IgG-HRPO conjugate (Sigma-Aldrich, Australia) diluted in TNTT to 1:10,000 dilution, was added to each well, and incubated for 1 hour at room temperature. The plates were washed another 3 times with PBST and 50 µl of o-phenylenediamine dihydrochloride substrate (OPD, Sigma-Aldrich SigmaFast OPD Peroxidase) dissolved in distilled water was added to each well and incubated in the dark at room temperature for 20 minutes. 25 µl of 2.5M sulphuric acid was added to each well to stop reaction. The optical density (OD) of the wells was measured using an ELISA plate reader (Benchmark) at a wavelength of 490 nm. Individual titres were calculated using formulas developed by Moredun Research Institute. The curvilinear relationship of absorbance and dilution was plotted and titres and $R^2$ values derived from the curvilinear association between dilutions between 1:200 and 1:1280 providing results in units of titre.

#### *2.3.3.2. Enzyme Linked Immuno-Absorbent Assay (ELISA) - H. contortus L3 antigens*

All blood samples were also tested by ELISA for the presence of IgG directed against *H. contortus* L3 antigens to assess naturally acquired immunity to *H. contortus* (Gill 1991). Preliminary analysis determined that dilution rate required to determine antibody titre in samples was 1:8000 diluted in PBS + tween (Sigma-Aldrich, Australia, PBST), and a standard curve was created by serially diluting 2.82 mg/mL *H. contortus* antigen (University of New England, Armidale) from 1:200 to 1:102,400. Microtitre plates (Costar EIA/RIA High-Binding 96well, USA) were coated with 100 µl/well using a sodium carbonate buffer (pH 9.6) mixed with stock *H. contortus* L3 antigen to

concentration 10 µg/mL, covered and incubated at 4°C for 24hours. Plates were then washed three times with a washing PBST. Duplicate standards, blanks and diluted samples were added to each well (100 µl/well) and incubated for 1 hour at 37 °C. Plates were washed a further three times with washing PBST and 100 µl of monoclonal anti goat/sheep IgG (Silenus Laboratories, Australia), diluted in PBST to 1:3000 dilution was added to each well, and incubated for a further 1 hour at 37°C. Plates were washed a further three times with washing PBST and 100 µl of OPD substrate (o-phenylenediamine + citric phosphate buffer + hydrogen peroxide) was added to each well and incubated in the dark at room temperature for 30 minutes. The reaction was stopped by the addition of 50 µl/well of 2N sulphuric acid. The optical density (OD) of the wells was measured using an ELISA plate reader (Benchmark) at a wavelength of 490 nm. Individual total antibody titres were calculated from the standard curve to provide a result in units of titre.

#### *2.3.3.3. Worm Egg Counts and larval differentiation*

WECs were completed using a modified McMaster method (Whitlock 1948). Approx. 2 g of faeces was diluted in a ratio of 1:5, faeces: water and mechanically mixed to form a homogenous sample. Using a 0.5 mL volume chamber Whitlock slide, 600 µl and 150 µl salt solution and faecal sample respectively, added to each chamber. The remaining faeces were pooled by treatment group and used for larval differentiation of genera following incubation as described below. Faeces were mixed with vermiculate (~¼ of the faeces volume) in glass jars and moistened with water. Jars were then placed into an incubator for 7 days at 24°C. The jars were removed filled with tap water to the meniscus, a petri dish placed on top and the jar and Petri dish inverted together. The Petri dish was half-filled with tap water and left for minimum of 1 hour. Liquid was aspirated from the Petri dish using a pipette and placed into a V-bottom centrifuge tube and larvae left to sink. Larval differentiation was completed to species level on sheathed L3 stained with dilute iodine. Larval count for each species was expressed as a percentage, revealing that *H. contortus* was the dominant species for the duration of the experiment (96-100%).

## 2.4. Weather Data

Throughout the experiment, cold nights were observed (below 4°C), with maximum temperatures highest in early autumn in March (32.5°C) continuing to decrease throughout late autumn, winter and early spring (Figure 2). Between March and May low rainfall totals were associated with reduced pasture growth. This was broken by atypical high rainfall in June followed by more typical rainfall increasing until the beginning of spring in September. A uniform view of pasture availability

in photos can be seen in figure 3, with April and May associated with very low pasture quantity and quality, whilst June and July green re-growth began to occur.

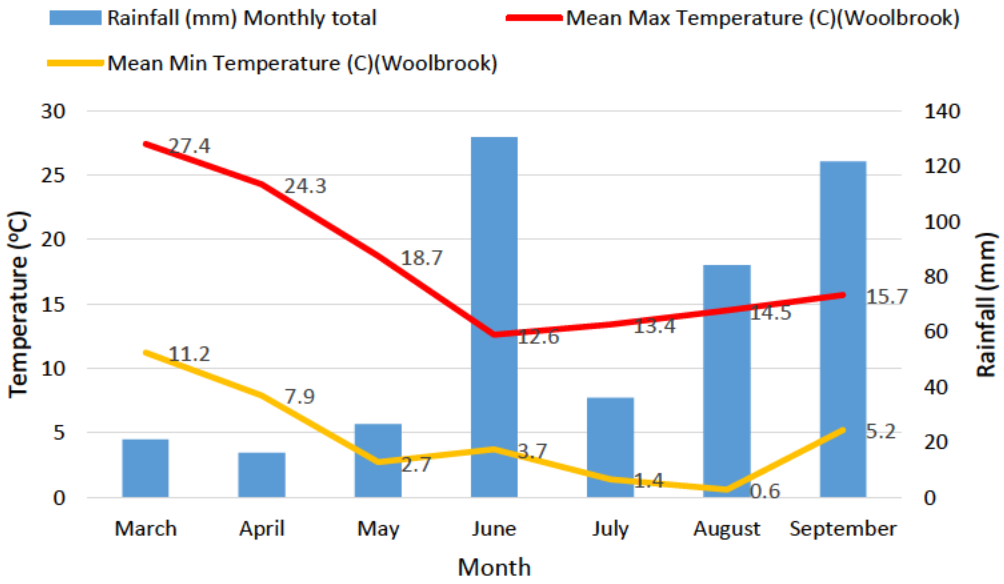


**Figure 2.** Monthly rainfall records from 'Congi Station' and temperature records (BOM) for nearby Woolbrook for the duration of the experiment.

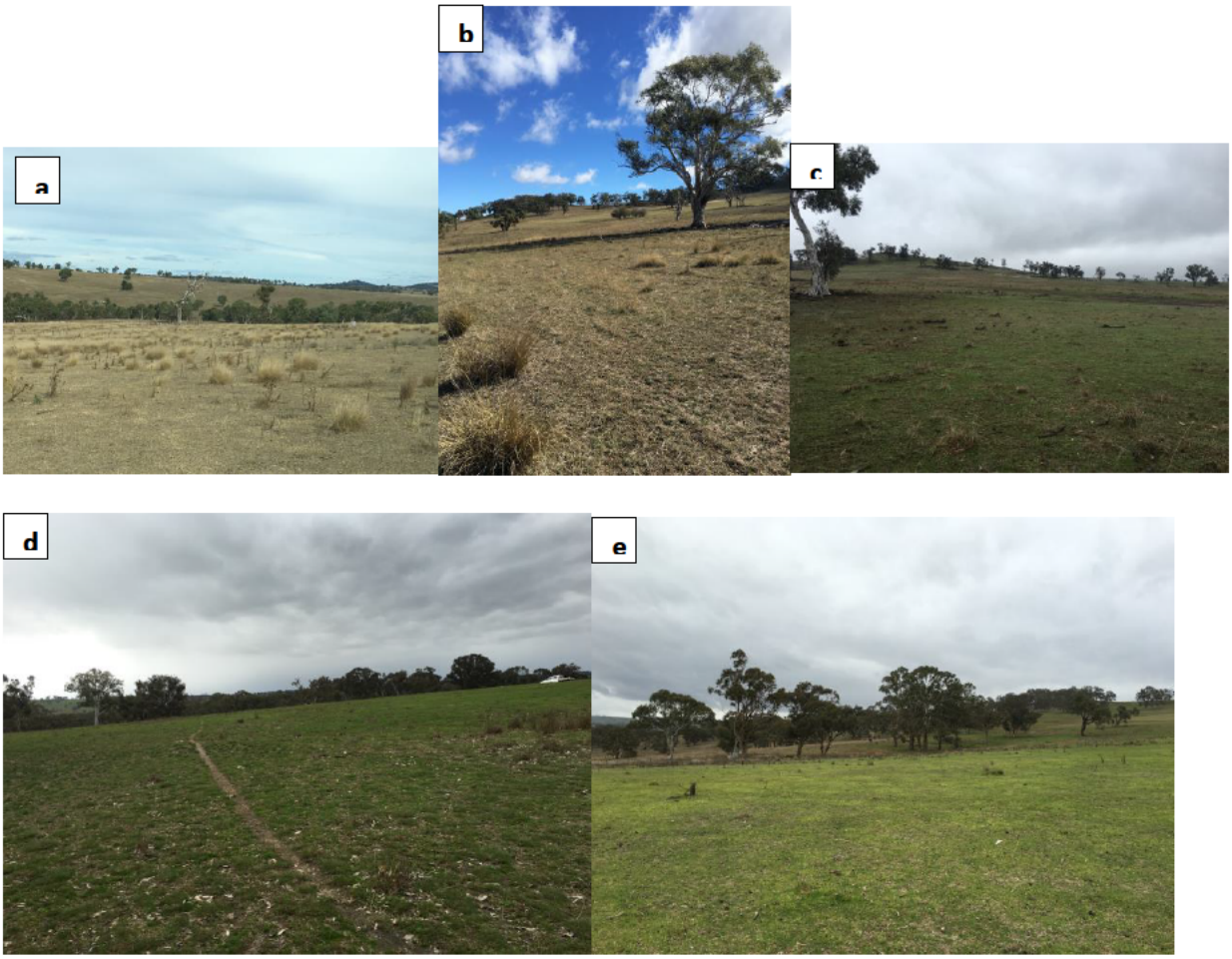


**Figure 3.** Photos of experimental ewe paddocks during (a) April, (b) May, (c) June, (d) August and (e) September. July missing due to the paddock being inaccessible due to very wet weather.

## 2.4. Data and statistical analysis

Data were analysed using JMP 13.1.0 software (2015 SAS Institute Inc). WEC data were cube root transformed and Barbervax® antibody titre Log10 transformed to remove association between the variance and the mean. *H. contortus* ELISA data were Log10 (y + 10) transformed. For variables measured only once (eg fleece and litter size variables) general linear models were fitted testing the effect of treatment group with covariates and interactions fitted where appropriate. For variables measured repeatedly from the same animals (eg. WEC, antibody titres) a mixed restricted maximum likelihood model (REML) was fitted with the individual sheep as a random factor and treatment, time (week) and their interactions fitted as fixed effects. Association between measured variables (eg. WEC and antibody titre) was explored using curve fitting, correlation and linear regression. A P value of $<0.05$ was used to determine significance. The

significance of differences between means within a significant treatment was determined using Student's t-test. Data are presented as least squares means (LSM) ± standard error of the mean (SE). Untransformed WEC data are also reported to show the actual epidemiological consequences of treatments in terms of egg contamination of pastures and to enable comparison with normal diagnostic results used by graziers and advisors for worm control decisions, which are not transformed. No animals or outliers were removed from any analysis, with the exception of the one ewe in the VACC treatment previously mentioned given an extra anthelmintic treatment due to high WEC. Fleece-free bodyweight at shearing was calculated by subtracting the fleece weight from the pre-shearing bodyweight.

Non-responders to the vaccine were defined as those whose WEC was above the lower 95% confidence interval of the control group. This is the method used to determine outliers in the Barbervax® registration trials. This concept was expanded to include the response for Barbervax® log10 antibody titre with non-responders defined as those whose log10 Barbervax® antibody titres were below the upper 95% confidence interval of the control group. The protective Index (PI) for Barbervax® vaccination was calculated using the following equation:

$$PI = \frac{Unvaccinated\ WEC\ (epg) - Vaccinated\ WEC\ (epg)}{Unvaccinated\ WEC\ (epg)} \times 100$$

# 3. Results

## 3.1. Persistence of immunity following year 2 vaccination course (Phase 1)

### 3.1.1. Antibody Titres – Barbervax®(BV) antigens

The effect of vaccination treatment was significant for BV antibody titre [P<0.0001,(VACC BV titre 4.48 ±0.07; UNVACC BV titre 4.09 ±0.06)], whilst those of time (weeks post final vaccination) (P=0.26) and the interaction between them were non-significant (P=0.92). Vaccinated ewes had the highest BV antibody titres at all sample points and the difference was significant between VACC and UNVACC at week 4 (P=0.006), 12 (P=0.013) and 16 (P=0.03) and trended towards significance at week 8 (P=0.09). BV titres of the vaccinated ewes did not vary significantly over time but tended to increase to week 8 before decreasing to weeks 12 and 16, whilst unvaccinated controls followed a similar pattern at a lower titre (Figure 4a).

### 3.1.2. Antibody Titres – *H. contortus* (HC*)* antigens

The effect of vaccination treatment was significant for HC antibody titre [P=0.03 (UNVACC HC titre 5.24 ±0.09; VACC HC titre 5.52 ±0.09)], however the effect of time (weeks post final vaccination) (P=0.07) and the interaction between them (P=0.80) were non-significant. VACC ewes had the highest HC titres at all sample points, and the difference between VACC and UNVACC was significant at week 8 (P=0.019) and approached significance at week 16 (P=0.066). HC titres of the vaccinated followed a similar pattern to the Barbervax® antibody titres, increasing to week 8 before decreasing to week 12 and 16, and UNVACC following a similar pattern but at a lower titre (Figure 4b).

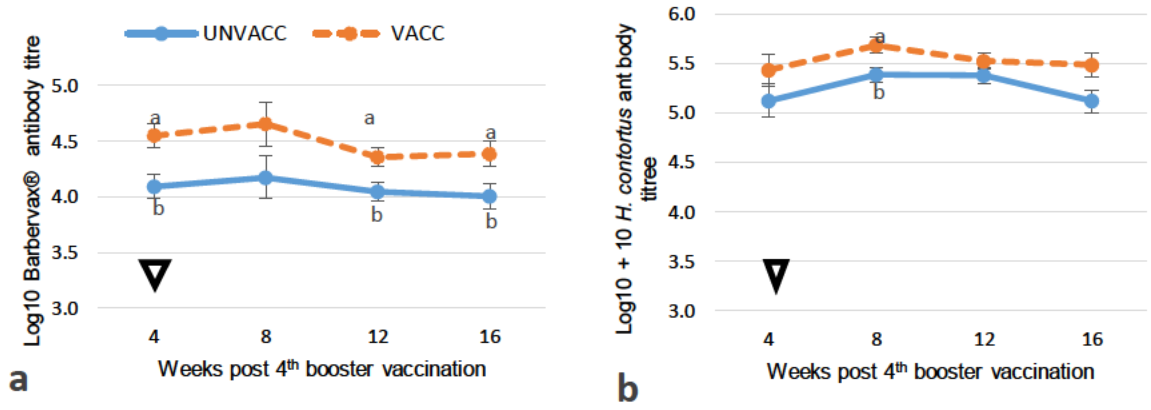


**Figure 4.** (a) Log10 transformed Barbervax® antibody titres and (b) *H. contortus* antibody titres (ls mean ± s.e.) of Barbervax® VACC and UNVACC Merino ewes subjected to a natural *H. contortus* challenge. Where letters[a,b] differ means within the time-point differ significantly (P<0.05). Open triangle: anthelmintic treatment to all ewes.

### 3.1.3. WEC

The effect of weeks post final vaccination was significant (P=0.025), whilst effects of vaccination (P=0.77) and the interaction (P=0.61) were non-significant. WEC's decreased from week 4 as a result of the anthelmintic treatment, before increasing to week 12 and 16 without significant differences between vaccination groups (Figure 5a). Despite the lack of significant difference between treatments, the untransformed WEC's were arithmetically lower in VACC ewes than controls at all weeks post the 4th booster vaccination (Figure 5b).

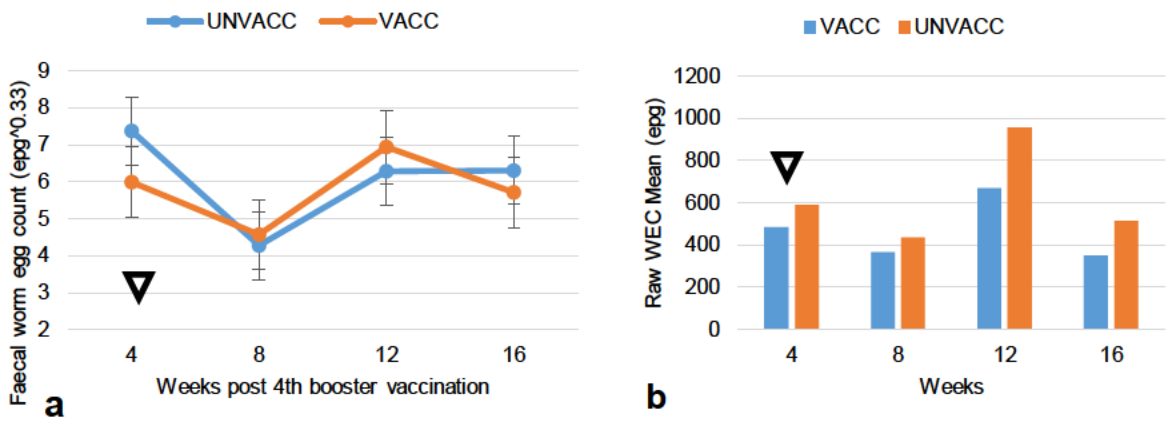


**Figure 5**. (a) cube-root transformed faecal worm egg count and (b) un-transformed faecal worm egg count (epg) (ls means ± s.e.) of Barbervax® VACC and UNVACC Merino ewes subjected to a natural *H. contortus* challenge. Open triangle: anthelmintic treatment.

**Table 1.** Larval differentiation results showing percentage of *H. contortus* from VACC and UNVACC ewes at weeks 4, 8, 12 and 16 after the final hogget booster vaccination.

| Week | VACC | | UNVACC | |
|---|---|---|---|---|
| | % *H. contortus* | % Other | % *H. contortus* | % Other |
| **4** | 100 | 0 | 96 | 4 |
| **8** | 100 | 0 | 100 | 0 |
| **12** | 100 | 0 | 100 | 0 |
| **16** | 99 | 1 | 100 | 0 |

### 3.1.4. Association between WEC and Barbervax® antibody titre

Analysis of the association between WEC and Barbervax® antibody titre in VACC and UNVACC ewes combined revealed weak negative associations overall and of VACC and UNVACC treatments that did not achieve statistical significance at any sampling time (Figure 6) or when data from all sample dates were combined (weeks 4, 8, 12, 16) (Figure 7). $R^2$ values revealed these associations to be very weak ($R^2$=0.0003-0.06).

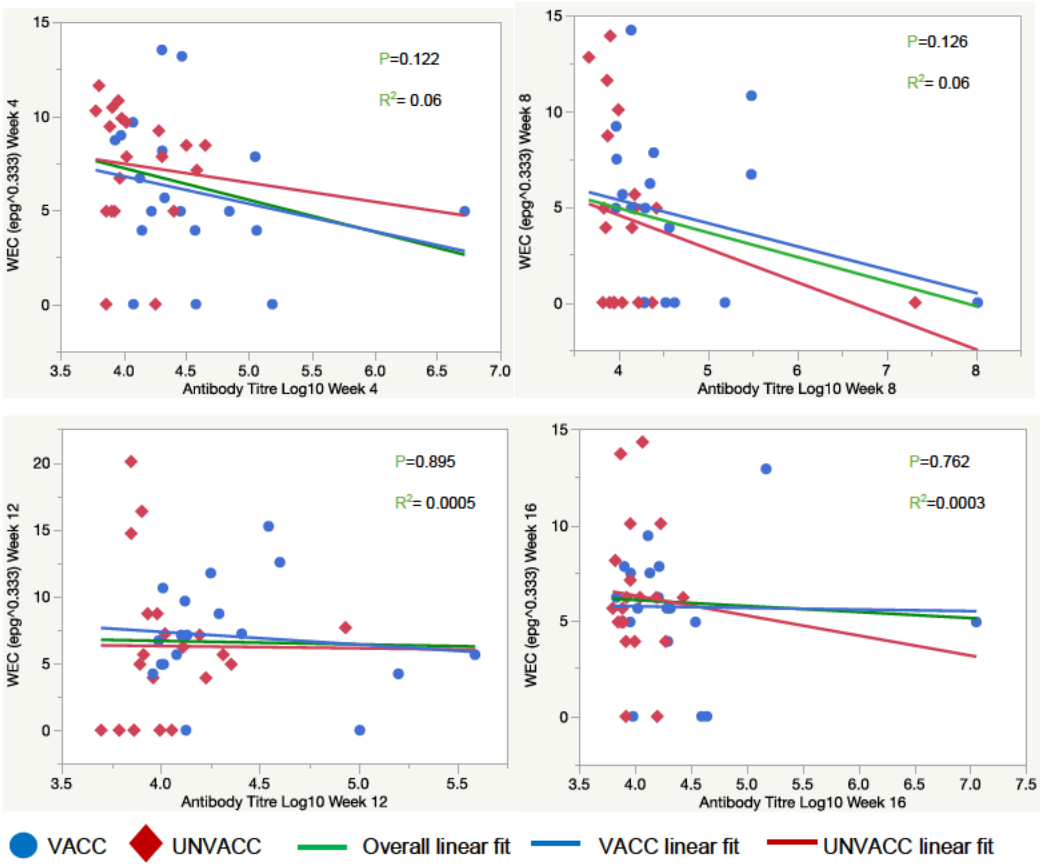


**Figure 6.** Bivariate fit of cube-root transformed VACC and UNVACC treatment faecal worm egg count and log10 transformed Barbervax® antibody titre from weeks 4 (top left), 8 (top right),12 (bottom left) and 16 (bottom right). Each point represents a single animal sampled on a given day. P-value and $R^2$ for overall linear fit is shown.

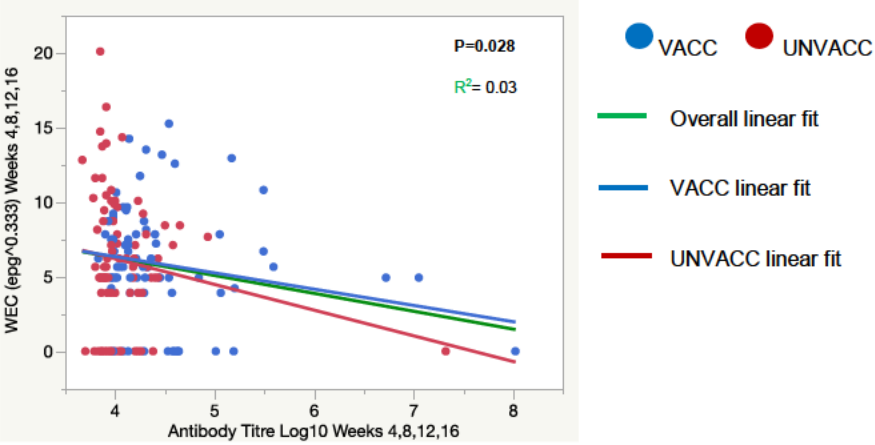


**Figure 7.** Bivariate fit of cube-root transformed VACC and UNVACC faecal worm egg count and log10 transformed Barbervax® antibody titre during the experiment from weeks 4,8,12 and 16

combined. Each point represents a single animal sampled on a given day. P-value and $R^2$ for overall linear fit is shown.

### 3.1.5. Association between WEC and *H. contortus* antibody titre

Analysis of the association between WEC and *H. contortus* antibody titre in VACC and UNVACC ewes combined revealed negative associations overall and of VACC and UNVACC treatments, that did not achieve statistical significance at any sampling time (weeks 4, 8, 12, 16) (Figure 8). Week 16 trended towards significance (P=0.08), with a negative association observed in the VACC treatments where high *H. contortus* antibody titre decreased WEC. $R^2$ values revealed these associations to be very weak ($R^2$=0.01-0.08).

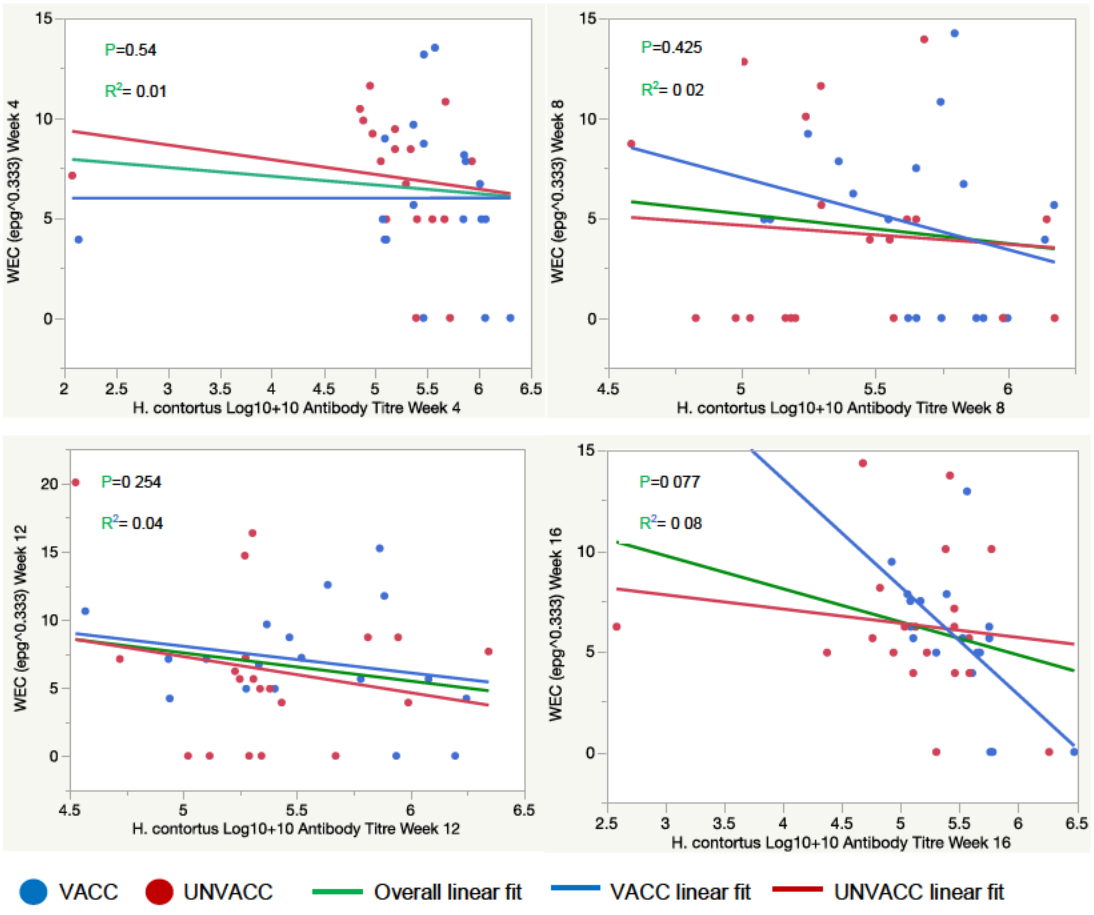


**Figure 8.** Bivariate fit of cube-root transformed VACC and UNVACC treatment faecal worm egg count and log10+10 transformed *H. contortus* antibody titre from weeks 4 (top left), 8 (top right),12 (bottom left) and 16 (bottom right). Each point represents a single animal sampled on a given day. P-value and $R^2$ for overall linear fit is shown.

### 3.1.6. Association between Barbervax® antibody titre and *H. contortus* antibody titre

Analysis of the relationship of Barbervax® antibody titre and *H. contortus* antibody titres in both vaccination treatments combined showed positive associations that were significant at week 8 (P=0.028) and 12 (P=0.0002), although not at week 4 and 16 (Figure 9). $R^2$ values revealed these associations were weak ($R^2$=0.0001 - 0.31). These associations should be treated with caution, due to leverage effects of individual animals. No reasonable justification could be found to remove animals from the analysis.

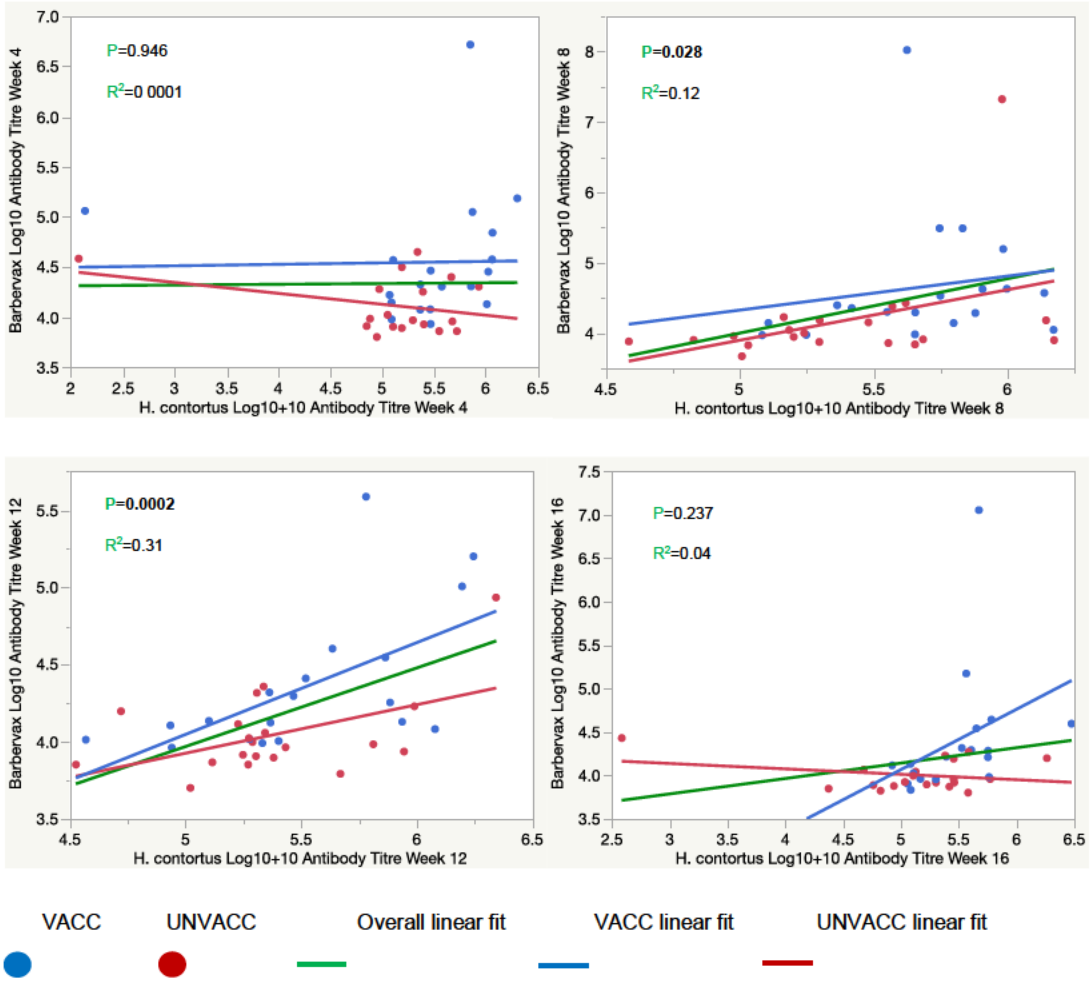


**Figure 9.** Bivariate fit of overall, VACC and UNVACC treatment Barbervax® Log10 and *H. contortus* Log10+10 antibody titre weeks 4 (top left), 8 (top right),12 (bottom left) and 16 (bottom right). Each point represents a single animal sampled on a given day. P-value and $R^2$ for overall linear fit is shown.

### 3.1.7. Protective Index of Barbervax®

Reduction in WEC ranged from 18-32% in the vaccinated ewes at each time-point (Table 2), with a 23% reduction over the entire experimental duration.

**Table 2**. Protective Index (%) provided to the vaccinated ewes for the duration of the experiment post the 4 h booster vaccination.

| | **Weeks post 4th booster vaccination (% WEC reduction)** | | | |
|---|---|---|---|---|
| | **4** | **8** | **12** | **16** |
| **Barbervax® vaccinated ewes** | 18 | 16 | 30 | 32 |

### 3.1.8. Non-Responders – WEC and Barbervax® antibody titre

At all weeks post the 4th booster vaccination, greater than 40% of the vaccinates were classified as 'non-responders' based on WEC and antibody titre (Table 3). Five animals (26%) 'non-responded' at all weeks and seven animals 'non-responded' at 3 occasions (37%) (Figure 10). No pattern was observed for 'non-responding' based on WEC or antibody titre, ie. responsiveness was rather random, with an animal classified as a 'non-responder' one week, but not the following rather than being consistently classified as a responder or non-responder.

**Table 3.** Number and percentage of 'non-responders' to Barbervax® at each time point post the 4th booster vaccination based on WEC and antibody titre.

| | **WEC** | **Antibody Titre** |
|---|---|---|
| **Week 4** | 8 (42%) | 9 (47%) |
| **Week 8** | 12 (63%) | 12 (63%) |
| **Week 12** | 17 (89%) | 10 (53%) |
| **Week 16** | 15 (79%) | 11 (58%) |

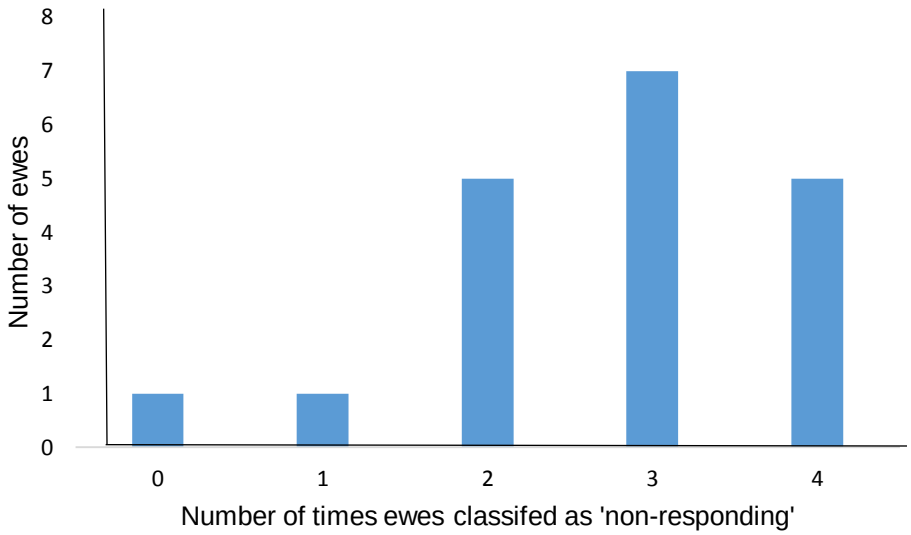


**Figure 10.** Number of ewes classified as 'non-responders' 0, 1, 2, 3 or 4 times (out of the 4 sample times) based on WEC values at weeks 4, 8, 12 and 16 post 4$^{th}$ booster vaccination.

## 3.2. Effect of suppressive anthelmintic treatment and exposure to continual natural challenge on response to pre-lambing Barbervax® booster (Phase 2)

### 3.2.1. Antibody Titres - Barbervax® antigens

The effect of time (weeks post pre-lambing booster vaccination) (P=0.0002), vaccination treatment [P=0.0003 (UNVACC 3.92 ±0.15, VACC 4.58 ±0.16, VACCMOX 4.84 ±0.15)] and the interaction between weeks and treatment (P=0.0003) were all highly significant. VACCMOX ewes had the numerically highest antibody titres at all sample points following the vaccination at week 21, with VACC ewe antibody titres slightly but not significantly lower than VACCMOX at any week. However, significant differences between vaccinated and UNVACC ewes occurred at weeks 22 (P<0.0001), 23 (P=0.0004), 24 (P=0.0023) and 25 (P=0.001). Ewes in the vaccinated treatments had an increase in antibody titre between weeks 21 and 22, before titres gradually decreased or plateaued over the following 3 weeks, whilst unvaccinated controls antibody titres continued to decrease from week 21 (Figure 11a).

### 3.2.2. Antibody Titres – *H. contortus* antigens

The effect of vaccination [P<0.0001 (UNVACC 5.15 ±0.04; VACC 5.47 ±0.04; VACCMOX 5.50 ±0.04)] and time (weeks post pre-lambing vaccination) (P=0.01) were significant, although the

interaction between them was not (P=0.17). VACCMOX and VACC ewes had similar titre levels at all sample points from week 21, and the difference between the vaccinated treatments was non-significant. Significant differences between vaccinated and unvaccinated treatments occurred at weeks 22 (P=0.0003), 23 (P<0.0001) and 24 (P=0.004). Following the pre-lambing booster at week 21, VACC and VACCMOX titres increased (Figure 11b), before decreasing at week 24. In comparison, UNVACC antibody titres decreased from week 21 to 23, before increasing to week 25.

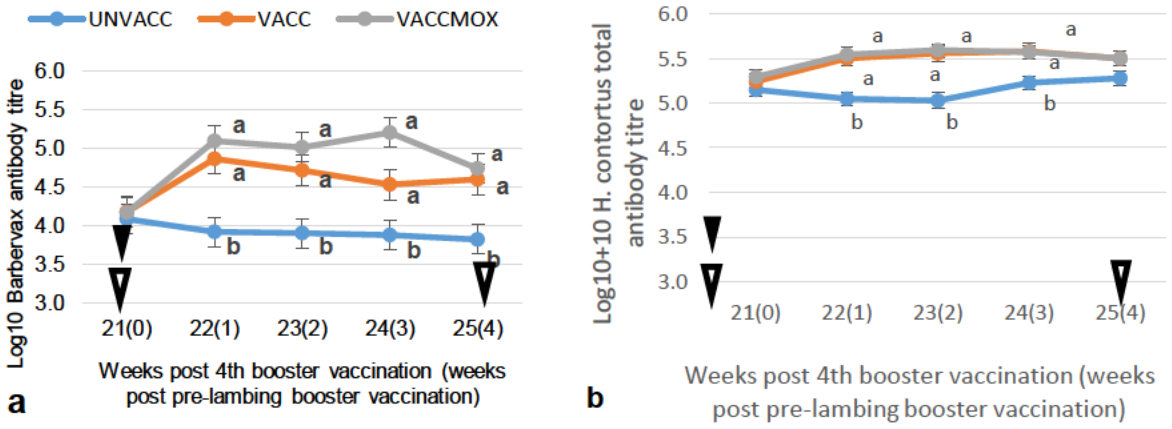


**Figure 11.** (a) Log10 transformed Barbervax® antibody titres and (b) *H. contortus* antibody titres (ls mean ± s.e.) of Barbervax® VACC and UNVACC Merino ewes subjected to a natural *H. contortus* challenge. Where letters[a,b] differ means within the time-point differ significantly (P<0.05). Solid filled triangles: Barbervax® vaccination (to vaccinated treatments); open triangle: anthelmintic treatment to all ewes.

### 3.1.5. Association between Barbervax® antibody titre and *H. contortus* antibody titre

Analysis of the relationship of Barbervax® antibody titre and *H. contortus* antibody titre showed positive linear associations whereby as Barbervax® antibody titre increased, an increase in *H. contortus* antibody titre also occurred. The linear fit trended towards significance at week 21 (P=0.094) and was significant for the next 4 weeks (P<0.0001-0.042) (Figure 12). $R^2$ values revealed these associations to be weak ($R^2$=0.05-0.28). These associations should be treated with caution, due to leverage effects of individual animals although no reasonable justification could be found to remove animals from the analysis.

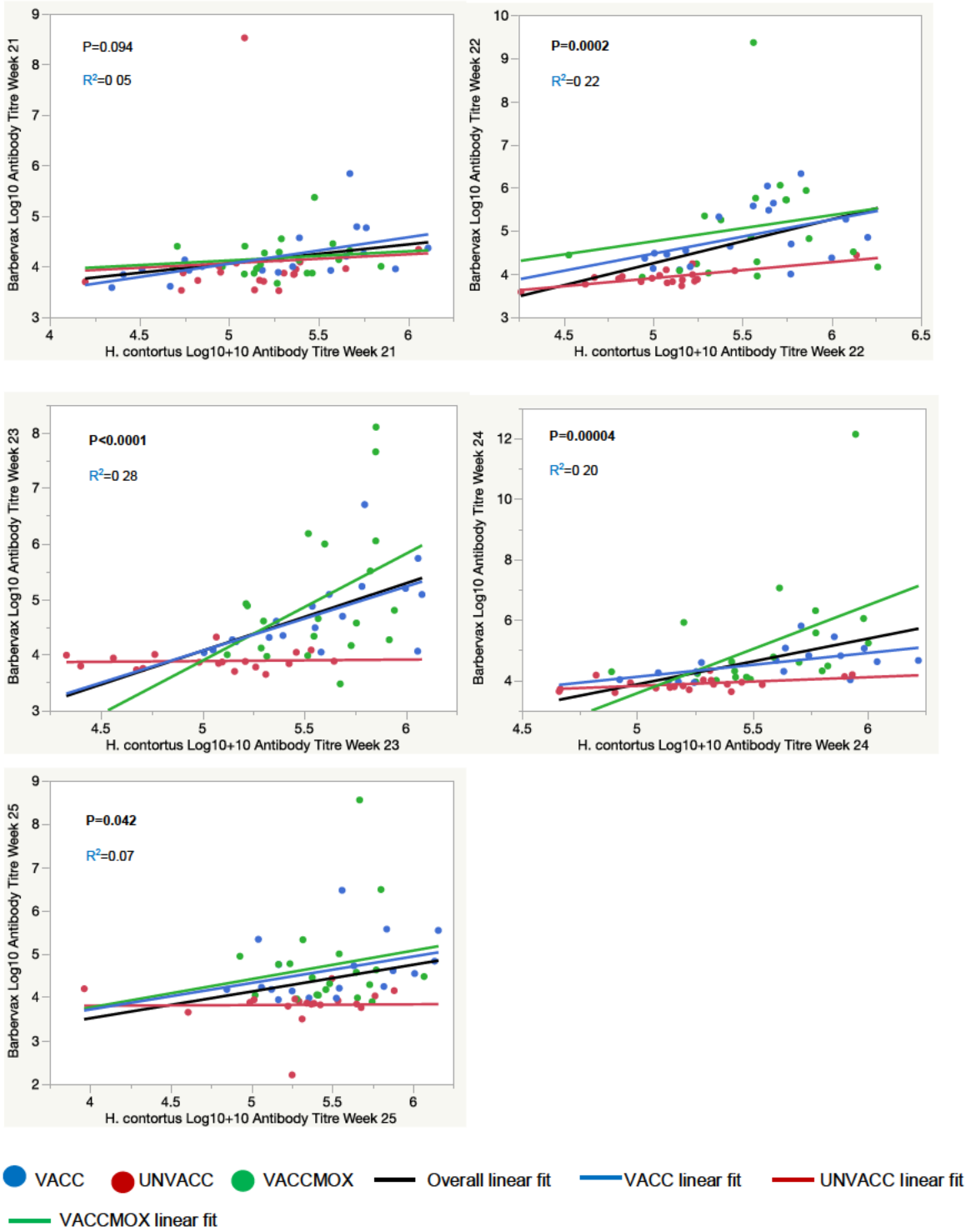


**Figure 12.** Bivariate fit of VACC and UNVACC Barbervax® Log10 and *H. contortus* Log10+10 antibody titre weeks 21, 22, 23, 24 and 25. Each point represents a single animal sampled on a given day. P-value and $R^2$ for overall linear fit is shown.

## 3.3. Effect of Barbervax® treatment on performance

The effect of Barbervax® treatment was non-significant for scanned litter size, fleece weight and mean fibre diameter. The overall mean, least square means and error for treatments and effects are shown in table 4. The co-variates ewe joining weight (P=0.0002) and BCS (P=0.022) had significant positive effects on scanned litter size resulting in an additional 0.039 and 0.268 of a lamb scanned per 1kg ewe bodyweight and 1 BCS respectively. There was no significant effect of shearing weight or BCS on fleece weight or fibre diameter. The significant effects of ewe joining weight and BCS were qualified by significant interaction between them and vaccination treatment (P = 0.0062 and 0.0014 respectively). These interactions revealed that the beneficial effects of increased ewe joining weight on scanned litter size were only observed in the vaccinated treatments (Figure 13a). Indeed, there were no multiple births in the control treatment. While the beneficial effect of pre-joining BCS were only observed in the VACCMOX treated ewes (Figure 13b). There was also significant interaction between the effects of vaccination treatment and BCS at shearing on GFW (P=0.0102) with a positive association in unvaccinated ewes and a negative association in vaccinated ewes. Fitting ewe Barbervax® treatment, joining bodyweight/BCS and the interactions on pregnancy status of ewes (pregnant/empty) revealed no significant chi square result for any effect (P>0.05).

**Table 4.** Performance data (scanned litter size, fleece weight minus belly (kg) and mean fibre diameter) of Merino ewes under varying Barbervax® treatments (VACC, UNVACC, VACCMOX) imposed for 25 weeks commencing 3 weeks before joining. Shearing was at week 19.

| Variable /Source | Scanned litter size | | Fleece weight minus belly (kg) | | Mean fibre diameter (µm) | |
|---|---|---|---|---|---|---|
| Covariate* | Ewe weight (kg) | Ewe BCS (1-5) | Ewe weight (kg) | Ewe BCS (1-5) | Ewe weight (kg) | Ewe BCS (1-5) |
| Overall mean | 1.08±0.04 | | 3.34±0.05 | | 16.86±0.14 | |
| Vacc Treat | P = 0.20 | P =0.11 | P =0.91 | P =0.86 | P =0.63 | P =0.64 |
| VACC | 1.15±0.07[A] | 1.1±0.06[A] | 3.32±0.09[A] | 3.31±0.08[A] | 16.83±0.26[A] | 16.83±0.25[A] |
| UNVACC | 1.0±0.07[A] | 1.0±0.07[A] | 3.33±0.09[A] | 3.34±0.08[A] | 17.04±0.26[A] | 17.03±0.25[A] |
| VACCMOX | 1.15±0.06[A] | 1.19±0.06[A] | 3.37±0.08[A] | 3.38±0.08[A] | 16.70±0.24[A] | 16.71±0.24[A] |
| Ewe Weight* | **P =0.0002** | | P =0.76 | | P= 0.76 | |
| Slope | 0.039+0.010 | | 0.005+0.016 | | 0.014+0.045 | |
| Ewe BCS* | | **P =0.022** | | P = 0.63 | | P = 0.91 |
| Slope | | 0.268+0.114 | | -0.108+0.225 | | 0.074+0.685 |
| Ewe scanned litter size | | | P =0.50 | P =0.69 | P =0.78 | P =0.74 |
| Slope | | | 0.136+0.199 | 0.064+0.163 | -0.158+0.570 | -0.167+0.498 |
| Vacc x Weight | **P= 0.0062** | | P = 0.33 | | P =0.82 | |
| Vacc x BCS | | **P= 0.0014** | | **P = 0.0102** | | P =0.68 |

* Weight or BCS at joining for scanned litter size, at shearing for fleece weight and mean fibre diameter.

[a,b]Where means share a common letter in the superscript they do not differ significantly (P<0.05).

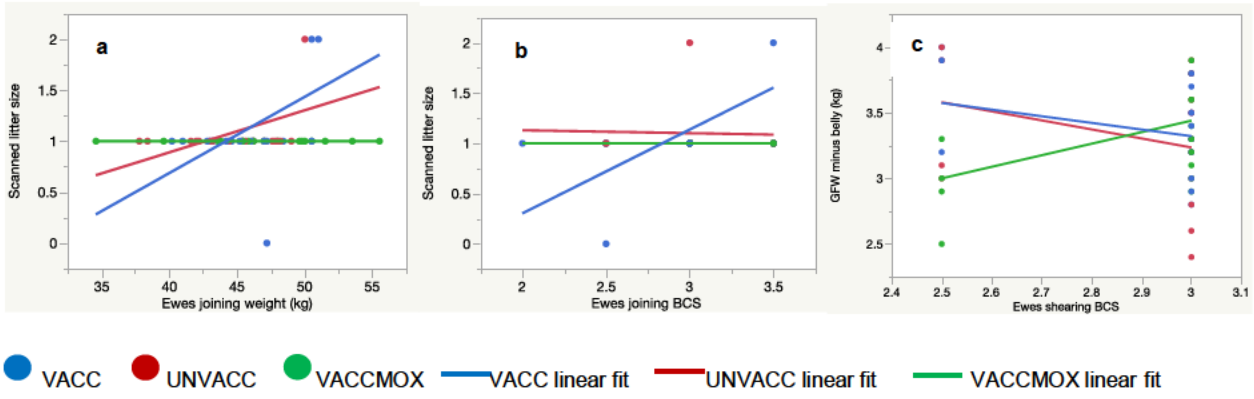


**Figure 13.** Plots illustrating the significant interaction between treatments and co-variates from Table 3.

# 4. Discussion

During phase 1 of the experiment, the final of four Barbervax® booster vaccinations to hogget ewes induced a small but sustained increase in Barbervax® antibody titre, with no significant effect on WEC. This resulted in very low protective indices of 18-32% during the 16-week post booster period relative to control ewes, under conditions of low *H. contortus* challenge. As no significant reduction in WEC was achieved, the hypothesis about the extended duration of protection following this booster vaccination could not be tested. The possible reasons for the failure of vaccinal protection are explored later in the discussion.

During phase 2 of the experiment, a pre-lambing booster Barbervax® vaccination resulted in a more marked increase in Barbervax® antibody titre with no difference between animals subjected to natural *H. contortus* challenge and those in which challenge was suppressed using sustained anthelmintic protection. WEC was not measured following this vaccination due to proximity to lambing and the pre-lambing anthelmintic treatment applied at the same time as the booster vaccination. These results do not support the hypothesis that natural exposure will enhance the antibody response to booster vaccinations, although this must be qualified by issues discussed more fully below.

Other findings of interest in this study include the lack of significant association between WEC and Barbervax® antibody titre, the apparent cross-reactivity between the ELISA detecting hidden antigen and the ELISA detecting pooled *H. contortus* L3 and the lack of a litter size response to heavier bodyweight in control ewes. These are discussed further below.

Some reasons for the failure to induce significant protection in phase 1 may include failure of correct vaccination, failure of Barbervax® to induce protection, sustained Barbervax® protection in the control ewes, low *H. contortus* burdens, inadequate experimental power to detect small reductions in WEC, running control and vaccinated ewes in the same mob or some combination of these.

In the experiment of Baker (2016) using 20 hogget ewes per treatment group, running concurrently with this experiment and vaccinated by the same personnel, a significant difference between WECs of vaccinated and control ewes following artificial challenge with *H. contortus* was observed at week 8. Significant differences in WEC between the two groups following natural challenge were also observed at 3 weeks post vaccination (Baker, 2016). This indicates both that it is unlikely there was failure of correct vaccination and that control ewes did not have a persistent high level of protection. This suggests that other reasons are responsible for the absence of significant protection following vaccination in this experiment.

*H. contortus* infection levels throughout our experiment were low to moderate (350-950 epg) primarily a result of the dry autumn conditions followed by cold winter temperatures, both of which have major negative effects on development of *H. contortus* from egg to L3 (O'Connor *et al.* 2006; Bailey *et al.* 2009a; Khadijah *et al.* 2013). Any lack of challenge would have been exacerbated by the anthelmintic treatment applied at week 4. The entire mob was treated when in retrospect, excluding the experimental ewes would have been preferable. During the Barbervax® hogget registration trials unvaccinated controls had average WEC's ranging from 1500 to over 2000 epg while vaccinated ewes have average WECs between 200 and 1000 epg indicating higher levels of natural infection (Smith 2014b). In any protection study, based on reduction of a disease indicator, low levels of the disease or indicator impair the ability to detect differences. The ewes in the present experiment may also have developed sufficient acquired immunity to suppress infection when challenge levels were low. The experimental animals had grazed naturally infected pastures 12 months prior to the beginning of the hogget booster vaccination course, enabling this to occur. Merino ewes 6-7 months old developed significant acquired immunity as seen by a reduction in worm burden and increase in haemagglutinating antibody during a 55-week long *H. contortus* infection (Adams and Beh 1981). However, following a re-infection, no further increase in antibody levels or increase in WEC compared to uninfected controls was observed, suggesting that once immunity was developed, the animal's antibody levels plateaued controlling further infection. A significant level of background immunity in the experimental flock is supported by the

relatively low WECs obtained following artificial *H. contortus* challenge with 5000 L3 to ewes in the same mob in the study of Baker (2016).

Due to the restriction of working with a commercial property, and other resource constraints there was a limited number of animals available for use in the experiment. Experiments with similar or smaller numbers of animals have found significant differences in WEC between treatments (Datta *et al.* 1998; Doyle *et al.* 2011; Baker 2016) so it wasn't unreasonable to expect significant WEC differences. However, power of experiment calculations using WEC variance data from the experiment, revealed the number of animals we used per group (n=20) would detect a real reduction in WEC of 50% from 1000 to 500 epg at the $P<0.05$ level in only 50% of cases. To increase power from 50% to 80% power, the number of animals needed was >41. The numbers of animals used in the registration trials for yearlings and peri-parturient ewes ranged from 25 to 60, with two studies on lactating ewes using vaccinated groups of 16 and 17 ewes respectively (Smith 2014b, 2014a).

Throughout this experiment controls and vaccinated grazed together. Vaccination protection trials typically compare vaccinated and unvaccinated animals given the same level of challenge and that was the design chosen for this experiment. Grazing the treatment groups together ensured that all animals were subject to the same level of challenge, nutrition and other environmental effects and enabled correct analysis of the data with animal as the replicate, rather than paddock mean. During the Barbervax® registration trials for lambs, hoggets and peri-parturient ewes, most experiments also included treatment groups that grazed together (Smith 2014a, 2014b). While testing the vaccine under conditions of uniform challenge this way has many advantages, it does not capture the full consequences of vaccination of whole mobs, in which reductions in WEC lead to resultant reductions in challenge amplifying the protective effects of the vaccine over time. Thus, in the present experiment, grazing together would have likely reduced the magnitude of differences between treatment groups, relative to grazing them separately and thus reduced the apparent protective effect.

The results of phase 2 of the experiment do not support the hypothesis that response to booster challenge, as assessed by Barbervax® antibody levels will be enhanced by constant natural challenge prior to a pre-lambing vaccination. Both vaccinated treatments had a marked response to booster vaccination irrespective of whether natural challenge was suppressed or not. Interpretation of the results of phase two needs to take into account the finding of Kelly *et al.* (2012) that despite the administration of a controlled release anthelmintic capsule (CRC), larval challenge with *T. colubriformis* was still immunogenic, significantly increasing both total antibody

and eosinophil counts compared to uninfected and uninfected-CRC animals. The VACCMOX ewes in this experiment were also ingesting larvae and most likely mounting an immune response despite suppression of further development. Thus, the experimental model chosen may not have enabled a true test of the hypothesis.

The effect of the vaccination treatments in phase 2 on WEC could not be measured due to the application pre-lambing anthelmintic treatment at the same time as the booster vaccination. This is in line with Congi farm practice and industry and Barbervax® recommendations. In the registration trials using peri-parturient and lactating ewes, reductions in WEC of previously vaccinated ewes given a pre-lambing boost ranged between 55-73%. The antibody titres in our experiment following a pre-lambing boost (>100,000) were higher than in the registration trials (~7000-25,000) so it might be inferred that WEC would also be reduced as it was in those trials, but this is purely speculative.

No significant relationship was found between WEC and Barbervax® specific antibody titre, and consequently antibody titre was not a good measure of vaccinal protection. The reasons for the poor association are unclear. The ELISA was completed closely following the method provided by Moredun. Baker (2016) also found no significant correlation between Barbervax® specific titre and protection at any point, however the relationships were in the expected direction, increased titres were associated with lower WEC. In a vaccine therapeutic trial, housed 9-month old and twice Barbervax® vaccinated lambs were given a bolus dose of 5000 *H. contortus* L3. By 16 days post V2, significant correlations found between antibody titre and WEC ($P<0.01$, $R^2=0.327$) and antibody titre and worms (<0.001, $R^2= 0.478$) (Smith Unpublished-b). LeJambre *et al.* (2008) also showed a significant inverse relationship exists between antibody titre and WEC of sheep vaccinated with Barbervax® antigens. This appears to be the only regression analysis completed during Barbervax® vaccine trials, with none of the registration trials formally reporting an association. Rather a descriptive approach was used to describe the association between WEC and antibody in vaccinated animals (Smith 2014b, 2014a). Other studies have also shown strong associations between increased immunoglobulins, primarily serum IgA, IgG, IgE and faecal IgA, with decreased WEC's, worm burden and worm length during *H. contortus* and *O. circumincta* infections (Gill *et al.* 1993a; Stear *et al.* 1995; Kooyman *et al.* 1997a; Amarante *et al.* 2005). Furthermore, the relaxation and decrease of immunity prior to lambing documented in both Barbervax® and *H. contortus* antibody analyses in phase 2 follows similar patterns as described by Sykes *et al.* (2007) and Beasley *et al.* (2010), with the peri-parturient relaxation of immunity prior to lambing. The decrease in immunity coincides with a known increase in WEC, and despite

having no WEC data for phase 2, the rise is well-documented (O'Sullivan and Donald 1973; Woolaston 1992; Kahn *et al.* 1999; Beasley *et al.* 2010).

Interestingly, titres from the *H. contortus* antigens ELISA increased significantly with Barbervax® vaccination with a significant correlation ($P<0.05$) between the two titres. This indicates cross-reaction between the two ELISA tests. The Barbervax® vaccine antigen is the same as that used in the ELISA and comprises whole *H. contortus* with purification to select the H11 and H-gal-GP proteins. The *H. contortu*s ELISA has purified whole L3 extract as the antigen and so it is not surprising that there may be some shared antigens between the two. However, given the poor association between antibody titres and WEC, the utility of either measurement is unclear.

The protective index calculated during the experiment ranged between 16 and 32%, which is markedly less than the 55-83% reduction in *H. contortus* egg counts that was provided by the vaccine in the hogget registration trials (Smith 2014b). Greater reductions were observed at the CSIRO (73-91%) trial site compared to VHR (55-84%) during the lamb and hogget registration trials with vaccinated and unvaccinated animals grazed separately, which were considered due to lusher pasture and more favourable climatic conditions resulting in higher *H. contortus* WEC counts at VHR's trial sites (Smith 2014a). Based on this it could be expected that greater or equal reductions would have been observed in our experiment, as the dry and cold climatic conditions experienced were unfavourable for *H. contortus* development (O'Connor *et al.* 2006). However, they are favourable for survival of existing L3 on pasture (O'Connor *et al.* 2006).

Non-responders based on WEC were determined during the registration trials using the same method as the present study, however few animals were classified as non-responders. It is unclear in the registration trials as to whether they were selected based on a single or multiple measures. In our experiment the proportion of non-responders each time-point, ranged between 42% and 89% with little consistency of status between samplings. Baker (2016) also found a larger number of non-responders in their trial compared to the registration trials with between 42% and 63% of animals classified as non-responders. The obvious cause for the large numbers of non-responders in these studies was the limited impact of vaccination on WEC overall.

Finally, Barbervax® treatment had no overall negative or positive effects on ewe performance measures, albeit with small numbers of replicates. Again, this is most likely due to the limited impact on WEC. However, the data revealed some interesting significant interactions. Interestingly a positive association between pre-joining weight and scanned litter size was only observed in vaccinated ewes, not controls for which no litter size above 1 was recorded. The

reasons for this are not clear. On the other hand, a positive association between BCS at shearing and fleece weight was only observed in unvaccinated ewes. Again, the reasons are not clear. No previous Barbervax® trials have looked at the effect of treatment on animal performance, so this data is novel and further research is required for firm conclusions to be drawn.

# 5. Conclusion

In conclusion, the hypothesis regarding the duration of vaccinal protection in hoggets following a full course of vaccination in year 2 persists longer than 6 weeks could not be tested as no significant effects of vaccination on WEC were observed and only minor effects on Barbervax® ELISA titres. Many factors may have contributed to this, most notably the comparatively low WEC counts induced by the level of natural challenge, the numbers of replicates (20) and the grazing together of the vaccinated and unvaccinated animals. The second hypothesis that natural challenge would enhance Barbervax® antibody titres following booster vaccination was not supported by the data, but again the hypothesis may not have been properly tested with the experimental model used, since suppressive anthelmintic treatment does not eliminate the immune response to L3 challenge. Interestingly, strong cross-reaction between the Barbervax® and *H. contortus* ELISA tests was observed, indicating that they may be insufficient to discriminate against naturally acquired immunity, and that acquired following vaccination with Barbervax®. Further research with a greater number of animals, a greater *H. contortus* challenge and treatments grazed simultaneously would be beneficial to add to these findings. Future experiments to test whether natural challenge enhances immunity induced by Barbervax® will require a different experimental model and use of WEC as an endpoint.

# CHAPTER 3

# Changing the Barbervax® vaccination regimen in lambs to evoke the same immunological protection against *Haemonchus contortus*, and effect on performance

## 1. Introduction

The inability of lambs to mount an effective immune response to *H. contortus* under 6 months of age is due to a delay in development of acquired immunity to this parasite (Colditz *et al.* 1996). Older lambs develop partial immunity provided they have continuous exposure to the parasite (Schallig 2000). The hypo-responsiveness in young lambs under 8 months is characterised by lambs having fewer CD4+ and CD8+ lymphocytes, less γ-interferon, low eosinophilia and a smaller antibody response to infection (Watson *et al.* 1994; Colditz *et al.* 1996; Schallig 2000; Alba-Hurtado and Munoz-Guzman 2013). One-month-old Merino lambs trickle infected with 10,000 L3 *H. contortus* over 7 weeks were anthelmintic treated and then re-infected with a bolus dose. There were no significant differences in IgG, IgM or IgE relative to uninfected controls, despite a 20-56% reduction in WEC, although not significant (McClure *et al.* 1998). In comparison, lambs infected with *T. colubriformis* had significantly increased IgG, IgE and IgA titres and a simultaneous 44-65% reduction in WEC evident for 6 weeks of infection (McClure *et al.* 1998). It was apparent that lambs in this age group were very inefficient in recognising *H. contortus* in contrast to *T. colubriformis* (McClure *et al.* 1998). This hypo-responsiveness to *H. contortus* infection is a significant barrier to the development of effective vaccination against *H. contortus* in young lambs.

Two approaches to overcome this barrier were the use of a range of adjuvants to stimulate and prolong the immune response and the use of hidden antigens extracted from gut microvilli in *H. contortus.* Because immune responsiveness is present in young lambs to antigens such as those present in routine lamb vaccines (clostridial toxoids and the caseous lymphadenitis bacterin) exposure to such hidden antigens has good prospects for inducing an effective immune response in contrast to the natural excretory and surface antigens that the host is exposed to during natural infection.

Vaccination with a DDA adjuvant and two ES *H. contortus* antigens failed to reduce WEC in 3-month old Texel lambs but was effective in 8-9 month old lambs, inducing 83-99.9% reduction in WEC for 3-4 weeks. However this did not stimulate an IgG response and only minor IgE and IgA antibody responses (Schallig *et al.* 1994; Kooyman *et al.* 2000; Vervelde *et al.* 2001). Use of Alhydrogel and Freund's adjuvants with *H. contortus* antigens in 3-month old Merino lambs stimulated an antigen-specific antibody response for 3-5 weeks and reduced WEC by 56-70% in (Jacobs *et al.* 1999; Smith *et al.* 1999). Whilst repeat experiments using these adjuvants and antigens were not successful, use of QuilA and hidden antigens were shown to extend the immune response up to 6-weeks and improve WEC reduction (Le Jambre *et al.* 2008; Smith *et al.* 2013; Smith 2014a).

A dose response trial for Barbervax® using 5 µg antigen in QuilA determined that vaccination was required every 6-weeks to maintain immunity, assessed by WEC (Smith Unpublished-b). Lambs vaccinated with QuilA and a total of 5 µg of two hidden antigens, H11 and H-gal-GP, had a mean protection level of approximately 90%. Attempts at increasing the 6-week duration of immunity by increasing the antigen dose to 100 µg of H11 and H-gal-GP antigen was trialled in lambs. Vaccinated lambs had a significant reduction (~75%) in WEC with protective immunity lasting up to 7 weeks (Le Jambre *et al.* 2008), 1 week longer than that induced by QuilA adjuvant and 5 µg antigen. However, in a separate experiment, lambs given 50 µg of hidden antigen H-gal-GP, had a lower reduction in WEC (57%) (Basetto *et al.* 2014) than lambs given 5 µg (71-91%) and duration of protection again lasted 6 weeks (Smith 2014a). It is unclear why lambs had varied responses to varying amounts of antigen, but 5ug was selected as the dose to use in the final commercial product. Furthermore it was determined that lambs required 3 priming doses before significant immunity was obtained. Further booster vaccinations were required every 6 weeks, with decreases in antibody levels coinciding with increases in WEC (Smith 2014a).

Based on these findings the Barbervax® on-label instructions are to administer the first priming (P) vaccination at lamb marking (P1), with a second priming vaccination 3-4 weeks later (P2), and final priming vaccination (P3) a further 3-4 weeks later at lamb weaning. The following 3 'booster' vaccinations boosters' (B1, B2 and B3) are administered at 6-weekly intervals to maintain immunity during the summer season. Marking and weaning are both common husbandry practices requiring a yarding and associated muster, however, P2 requires an extra muster and yarding that is timely, costly and increases the risk of mis-mothering as lambs are still at foot of their dam's. Mis-mothering, particularly among Merinos, is a common reason for lamb mortality (Hinch and Brien 2014) and 25% of neonatal lamb deaths are thought to be caused by this

(Refshauge *et al.* 2015). On the property this experiment was conducted on, the cost to muster a mob of 2000 ewes plus 1800 lambs and time spent in the yards amounts to $0.53/lamb. This cost involves the use of 4 staff, a 9-hour working day and machinery costs equaling a daily total of approximately $950.00 (Uren 2018). Feedback from commercial sheep producers using the vaccine, find this extra muster to be problematic. Simply omitting the vaccination runs the risk of exceeding the recommended priming interval of 3-4 weeks and delaying the onset of protective immunity. For producers, the delay in immunity would be preferable than the extra costs and welfare issues associated with the extra muster, particularly as a strategic anthelmintic treatment is typically administered at weaning, providing a measure of chemical protection. Commonly the marking to weaning interval on-farm is ~8 weeks. This experiment tested the general hypothesis that by reducing the time interval between marking and weaning to 6 weeks, and providing a double dose of vaccine at marking, weaning or both, could remove the requirement for P2 between marking and weaning.

# 2. Materials and Methods

## 2.1. Experimental Site

The experiment was conducted at TA Fields 'Congi Station' near Woolbrook, NSW (latitude: 30.91$^{o}$S, Longitude: 151.29$^{o}$E, Altitude: approx. 975 m), from November 2016 to early March 2017 and was approved by the University of New England's Animal Ethics Committee (Approval number: AEC16-048). The region experiences summer dominant rainfall, with major rain events occurring during December, January and February, and coincides with increased *H. contortus* burdens and haemonchosis outbreaks. Lambs grazed native pastures for the duration of the experiment and were housed in a 71-hectare paddock with their dam's until weaning, where they were subjected to parasite challenge, predominately [99-100% (Table 2)] *H. contortus*. All lambs were managed by farm personnel according to 'Congi' farm best practice and kept separate to other flocks. Weather and pasture measurements were obtained for the duration of the experiment and are reported.

## 2.2. Experimental design and application of treatments

The experiment used 175 lambs born to the maiden ewe flock described in chapter 2. Lambing commenced on 11$^{th}$ September 2016 and concluded 16$^{th}$ October 2016. At marking (8$^{th}$ November 2016, week -6), whilst lambs were still at foot, they were randomly split into five treatment groups (n=35). Primer vaccinations include P1 at marking (week -6), P2 3-weeks post marking and P3 at

weaning (week 0). The treatment groups are summarised in Table 1 and each treatment group will be referred to by their treatment code.

**Table 1.** Lamb treatment groups, Barbervax® primer vaccination protocols and time between treatments.

| **Treatment code** | **Barbervax® primer vaccinations (✓ - single dose, ✓✓ - double dose)** | | | **Vacc. Interval (wk)** | **Comment** |
|---|---|---|---|---|---|
| | **Marking** | **3 wk. post marking** | **Weaning** | | |
| | *P1* | *P2* | *P3* | | |
| **UV** | - | - | - | - | Unvaccinated control |
| **VP** | ✓ | ✓ | ✓ | 3 | Recommended protocol (vaccinated control) |
| **MddW** | ✓✓ | - | ✓ | 6 | Double then single dose |
| **MWdd** | ✓ | - | ✓✓ | 6 | Single then double dose |
| **MddWdd** | ✓✓ | - | ✓✓ | 6 | Double then double dose |

A single 1 mL dose of Barbervax® was injected subcutaneously under the ear on the right side. A double dose was administered by giving a single dose on both sides of the animal. Control lambs were given 1 mL of saline solution in the same administration as Barbervax® treatment. Lambs were also vaccinated against contagious ecthyma (Scabiguard®) five clostridial diseases and contagious lymphadenitis (Glanvac 6 in1®) and given preventive blowfly treatment using dicyclanil pour on (Clik®) at marking and weaning. All lambs including the controls were treated with an anthelmintic [TriGuard® (Abamectin 1.0 g/L, Oxfendazole 22.7 g/L and Levamisole 33.9 g/L)] at weaning and at the experiment conclusion, with dose based on the heaviest animal. All vaccinated treatments were given 1 mL Barbervax® booster vaccinations at weeks 5 (B1) and 10 (B2) post weaning. Multiple batches were used throughout the experiment and are as follows; at marking (Batch No: 10, Exp: Jan 2017), Week -3 (Batch No: 9, Exp: Feb 2017), weaning (Batch No: 10, Exp: Feb 2017), Booster 1 (Batch No: 11/1, Exp: Feb 2017) and Booster 2 (Batch No: 12, Exp: Jan 2018).

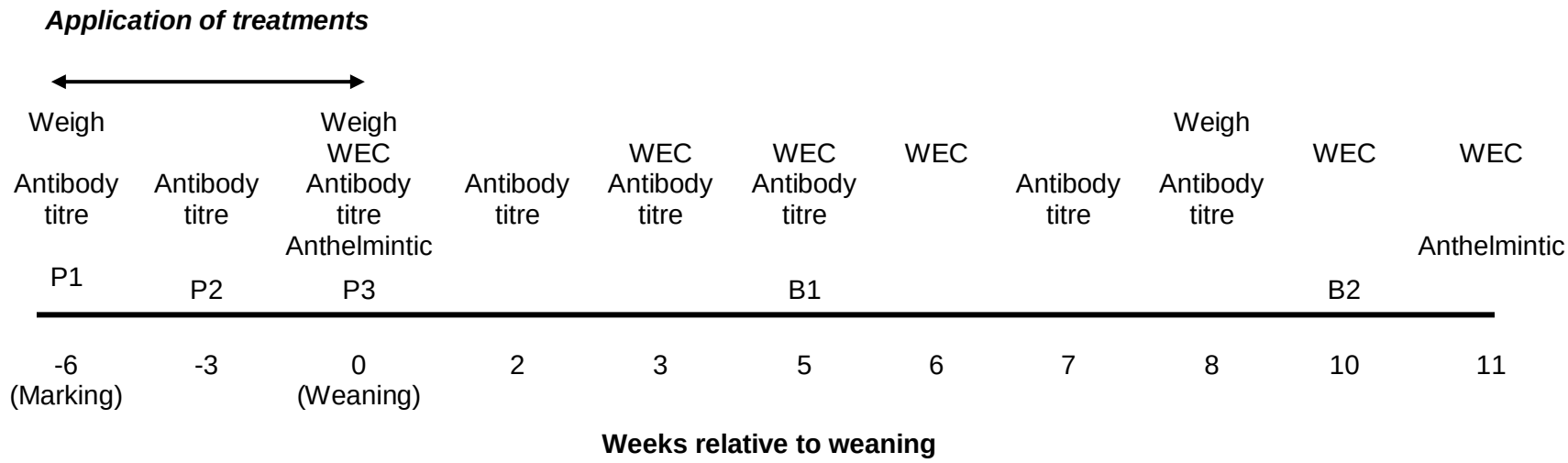


**Figure 1.** Timeline of treatments and sample collections for all treatment groups (not to scale)

## 2.3. Animal Measurements

### 2.3.1. Sample collection

#### *2.3.1.1. Blood samples*

Blood samples were collected from all lambs via jugular venepuncture into 1 x 5 mL SST Vacutainer® tubes at lamb marking (week -6), -3 weeks and at weaning (week 0). Animals were then bled at intervals as outlined in Figure 1. Samples were spun on day of collection at 2763 x gravity at 4 $^{o}$C for 15 minutes using the Beckham Coulter Allegra X-15R Centrifuge and serum were stored in 2 replicated 5 mL pots at -20 $^{o}$C until analysis.

#### *2.3.1.2. Faecal samples*

Individual faecal samples were collected per rectum using new disposable gloves for each collection. Faecal samples were collected at weaning and intervals as outlined in Figure 1.

#### *2.3.1.3. Bodyweight*

Individual lamb bodyweights were collected using Ruddweigh scales at marking (week -6), weaning (week -3) and 8 weeks post-weaning (week 8) (Figure 1). All animals were weighed off feed.

### 2.3.2. Laboratory Methods

#### *2.3.2.1. Enzyme Linked Immuno-absorbent Assay (ELISA) – Barbervax® antigens*

Analysis of lamb serum samples were done using the same Barbervax® ELISA method as described in chapter 2, section 2.3.3.1.

#### *2.3.2.2. Worm Egg Counts*

Analysis for larval differentiation and WEC were done using the method described in chapter 2, section 2.3.3.3.

## 2.4. Weather Data

Weather data are summarised in Table 2 and reflect typical summer values in this summer-rainfall environment. Figure 3 shows a paddock photo for each month of the experiment revealing generally favourable pasture conditions.

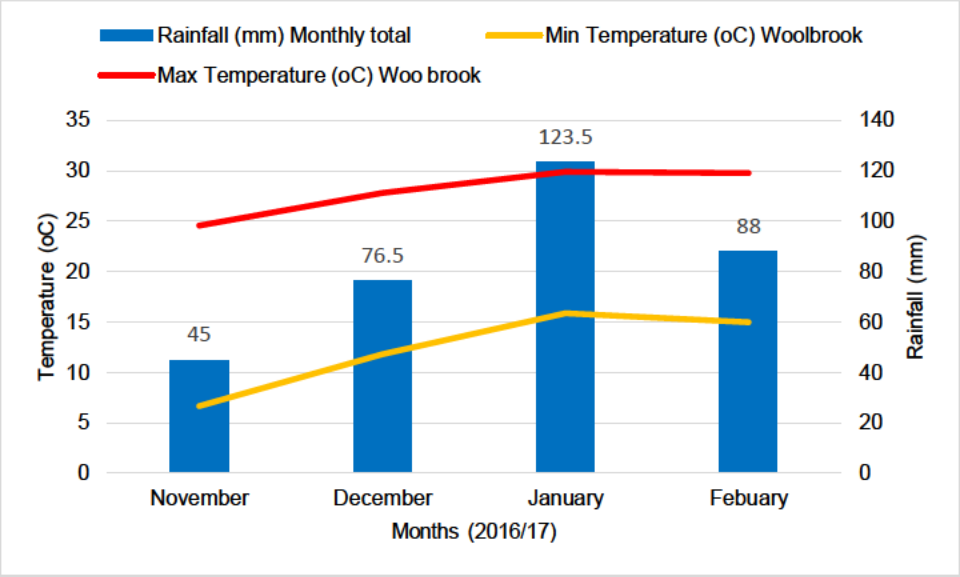


**Figure 2.** Monthly rainfall records from 'Congi Station' and temperature records (BOM) for Woolbrook for the duration of the experiment.

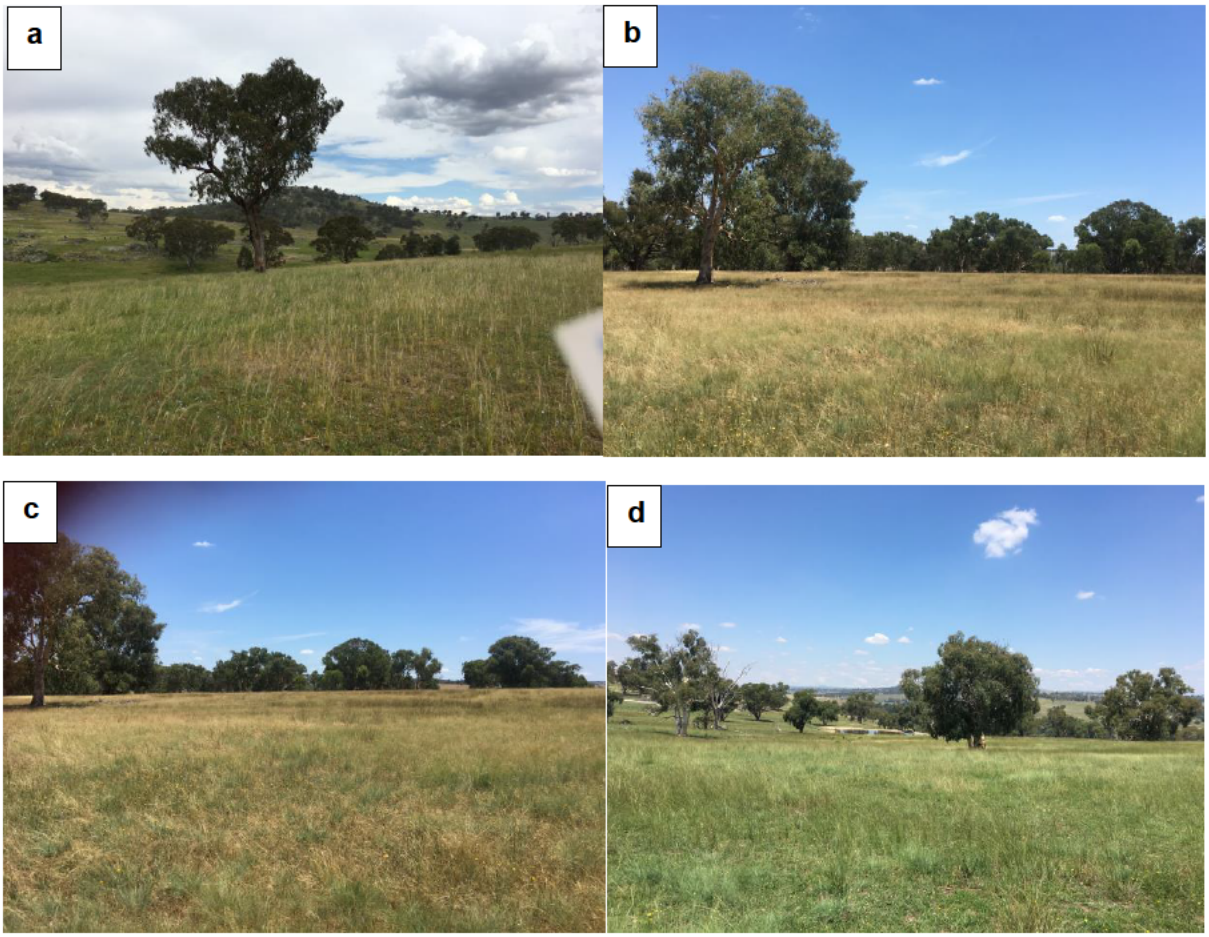


**Figure 3.** Photos from lamb paddocks during (a) November, (b) December, (c) January and (d) February.

## 2.5. Data and statistical analysis

Data were analysed using JMP 13.1.0 software (SAS Institute Inc). WEC data were cube root transformed and Barbervax® antibody titre Log10 transformed to remove association between the variance and the mean. Lamb bodyweight was analysed within time period fitting the effect of treatment group with covariates and interactions where appropriate. For variables WEC and antibody titres repeated measures were taken into account by fitting a mixed restricted maximum likelihood model (REML) with individual sheep as a random factor and treatment, time (week) and their interaction fitted as fixed effects. Association between measured variables (eg. WEC and antibody titre) were explored using curve fitting, correlation and linear regression. A P-value of $<0.05$ was used to determine significance. The significance of differences between means within a significant treatment was determined using Student's t-test. Data are presented as least squares means (LSM) ± standard error of the mean (SE). Untransformed WEC data are also reported to

show the actual epidemiological consequences in terms of egg contamination of pastures and to enable comparison with normal diagnostic measures, which are not transformed. Untransformed WEC values are used by graziers and advisors for worm control decisions. A total of 9 lambs died prior to weaning throughout the experiment and were excluded from the analyses. No outliers or other animals were removed from the analyses.

Non-responders to the vaccine were defined as those whose WEC was above the lower 95% confidence interval of the control group. This is the method used to determine outliers in the Barbervax® registration trials. This concept was expanded to include the response for Barbervax® log10 antibody titre with non-responders defined as those whose log10 Barbervax® antibody titres were below the upper 95% confidence interval of the control group. The protective Index (PI) for Barbervax® vaccination was calculated using the following equation:

$$PI = \frac{Mean\ unvaccinated\ group\ WEC\ (epg) - Vaccinated\ individual\ WEC\ (epg)}{Mean\ unvaccinated\ group\ WEC\ (epg)} \times 100$$

# 3. Results

## 3.1. Antibody Titres – Barbervax® (BV) antigens

Vaccination treatment (P<0.0001), time (P<0.0001) and the interaction between them (P<0.0001) were all highly significant. Within the vaccination treatment the VP, the three alternate treatments (MddW, MWdd, MddWdd) and UV were significantly different from each other, however no difference existed between the three alternative treatments.

BV antibody titres of the UV ewes continued to decrease for the duration of the experiment. Increases in BV titre were observed following the 2$^{nd}$ vaccination in all vaccinated treatments (week -3 in the VP and week and at week 0 in the alternative treatments) with the magnitude of the response much greater in those receiving the 2$^{nd}$ vaccination at weaning. Treatments did not differ significantly until weaning (week 0), where VP BV titres were significantly higher than the remaining 4 treatments (P= 0.0004). From weaning antibody titres of the 3-alternate treatment increased to that of the VP and all vaccinated treatments were significantly different to the UV. There was no significant difference between the vaccinated treatments until the end of the blood sampling at week 8 after weaning (P<0.0001) (Figure 4).

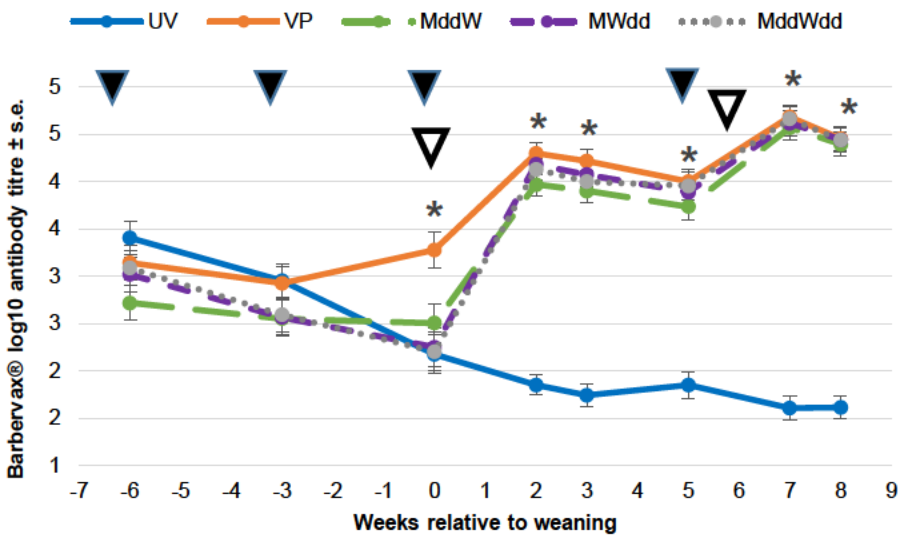


**Figure 4**. Log10 transformed Barbervax® antibody titres (ls mean ± s.e.) from Merino lambs subjected to a natural *H. contortus* challenge and differing treatment regimens; unvaccinated controls (UV), recommended protocol [vaccinated control (VP)], double then single dose (MddW), single then double dose (MWdd) and a double then double dose (MddWdd). *means within time-points differ significantly (P<0.05). Solid filled triangles: Barbervax® vaccination (to relevant treatment groups); open triangles: anthelmintic treatments (to all treatment groups).

## 3.2. Faecal worm egg count and larval differentiation

The effects of vaccination treatment (P<0.0001), week (P<0.0001) and the interaction between them (P=0.0024) on WEC were all highly significant. Vaccinated treatments (VP, MddW, MWdd, MddWdd) had significantly lower overall WEC than the UV, with no significant difference between the vaccinated treatments at any time point.

The WEC of all 5 treatment groups decreased immediately after weaning (week 0) as a result of the weaning anthelmintic treatment before increasing sharply between weeks 3 and 5. Vaccinated and unvaccinated treatments differed significantly at weeks 5 (P=0.0150), 6 (P=0.0010), 10 (P=0.0003) and 11 (P<0.0001) (Figure 5a). Following the first booster vaccination at week 5, WEC decreased in the vaccinated animals at week 6 when an anthelmintic treatment was applied to all animals. WEC increased from week 6 to week 10, when a second booster vaccination was given. Unlike the booster at week 5, this failed to reduce WEC which continued to increase to the last sampling at week 11 reaching an untransformed average of 15,500 and 6000 epg in the unvaccinated and vaccinated treatments respectively (Figure 5b). The

untransformed WEC data revealed that reductions in the vaccinated WEC ranged from 67 epg at week 3, to >8800 epg at week 17 (Figure 5b). Species differentiation showed *H. contortus* to be the dominant species for the entirety of experimentation (Table 2).

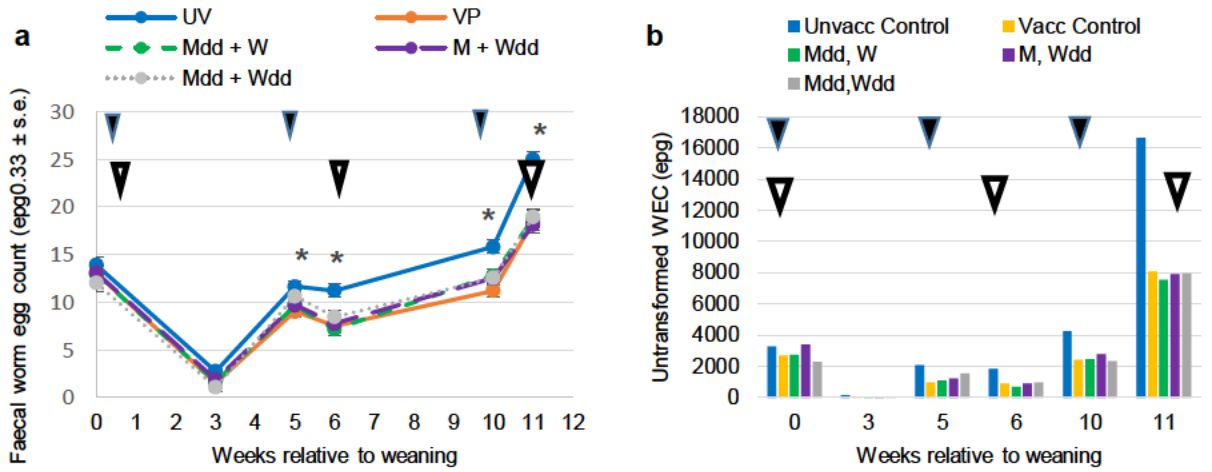


**Figure 5**. (a) Cube-root transformed worm egg counts (ls mean ± s.e.) and (b) untransformed worm egg counts (epg) taken from Merino lambs subjected to a natural *H. contortus* challenge and differing treatment regimens; unvaccinated controls (UV), recommended protocol [vaccinated control (VP)], double then single dose (MddW), single then double dose (MWdd) and a double then double dose (MddWdd). *means within time-points differ significantly ($P<0.05$). Solid filled triangles: Barbervax® vaccination (to relevant treatment groups); open triangles: anthelmintic treatments (to all treatment groups).

**Table 2.** Larval differentiation for percentage of *H. contortus* and other species from lambs for weeks 0, 3, 5, 6, 10 and 11 post-weaning.

| Wk | Species | Percentage of each species | | | | |
|---|---|---|---|---|---|---|
| | | UV | VP | MWdd | MddW | MddWdd |
| 0 | *H. contortus* | 100 | 99 | 100 | 99 | 100 |
| | Other | 0 | 1 | 0 | 1 | 0 |
| 3 | *H. contortus* | 100 | 100 | 100 | 100 | 99 |
| | Other | 0 | 0 | 0 | 0 | 1 |
| 5 | *H. contortus* | 100 | 100 | 100 | 100 | 100 |
| | Other | 0 | 0 | 0 | 0 | 0 |
| 6 | *H. contortus* | 100 | 100 | 100 | 100 | 100 |
| | Other | 0 | 0 | 0 | 0 | 0 |
| 10 | *H. contortus* | 100 | 100 | 100 | 100 | 100 |
| | Other | 0 | 0 | 0 | 0 | 0 |
| 11 | *H. contortus* | 100 | 100 | 100 | 100 | 100 |
| | Other | 0 | 0 | 0 | 0 | 0 |

## 3.3 Protective efficacy of Barbervax®

Reduction in WEC ranged from 14-64% in the vaccinated treatments. Due to the anthelmintic treatment at weaning (week 0), the protective index at week 3 may be compromised. However, the VP protective index ranged from 44 to 53% between weeks 5 and 11. The protective index of the remaining treatments had a greater range, particularly the MddWdd treatment which ranged between 27 to 52%. Combining the three alternate treatments the PI between these and the VP were similar (Table 3). No significant differences between protective indexes of vaccinated treatments were observed at any time-point ($P>0.05$).

**Table 3**. Mean protective Index calculated from individual lambs (shown in percentage) provided to the vaccinated controls and altered vaccination treatments for the duration of the experiment.

| Treatment | Weeks post weaning (% WEC reduction) | | | | | |
|---|---|---|---|---|---|---|
| | **0** | **3** | **5*** | **6** | **10*** | **11** |
| ***P-value*** | *0.66* | *0.99* | *0.36* | *0.94* | *0.93* | *1.00* |
| **VP** | 18 ±14.42 | 64 ±19.81 | 53 ±8.58 | 52 ±9.77 | 44 ±7.99 | 53 ±6.12 |
| **MWdd** | -4 ±12.85 | 44 ±19.51 | 41 ±8.71 | 52 ±9.77 | 35 ±7.86 | 52 ±6.21 |
| **MddW** | 16 ±12.85 | 60 ±19.51 | 48 ±8.85 | 62 ±9.62 | 42 ±7.74 | 55 ±6.31 |
| **MddWdd** | 29 ±13.57 | 61 ±19.51 | 27 ±8.71 | 47 ±10.09 | 47 ±7.74 | 52 ±6.12 |
| **MWdd,MddW,MddWdd** | 14 ±7.55 | 55 ±11.26 | 39 ±5.06 | 54 ±5.67 | 42 ±4.49 | 53 ±3.59 |
| **All vaccinated treatments** | 14 ±6.69 | 57 ±9.79 | 42 ±4.36 | 54 ±4.90 | 42 ±3.92 | 53 ±3.1 |

* Barbervax® booster vaccination

## 3.4 Relationship between WEC and Barbervax® antibody titre

There was a significant linear negative association between WEC and Barbervax® specific antibody titre at weeks 3 (P =0.0008) and 5 (P = 0.002), but not at week 0 (weaning) (P=0.14) (Figure 6). However, $R^2$ values revealed that the antibody titres explained a low proportion of the variation in WEC ($R^2$=0.02-0.06).

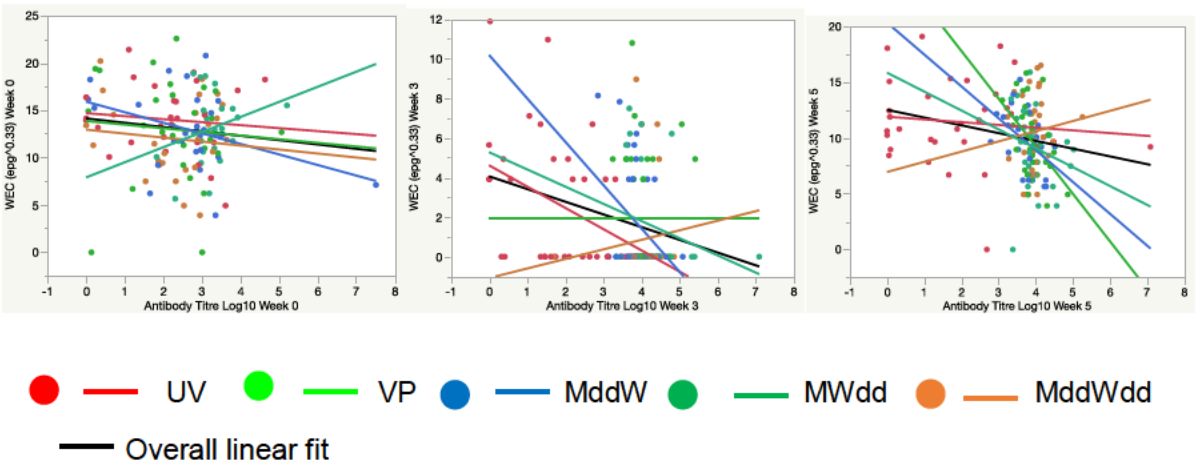


**Figure 6**. Bivariate fit of unvaccinated controls (UV), recommended protocol [vaccinated control (VP)], double then single dose (MddW), single then double dose (MWdd) and a double then double dose (MddWdd) Barbervax® Log10 antibody titre and faecal worm egg count at weeks 0 (left), 3 (middle) and 5 (right). Each individual point is a single animal, colour markers indicate the treatment, and lines indicating the linear fits, overall and within treatments.

## 3.5 Non-Responders

There was 1 non-responder based on antibody titre at week 3, totaling 8%. There were no other non-responders based on titre at any other week (Table 4). The percentage of non-responders based on WEC ranged from 21% to 38% at different time points. A significant effect of treatment on Barbervax® antibody titres of non-responders was only observed at week 0 relative to weaning ($P<0.0001$) at which time the lambs given the recommended protocol and had received 2 prior vaccinations had a lower proportion of non-responders (3/30) than the other vaccinated treatments combined (48/94). The percentage of non-responders declined at the samplings following booster vaccinations at week 5 and 10 (Table 4). There were no significant effects of treatment on WEC non-responders at any time point (P range 0.19 - 0.86).

As shown in Figure 7 non-responders based on WEC were not consistently non-responders, rather different animals were responding to vaccination at different times. This calls into question whether the term refers to a particular trait of the animal. The proportion of lambs which non-responded 4 or 5 times and may represent true non-responders was only 11 (6.6 %) lambs. On the other hand, 42 lambs (25 %) were classified as responders to vaccination at all time-points based on WEC at post-weaning (Figure 7). It was apparent that lambs which non-responded between 1 and 3 times did so in no particular order. Furthermore, lambs classified as non-responders post weaning were 71% likely to respond post the first booster vaccination (week 5),

whilst lambs who responded post-weaning, were 83.3% likely to respond in the second period and this trended towards significance ($\chi^2$ P=0.07) (Table 5).

**Table 4.** Number and percentage of 'non-responders' to the vaccine based on WEC and antibody titre.

| | WEC | Antibody Titre |
|---|---|---|
| **Week 3** | 35 (27%) | 1 (8%) |
| **Week 5** | 48 (38%) | 0 (0%) |
| **Week 6** | 40 (31%) | 0 (0%) |
| **Week 10** | 39 (30%) | 0 (0%) |
| **Week 11** | 27 (21%) | 0 (0%) |

**Table 5.** Concordance of non-responder (NR) and responder (R) status following booster vaccinations at weaning and 5 weeks later.

| | | Post first booster vaccination (week 6 to week 11) | | |
|---|---|---|---|---|
| **Post-weaning vaccination** | | **NR** | **R** | **Total** |
| | **NR** | 15 | 36 | 51 |
| | **R** | 16 | 80 | 96 |
| | **Total** | 31 | 116 | 147 |

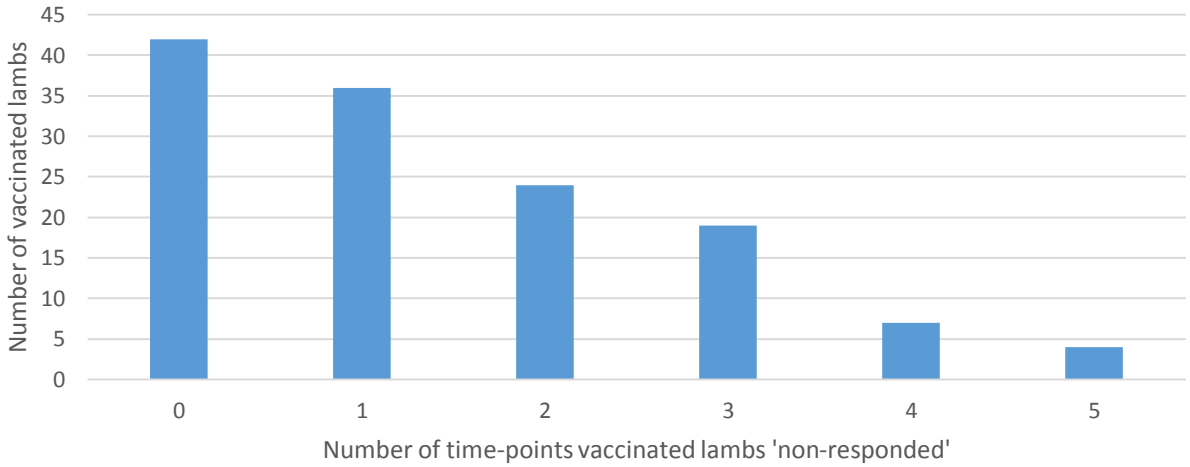


**Figure 7**. Number of time-points and lambs who 'non-responded' based on WEC

## 3.6 Body Weight

Body weight of all lambs increased over the duration of the experiment (Table 6). No significant differences were observed in bodyweights between treatments at any time point (P>0.05). No significant differences were observed in weight gain between treatments throughout the experiment (P>0.05) (Table 6).

The effect of Barbervax® treatment was non-significant for bodyweight at weaning and week 8 (Table 7). The covariate of lamb marking bodyweight was significant and had positive effects on lamb weaning (P<0.0001) and week 8 (P<0.0001) bodyweights resulting in an additional 1.05kg and 1.06kg respectively. Lamb rear type (single/twin) was fitted as an effect in the model, however it was not significant and removed from the analysis.

**Table 6.** Post marking weight gain of lambs in the different treatment groups (kg/day ± s.e.) over the duration of the experiment (week -6 to week 8), from marking (week -6) to weaning (week 0) and from weaning (week 0) to week 8. Average bodyweights (kg ± s.e.) at marking and week 8 in the different treatment groups. There were no significant treatment effects for any of these variables.

| | **UV** | **VP** | **MWdd** | **MddW** | **MddWdd** |
|---|---|---|---|---|---|
| ***Average bodyweight at marking (kg)*** | *16.98±0.529* | *17.76±0.534* | *18.02±0.457* | *17.73±0.517* | *16.68±0.452* |
| **Week -6 to week 8 (kg/day)** | 0.109±0.004 | 0.106±0.004 | 0.105±0.004 | 0.114±0.004 | 0.111±0.004 |
| **Week -6 to week 0 (kg/day)** | 0.141±0.007 | 0.132±0.008 | 0.140±0.008 | 0.142±0.008 | 0.154±0.008 |
| **Week 0 to week 8 (kg/day)** | 0.083±0.011 | 0.097±0.011 | 0.082±0.011 | 0.101±0.011 | 0.078±0.011 |
| ***Average bodyweight at week 8 (kg)*** | *27.48±0.707* | *28.05±0.700* | *28.36±0.574* | *28.65±0.684* | *27.45±0.536* |

**Table 7.** Performance data (weaning and 8-weeks post-weaning bodyweight of Merino lambs under varying Barbervax® treatment groups [unvaccinated controls (UV), recommended protocol [vaccinated control (VP)], double then single dose (MddW), single then double dose (MWdd) and a double then double dose (MddWdd)]

| Variable /Source | Weaning Weight (kg) | Week 8 weight (kg) |
|---|---|---|
| **Overall mean** | 23.31 ± 0.28 | 27.98 ± 0.29 |
| **Vacc Treatment** | P = 0.63 | P =0.72 |
| **UV** | 23.41 ± 0.31$^{A}$ | 28.07 ± 0.37$^{A}$ |
| **VP** | 23.07 ± 0.30$^{A}$ | 27.73 ± 0.36$^{A}$ |
| **MddW** | 23.61 ± 0.30$^{A}$ | 28.35 ± 0.36$^{A}$ |
| **MWdd** | 23.28 ± 0.32$^{A}$ | 27.76 ± 0.38$^{A}$ |
| **MddWdd** | 23.68 ± 0.30$^{A}$ | 28.16 ± 0.38$^{A}$ |
| **Lamb marking weight*** | **P <0.0001** | **P <0.0001** |
| ***Slope*** | 1.047 + 0.047 | 1.057 + 0.058 |
| **Vacc x Marking weight** | P= 0.18 | P = 0.37 |

* Weight at marking for weaning weight, and marking and weaning for week 8 bodyweight

$^{a,b}$Where means share a common letter in the superscript they do not differ significantly (P<0.05).

## 3.7. Animal Deaths

Lambs were considered to have died when they ceased being present at measurements. Nine lambs were assumed to have died prior to weaning and were excluded from the analysis. No mortality was recorded post weaning. The pre-weaning mortality of 5% is within the normal pre-weaning mortality range (Thompson *et al.* 2011; Hinch and Brien 2014). Number of lambs that died and their treatment group were as follows: UV = 1, VP = 2, MddW = 2, MWdd = 2 and MddWdd = 2.

# 4. Discussion

The findings of this experiment provide support for the deferral of the second priming vaccination in the registered Barbervax® regimen until weaning. In a situation where the time interval between marking and weaning was reduced to 6 weeks, and a typical anthelmintic treatment at weaning was given, removing this priming vaccination delayed the antibody response to P2 until the post weaning primer, but had no effect on WEC. The rise in antibody titre following the 2$^{nd}$ priming vaccination was much greater when the primer was given at weaning, than at 3 weeks after marking indicating that the 6-week interval did not compromise the response to this vaccination but enhanced it. This may be due to maturation of the immune system or the increased priming vaccine doses used at marking and/or weaning. In antibody terms the two primers given at marking and weaning, achieved the same antibody levels at 2 weeks post weaning as did the recommended 3 primers between marking and weaning. The role of vaccine dose in the enhanced response to P2 given at weaning is unclear as the experimental design did not include a treatment in which only single doses were applied at marking and weaning. However, the fact that the three alternative vaccination regimens all provided a similar response, irrespective of the timing and number of double doses suggests that dose did not play a critical role in the observed responses.

Vaccination significantly reduced *H. contortus* infection in the vaccinated lambs from 5-weeks post weaning by approximately 50%. This reduction is lower than that found by Dobson *et al.* (2013) who showed in a modelling study, a minimum vaccine efficacy of >65% is required to have an epidemiological effect and control haemonchosis. Efficacy of >65% results in a 4.5% mortality rate in vaccinates, compared to weaners drenched 4 times/year and a 27.7% mortality rate (Smith 2015). This reduction was lower than reported in previous Barbervax® trials (Smith 2014a). Protection between 65-91% was found in vaccinated lambs, and with an overall claim by Barbervax® of approximately 85% reduction in lambs (Smith 2014a). Vaccination trials conducted by CSIRO were found to have the largest WEC reductions compared to those conducted by Invetus (formerly VHR), with the difference thought to be due to lusher pasture conditions and local climatic conditions at Invetus site, resulting in larger infections (Smith 2014a). However, in this experiment lush pasture conditions and large infections were also present and protection was still evident, so it is not the level of challenge that is affecting protective index. During Barbervax® trials, lambs were subjected to burdens ranging between ~1000 and ~5000 epg. In the latter stages of this experiment lambs were subjected to a much greater burden, particularly at week 10 and 11. There is anecdotal evidence from other properties at around this time that under

conditions of high challenge, Barbervax® can be overwhelmed (Besier, B.B. 2017 pers. comm.) and this appears to have been the case in the current experiment. However, this was not associated with a decline in protective index, which remained above 50% in all vaccinated treatments at week 11. The results indicate simply that at very high levels of challenge reductions of this magnitude are insufficient to prevent very high WEC in vaccinated animals. Vaccinations were generally followed by an increase in protective index at the next WEC measurement, particularly following the vaccination at weaning. This same finding occurred in the registration trials with a increases (1-20%) in protection following booster vaccinations (Smith 2014a).

At week 3 post weaning, a decrease in WEC in vaccinated animals occurred. However due to short-acting anthelmintic treatment at weaning it is unclear as to the true reduction in WEC courtesy of Barbervax vaccination, although clear there is some protection at week 3. One week after the first booster vaccination at week 5 post weaning, a decrease in WEC occurred in vaccinated groups but the response was not assessed again until week 10 due to anthelmintic treatment at week 6. By week 10 (5 weeks post B1) WEC values had approximately trebled in the vaccinated animals despite only a modest decline in protective index from 54% to 42% over the same period in the vaccinated treatments. In the registration trials booster vaccinations similarly caused a decrease in WEC 2-3 weeks post vaccination (Le Jambre *et al.* 2008; Smith 2014a) with protective immunity lasting 7 (Le Jambre *et al*., 2008) and 6 weeks (Smith 2014a). This duration of effective protection was not observed following the 5-week post weaning booster vaccination in the present experiment. Following a second booster vaccination at week 10, the WEC of all animals (vaccinated and unvaccinated) increased markedly at week 11. Interestingly, as reported by the property manager Barbervax® vaccinated animals across all flocks demonstrated a similar lack of response to vaccination at this time. Most importantly this increase was accompanied by an increase in protective index in vaccinated animals by approximately 10% to 54% but this was insufficient to provide effective control. This increase in WEC was due to antibody titre and immunity levels insufficient to weaken or kill large numbers of incoming *H. contortus,* or climatic conditions favouring *H. contortus* development such that the rate of worm intake by the weaners, exceeded the speed of worm mortality. Natural immunity to *H. contortus* is also likely to have been overwhelmed by the very high level of challenge and not provided significant complementary immunity.

During the Barbervax® registration trials lower WEC were observed when vaccinated animals grazed pastures previously grazed by vaccinated animals demonstrating the cumulative epidemiological consequences of vaccination. In the present experiment, treated and non-treated

animals grazed together ensuring the same level of challenge, nutrition and other environmental effects for all treatments and removing paddock effects, enabling analysis using animal as the replicate. During Barbervax® registration trials for lambs, hoggets and peri-parturient ewes, most experiments also grazed treatments together (Smith 2014a, 2014b). Despite the advantages of testing the vaccine using uniform challenge, this did not reveal the true reduction in WEC over time of vaccinating the entire mob. Grazing the treatments together has undoubtedly increased the level of parasite challenge vaccinated lambs were exposed to, particularly towards the end of the experiment. This is evident from the significantly higher WEC values observed in unvaccinated animals which would translate into additional challenge.

The reduction in WEC coincides with a high level of Barbervax® specific-antibody titre, with vaccinates having a significantly higher titre than their counterparts. As expected, in no treatment was there evidence of an antibody response to P1 at marking. In the VP treatment, there was a stepwise increase titre following P2 (week -3) and P3 (weaning). In the remaining vaccination treatments, the increase in titre after P2 (at weaning) was very large resulting in similar titres to that achieved after P3 in the VP treatment. This response to P2 at weaning was protective, resulting in significantly reduced WEC 5 weeks later. Following the peak in antibody levels two weeks post weaning vaccination there was a decline in antibody titre over the next 3 weeks prior to a further booster vaccination at week 5 and further increase in titre. However the decline in titre was gradual and (<0.5 log) and titres never fell to pre-boost levels.

The pattern of antibody response was broadly consistent with that observed during Barbervax® vaccination trials (Smith 2014a), and early Barbervax® dose response trials, which found the two-fold response to vaccination followed by a steady decrease in antibody titre over a 6-week period, although titre levels did remain two-fold higher than pre-booster levels. The decrease in antibody titre is the result of the 'hidden antigen' where frequent vaccination is required to maintain immunity and levels of anti-H11 and H-gal-GP antibodies (Smith *et al.* 2001a; Smith *et al.* 2001b; Le Jambre *et al.* 2008).

The hidden antigen approach is based on circulating antibody being ingested in blood by worms and this leading to impairment of their function. Based on this, and the broad concordance of higher Ab levels with reduced WEC one would predict a strong negative association between antibody titre and WEC within individual animals. However, this was not observed in the present experiment with no relationship between Barbervax® antibody titre and WEC at weaning and only weak but significant negative associations at weeks 3 and 5. This contrasts with stronger associations observed in other studies. A Barbervax® vaccine therapeutic trial using 5 µg antigen

found strong and highly significant negative associations between specific-antibody titres, WEC and worm recovery ($P<0.001$, $R^2=0.33$-$0.64$) (Smith Unpublished-b). Le Jambre *et al.* (2008) used 100 µg of native *H. contortus* antigen, also found significant ($P<0.05$) inverse correlations between total vaccine antibody (IgG1) titres and WEC immediately prior to weaning and the first booster vaccination. The authors suggested this earlier correlation prior to weaning may exist due to the total antigen content of the experimental vaccine, as it was 20 times that of Barbervax®. This allowed animals to produce a larger and faster antibody response. However, studies using lower levels of the hidden antigen at 50 µg or 5 µg in lambs, found lambs which received 50 µg, had a lower WEC reduction (57%) than those receiving 5 µg (72%) (Basetto *et al.* 2014). A therapeutic trial using Barbervax® found a significant negative association between titre, WEC and worm burden (Smith Unpublished-b), however in further Barbervax® studies the association between titre and WEC has not been reported. The weak association between WEC and Barbervax® Ab titre in the present experiment is unlikely to be a result of the ELISA results, as the Moredun method was followed exactly and samples which titres read abnormally high were re-analysed. Therefore, the reasons for the weak association are unclear. However, they do indicate that Barbervax® Ab titre has some significant limitations as a marker of Barbervax® protection in individual animals.

The Barbervax® registration trials document the notion of animals classifiable as non-responders to vaccination. Non-responders are not mentioned in early publications using irradiated larvae, natural or 'hidden' antigen *H. contortus* vaccination experiments. However, separation of lambs into responders and non-responders (NR) enabled consistent WEC reductions (66-99%) to be observed in responders following vaccination with irradiated *T. colubriformis* larvae (Dineen *et al.* 1978; Dineen and Windon 1980; Windon *et al.* 1980). However, it was not mentioned whether it was the same animals repeatedly classified as a responder or non-responding. Throughout the experiment, the percentage of non-responders based on WEC and antibody titre decreased as the lambs matured. Dineen *et al.* (1978) documented a similar pattern in percentage of animals responding at 17 weeks of age (63%) versus 37 weeks of age (86%), suggesting that lambs can be 'primed' but are unable to mount an effective immune response until later.

In the vaccination cycle, immediately post-weaning, repeatable significant associations between antibody titre and WEC of non-responders and responders existed. Lambs which responded had higher titres and lower WEC compared to non-responders. However, in the second vaccination cycle (post week 5 booster) there was poor concordance of classification with the first cycle, indicating that response or non-response was not an inherent trait of individual animals.

Interestingly, analysis of responders based on antibody titres rather than WEC revealed that all lambs were classified as responders. No previous work has analysed whether animals can be classified as a non-responder based on vaccine specific antibody titre. The evidence from the present experiment would support assessment of non-responders based on WEC with vaccine-antibody titre is indicative of a vaccination response rather than a protective response. The results of this experiment also clearly indicate that response to Barbervax® vaccination is not an inherent trait of individual animals, but rather an indicator of vaccination success only following an individual vaccination event. Moreover, animals may frequently be classified as responders or non-responders at different times following an individual vaccination event. This casts doubt on the utility of this measure.

This experiment provided some evidence of passive maternal transfer of Barbervax® antibodies to lambs. At marking (week -6), serum specific-antibody titres from all treatments were comparatively high despite to the lambs having no prior exposure to vaccination. In the unvaccinated lambs a steady decline of almost 2 logs throughout the experiment then followed. This most likely due to passive transfer of maternal antibodies as has been observed previously following vaccination with a hidden *H. contortus* antigen (Andrews *et al.* 1995). The likely consequences of such transfer on susceptibility to *H. contortus* in lambs, or interference with Barbervax® vaccination are discussed in Chapter 4.

Finally, bodyweight and weight gain (g/d) were not significantly affected by Barbervax® treatment as was the case in the registration trials (Smith 2014a). This confirms the safety of the vaccine and lack of likely effect on performance traits.

# 5. Conclusion

In conclusion, the hypothesis that by reducing the time interval between marking and weaning to 6 weeks, and providing a double dose of vaccine at marking, weaning or both, supported that the requirement for P2 could be removed between marking and weaning. By providing a shortened marking to weaning time interval to 6 weeks, and either doubling the dose at marking or weaning, a high antibody titre and WEC reduction equal to vaccinated controls is achieved. Importantly, removal of P2 removes the need for the extra muster and reduces associated costs, risks of mis-mothering, stress and welfare for lambs and ewes. Interestingly the alternative vaccination treatments induced a large and protective antibody response following the second vaccination at weaning. In this case the vaccine caused an effect comparable to traditional inactivated vaccines with an anamnestic response and protective immunity following boosting after a single priming

non-protective dose at marking. It is important to determine in the future if equivalent results can be obtained with single doses of vaccine at marking and weaning, 6 weeks later. In the interim any one of the alternative treatments proposed could be used. Another important finding of this experiment was that protective index was relatively independent of WEC level and insufficient to provide adequate control of *H. contortus* under conditions of high challenge. Further research is required to improve Barbervax® protection during high challenge periods and better understand factors affecting it. Other notable findings were that there was little utility in classifying animals as responders or non-responders, as this didn't appear to be a repeatable trait of an individual sheep, and that Barbervax® antibody levels were poor predictors of WEC, and thus protection in individual sheep.

# CHAPTER 4

# Effects of Barbervax® vaccination on wellbeing and performance of maiden Merino ewes and their progeny, including passive transfer of maternal antibody to lambs.

## 1. Introduction

Use of preventative and control treatments, particularly in sheep parasitically challenged, reduces the disease burden and stress and allows the sheep to partition nutrition more towards production due to lower maintenance needs (Coop and Holmes 1996). In addition, improved worm control will result in increased feed intake due to removal inhibitory effects of gastrointestinal nematode infection on voluntary feed intake (Steel *et al.* 1980). Success of anthelmintic treatment in removing parasitic burdens and increasing live-weights, wool quantity and pregnancy performance is well-documented. In *T. colubriformis* infected Merino ewes and lambs given a controlled-release Albendazole capsule (CRC), egg counts were reduced by 70-97% in ewes over the period of lambing to weaning. Ewes treated with the CRC lost less weight and grew 12% more wool during lactation than untreated ewes, whilst untreated lambs had 16-25% reduction in clean fleece weight compared to CRC lambs (Barger *et al.* 1992). In a summer rainfall environment, Merino weaners suppressively treated with 5, 10 and 16 anthelmintic treatments, increased wool production by 18.4, 25.0 and 34.2% and live weight gains of 20.7, 23.7 and 24.7 kg respectively (Johnstone *et al.* 1979). Furthermore, ewes anthelmintic treated pre- and post-lambing had lambs that grew at a faster rate and were 6 kg heavier by 18 weeks of age compared to lambs from non-treated ewes (Darvill *et al.* 1978). Given these positive results for increases in production traits of treated sheep, it would be expected that provision of vaccination, suppressing *H. contortus* infection, may have desirable effects.

It is well documented that better ewe condition during joining, late gestation and lactation benefits scanning rates, lamb birth weight and lamb growth and survival respectively (Gibb and Treache 1980; Russel *et al.* 1981; al-Sabbagh *et al.* 1995; Morris and Kenyon 2004). In peri-parturient ewes, the peri-parturient rise in worm egg counts is caused by a decrease in acquired immunity to nematode infection (Barger 1993). Anthelmintic treatments prior to lambing reduce this risk and allow the ewe to partition additional nutrition and bodily resources to the foetus, lamb and milk

production. It could therefore be predicted that controlling gastrointestinal nematode infection in peri-parturient ewes using Barbervax® would similarly allow the ewes to maintain or improve performance and production.

During the antigen trials conducted prior to 2010 and the Barbervax® registration trials, investigations focused on developing a protective immune response, with vaccination effects on ewe and lamb performance not reported. The maiden ewe mob of approximately 200 ewes and their lambs used in this experiment provided an opportunity to address this situation, as the animals received a variety of Barbervax® treatments in the studies reported in this thesis and Baker (2016), and were subject to intensive measurement for a sustained period. While vaccination effects on performance of ewes and lambs in specific experiments is reported in Chapters 2 and 3, this chapter investigates the production and performance effects of Barbervax® in Merino ewe hoggets in the entire mob, and on their progeny, with a particular focus on greasy fleece weight (GFW), fleece diameter, bodyweight and body condition scores (BCS), scanned litter sizes and progeny survival (rear type) and maternal transfer of Barbervax® antibodies in lambs.

# 2. Materials and methods

## 2.1. Experimental Site

The experiment was conducted at TA Fields 'Congi Station' near Woolbrook, NSW (latitude: 30.91˚S, Longitude: 151.29˚E, Altitude: approx. 975 m), from April 2016 to the end of February 2017, during which the experiments reported in chapters 2 and 3 ran. The region experiences summer dominant rainfall, with major rain events occurring during December, January and February, coinciding with increased *H. contortus* burdens and haemonchosis outbreaks. Ewes and lambs grazed native pastures for the duration of the experiment where they were subjected to natural gastrointestinal nematode challenge, predominately (96-100%) *H. contortus* (Chapters 2 and 3). Prior to lambing, ewes were grazed in 50 and 63 hectare paddocks, then moved to a 71 hectare paddock for lambing, where lambs remained after weaning. At weaning the ewes were returned to a larger ewe mob and all recording ceased. The weaned lambs were managed by farm personnel according to Congi farm practice and kept separate to other flocks. Weather measurements during the experiment are reported in chapters 2 and 3.

## 2.2. Experimental design, ethics approval and application of treatments

The experiment included 198 2014-born fine-wool Merino hogget ewes and their 2016-born progeny, 175 lambs, for a total of 44 weeks, and was approved by the University of New England's Animal Ethics Committee (Approval numbers: AEC16-048, AEC16-049). Day 0 was the day of joining on 29th April 2016 (week 0). Ewes were followed from the commencement of the experiment until lamb weaning (week 34), and lambs followed from marking until the conclusion of the experiment at week 45 (6$^{h}$ March 2017). Ewe joining concluded 3rd June 2016 (week 5), and pregnancy ultrasound scanning (to obtain foetal number) and shearing (for mean fibre diameter and fleece weight) were on the 14th July (week 11) and 21st July 2016 (week 12) respectively. Lambing commenced 11th September 2016 (week 19) and concluded the 25th October 2016 (week 26). Finally, lamb marking and weaning were completed on 8th November 2016 (week 28) and 20th December 2016 (week 34) respectively (Figure 1).

Ewes and lambs were a part of 3 separate experiments (Baker 2016; Broomfield *et al.* 2017a, 2017b) which ran concurrently. The animals were given different treatment regimens of Barbervax®. All ewes were given a full course of Barbervax® entailing 6 vaccinations as lambs in 2014, and 100 of these ewes received 4 Barbervax® booster vaccinations in year 2 on 4th December 2015, 8th January, 5th February and 11th March 2016. Of these 100 vaccinated ewes, 20 ewes received a long-acting Moxidectin (LA-Moxi) for 125 days "worm-free", prior to a Barbervax® pre-lambing booster vaccination on 4th August 2016 (VACCMOX + PLBOOST). An additional 19 vaccinated ewes (VACC + PLBOOST) also received a Barbervax® pre-lambing booster vaccination on the 4th August 2016, 6 weeks prior to lambing. The remaining 61 vaccinated ewes received no pre-lambing booster vaccination (VACC). Finally, the other 98 ewes were unvaccinated (UNVACC) for the duration of the experiment. Of the ewes' progeny, 140 lambs were treated as described in chapter 3. Due to the lack of significant difference between lamb Barbervax® treatments, for this chapter the treatments were combined into vaccinated (VACCLAMBS) and unvaccinated (UNVACCLAMBS). All animals were randomly allocated into treatment groups as described in chapters 2 and 3. The different treatment groups are shown in table 1.

**Table 1.** Ewes treatment groups for this experiment.

| Animal | Treatment group | Number (n) | Treatment label |
|---|---|---|---|
| **EWES** | Unvaccinated controls (Barbervax® vaccination as lambs only) | 100 | UNVACC |
| | Barbervax® vaccination (lambs and hoggets) | 58 | VACC |
| | Barbervax® vaccination (lambs, hoggets + pre-lambing booster) | 19 | VACC + PLBOOST |
| | Barbervax® vaccination (lambs, hoggets, pre-lambing booster + LA Moxidectin) | 20 | VACCMOX + PLBOOST |
| **LAMBS** | Unvaccinated controls | 34 | UNVACCLAMBS |
| | Barbervax® vaccination (3 priming + 2 booster vaccinations) | 132 | VACCLAMBS |

When vaccinated, ewes and lambs were given a single dose of Barbervax® as recommended (1 mL regardless of bodyweight) injected subcutaneously behind the ear. Where lambs were given two doses, one dose was applied behind each ear. Unvaccinated control ewes and lambs were injected with 1 mL saline solution at the same site. All ewes including the controls were given anthelmintic treatments [TriGuard® (Abamectin 1.0 g/L, Oxfendazole 22.7 g/L and Levamisole 33.9 g/L)] at weeks -3 and 14 in response to rising worm egg counts and a strategic pre-lambing drench at week 18 [Q-Drench® (Abamectin1.0 g/L, Albendazole 25.0 g/L, Closantel 37.5 g/L, Levamisole hydrochloride 40.0 g/L)]. One individual from the VACC group was given an extra precautionary anthelmintic treatment due to a high and increasing WEC and excluded from the statistical analysis.

Lambs were vaccinated against contagious ecthyma (Scabiguard®) five clostridial diseases and contagious lymphadenitis (Glanvac 6 in1®) and given preventive blowfly treatment using dicyclanil pour on (Clik®) at marking (week 28) and weaning (week 34). These treatments were administered by farm staff to meet farm requirements. All lambs including the controls were treated with an anthelmintic [TriGuard® (Abamectin 1.0 g/L, Oxfendazole 22.7 g/L and Levamisole 33.9 g/L)] dosed to the heaviest animal at weaning and at the conclusion of the experiment (week 44). No ewes died during the experiment, however 9 lambs died prior to weaning. These were kept in the analysis, due to having some performance measures. The pre-weaning mortality of 5% was considered to be within the normal pre-weaning mortality range.

Individual body condition scores and body weights were recorded from the ewes pre- (week 0) and post-joining (week 5), shearing (week 12), marking (week 28) and weaning (week 34), and lambs bodyweights collected at marking, weaning and 8-weeks post-weaning (week 41). Barbervax® specific antibody titres of the lambs were obtained as described in Chapter 3. The timeline of major husbandry events during the experiment are shown in figure 1.

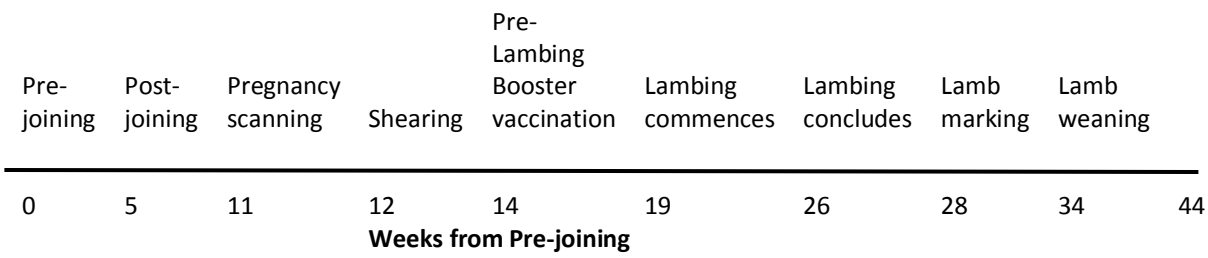


**Figure 1.** Timeline of major husbandry dates relative to the commencement of the experiment at pre-joining (timeline not to scale).

## 2.3. Animal Measurements

### 2.3.1. Sample and collection

#### *2.3.1.1. Blood samples*

Blood samples were collected from all lambs via jugular venepuncture into 1 x 5 mL SST Vacutainer® tubes and serum collected as per chapter 3, section 2.3.1.1 from weeks 28 to 41.

### 2.3.2. Laboratory Methods

#### *2.3.2.1. Enzyme Linked Immunoassorbent Assay (ELISA)*

Analysis of lamb serum samples were done-so using the same Barbervax® ELISA method as described in chapter 2, section 2.3.3.1 Enzyme Linked Immuno-Absorbent Assay (ELISA) – Barbervax® antigens.

### 2.3.3. Performance Measures

#### *2.3.3.1. Body weight and Body Condition Scores (BCS)*

Individual ewe bodyweights and BCS were recorded at pre- and post-joining, shearing, lamb marking and weaning. Method of weighing and assessing BCS are as described in chapter 2 section 2.3.2.1. Lambs were individually weighed at marking, weaning and 8-weeks post-weaning using the same method as in chapter 3 section 2.3.2.1.

#### *2.3.3.2. Pregnancy scanning and fleece scanning*

Individual ewes were pregnancy scanned for foetal number and shorn at week 11 and 12 respectively, as per methods described in chapter 2 section 2.3.2.2.

#### *2.3.3.3 Lamb Parentage*

Parentage was determined by DNA SNP analysis using the test offered by Sheep Genetics®. Blood samples were collected as per Sheep Genetics instructions (ear nick following alcohol wipe) and a drop of blood placed on a labelled Sheep Genetics® blood card and allowed to dry. Rams, ewes and lambs were sampled at weeks 1,11 and 34 respectively, and all cards were sent to Sheep Genetics® for parentage analysis (SheepCRC 2014).

## 2.4. Weather data

The weather data and pasture for this experiment is the same as that documented in chapters 2 and 3 sections 2.4.

## 2.5. Data and statistical analysis

Data was analysed using JMP 13.1.0 software (2015 SAS Institute Inc). Barbervax® antibody titres were Log10 transformed to remove association between the variance and the mean. For performance variables general linear models were fitted testing the fixed effect of ewe or lamb treatment group model, with covariates and interactions with them fitted where appropriate. A P-value of $<0.05$ was used to determine statistical significance. Data are presented as least squares means (LSM) ± standard error of the mean (SE). The significance of differences between means within a significant treatment was determined using Student's t-test. No outliers or dead lambs were removed from the analysis, only the anthelmintic treated ewe previously mentioned. All data was recorded directly apart from ewe fleece-free bodyweight at shearing which was calculated by subtracting the fleece weight from the pre- shearing bodyweight.

# 3. Results

## 3.1. Effect of ewe Barbervax® treatment on ewe performance

### *Bodyweights*

There was no significant effect of Barbervax® vaccination on ewe bodyweight at shearing and weaning, however there was a significant effect on ewe bodyweight at lamb marking (P=0.0262) (Table 3). VACCMOX + PLBOOST (47.37 kg ±0.69) ewes were heaviest at lamb marking and VACC + PLBOOST (45.0 kg ±0.67) ewes the lightest, and the difference significant. UNVACC(46.88 kg ±0.29) and VACC + NOPLBOOST (46.09 kg ±0.39) treated ewes fell between the VACCMOX + PLBOOST and VACC + PLBOOST ewes at marking, however the differences between treatments weren't significant. The covariates post-joining weight (P =0.0427) and pre-joining BCS (P =0.0181) had positive associations with ewe shearing weight with an additional 0.47 kg and 3.78 kg, per kg ewe body weight post-joining and BCS pre-joining respectively (Table 3). Furthermore, for ewe shearing weight with bodyweight fitted as a covariate, scanned litter size trended towards significance (P =0.09), whilst with BCS as a covariate, scanned litter size was a highly significant effect (P=0.0019), equaling 3.278kg extra per lamb scanned.

For ewe marking bodyweight, the covariate post-joining weight (P<0.0001) had a significant positive effect with an additional 0.92 kg at marking per kg ewe bodyweight post-joining. In comparison, the effect of lamb number reared had a significant negative effect on marking bodyweight, with -2.78 kg per lamb reared (Table 3). Similarly, covariate BCS post-joining (P =0.0078) had a highly significant positive effect on ewe marking weight with an additional 4.27 kg marking weight per BCS post-joining. In comparison, the effect of lamb number reared had a significant negative effect on marking bodyweight (P =0.0013), with -1.928 kg per lamb reared. There was a significant interaction between the effects of vaccination and shearing BCS on ewe bodyweight at marking (P =0.0476) (Table 3). This reflected that the benefit of increased BCS was most apparent in VACC + NOBOOST ewes, with a negative association seen in VACC+ PLBOOST ewes (Figure 2c). Finally, there were no significant effects of weight, BCS, scanned litter size or interactions on ewe bodyweights at weaning.

**Table 2**. Significance, LSM±SEM for fixed treatment effects and slopes for covariate effects from analyses of key performance variables (scanned litter size, fleece weight minus belly (kg) and mean fibre diameter) of Merino ewes subjected to different Barbervax® treatments (UNVACC, VACC + NOPLBOOST, VACC + PLBOOST, VACCMOX + PLBOOST). Significant P values are in bold. Treatment means within an effect sharing a letter in the superscript, do not differ significantly.

| **Variable** | **Scanned litter size** | | **Fleece weight minus belly (kg)** | | **Fibre diameter (μ)** | |
|---|---|---|---|---|---|---|
| **Covariate fitted*** | **Ewe weight (kg)** | **Ewe BCS (1-5)** | **Ewe weight (kg)** | **Ewe BCS (1-5)** | **Ewe weight (kg)** | **Ewe BCS (1-5)** |
| **Overall mean** | 1.06±0.02 | | 3.30±0.03 | | 16.78±0.07 | |
| **Vacc Treat** | P = 0.46 | P =0.50 | P =0.62 | P =0.34 | P =0.95 | P =0.83 |
| **UNVACC** | 1.05±0.03$^{A}$ | 1.06±0.03 $^{A}$ | 3.27±0.04 $^{A}$ | 3.27±0.04 $^{A}$ | 16.76±0.10 $^{A}$ | 16.76±0.103 $^{A}$ |
| **VACC + NOPLBOOST** | 1.02±0.04 $^{A}$ | 1.02±0.04$^{A}$ | 3.32±0.05 $^{A}$ | 3.32±0.05 $^{A}$ | 16.80±0.13 $^{A}$ | 16.84±0.134 $^{A}$ |
| **VACC + PLBOOST** | 1.13±0.08 $^{A}$ | 1.1±0.08 $^{A}$ | 3.32±0.09 $^{A}$ | 3.37±0.10 $^{A}$ | 16.83±0.23 $^{A}$ | 16.89±0.257 $^{A}$ |
| **VACCMOX + PLBOOST** | 1.13±0.07 $^{A}$ | 1.14±0.07 $^{A}$ | 3.38±0.09 $^{A}$ | 3.45±0.10 $^{A}$ | 16.66±0.24 $^{A}$ | 16.60±0.270 $^{A}$ |
| **Ewe Weight*** | **P <0.0001** | | P =0.79 | | P= 0.80 | |
| ***Slope*** | 0.035+0.008 | | -0.003+0.01 | | -0.007+0.026 | |
| **Ewe BCS*** | | **P=0.0062** | | P =0.07 | | P =0.55 |
| ***Slope*** | | 0.250+0.091 | | -0.284+0.156 | | 0.246+0.415 |
| **Ewe scanned litter size*** | | | **P =0.0255** | **P =0.0190** | P =0.97 | P = 0.75 |
| ***Slope*** | | | 0.191+0.084 | 0.190+0.080 | -0.010+0.223 | -0.068+0.214 |
| **Vacc x Weight** | **P= 0.0309** | | P = 0.68 | | P =0.47 | |
| **Vacc x BCS** | | **P= 0.0033** | | P =0.22 | | P =0.48 |

*ewe weight and BCS at joining for scanned litter size, ewe fleece-free shearing weight and BCS for fleece weight and fibre diameter

$^{a,b}$Where means share a common letter in the superscript they do not differ significantly (P<0.05).

**Table 3.** Performance data (ewe shearing, marking and weaning bodyweight) of Merino ewes under varied Barbervax® treatments (UNVACC, VACC + NOPLBOOST, VACC + PLBOOST, VACCMOX + PLBOOST)

| Variable /Source | Ewe shearing weight | | Ewe marking weight | | Ewe weaning weight | |
|---|---|---|---|---|---|---|
| **Covariate*** | **Ewe weight (kg)** | **Ewe BCS (1-5)** | **Ewe weight (kg)** | **Ewe BCS (1-5)** | **Ewe weight (kg)** | **Ewe BCS (1-5)** |
| **Overall mean** | 42.67±0.37 | | 46.49±0.33 | | 45.53±0.44 | |
| **Vacc Treat** | P = 0.68 | P =0.75 | **P =0.0262** | P =0.11 | P =0.45 | P =0.41 |
| **UNVACC** | 42.41±0.40 A | 42.57±0.48 A | 46.88±0.29 A,B | 47.03±0.43 A | 45.53±0.397 A | 45.57±0.563 A |
| **VACC + NOPLBOOST** | 42.96±0.50 A | 42.84±0.64 A | 46.09±0.39 A,B | 46.06±0.57 A | 45.92±0.53 A | 45.44±0.747 A |
| **VACC + PLBOOST** | 43.10±0.93 A | 42.36±1.10 A | 45.0±0.67 B | 45.23±1.06 A | 44.27±0.99 A | 43.40±1.395 A |
| **VACCMOX + PLBOOST** | 43.36±0.98 A | 43.84±1.13 A | 47.37±0.69 A | 48.43±1.12 A | 46.15±0.95 A | 46.64±1.445 A |
| **Ewe Weight*[1]** | P =0.09 | | P =0.49 | | P =0.58 | |
| ***Slope*** | 0.402+0.239 | | -0.111+0.161 | | 0.063+0.114 | |
| ***Ewe Weight*[2]*** | **P =0.0427** | | **P <0.0001** | | P <0.0001 | |
| ***Slope*** | 0.465+0.228 | | 0.919+0.159 | | 1.024+0.111 | |
| **Ewe BCS*[1]** | | **P=0.0181** | | P =0.61 | | P=0.75 |
| ***Slope*** | | 3.781+1.587 | | -1.000+1.963 | | -0.720+2.279 |
| **Ewe BCS*[2]** | | P =0.51 | | **P =0.0078** | | P =0.202 |
| ***Slope*** | | 1.259+1.892 | | 4.266+1.587 | | 5.055+2.157 |
| **Ewe scanned** | P =0.09 | **P =0.0019** | | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| **litter size** | | | | | | |
| ***Slope*** | 1.480+0.860 | 3.278+1.039 | | | | |
| **Ewe number lamb reared** | | | **P <0.0001** | **P =0.0013** | P =0.57 | P =0.65 |
| ***Slope*** | | | -2.782+0.417 | -1.928+0.590 | -0.333+0.590 | -0.381+0.851 |
| **Vacc x Weight*[1]** | P= 0.93 | | P =0.69 | | P=0.75 | |
| **Vacc x Weight*[2]** | P =0.99 | | P =0.53 | | P =0.79 | |
| **Vacc x BCS*[1]** | | P =0.43 | | **P =0.0476** | | P=0.10 |
| **Vacc x BCS*[2]** | | P =0.76 | | P =0.42 | | P =0.09 |

* Pre-joining[1] and post-joining[2] weight and BCS for shearing weight; fleece-free shearing[1] and post-joining[2] weight and BCS for marking weight; fleece-free shearing[1] and marking[2] weight and BCS for weaning weight

[a,b]Where means share a common letter in the superscript they do not differ significantly (P<0.05).

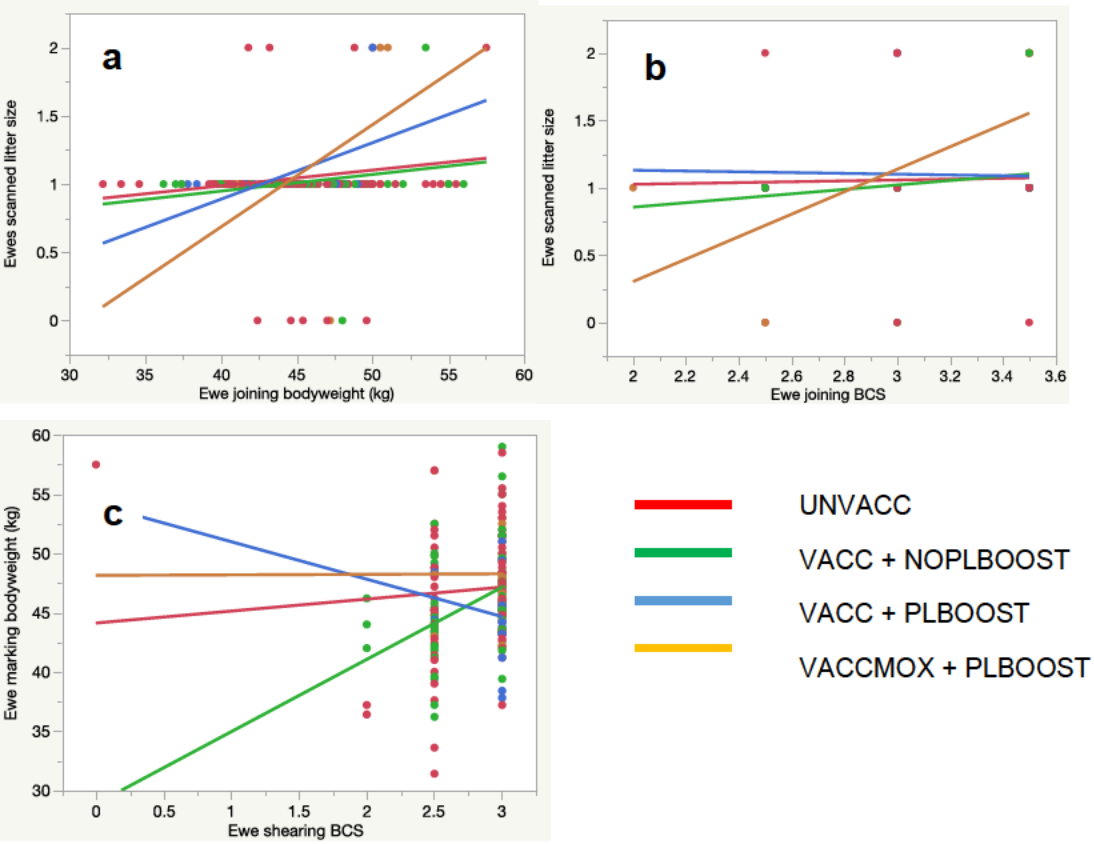


**Figure 2.** Plots illustrating the significant interaction between treatments and co-variates from Tables 2 and 3.

### *Scanned litter size, fleece weight and fibre diameter*

The overall mean, least squares means and standard errors for treatments and effects, together with P values are shown in table 2. There was no significant effect of Barbervax® treatment on scanned littler size, fleece weight or mean fibre diameter. The covariates ewe joining weight (P <0.0001) and BCS (P =0.0062) had significant positive effects on scanned litter size, with an additional 0.035 and 0.250 lamb scanned per 1kg ewe bodyweight and 1 BCS respectively (Table 2). There was no significant effect of shearing weight or BCS on fleece weight or fibre diameter, however there was a significant positive effect of scanned litter size on fleece weight in both models using weight (P =0.0255) and BCS (P =0.0190) as additional covariates (Table 2).

There was significant interaction between the effects of vaccination treatment and ewe joining weight and BCS on scanned litter size (P =0.0309 and 0.0033 respectively). The beneficial effects of ewe joining weight on scanned litter size were observed in all treatments, although was greatest in the VACCMOX + PLBOOST treatment (Figure 2a), whilst the beneficial effect of joining BCS was observed only in the VACCMOX + PLBOOST ewes (Figure 2).

## 3.2. Effects of ewe Barbervax® treatment on lamb performance

### *Lamb bodyweight*

There was no significant effect of Barbervax® vaccination or ewe bodyweights on lamb weight at marking, weaning and 8-weeks post-weaning (Table 4). The effect of number of lambs reared was highly significant on lamb marking weight (P<0.0001), lamb weaning weight (P =0.004) and lamb weight 8-weeks post-weaning (P<0.0001) (Table 4). The effects were negative with -2.293kg, -2.713kg and -0.333kg lamb weight lost per lamb reared, indicating a reduction in the magnitude of the effect post weaning. No interactions of vaccination treatment and weight were observed on lamb weight.

### *Maternal transfer of Barbervax® specific antibody to lambs*

There was a significant effect of the ewe Barbervax® treatment on lamb antibody titres at marking (P =0.0139) (Table 4). Lambs from vaccinated ewes had significantly higher antibody titres (VACCMOX + PLBOOST 3.62+0.32, VACC + PLBOOST 3.50+0.29, VACC + NOPLBOOST 3.16+0.15 than those from unvaccinated ewes (2.78+0.12), but no significant difference existed between lambs born to ewes from the different vaccination treatments (Table 4). There were no significant effects of ewe fleece-free weight at shearing, scanned litter size or their interaction on lamb marking antibody titre.

At marking, lamb Barbervax® vaccination treatments were applied and lamb treatment was fitted as an effect in the model, in addition to dam vaccination treatment. There was a significant effect of dam vaccination treatment (P=0.01) on lamb antibody titres, with lambs from the 3 vaccinated treatments having higher titres than lambs from unvaccinated ewes (Table 4). There was a significant effect of the ewe Barbervax® treatment on lamb antibody 3-weeks post marking (P=0.004), although lambs from vaccinated and pre-lambing boosted ewes titres the highest (Table 5). At weaning, neither ewe or lamb treatment was a significant effect on antibody titre. However from 2 weeks post weaning (not shown) the lambs treatment remained to be highly significant effect on lamb antibody titre (P<0.0001) (Table 5).

**Table 4.** Performance data (lamb marking, weaning, 8-weeks post weaning bodyweight, lamb marking antibody titre) of Merino lambs under ewe Barbervax® treatments (UNVACC, VACC + NOPLBOOST, VACC + PLBOOST, VACCMOX + PLBOOST)

| Variable /Source | Lamb marking weight | Lamb weaning weight | Lamb 8-week post-weaning weight | Lamb marking antibody titre |
|---|---|---|---|---|
| **Overall mean** | 17.49±0.23 | 23.31±0.28 | 27.96+0.29 | 3.04+0.09 |
| **Total lambs (n)** | 171 | 167 | 164 | 168 |
| **Vacc Treat** | P = 0.13 | P =0.13 | P =0.40 | **P =0.0139** |
| **UNVACC** | 17.68±0.31 [A] | 23.37±0.38 [A] | 27.97±0.40 [A] | 2.78+0.12 [B] |
| **VACC + NOPLBOOST** | 17.67±0.36 [A] | 23.84±0.46 [A] | 28.47±0.48 [A] | 3.16+0.15 [A] |
| **VACC + PLBOOST** | 17.06±0.83 [A] | 22.95±1.05 [A] | 27.44±1.07 [A] | 3.50+0.29 [A] |
| **VACCMOX + PLBOOST** | 15.80±0.77 [A] | 21.25±1.00 [A] | 26.64±1.03 [A] | 3.62+0.32 [A] |
| **Ewe Weight*[1]** | P =0.0140 | P =0.18 | P =0.27 | P =0.47 |
| ***Slope*** | 0.235+0.095 | -0.260+0.191 | -0.223+0.200 | -0.024+0.033 |
| **Ewe Weight*[2]** | P =0.34 | P =0.06 | P =0.11 | |
| ***Slope*** | -0.099+0.104 | 0.329+0.172 | 0.289+0.178 | |
| **Ewe scanned litter size** | | | | P =0.10 |
| ***Slope*** | | | | 0.532+0.322 |
| **Ewe number lamb reared** | **P <0.0001** | **P =0.0004** | **P <0.0001** | |
| ***Slope*** | -2.293+0.574 | -2.713+0.749 | -0.333+0.590 | |
| **Vacc x Weight*[1]** | P= 0.44 | P =0.11 | P=0.17 | P = 0.98 |
| **Vacc x Weight*[2]** | P =0.36 | P =0.30 | P =0.54 | |

* Ewe fleece-free shearing[1] and marking[2] weight for lamb marking weight; ewe marking[1] and weaning[2] weight for lamb weaning weight; ewe marking[1] and weaning[2] weight for lamb 8-weeks post-weaning weight; ewe fleece-free shearing weight for lamb marking antibody titre.

[a,b]Where means share a common letter in the superscript they do not differ significantly (P<0.05).

**Table 5.** Performance data (lamb antibody titre 3 weeks post marking, weaning and 2 weeks post weaning) of Merino lambs under ewe Barbervax® treatments (UNVACC, VACC + NOPLBOOST, VACC + PLBOOST, VACCMOX + PLBOOST)

| Variable /Source | Lamb 3 weeks post marking antibody titre | Lamb weaning antibody titre | Lamb antibody titre 2 weeks post weaning |
|---|---|---|---|
| **Overall mean** | 2.70±0.08 | 2.46 0.10 | 3.67+0.09 |
| **Total lambs (n)** | 156 | 154 | 154 |
| **Ewe vaccination treatment** | **P =0.0044** | P =0.11 | P =0.91 |
| **UNVACC** | 2.60±0.14 [B] | 2.14± 0.16 [B] | 3.03±0.09 [A] |
| **VACC + NOPLBOOST** | 2.70±0.15 [B] | 2.57±0.18 [A] | 2.98±0.09 [A] |
| **VACC + PLBOOST** | 3.36±0.28 [A] | 2.53±0.35 [A,B] | 3.12±0.20 [A] |
| **VACCMOX + PLBOOST** | 3.44±0.28 [A] | 2.78±0.32 [A,B] | 3.01±0.18 [A] |
| **Lamb vaccination treatment** | P =0.23 | P =0.19 | **P <0.0001** |
| **UNVACCLAMB** | 3.15±0.20 [A] | 2.34± 0.23 [A] | 1.91±0.13 [A] |
| **VACCLAMB** | 2.90±0.12 [A] | 2.67±0.14 [A] | 4.16±0.08 [B] |

[a,b]Where means share a common letter in the superscript they do not differ significantly (P<0.05).

# 4. Discussion

Barbervax® was made commercially available in Australia in 2014, with the Barverax® trial work focused on demonstrating efficacy in reducing *H. contortus* worm egg count and worm burden in lambs, hoggets and peri-parturient ewes. There are no reports on the effect of vaccination on ewe or lamb performance so these findings are novel.

Ewe Barbervax® vaccination effected ewe marking bodyweight, although not shearing and weaning weights. Interestingly, there appeared to be no pattern in the weights of ewes vaccinated or not, with vaccinated ewes given a pre-lambing booster and long-acting Moxidectin the heaviest of all treatments, whilst vaccinated ewes given a pre-lambing booster the lightest. Furthermore, unvaccinated and vaccinated ewes fell in between the aforementioned treatments. It could be assumed that application of a preventative treatment would allow the animals to partition energy to maintain ewe bodyweight rather than controlling parasitic burden. Whilst this is apparent in the vaccinated and Moxidectin treated ewes, where 4-months worm-free exposure has caused these animals to be 1.5-2.5kg heavier than the remaining ewe treatments. However, this theory has not occurred in the remaining treatments, with unvaccinated ewes numerically performing better than vaccinated ewes. Barbervax® registration trials showed that numerically bodyweights were higher in vaccinated ewes than unvaccinated (Smith 2014b). The unclear result in our experiment may be a result of the treatments grazing together, which Barbervax® states that treatments should be grazed separately.

Ewe shearing bodyweight was effected by post-joining weight and pre-joining BCS, whilst marking bodyweight was effected by bodyweight and BCS post-joining. Neither bodyweight and BCS were significant at any one time, which was unexpected as bodyweight and BCS are positively correlated with 1 BCS unit equating to 7kg of ewe live weight (Kenyon *et al.* 2014). Despite this, body weight and condition score measurements obtained at key husbandry points, do affect body weight and body condition later on. As expected bodyweight and BCS were positive effects, with 465 g and 919 g extra on shearing and marking weight respectively per 1 kg of body weight at pre- and post- joining.

Ewes marking bodyweight was effected by lamb number reared as well as the interaction between vaccination treatment and shearing BCS. Lamb number reared negatively affected the ewes marking weight, with a loss of 2.78 kg per lamb reared. Whilst this isn't a surprising result, as the ewe is partitioning many of its resources to its lamb, it is a significant amount of lost weight and loss of production efficiency. As well as this, during the post-weaning period the ewe must

replenish this lost weight and return to pre-lamb performance, with time dependent upon the quality of nutrition available. However, by weaning lamb rearing was no longer an effect to body weight, with the ewe partitioning energy and nutrition back towards it's own requirements. Furthermore, marking bodyweight was also affected by the interaction of post-joining BCS and vaccination treatment. Interestingly, vaccinated ewes who received no pre-lambing booster had a strong positive association and positive effect to marking bodyweight, whilst ewes vaccinated and pre-lambing boosted bodyweight at marking was negatively affected. The unvaccinated and vaccinated plus Moxidectin treated ewes bodyweight remained unaffected. The reason for these treatment differences are unclear, although perhaps also a result of treatments grazing together.

Ewe Barbervax® treatment had no effect on scanned litter size, fleece weight and mean fibre diameter. Instead scanned litter size was affected by ewe joining bodyweight and BCS and the interactions. Number of lambs scanned increased per 1 kg and BCS, with the best scanning rates in the vaccinated, pre-lambing booster +/- Moxidectin treated ewes. It appears the more preventive and suppressive treatments applied, the more positively ewes responded. This is in agreeance that better body condition of ewes at joining and lambing improves scanning rates, lambing and marking percentages (Kenyon *et al.* 2004). In Merino ewes, 1 BCS score can equal 13 extra lambs born per 100 ewes joined, with '3' (on a scale of 1-5) optimal for high lamb marking percentages (Marchant 2007). Furthermore, lambs which suckle from ewes under ideal condition score (3.2), have a greater daily growth rate and overall weight gain than lambs which suckle ewes with less condition (2.4) (Gibb and Treache 1980). Whilst vaccination didn't significantly provide any additional effects to scanned litter size, the results do agree that better ewe condition at joining improves the success of joining and scanning percentages.

GFW was only affected by scanned litter size with an increase of 190 g more per lamb scanned. The ewes on 'Congi', as fine wool Merinos, are bred and selected for fleece values, including GFW and diameter. Despite the pregnancy status, the ewes were still actively partitioning some nutrients to fleece weight. In comparison the relationship between litter size and GFW has shown to be negatively correlated (Safari *et al.* 2007). Maiden ewes scanned and rearing a single lamb had a significantly lighter clean fleece weight than their adult counterparts and adult twin-bearing ewes (Kelly *et al.* 1992). Furthermore, Merino ewes joined at 8-months produced 7% less weight hogget fleece weight, although gained 0.75/pregnant ewe (Tyrrell 1976). In our experiment it remains unclear why ewes had an increase in fleece weight for each lamb scanned. Fleece diameter was unaffected by any variable in our experiment, which agrees with the literature,

where nutrition and pregnancy have no effect (Kelly *et al.* 1992; Ferguson *et al.* 2011), and is genetically determined (Morley 1955; Fogarty 1995; Huisman and Brown 2008).

Barbervax® treatment had minimal negative effect on performance variables, continuing with lamb body weight at marking, weaning and 8-weeks post-weaning also not affected. However, the lambs weight at marking and weaning was greatly affected by number of lambs reared, with 2.29 kg and 2.71 kg lost per lamb reared respectively. Lamb body weight of twins and triplets can be 0.9-1.9 kg lighter at birth compared to singles (Hinch *et al.* 1985; Morris and Kenyon 2004). The effect of rear type on lamb weight is not a surprising result, as multiple lambs are competing against each other on the dam, restricts resource availability. The effect of rear type on lamb weight 8-week post weaning, although still significant, decreased to a loss of 333 g per lamb reared. This can be explained that after weaning the lamb becomes an individual (if a twin) and no longer competing for the dam's resources. It is likely that had the progeny been followed for longer, the effect of rear-type would eventually become non-significant and have zero effect on lambs weight. Interestingly ewe bodyweight in this experiment did not have a significant effect, on lamb weight in contrast to Kenyon *et al.* (2009) who found that lambs born to ewes heavier and in optimal condition are 1-1.5 kg heavier at birth and by day 100 of lactation, compared to lambs from lighter ewes. However, due to the mob of ewes treated as a whole, per farm practice and all were of similar weights and condition, it is likely this is the reason the ewes weight was not a significant effect.

The lamb Barbervax® IgG antibody titre at marking was affected by dam vaccination treatment. Lambs from vaccinated ewes had significantly higher titres to Barbervax® antibodies than those from unvaccinated ewes, indicating sufficient maternal transfer of antibodies to remain elevated at 2-7 weeks of age. This same finding has been shown in pregnant ewes vaccinated with an enriched H11 antigen fraction, one of two main antigens also in Barbervax®. Lambs born to vaccinated ewes had significantly elevated H11 antibody levels up to 9-weeks of age, with levels peaking at 3-weeks of age, before decreasing (Andrews *et al.* 1995). The findings are consistent with a half-life of maternally transferred IgG in lamb serum of around 3 weeks (Watson 1992). Whether this difference in antibody levels is sufficient to induce differences in susceptibility to worm infection or to interfere with the V1 vaccination can only be speculated upon.

Following on from the maternal effect to antibody titre at marking, the same effect was evident at 3-weeks post-marking and weaning. However, from weaning there was no effect of dam Barbervax® treatment on the lambs antibody response, with the exception of 7-weeks post-weaning. The result 7-weeks post-weaning is possible noise within the analysis or statistical

output, however due to no further samples taken and any pattern that may have continued could not be observed. Interestingly, at 3-weeks post marking and weaning, lambs from vaccinated, pre-lambing boosted and Moxidectin treated ewes had the highest titres, followed by vaccinated and pre-lambing boosted ewes. Lambs from unvaccinated ewes, as expected, had the lowest titres. Whilst there appears to be a maternal effect on the lamb's titres, it appears to have not hindered their own ability to mount a vaccinal immune response, but rather improved their response when born to vaccinated ewes. Without WEC samples at marking and 3-weeks post-marking, the difference this antibody titre has made to protection is unclear. It is unlikely there would be a reduced WEC effect to the younger lambs prior to weaning, with possibility of lowered WEC's in the older lambs. Although even if there weren't a great reduction in the lambs WEC, this antibody titre could be assisting in reducing pasture contamination. Further experimentation into the effect of maternal antibody titre on the lambs titres and whether there is WEC protection evident earlier on would be highly valuable. If maternal antibodies are helping control infection at marking, it may be possible the first vaccination could be delayed as to not muster in young lambs who have been born towards the end of lambing period.

# 5. Conclusion

In conclusion Barbervax® treatment had no overall effect on ewe production or performance traits measured confirming safety of use. Furthermore, ewe vaccination had no effect on lamb bodyweight, although there was a significantly higher Barbervax® specific-antibody titre at marking, indicative of maternal transfer. Indirect evidence from this experiment suggests that this level of maternal antibody is unlikely to be protective against worm infection at this age, or interfere with the response to the first Barbervax® vaccination in lambs, given at marking. Further research would include repeating this work on larger numbers and multiple flocks vaccinated with Barbervax®. More detailed studies into factors affecting the passive transfer of maternal Barbervax® antibodies to lambs the consequences of this are also indicated.

# CHAPTER 5

# GENERAL DISCUSSION

## 5.1. Introduction

The increase in prevalence of anthelmintic resistance has intensified the need for alternative parasite control methods on-farm. The release of Barbervax® in 2014 to control *H. contortus*, offers producers an effective and alternate control option. The vaccine is registered for use in all sheep ages and the vaccination schedule requires frequent booster vaccinations to maintain immunity during the summer season.

Several hypotheses were tested in this thesis using maiden hogget Merino ewes and their progeny. The work reported in Chapter 2 failed to effectively test that duration of vaccinal immunity lasts longer than 6 weeks because no significant vaccinal immunity was induced. It showed that antibody titres following pre-lambing treatment were not improved by natural challenge. The work reported in Chapter 3 demonstrated that the second priming dose in the initial vaccination regime in lambs could be removed with adjustments to marking to weaning interval and vaccine dose. This would eliminate the risk of mis-mothering and high cost of re-mustering ewes and lambs between marking and weaning. Finally, the results reported in Chapter 4 showed that there were no negative effects of vaccination on ewe or lamb performance and indicated the presence of maternally derived Barbervax® antibodies at lamb marking. A disappointing aspect of both the ewe and the lamb experiments was that the protection levels obtained were below those reported in the registration trials resulting in inadequate protection during high challenge in the case of the lambs post weaning.

## 5.2. Barbervax® in the Merino hogget

The duration of protection claimed by Barbervax® in boosted ewes is 6 weeks after each booster, however it is possible that after multiple boosters the duration of protective immunity at the end of the season may be longer. The attempt to test this in Chapter 2 failed due to anthelmintic treatment 4 weeks after the final booster. However, Barbervax® induced significant increases in antibody titres but not significant reductions in WEC. Using artificial challenge Baker (2016) working within the same mob of 200 maiden ewes did detect a significantly lower WEC in vaccinated ewes 8 weeks post final booster vaccination. This suggests vaccinal protection may last longer than 6-weeks. The lack of significant results in our experiment is likely to be a result of

a combination of low natural challenge (<900 epg) modest experimental power to detect differences in WEC, the fact that vaccinated and unvaccinated sheep ran together in the same mob and development of significant natural immunity due to chronic challenge. In the experiments of Baker (2016) and the Barbervax® registration trials WEC above 2000 epg were present in control animals. It appears that the ewes naturally acquired immunity was adequate to control the low infection they were exposed to in Chapter 2 and a greater infection would be required to observe vaccinal protection. High levels of background natural immunity against *H. contortus* reduce the additional impact that Barbervax® vaccination will have, thus reducing the protective index.

In the registration trials, antibody titres of yearling ewes, vaccinated the year prior, were over 2000 units higher than naïve controls some 8-9 months post their last vaccination (Smith 2014b). Given the short duration of the antibody response to Barbervax® vaccination and the half-life of circulating IgG of only 14 days these results suggest that the previously vaccinated ewes receive a stimulus to maintain antibody titres other than vaccination. Phase 2 in Chapter 2 attempted to test whether exposure to natural challenge could help maintain Barbervax® titres and be responsible for the higher titres observed for long periods in previously vaccinated ewes. The results did not support the hypothesis with no difference in ELISA titres between ewes vaccinated with Barbervax® with or without suppression of worm infection using Moxidectin LA. However since suppressive anthelmintic treatment has been shown to not prevent an immune response to L3 infection (Kelly *et al.* 2010) the experimental design may not have enabled a proper test of the hypothesis.

Interestingly, analysis of serum samples in ELISA tests using both Barbervax® antigens and *H. contortus* L3 antigens revealed a high level of correlations between the two tests. This is suggestive of cross-reaction between the two tests, resulting in the tests possibly insufficient to distinguish between naturally acquired and vaccinal immunity. This cross reaction may be the result of the presence of *H. contortus* antigens other than H11 and H-gal-GP in the vaccine and/or the presence of H11 and H-gal-GP antigens in the *H. contortus* L3 antigen preparation used in the *H. contortus* ELISA test. This cross reaction may help explain the low level of protection observed in Chapter 2, with high levels of natural immunity antibodies cross reacting with non-Barbervax® antigens on the ELISA plate. However, this would not explain the prolonged elevation of Barbervax® antibody levels in fully vaccinated animals beyond those predicted by a hidden antigen response. In this case, the elevation is likely due to low level antibody production

by memory cells, or indeed, during natural infection some exposure to H11 and H-gal-GP occurs, sufficient to maintain higher antibody levels in vaccinated animals.

As expected no systematic effects of Barbervax® vaccination on production were detected in ewes. The lack of negative effects confirms the apparent safety of the vaccine while the lack of positive effects probably results from the failure to significantly affect WEC.

## 5.3. Barbervax® in lambs

Producers who would benefit from implementing Barbervax® are reluctant to begin using it due to the frequency of vaccinations and in particular the P2 primer between marking and weaning. The results of chapter 3 supported the hypothesis that P2 could be removed by reducing the marking to weaning interval to 6 weeks and a double dose at marking and/or weaning. Interestingly, lambs in the alternative treatments produced a full response to a second vaccination at weaning, equivalent in magnitude to the response in lambs given two primers beforehand. Given this and the use of a weaning drench in lambs, the risks associated with the alternative approach appear to be minimal. Removal of P2 eliminates the cost and time of the P2 muster, stress to ewe and lambs, and the risk of mis-mothering. Since commencing Barbervax® use in lambs, with the exception of the first year of its use, Congi Station has reduced the marking to weaning interval and not provided lambs P2 with apparent success. Further experimental work is required to confirm if a single dose at marking and weaning 6 weeks later is sufficient to induce the responses observed in the present experiment.

Despite the removal of P2, lambs given the altered vaccination protocols fared no worse nor better than animals given the vaccination schedule as per Barbervax® instructions. Prior to experimentation, it was thought that lambs which received a double dose at marking may exhibit a larger antibody response. However, this was not the case with the alternative vaccination treatments antibody response only increasing to the same levels as the Barbervax® protocol following the weaning vaccination. Removal of P2 also did not impact the lamb's protective immunity, with the vaccinal immune response more dependent on timing rather than on antigen dose.

Protection throughout experimentation in chapter 2 and 3 was approximately 50%. Despite this protection, it was still inadequate to control high challenge towards the end of experimentation in vaccinated lambs. Modelling studies revealed that a minimum of 65% efficacy was required for effective control, and below this vaccination cannot provide effective control against haemonchosis and mortality. Vaccine efficacy of 65% and above results in 4.5% vaccinated

weaner mortality rate, compared to weaners anthelmintic treated 4 times/year and a 27.7% mortality rate (Smith 2015). Reasons for this poor protection are not due to experimental reasons but may be a result of faulty vaccine batches. Within and between batch variation is likely to occur during the Barbervax® production process. Each batch wouldn't be tested in vivo and it is possible not every batch is analysed by ELISA method either. Commercially, Barbervax® sales are increasing and with a bigger demand for the vaccine, larger batches are being made with possible less time for quality control and attention to the process.

Furthermore, challenge level has not affected the degree of protection. This was demonstrated by the ~50% reduction in WEC of lambs at the end of experimentation where lambs were exposed to large infections (controls ~5,000 epg; vaccinates ~6000 epg) (Chapter 3). Barbervax® registration trials on lambs found a greater reduction at CSIRO's (73-91%) experimental sites than Invetus (55-84%). This difference was thought to be due to a greater infection favoured by lusher pasture conditions at Invetus sites (Smith 2014a). However, despite lusher pasture conditions and a much greater challenge level than Invetus', protection was still achieved in the lambs.

In Chapter 2 and 3, lack of significant associations between WEC and antibody titre were found. In comparison, a vaccine therapeutic trial found a strong and significant relationship between antibody titre and WEC 9 days pi. It is unclear why we had poor associations as ELISA method provided by Moredun was closely followed. The ELISA may be contaminated by the cross-reaction between Barbervax® and H. contortus antigen ELISA. Variation during the ELISA process may also be a reason, due to minor time differences between each plate analysis and variations in equipment used between Moredun and UNE (eg. ELISA plate reader).

Furthermore, in Chapter 2 and 3 a lack of repeatability of responders was discovered. The registration trials documented the notion of animals classifiable as non-responders to vaccination based on WEC. Separation of lambs into responders had higher titres and lower WEC compared to non-responders, however the repeatability of this was poor. Lambs which responded in the first cycle (post-weaning vaccination) did not all respond in the second cycle (post first booster vaccination). It was clear that a vaccination response of the individual was not an inherent trait, but rather an indicator of vaccination success. Furthermore, animals can be classified as responding or not at different times following vaccination. This casting doubt as to the utility of measuring responders and non-responders.

An important finding in Chapter 4 was that lamb Barbervax® antibody titre was affected by ewe vaccination treatment at marking and 3 week post marking with a trend towards an effect at weaning. Lambs from vaccinated ewes had higher titres than their counterparts indicating detectable levels of maternal ab at 2-7 weeks of age. Despite this, it did not hinder the lambs ability to mount their own vaccinal immune responses. It was unclear whether these antibody levels of lambs from vaccinated ewes was sufficient to cause differences to level of worm infection. Furthermore, analysis of bodyweight (Chapter 3) showed there to be no effect of vaccination on bodyweight, as was the case in the registration trials (Smith 2014a), confirming the safety of the product and lack of any effect to future lamb performance.

## 5.4. Comments on experimental design

An important point to make is the unvaccinated control ewes throughout the experiments were not true unvaccinated animals. Congi began to implement Barbervax® in lambs shortly following its release, with the ewes (then lambs) receiving a full vaccination course of Barbervax®. The ewes also received their first booster vaccination as hoggets prior to confirming experimental design and commencement of the experiment. Discussions concluded that ewes not vaccinated as hoggets could be used as 'unvaccinated controls'. It was unlikely the ewes would still have high antibody titres 10 months following their previous vaccination as lambs, however ELISA analysis revealed these animals still had high antibody titres 12 months later. In future experimentation, true unvaccinated ewes should be used.

Whilst working on-farm was an ideal situation it restricted the availability of animals. A mob of 198 ewes was provided by Congi Station's manager, although due to multiple experiments running simultaneously there were several treatment groups of ewes and lambs, all with varied sample sizes. Calculation of the experimental power revealed that the sample size of 20 ewes/treatment used during Chapter 2 was too low, with a minimum 40 ewes required to provide 80% power to detect a significant reduction in WEC of $P<0.05$. Use of 20 animals per treatment wasn't an unreasonable number to have used given as few as 16 animals per treatment group used in some of the ewe and lamb registration trials (Smith 2014a, 2014b) and 20 animals used previous experimental studies with WEC as key variable. However, future experimentation should involve more animals per treatment group, particularly further experimentation on performance (Chapter 4).

The experimental design called for treatment groups to be grazed together. This ensured animals (ewes and lambs) were subjected to the same level of challenge, nutrition and environmental

effects, reducing any paddock effects had the treatments grazed separately. Furthermore, grazing the treatments together, the animal becomes the experimental unit, rather than the paddock. However, because the animals grazed naturally infected pastures, the results could be affected by the varied challenge level. Grazing the treatments together, as discussed in Chapter 2, almost certainly reduced the magnitude of differences between the groups because unvaccinated ewes are contributing additional pasture contamination that the vaccinated ewes would not otherwise be exposed to and vice versa. Similarly, in Chapter 3, this approach potentially exposed the vaccinated lambs to a greater challenge. However, there were 34 unvaccinated lambs grazing with 132 vaccinated animals so the magnitude of the effect would not have been great. The magnitude of the reduced challenge faced by unvaccinated lambs would be very much greater than if they were grazed on their own at the same stocking rate. Whilst grazing the treatment groups together didn't allow us to capture the epidemiological consequences of vaccination over time, significant reduction in WEC was observed, particularly in the lambs. Vaccine studies (including the Barbervax® registration trials) test protection against a uniform challenge, and many of the registration trials grazed vaccinated and unvaccinated treatments together.

The routine practice of administering a pre-lambing anthelmintic treatment at Congi Station (a recommended practice) prevented measurement ewe WEC after the pre-lambing vaccination, and therefore the effect of vaccination on the peri-parturient rise in WEC. The ability to follow WEC of the ewes through lambing and lactation would have been beneficial, however for this to occur, we would have needed easy and frequent access to a mob. Furthermore, the anthelmintic treatment applied in April (Phase 1, Chapter 2), was 4-weeks post the start of the experiment and impaired the ability to detect the duration of protection following vaccinations in hoggets. This was an oversight during the experimental period.

The vaccinated + Moxidectin treated ewes were to approximate 'worm-free' sheep but with ingestion of larvae still proven to stimulate an immune response in sheep given suppressive anthelmintic treatment (Kelly *et al.* 2012) this experimental model compromised the ability to determine whether exposure to natural challenge helps maintain Barbervax® antibody titres. To properly compare the effect of constant natural challenge on Barbervax® antibody titres ewes should remain housed and vaccinated ewes naïve to parasitic exposure and trickle infected with Haemonchus should be compared.

As discussed in Chapter 3, it was an oversight to not include a treatment group consisting of a single dose at marking and weaning. At the time it was felt that a single dose 6 weeks apart would be insufficient to mount an adequate response but the results of the lamb experiment and two

years of practice at Congi where only single doses were given at marking and weaning, suggest that this was an incorrect assessment and using single doses at marking and weaning with no P2 between is worthy of further investigation.

## 5.5. Thoughts and future experimentation

The results in Chapter 2 weren't ideal, and one error in particular was the anthelmintic treatment at week 4 in Phase 1. Re-running the first experiment with a greater natural challenge and number of animals would assist in defining the true duration of protection following a full vaccination course in year 2. Further experimentation to test whether natural challenge enhances immunity induced by Barbervax® would be ideal, although a different model will be required. Removing anthelmintic treatments that impair testing of hypotheses without compromising animal welfare will be important in future work. The findings of this experimentation might make it possible to extend vaccination intervals during booster courses, particularly during summers with smaller parasite infections, or eliminate booster vaccinations if WEC are closely followed on-farm. Additionally, it might be possible to recommend treatment strategies prior to pre-lambing vaccinations.

The results discussed in Chapter 3 clearly indicated the ability to remove the second priming vaccination in lambs. Despite a delay in increasing antibody titre of the alternate vaccinated treatments until after the second vaccination at weaning, the marked titre response revealed that development of the immune response to vaccination is time rather than dose dependent. Further work to determine whether equivalent results can be obtained with single doses at marking and weaning with a 6-week interval are imperative, given the cost savings of eliminating one dose from the first year schedule. Success of this would eliminate both the cost of the extra vaccination (double dose) and the cost of the muster/yarding. In effect, the weaning (third priming) vaccination as per the Barbervax® protocol becomes the new second and final priming vaccination. Thereafter, booster vaccinations could occur as per protocol.

The reason for the large antibody response to the 2$^{nd}$ vaccination at weaning in the alternative treatments is unclear. Whilst antibody titres of the VP treated lambs increased gradually, titres of the alternative treatments decreased between marking and weaning (decline of maternal antibody) before increasing to sharply the same level post weaning (anamnestic active immune response to booster vaccination). Neither dose nor the frequency of priming vaccinations appeared to effect antibody titre in the vaccinated lambs but the experimental design was not factorial and did not allow clear separation of these effects. It is possible the better antibody response to the weaning vaccination may be a result of the immune system maturing or memory

cells have had a greater ability to respond to the weaning vaccination. It would be interesting to see the antibody response following a single dose at marking and weaning and see if the same response following weaning occurs.

The marked increase in lamb's WEC towards the end of this experiment was concerning, clearly demonstrating that Barbervax® can be overwhelmed. Whilst it was not our intention to show this a 50% reduction in WEC did still occur, but was insufficient with ~6000 epg in vaccinated weaners being highly concerning. Dobson *et al.* (2013) using modelling studies, found that >65% efficacy was required to better control haemonchosis and reduce the mortality rate. The results in Chapter 2 and 3 showed all protection fell below the minimum efficacy for successful control. Further studies investigating Barbervax® protection at a wide range of challenge levels would be beneficial with a view to ensuring adequate protection under conditions of high challenge would be highly beneficial.

All sheep subjected to vaccination receive the same 5 µg antigen in 1 mL, and most animals develop an antibody response. During large burdens, the antibody response remained high in all animals with a slow and similar decline over the following 5 weeks. This decline was no different that seen during the registration trials (Smith 2014a). It may be possible that during large burdens, there are greater number of worms competing for sites, and therefore consuming less blood and not being as damaged by antibodies. Importantly, the vaccine did not cease to work at high challenge levels, but rather protection was much lower in these experiments than the registration trials. Perhaps this problem be solved by more frequent vaccinations with greater antigen amount during wet summers. It could be questioned as to how much more antigen is required to stimulate a larger immune response to cope with large burdens. It has been discussed that for a large antibody response, a much greater amount of antigen is required, although vaccination of 9-month old lambs with 50 µg had less of a reduction in WEC, than lambs which received 5 µg (Basetto *et al.* 2014). Increasing the antigen amount or frequency of dosage would increase cost to produce and buy the vaccine.

Analysis of responders and non-responders during Chapters 2 and 3 showed that this trait was not highly repeatable, and so the utility of this measure is doubtful. In both of the experiments classifying ewes and lambs as non-responders based on WEC was challenging with little consistency or repeatability of classification in vaccinated animals. Interestingly all lambs were responders based on antibody titre regardless of whether they were responders or not based on WEC. No previous work has analysed whether animals are classified as a responder or not based on antibody titre, and while it appears that antibody titre is indicative of a vaccination response,

the lack of association between antibody titre and WEC in the current work indicate that and non-responders should be classified based on WEC which is likely a better predictor of worm burden.

Further experimentation on the maternal transfer of Barbervax® antibodies and the consequences of this for lambs would be optimal. Based on data from Chapter 3 it appears that it is unlikely to interfere with the lamb vaccination response, although more studies replicating this work on greater numbers and different properties will help to confirm the extent and factors affecting the transfer of Barbervax® antibodies. It may be possible that the marking vaccination could be delayed if the lamb's antibody levels are sufficiently elevated before the first priming vaccination. This would mean the lambs could be older and the younger ones less at risk, particularly for lambs who are born later during the lambing season.

Few strong conclusions can be drawn on the results from Chapter 4 although it was clear that there were no negative effects of vaccination. It would be expected that provision of a preventative treatment would allow animals to partition nutrients preferentially to performance. This was apparent in the vaccinated and Moxidectin treated ewes who were 1.5-2.5 kg heavier at marking, however unvaccinated ewes were numerically heavier than the remaining two vaccinated treatments clearly indicating a lack of effect of vaccination per se. Further and repeat experimentation on production and performance values of ewes (ie. fleece quality and quantity, bodyweight, scanned litter size, progeny survival) is required, as well as following their progeny closely and for a longer period of time to observe their production and performance.

## 5.6. Industry Application

The release of Barbervax® is already providing producers with an alternative and successful control option for *H. contortus*. Whilst Barbervax® is not a suppressive treatment like effective anthelmintics, used correctly it is has a role as a preventative treatment against which resistance is unlikely to develop. Ongoing experimentation on Barbervax® will further our knowledge and the use of the vaccine on-farm. Scientists, in response to questions and feedback from users of the vaccine, are turning their attention towards stream-lining Barbervax® use on-farm documenting its effects on production and investigating more on its mode of action.

Particularly for lambs, the findings from Chapter 3 are highly significant for the industry, because if implemented they would reduce costs and welfare risks significantly at the individual farm level and to the industry as a whole. Calculation of the cost for the extra muster as per Chapter 3 revealed a cost of AUD $0.53 per lamb. The cost of the dose used at P2 in the Barbervax® protocol hasn't been eliminated, as it has been moved to either marking or weaning. Regardless

of this, savings of thousands of dollars have been achieved by removing the extra and unnecessary mustering and yarding. Further research may yet reveal that the double dose at marking and/or weaning is not required.

For Merino's, eliminating the chances of mis-mothering and stress to both lambs and ewes is important to producers, scientists and a world focusing more on animal production and welfare. For larger properties animals may have to be mustered kilometres to sheep yards. Mustering large distances with lactating ewes and their progeny is use of the animals' energy and body reserves, which is best used towards growth, milk production and maintaining bodyweight. Therefore, removal of P2 and its associated muster, risks and costs may assist in adoption of Barbervax® by producers requiring alternate control methods.

Results from future experimentation on Barbervax® will determine if double doses are required when P2 is eliminated, define optimal vaccination and other control practices during high challenge and better define the potential positive and negative consequences of maternal transfer of Barbervax® antibodies to lambs.

## 5.7. Conclusion

This thesis has provided a detailed analysis into protection against *H. contortus* provided by Barbervax® vaccination in Merino hoggets and their progeny and discovered practical alternatives to eliminate the difficult and unpopular P2 priming vaccination. This thesis has begun to highlight the effects of vaccination on production and performance in hoggets and the effect on lambs from varied hogget treatments. This thesis has produced interesting results and provoked thoughts and questions for further experimentation. The vaccine is a highly beneficial addition to armoury available to control *H. contortus*. This thesis has uncovered ways of improving its uptake but at the same time revealed some limitations in the level of protection achieved, which proved insufficient to deal with conditions of high challenge.

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